Studies on regulation of glutamine synthetase isoforms during water deficit in differentially drought tolerant rice (*Oryza sativa* L.) cultivars and in a resurrection plant, *Selaginella bryopteris* L.

Thesis submitted to the University of North Bengal
For the Award of Doctor of Philosophy
in
Biotechnology

By

KAMAL KRISHNA SINGH

Supervisor

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West Bengal, India -734013

August 2013

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No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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Date: 06.08.2013

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Abstract

Water deficit (WD) has adverse effect on rice (*Oryza sativa* L.) and acclimation requires essential reactions of primary metabolism to continue. Rice plants utilize ammonium as major nitrogen source, which is assimilated into glutamine by the reaction of Glutamine synthetase (GS, EC 6.3.1.2). Rice plants possess one gene (OsGS2) for chloroplastic GS2 and three genes (OsGS1;1, OsGS1;2 and OsGS1;3) for cytosolic GS1.Here, we report the effect of WD on regulation of GS isoforms in drought-sensitive (cv.IR-64) and drought-tolerant (cv. Khitish) rice cultivars. Under WD, total GS activity in root and leaf decreased significantly in IR-64 seedlings in comparison to Khitish seedlings. The reduced GS activity in IR-64 leaf was mainly due to decrease in GS2 activity, which correlated with decrease in corresponding transcript and polypeptide contents. GS1 transcript and polypeptide accumulated in leaf during WD, however, GS1 activity was maintained at a constant level. Total GS activity in stem of both the varieties was insensitive to WD. Among GS1 genes, OsGS1;1 expression was differently regulated by WD in the two rice varieties. Its transcript accumulated more abundantly in IR-64 leaf than in Khitish leaf. Following WD, OsGS1;1 mRNA level in stem and root tissues declined in IR-64 and enhanced in Khitish. A steady OsGS1;2 expression patterns were noted in leaf, stem and root of both the cultivars. Results suggest that OsGS2 and OsGS1;1 expression may contribute to drought tolerance of Khitish cultivar under WD conditions.

Resurrection plants have evolved a wide spectrum of adaptations to cope with the challenges of environmental stress and can serve as a system for studying metabolic events during dehydration and rehydration. *Selaginella bryopteris* belonging to the family Selaginellaceae, is a resurrection lycophyte growing in the hilly area of tropical regions. In present investigation attempts were made to isolate and characterize GS from S. *bryopteris*. The regulation of GS expression during dehydration and rehydration of *Selaginella* fronds and its relationship with other enzymes of ammonium metabolism were also determined. The RWC of the *S. bryopteris* fronds decreased from 95 to 5 % within 24 h of dehydration treatment. Dehydration condition resulted in decrease in the total protein content. The decline in protein level paralleled a significant increase in free ammonium content. The level of proline also enhanced during dehydration. The effects

were reversible with concentrations returning to level comparable with that of full turgor state 24 h after the water deficit condition was alleviated. Anion-exchange chromatography of *S. bryopteris* frond extract revealed the presence of only cytosolic GS isoform. Furthermore, immunoblot analysis showed the presence of single band of molecular size about 39 kDa. The GS enzyme was partially purified from *S. bryopteris* fronds by ammonium sulphate precipitation, gel-filtration and ion-exchange chromatography. The partially purified enzyme showed pH optima of around 5.5. The enzyme was remarkably thermostable as it didn't lose activity on pre-incubation at temperature upto 60 ^{o}C. GS from *S. bryopteris* showed considerably higher temperature optima of about 60 ^{o}C. The Km value (2.4 mM) for glutamate was lower than that reported for enzymes from leaves of higher plants. In dehydration treated fronds, the activity of glutamine synthetase and glutamate synthase (GS /GOGAT), the main enzymes for ammonium assimilation remained largely unaltered. In contrast, glutamate dehydrogenase (GDH) aminating activity and polypeptide content increased from the beginning and became almost two fold at 24 h of dehydration treatment thus providing an alternative route for assimilation of ammonia. Results suggested that GS/GOGAT along with GDH may play important role in amelioration of ammonia toxicity and enhanced production of proline observed during dehydration.

PREFACE

I joined Dr. Ghosh's lab at Department of Biotechnology, University of North Bengal in August 2007. Here, I interacted routinely with lots of experiments and tried to take a tangential taste of intermingled problems of biological science, especially plants. Living beings are fast reactive because when a seed is left in the environment, it interacts with moisture, air, light and enzymatic thunderings inside it gives root and shoot outside. On the other hand, plants are authorized by nature in this universe to trap solar energy and supply it to us; so we can call them, Almighty in terms of energy. In spite of adverse conditions, they survive for years at the same place due to cellular brilliancy of stress management, unfortunately they are also caught by stresses at times. Similarly, the work was also perturbing and stressful for me, though I was not sessile like plants. Anyway, I enjoyed the job and it's my pleasure to have the opportunity to express my gratitude to all of them who assisted me.

I am highly grateful to my supervisor Dr. Shilpi Ghosh, Assistant Professor, Department of Biotechnology, North Bengal University, by whose grace I completed my research work successfully. It gives me immense pleasure to express my indebtedness to her for giving valuable suggestions and constant guidance during the entire course of study. It was not possible to complete this journey without her guidance. It is difficult for me to write in few words my gratitude towards her.

I would like to pay my sincere thanks to the H.O.D., Dr. Ranadhir Chakraborty, Department of Biotechnology, North Bengal University, who inspired me always to do best in life. And also, thanks to the faculty members; Dr. Dipanwita Saha and Dr. Anoop Kumar, Department of Biotechnology, University of North Bengal for their active support to carry out the research.

I express my sincere thanks to the Vice chancellor, Registrar, Science Dean, University of North Bengal, for granting me opportunity to carry out the research work in this university.

I am very grateful to Dr. Joydeb Pal, Dr. Tapas Kumar Chaudhuri and Dr. Min Bahadur, Department of Zoology, University of North Bengal; Dr. Arnab Sen, Department of Botany and Dr. Ashish Nanda, Department of Chemistry, University of North Bengal, for their kind support and infrastructural aid.

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Singh, Mr. Sunil Kumar, Mr. Kripa Shankar Singh and Dr. Chandramani Kumar, Mr. Chandan Kumar for being with me when nothing seemed to be going right.

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Heartiest thanks to my aunty Late Shanti Devi and Jiju, Late Shivvash Singh; your absence will always be a matter of immense regret for me. And I extend the hand of special thanks to my family members; Sri Shivpujan Singh, Sri Shambhu Nath Singh, Sri Prem Chand Singh, Sri Manik Chand Singh and rest of the family members for their munificent support during my nasty situation.

Finally, I would like to thank and express 'inexpressible' gratitude to my father Sri Arjun Singh and my mother Smt. Roopkali Devi for perpetual support in all directions.

The victory really becomes more pleasurable when you strike the target in very tough situations. Anyway, the moment becomes truly exceptional, when, imaginings turn into reality.

Kamal Krishna Singh

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CHAPTER 1

General introduction and literature review

1.1. INTRODUCTION

Nitrogen is an essential nutrient for plant growth and productivity. In higher plants inorganic nitrogen is assimilated predominantly via Glutamine synthetase (GS; EC 6.3.1.2)/ Glutamate synthase (Glutamate oxo-glutarate ammonium transferase, GOGAT; EC 1.4.7.1) pathway. GS catalyses the ATP-dependent condensation of ammonium (NH_4^+) with glutamate to yield glutamine. The enzyme GOGAT catalyses the conversion of glutamine and 2-oxoglutarate to two molecules of glutamate. The net outcome of the GS-GOGAT cycle is the production of glutamate, which can then be incorporated into other amino acids through the action of aminotransferases or transaminases (Forde and Lea 2007; Bernard and Habash 2009). Specific amino acids subsequently become precursors for all nitrogen-containing organic molecules, such as proteins, chlorophyll, cytochrome/phytochrome, secondary metabolites and nucleic acids. In higher plants, GS exists in multiple isoforms that are either cytosolic (GS1) or plastidic (GS2). The relative abundance of GS isoforms varies within different plant species and within different organs of the same plant, depending on their photosynthetic type (McNally and Hirel 1983; McNally et al. 1983; Bernard and Habash 2009). GS2 is encoded by one gene and GS1 is encoded by three to five genes depending on plant species (Forde and Cullimore1989; Miflin and Habash 2002). Phylogenetic studies on GS nucleotide or amino acid sequences have shown that chloroplastic and cytosolic GS form two sisters groups (Pesole et al. 1991) and have evolved through gene duplication (Doyle 1991; Swarbreck et al. 2011). These two major GS isoforms play primarily nonoverlapping roles in plant nitrogen assimilation. GS2 is the predominant isoform in the leaf, where it assimilates ammonia originating from

nitrate reduction and photorespiration (Wallsgrove et al. 1987; Bernard et al. 2008). Immunocytochemical studies have demonstrated that GS1 protein is localized in the vascular tissue, suggesting its metabolic role in assimilation of NH_4^+ to glutamine for transport and distribution throughout the plant (Pereira et al. 1996; Bernard and Habash 2009). In root, GS1 facilitates the assimilation of NH_4^+ taken from the soil or from symbiotic nitrogen fixation (Rai et al. 2000; Tobin and Yamaya 2001). GS has also been implicated in nitrogen remobilization during seed germination and regulating biosynthesis of proline, an amino acid with increased accumulation in response to osmotic stress (Brugiere et al. 1999; Silveira et al. 2003).

GS is regulated in a developmentally controlled manner during vegetative and reproductive plant growth stages and particularly through senescence. GS isoforms are also differentially regulated in response to environmental signals, such as, nitrogen status, light, biotic and abiotic stress (Oliveira and Coruzzi 1999; Bernard and Habash 2009). Although transcriptional control of GS is important in determining polypeptide abundance, the enzyme is also regulated by post-transcriptional and post-translational mechanism or by protein turnover (Bernard and Habash 2009). Moreover, phosphorylation and 14-3-3 interaction have been implied in the modulation of GS activity in plants (Man and Kaiser 2001; Riedel et al. 2001; Lima et al. 2006).

Drought, heat and salinity are the most important environmental stress factors that influence plant growth and development and place major limits on plant productivity in cultivated areas worldwide. Drought is a composite stress condition that includes soil water deficit, increased daytime temperature and reduced nutrient availability. However, the most important factor limiting growth and impairing plant productivity is the drop in water availability to the plant (Oliver et al. 2011). The specific plant responses to water deficit (WD) are dependent on the rate and amount of water loss, the duration of stress and the stage of plant development. Plants can perceive WD and elicit appropriate responses at the morphological, physiological, biochemical and molecular levels leading to altered metabolism, growth and development. Several features, like attenuated growth, stomatal closure, rolling of leaf, deep rooting, limited transpiration and reduced photosynthesis, and osmotic adjustments were found to be linked with drought tolerance (Ludlow and Muchow

1990). Adaptation to drought at biochemical and molecular levels undoubtedly involves the activation/increased expression of several genes, transient increases in ABA levels, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways. However, no consensus has been reached in defining the key processes determining tolerance and the secondary follow-up processes (Shinozaki and Yamaguchi-Shinozaki 2000; Bartel and Sunkar 2005).

During prolonged period of WD, decrease in water availability for transport associated process leads to limited uptake of nitrogen from soil and reduced availability of CO_2 for photosynthesis as stomata are induced to close followed by disturbances in carbon and nitrogen metabolism (Foyer et al.1998; Xu and Zhou 2006). Acclimation to WD requires responses that allow essential reaction of primary metabolism to continue. Recent studies have shown nitrogen assimilation to be critical for plant acclimation to stress conditions. It has been demonstrated that GS isoforms are regulated both at transcript and protein levels in response to plant status as well as environmental cues. The enzyme constitutes a regulatory point at which environmental signals are integrated and translated into a plant response in terms of growth and seed production (Swarbreck et al. 2011).

WD is one of the major constraint depressing rice (*Oryza sativa* L.) production. The effect of WD varies with the variety, degree, and duration of stress and its coincidence with different growth stages (Gao et al. 2007). Poor seedling vigour, fertility and consequent reduction in yield have been the major problems provoked by the stress condition. NH_4^+ is the main form of nitrogen available to the young rice plants, which is assimilated by GS to glutamine. Glutamine serves as the main form of organic-nitrogen for transport through vascular tissues (Ishiyama et al. 2004b). Rice plants possess one gene (OsGS2) for chloroplastic GS2. The cytosolic GS1 gene family consists of three isoforms encoded by OsGS1;1, OsGS1;2 and OsGS1;3. OsGS1;1 and OsGS1;2 are expressed in all organs with higher expression in leaf blades and roots. In leaf, they are present as minor form in comparison to GS2. OsGS1;3 is expressed mainly in spikelets (Tabuchi et al. 2005). These isoforms have been shown to be regulated in a developmentally controlled manner as well as by light and nitrogen nutrition (Kamachi et al. 1991; Ishiyama et al. 2004a, b; Tabuchi et al. 2007; Kusano et al. 2011). However, the regulation of these isoforms during WD has

not been investigated in detail. The role of GS isoforms in controlling nitrogen metabolism during WD can be understood by studying their regulation in differentially drought tolerant rice varieties.

Resurrection plants have evolved a wide spectrum of adaptations to cope with the challenges of environmental stress (Oliver et al. 2011; Yobi et al. 2012). They have unusual capability to survive long dry period. They are able to recover complete physiological activity following repeated protoplasmic dehydration of fully differentiated tissues and hence, can serve as a system for studying metabolic events during dehydration and rehydration. *Selaginella bryopteris* belonging to the family Selaginellaceae, is a resurrection lycophyte growing in the hilly area of tropical regions. It is a creeping plant with simple scale-like leaves on branching stems from which roots also arises. It is capable of surviving almost complete dehydration for prolonged period (Deeba et al. 2009). A useful insight into the role of GS isoforms under WD conditions can be obtained by studying the enzyme in resurrection plants, *S. bryopteris*. With all these in view, the present investigation was undertaken with the aim of studying the effect of WD on GS isoforms in *Oryza sativa* and *Selaginella bryopteris*.

1.2. OBJECTIVES

1. To screen rice cultivars for drought tolerance.
2. To determine GS isoform pattern in leaf, stem and root of rice seedlings by Ion-exchange chromatography, RT-PCR and Immunoblot analysis.
3. To study the effect of WD on activities of GS isoforms in leaf, stem and root of drought sensitive and drought tolerant rice cultivars.
4. To investigate the transcriptional and translational regulations of GS isoforms in leaf, stem and root of drought sensitive and drought tolerant rice cultivars in response to WD.
5. To determine GS isoform pattern of *S. bryopteris* fronds.
6. To purify the GS isoforms from *S. bryopteris* fronds and determine their biochemical characteristics.
7. To study the regulation of GS isoforms during hydration cycle of *S. bryopteris* fronds.

1.3. LITERATURE REVIEW

Nitrogen is an essential nutrient for plant growth and productivity. Plant species have evolved specific strategies to acquire nitrogen from their environments and assimilate it into organic compounds. The nitrogen sources directly available to plants include inorganic nitrogen compounds, such as nitrate (NO_3^-) and NH_4^+, as well as organic compounds, such as amino acids (e.g. glycine, alanine, glutamic acid, aspartic acid) and small peptides (Schimel and Bennett 2004).

The main pathway of inorganic-nitrogen assimilation to organic form is the Glutamine synthetase - Glutamate synthase (GS-GOGAT) cycle (*Figure 1.1*).

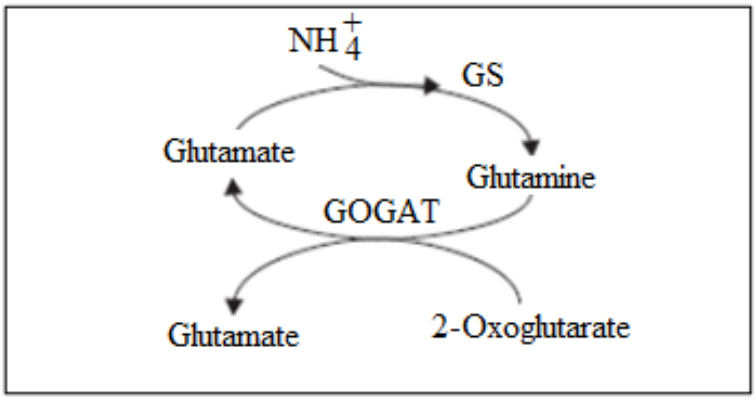

Figure 1.1. The glutamine synthetase-glutamate synthase (GS-GOGAT) cycle.

GS catalyzes the ATP-dependent condensation of NH_4^+ with glutamate to yield glutamine; GOGAT transfers the amide group of glutamine to α-ketoglutarate to subsequently produce glutamate (Bernard and Habash 2009). GS is a ubiquitous enzyme found in all higher plants, pteridophytes, bryophytes, algae, fungi and bacteria. The enzyme is involved in the assimilation of NH_4^+ produced via nitrate reduction, biological nitrogen fixation and catabolism of amino acids and, in the photosynthetic organism, in reassimilation of NH_4^+ evolved during photorespiration. In view of its key role in nitrogen metabolism, it has been studied quite extensively from varieties of prokaryotic and eukaryotic organisms. Information relevant to the present investigations is reviewed in the following subheadings.

1.3.1. GS isoforms in plants

GS exists in multiple isoforms in various plant organs such as leaves, roots, nodules, cotyledons and seeds. Leaves of several higher plants such as, barely (Mann et al. 1979), rice (Hirel and Gadal 1980), pumpkin (Kretovich et al. 1981), sorghum (Hirel and Gadal 1982), spinach (Hirel et al. 1982), potato (Teixeira et al. 2005) and wheat (Bernard et al. 2008) have been reported to contain two isoforms of GS that are either cytosolic (GS1) or plastidic (GS2). The cytosolic (GS1) isoform was the first to be eluted, followed by the plastidic (GS2) form (Becker et al. 1993; Tobin and Yamaya 2001), although the opposite elution pattern has been described in some species (Woodall et al. 1996; Orea et al. 2002). The cytosolic and chloroplastic localization of GS1 and GS2 has been further confirmed by immunological and molecular studies (Becker et al. 1993; Pereira et al. 1996; Bernard and Habash 2009). The relative abundance of GS isoforms varies within different organs of the same plant and within different plant species, depending on their photosynthetic type. McNally et al (1983) examined the relative abundance of GS1 and GS2 in leaves of a wide range of higher plants and on the basis of results obtained, classified plants into four different groups. Leaves of first group of plants contained only cytosolic form (GS1) of the enzyme, a pattern which is found in achlorophyllous higher plant parasites. The second group was characterized by having only the chloroplastic enzyme (GS2) as in tomato, spinach and mustard. The third group is represented by plants whose leaves contain GS1 as the minor component with chloroplastic isoform being the major form. Such a distribution profile of GS1 and GS2 is frequently encountered in C3 plants like barley (Mann et al. 1979), rice (Hirel and Gadal 1980), wheat (Tobin et al. 1985), sunflower (de la Haba et al. 1992) and Arabidopsis (Ishiyama et al. 2004a). The fourth group contains almost equal levels of GS1 and GS2 and such a situation is prevalent in most of the C4 species. Moreover, the relative proportion of GS1 and GS2 has been shown to be regulated in a developmentally controlled manner during vegetative and reproductive plant growth stages and particularly through senescence (Tobin et al. 1985; Kamachi et al. 1992; Kichey et al. 2005). They are also differentially regulated in response to environmental signals, such as, nitrogen status, light, biotic and abiotic stress (Oliveira and Coruzzi 1999; Bernard and Habash 2009). Majority of the studies on GS in roots indicate the presence of only cytosolic form of the enzyme (Mann et al. 1979; Ishiyama et al. 2004a; Ishiyama et al.

2004b; Bernard et al. 2008) though according to few reports substantial activity of GS was also reported to be associated with the plastid fraction of the root cells as in case of pea and alfalfa (Vezina et al. 1987). In fact, in roots of pea and alfalfa as high as about 50% of the total GS activity was recovered in the plastidic fraction when the plants were grown in NO_3^- containing media (Vezina et al. 1987). The isozymes of GS are not only found in leaves, stems, roots and seeds of plants but also recognized in root nodules of several leguminous plants such as, peanuts (Bonald et al. 1978; Tingey et al. 1987), alfalfa (Dunn et al. 1988), beans (Ortega et al. 1992), medicago (Stanford et al. 1993; Lima et al. 2006) and soybeans (Temple et al. 1996). In root nodules, the primary function of GS is the rapid assimilation of NH_4^+ released into the cytosol of the infected cells by N_2-fixing bacteroids (Atkins 1987). A marked increase in activity of GS during the development of nodule coinciding with the appearance of nitrogenases and legheamoglobin has been noted by several investigators (Cullimore and Benette 1988; Temple et al. 1996; Carvalho et al. 2003).

1.3.2. Structural and catalytic properties

1.3.2.1. Structural properties

GS has been purified and characterized from a variety of plant species and tissues such as leaves of pea rice (Hirel and Gadal 1980), spinach (Ericson 1985), jack pine (Vezina et al. 1988) and rapeseed (Ochs et al. 1995); roots of pea (Emes and Fowler 1979), rice (Hirel and Gadal 1980; Ishiyama et al. 2004b) douglas-fir (Bedell et al. 1995), *Arabidopsis* (Ishiyama 2004a); nodules of soybean (Mc Parland et al.1976), alfalfa (Groat and Schrader 1982), lupin (Mc Cormack et al. 1982) and bean (Cullimore et al. 1983). Molecular weight of the native GS protein from plant sources ranges from 350-400 kDa and it comprises of eight subunits of molecular mass of 38 to 43 kDa for the cytosolic GS and that of 44 to 45 kDa for the chloroplastic isoform. These subunits are arranged in a parallel, biplane, tetrahedral symmetry to form an octameric enzyme. The octameric as well as tetrameric states of both cytosolic and chloroplastic forms of enzymes from leaves of *Beta vulgaris* were found to be active whereas in roots only octameric form was enzymatically active (Mack and Tischner 1990). The atomic structure of maize cytosolic GS has recently been elucidated at 2.63-, 3.50- and 3.80-Å resolutions, indicating that plant

GS polypeptides (Type II) form decamers (Unno et al. 2006), which differ from the dodecameric structure of bacterial GS (Type I) (*Figure 1.2*).

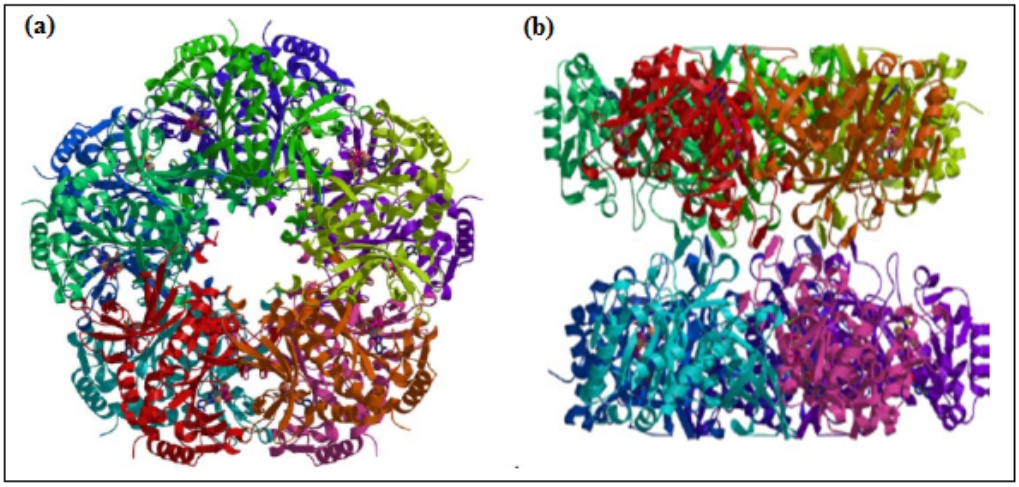

Figure 1.2. Structure of cytosolic GS from *Zea mays* (Maize) composed of two face-to-face pentameric rings of subunits contain 10 active sites, each between two neighbouring subunits within each ring. Source: Unno et al. 2006

1.3.2.2. Catalytic properties

In situ, the enzyme catalyses the following reaction which involves ATP-dependent amination of glutamate to glutamine:

$$\text{Glutamate} + \text{ATP} + NH^{+}_{4} \xrightarrow{Me^{2+}} \text{Glutamine} + \text{ADP} + \text{Pi} \qquad \text{(Reaction 1)}$$

Where, Me^{2+} can be magnesium or manganese. The reaction has been termed the 'biosynthetic' reaction and is considered the most physiologically relevant reaction that GS catalyzes.

The reaction is believed to proceed in two stages. In the first step γ-carboxylic group undergoes phosphorylation to yield γ-glutamylphosphate and in the next step phosphate group is replaced by amino group to form glutamine (*Figure1.3*). In addition of above physiological activity, isolated GS also catalyses the formation of γ-glutamylhydroxamate through either an ATP dependent semi-synthetase reaction (Reaction 2) or a transferase reaction (Reaction 3).

Glutamate + ATP + NH_2OH ⟹ γ-glutamylhydroxamate + ADP + Pi

(Reaction 2)

Glutamine + NH_2OH $\xrightarrow[Mn^{2+}, ADP]{Arsenate}$ γ-glutamylhydroxamate + NH_4^+

(Reaction 3)

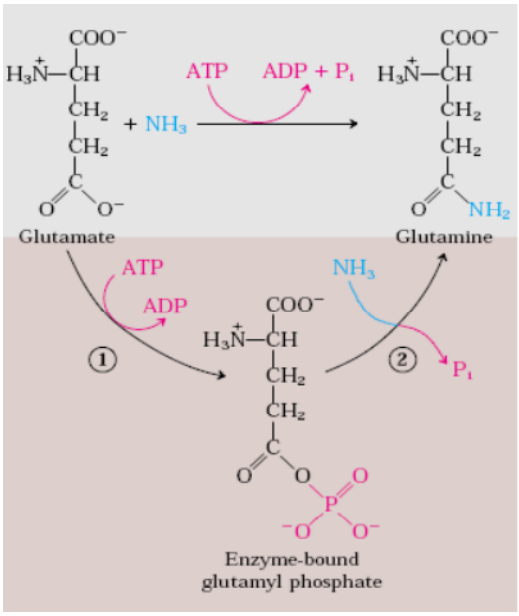

Figure 1.3. Reaction catalysed by glutamine synthetase.
Source: Nelson and Cox (2004)

The biosynthetic reaction of the enzyme has rarely been used for determination of GS activity in plants principally because, as compared to the semi-synthetase and transferase reactions, this activity is very low. Moreover, this assay is not suitable for use with unpurified extracts because of the presence of high activities of interfering enzyme such as glutaminase, ATPase, phosphatases and nucleotidases. On the other hand, semi-synthetase and transferase reactions have been generally used for determination of GS activity, since several folds higher activity of these reactions than biosynthetic activity offers a distinct advantage of increased sensitivity of the assay procedure (Lea et al. 1990).

1.3.3. Molecular characteristics

The primary translation product of GS2 has been shown to contain an N-terminal transit peptide which ostensibly facilitates its transport into chloroplasts and this transit

peptide is cleaved off after the enzyme is transported into the chloroplast. In contrast, such transit sequences have been shown to be absent in mRNA for GS1 isoform of the enzyme. Some key domains of the GS protein have been characterized, such as ATP-binding and glutamate-binding sites (Unno et al. 2006).

In angiosperms, GS2 is encoded by one gene and GS1 is encoded by multigene family consisting of three to five genes. Studies on GS1 gene families were first carried out in legume species, particularly *Phaseolus vulgaris* (Lara et al. 1983), *Pisum sativum* (Tingey et al. 1987) and *Medicago truncatula* (Stanford et al. 1993). GS gene families have also been described in non-legume plant species, such as *Zea mays* (Li et al. 1993), *Arabidopsis thaliana* (Ishiyama et al. 2004a), rice (Ishiyama et al. 2004b), potato (Teixeira et al. 2005), sugarcane (Nogueira et al. 2005) and wheat (Bernard et al. 2008). In cereal crops, the number of genes encoding cytosolic GS varies between three and five; wheat (GS1, GSr and GSe), rice (GS1;1, GS1;2. GS1;3), and maize (Gln1-1, Gln1-2, Gln1-3, Gln1-4 and Gln1-5). These genes are differentially expressed both in developmental and organ specific manner (Ishiyama et al. 2004b; Bernard et al. 2008).

GS gene and protein sequences are well conserved both within and across species. At the nucleotide level, cytosolic GS-encoding sequences are between 76 % and 82 % identical. At the amino acid level, the percentage similarity varies between 79 and 83 %, while the percentage identity varies between 88 and 93 %. Phylogenetic analyses of GS nucleotide or protein sequence have shown that chloroplastic and cytosolic GS emerged from gene duplication. The duplication occurred earlier than the monocot-dicot divergence (Bernard et al. 2008). Phylogenetic analyses of GS sequences retrieved from GenBank and plant transcript assembly databases gave further information on the evolution of the GS genes, in particular those encoding the cytosolic isoform. The topologies of the trees obtained with distance, parsimony, and likelihood phylogeny inference methods were overall similar (Pesole et al. 1991; Biesiadka and Legocki 1997; Swarbreck et al. 2011). The consensus tree obtained using maximum likelihood method indicated five monophyletic groups supported with high bootstrap values (*Figure 1.4*). One clade corresponded to the GS2 type while four additional clades included sequences encoding GS1.

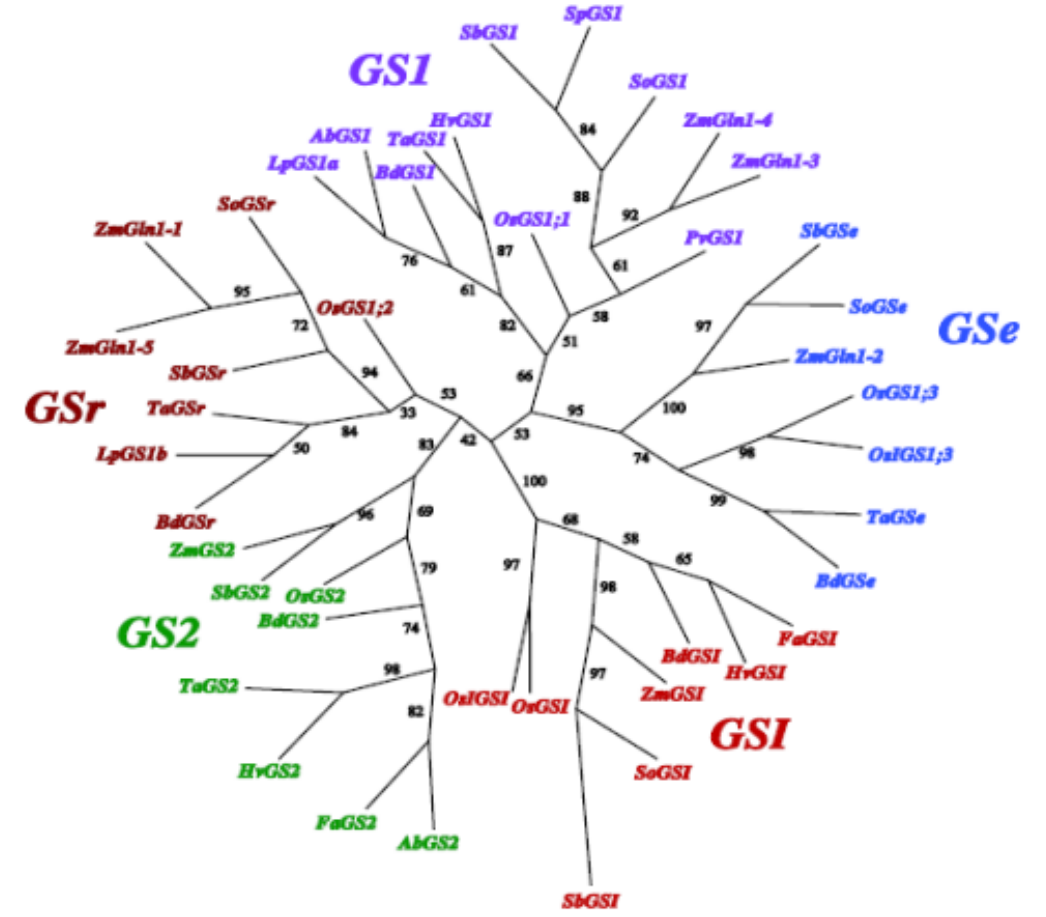

Figure 1.4. Unrooted phylogenetic tree of GS protein sequences from plant species of Poaceae family. The tree was calculated by using maximum likelyhood method. The reliabilities of each branch point were assessed by bootstrap analysis (100 replicates), and bootstrap values are displayed on the tree. GenBank accession number and transcript assembly number for each sequence used in the tree are listed below. *Brachypodium distachyon*: BdGS1, Bradi3g59970; BdGS2, Bradi5g24550; BdGSe, Bradi1g11450; BdGSI, Bradi3g27880; BdGSr, Bradi1g69530. *Festuca arundinacea*: FaGS2, TA701_4606; FaGSI, TA2341_4606. *Hordeum vulgare*: HvGS1, Q06378; HvGS2, P13564;HvGSI, AK252215. Lolium perenne: LpGS1a, ACR45959; LpGS1b, ACR45960. *Oryza sativa*: OsGS1;1, Os02g50240; OsGS1;2,Os03g12290; OsGS1;3, AAK18848; OsGS2, Os04g56400; OsGSI, Os10g31820. *Oryza sativa* Indica: OsIGln1;3, EEC76061; OsIGSI, EEC67088. *Panicum virgatum*: PvGS1, TA1886_38727. *Sorghum bicolor*: SbGS1, Sb04g028690; SbGS2, Sb06g031460; SbGSe,Sb01g010270; SbGSI, Sb01g020230; SbGSr, Sb01g042450. *Saccharum officinarum*: SoGS1, AAW21273; SoGSe, AAW21275; SoGSI, AAW21277; SoGSr, TA36550_4547. *Sorghum propinquum*: SpGS1, TA3613_132711. *Triticum aestivum*: TaGS1, AAZ30057; TaGS2, AAZ30060; TaGSe, AAR84349; TaGSI, TA75518_4565; TaGSr, AAR84347. *Triticum monococcum*: TmGS2, TA2112_4568. *Zea mays*: ZmGln1-1, P38559; ZmGln1-2, CAA46720; ZmGln1-5, P38563; ZmGln1-3, CAA46721; ZmGln1-4, CAA46722; ZmGS2, CAA46724; ZmGSI, GRMZM2G115646. *Avena barbata*, AbGS2, AbGSr, and AbGS1 were assembled from ESTs. Source: Swarbreck et al. 2011

The cytosolic GS genes are clustered into three phylogenetically distinct groups corresponding to GS1, GSr and GSe and an additional clade including GS1 and GSe groups, suggesting that these two groups are more related to each other than to the GSr group (Swarbreck et al. 2011).

1.3.4. Regulation of GS isoforms by light

Activity of GS in plant tissues is influenced by several environmental factors. Light is one of the important factors controlling the level of GS in photosynthetic tissues. An enhancement in GS activity during greening of leaves has been reported by several workers (Hirel et al. 1982; Canovas et al. 1986; Edward and Coruzzi 1989; Ghosh 2010). In all these investigations, light was found to exert a more profound influence on the appearance of GS2. In etiolated leaves of sorghum GS activity was detectable only in the cytoplasmic fraction and during greening increase in total activity was largely associated with the appearance of activity in the chloroplastic fraction (Hirel and Gadal 1982). On the other hand, etiolated sunflower cotyledons (de la Haba et al. 1992), and etiolated leaves of maize (Sakakibara et al. 1992) and *Pennisetum glaucum* (Ghosh 2010) were found to contain both GS isoforms and, on exposure to light the level of GS1 showed marginal increase and level of GS2 increased significantly. Functional expression analysis of the GS1 promoter in transgenic *Arabidopsis* also indicated the presence of regulatory sequences involved in the response to light (Avila et al. 2001).

In etiolated pea leaves, the steady-state levels of both the chloroplast-specific GS2 polypeptide and the GS2 mRNA increased during greening and are regulated by phytochrome (Tingey et al. 1987; 1988). Moreover, the fact is supported by the expression study of GS2 in mustard (Schmidt and Mohr 1989), wheat (Edwards and Coruzzi 1989), maize (Sakakibara et al. 1992), *Phaseolus* (Cock et al. 1991) and *Arabidopsis* (Peterman and Goodman 1991). In these studies GS2 was shown to be tightly regulated by light in a process at least in part mediated by phytochrome. However, Edward and Coruzzi (1989) noted and emphasized that white light elicited accumulation of GS2 mRNA several fold higher than that obtained with brief pulse of red light. Hence, they deduced that besides acting through phytochrome, light also exerted additional effect probably via its influence

in chloroplast metabolism. The level of GS1 gene expression was not significantly affected by light (Oliveira and Coruzzi 1999). According to some reports, light mediated enhancement in GS activity might involve activation of previously synthesized enzyme which was stored in inactive form in the plant tissue (Canovas et al. 1986; Ghosh 2010). The light dependent appearance of GS in *Pennisetum* seedling was inhibited by the photosynthetic inhibitor, dichlorophenyl dimethyl urea (DCMU) (Ghosh 2010). Recent studies on regulation of GS have also indicated the role of photosynthetic reactions in light dependent stimulation of GS. Exposure of sunflower plant to increasing CO_2 concentration in light caused concomitant increase in leaf starch and sugar contents and activities of both GS1 and GS2 (Larios et al. 2004).

1.3.5. Regulation of GS by nitrogen status

GS responds to nitrogen availability in the external medium as well as plant nitrogen status. Thus far, it has been difficult to establish a global model of GS response to variation in nitrogen availability in both roots and leaves. While NH_4^+ had no influence on appearance and activity of GS in sunflower cotyledons (de la Haba et al. 1988), it was reported to suppress the activity of GS in mustard cotyledons (Schmidt and Mohr 1989) and wheat cells (Fricke 1993).

Investigations have also been carried out on the changes in activities and expression of GS isoforms in response to nitrogen nutrition. In barley seedlings, the specific activity of GS was found to be much higher in leaves but declined in root in presence of NH_4^+ as compared to specific activities in respective organs of NO_3^- fed or N-free grown plants. With higher concentration of NH_4^+, the specific activity of GS1 rose in leaves but fell in roots. The activity of GS2 in leaves was also elevated with increasing concentration of NH_4^+ in the nutrient medium. The alterations in activities of GS1 and GS2 were correlated with changes in the subunit composition of the active holoenzyme (Mack 1995). NH_4^+ is supposed to substrate-induce the GS1 promotor of rice (Kozaki et al. 1991) and soybean (Hirel et al. 1987; Miao et al. 1991). Kozaki et al. (1992) showed that the GS2 promotor of rice is also activated by NH_4^+. Although some cytosolic GS gene members are up-regulated by the addition of NH_4^+, some are down-regulated or do not respond (Sakakibara et al. 1992; Ishiyama et al. 2004 a,b; Hirel et al. 2005; Kusano et al. 2011). In *Arabidopsis*,

GS1 mRNA and polypeptide accumulated in roots when plants were supplied with NH_4^+, however, the GS activity was maintained at a constant level. The discrepancy between the protein content and enzyme activity of GS1 was attributed to the kinetic properties and expression of four distinct isoforms encoded by *GLN1;1*, *GLN1;2, GLN1;3* and *GLN1;4* genes that function complementary to each other in *Arabidopsis*. GLN1;2 was significantly up-regulated by NH_4^+ and correlated with the rapid increase in total GS1 protein. However, GLN1;2 exhibited lower affinity to the substrates NH_4^+ and glutamate. In contrast, high affinity enzyme GLN1;1 was abundantly expressed in surface layer of root during nitrogen limitation and down regulated by NH_4^+ excess (Ishiyama et al. 2004a). Similarly in rice root the cytosolic OsGS1;1 and OsGS1;2 transcripts showed reciprocal response to NH_4^+ supply in the surface cell layers of roots. OsGS1;1 accumulated in the dermatogens, epidermis and endodermis under nitrogen limited conditions. By contrast, OsGS1;2 was abundantly expressed in the same cell layers under nitrogen sufficient conditions replenishing the loss of OsGS1;1 following NH_4^+ treatment (Ishiyama et al. 2004b). Recent study on quantitative comparative analyses between the metabolite profiles of a rice mutant lacking OsGS1;1 and its background wild type (WT). The mutant plants exhibited severe retardation of shoot growth in presence of NH_4^+ compared with the WT. Overaccumulation of free NH_4^+ in the leaf sheath and roots of the mutant indicated the importance of OsGS1;1 for NH_4^+ assimilation in both organs. The metabolite profiles of the mutant line revealed: (i) an imbalance in levels of sugars, amino acids and metabolites of the tricarboxylic acid (TCA) cycle, and (ii) overaccumulation of secondary metabolites, particularly in the roots under a continuous supply of NH_4^+. Metabolite-to-metabolite correlation analysis revealed the presence of mutant-specific networks between tryptamine and other primary metabolites in the roots. These results demonstrated a crucial function of OsGS1;1 in coordinating the global metabolic network in rice plants grown using NH_4^+ as the nitrogen source (Kusano et al. 2011).

NO_3^- had little effect on appearance of GS in mustard cotyledons (Schmidt and Mohr 1989) and scot pine seedlings, whereas in sunflower cotyledons (de la Haba et al. 1992) and maize leaves (Aguera et al. 1987) it strongly enhanced the level of the enzyme. NO_3^- enhanced the GS2 activity approximately by 4 fold in mesophyll cells and by 1.3 fold in bundle sheath cells of maize leaves but no enhancement was detected in GS1 level

(Sakakibara et al. 1992). Since the primary reaction of NO_3^- assimilation in C4 plants occur in mesophyll cells, the elevated activity was attributed to increased demand for assimilation of ammonia produced from nitrate reduction of these cells. An increase in the GS2 polypeptide content caused by NO_3^- nutrition was also found in pea roots (Vezina and Langlois 1989) and cultured rice cells (Hayakawa et al. 1990). Transcript stability is another means of GS1 regulation in response to nitrogen nutrition (Ortega et al. 2006). However, it is not clear whether plant nitrogen status or NO_3^- molecules interact with the cis-acting element at the 3' end of the GS1 transcript.

1.3.6. Regulation of GS isoforms by abiotic and biotic stress

Environmental stresses such as abiotic (drought, salinity and low or high temperatures) and biotic stresses are important factors which limit plant distribution and productivity (Hare et al.1999). Abiotic stress has toxic effects on plants and lead to metabolic changes, like loss of chloroplast activity, decreased photosynthetic rate and increased photorespiration rate (Winicov 1993). Teixeira and Fidalgo (2009) have suggested that nitrogen assimilation is more sensitive to water stress than CO_2-photosynthetic assimilation. The effect of abiotic stress on plant nitrogen metabolism has been studied with inhibition of protein synthesis, increased protein degradation and accumulation or depletion of protein and non-protein amino acids in a variety of monocots, dicots (Gilbert et al. 1998; Martinelli et al. 2007). Under drought conditions the expression of gene encoding ubiquitin related proteins and various proteases were found to be induced or enhanced, consistent with the requirement of protein degradation under stress conditions. All these processes ultimately lead to either acclimation to the stress conditions or to senescence and subsequent cell death. The response of GS to salt and drought stress hasn't been studied in much detail. During abiotic stress the abundance of GS2 polypeptide and its activity declined, whereas GS1 tended to increase or maintain the same level in the leaves (Bauer et al. 1997; Santos et al. 2004; Martinelli et al. 2007). The work of Hoshida et al (2000) also showed the importance of GS2 in salinity tolerance in *Oryza sativa*. Plant transformed with GS2 gene had increased photorespiratory capacity conferring resistance to salinity stress (Hoshida et al. 2000). The response of GS to abiotic stress in the roots is less clear, with studies on rice seedlings and potatoes showing decline in total GS activity in response to salt stress (Teixeira and Pereira 2007; Teixeira and Fidalgo 2009).

The accumulation of metabolite such as, proline, glycine betaine, polyols, polyamines and ions (i.e. potassium), is one of the mechanisms for stress tolerance (Kishor et al. 1995; Bajji et al. 2000). They act both by contributing to osmotic adjustment and by protecting proteins and cellular membranes. The studies of Brugiere et al (1999) have shown that silencing of a phloem specific GS (*Gln* 1-5) decreased proline production. GS thus plays a major role in regulating proline production consistent with the function of proline as a nitrogen source and a key metabolite synthesized in response to water stress. In potato leaf, in response to high salt levels GS2 regulation resides at the post-translational level while root GS1 is mainly regulated at a transcriptional level, with a differential expression of the GS1-encoding genes (Teixeira and Pereira 2007; Teixeira and Fidalgo 2009).

Nitrogen nutrition has a significant impact on plant disease development. Reduced availability of nitrogen often increases the susceptibility of plant to diseases. Some evidences exist for the induction of GS gene in infected leaf cells in plants under pathogen attack (Olea et al. 2004; Pageau et al. 2006; Tavernier et al. 2007). GS1 was upregulated in leaves of tobacco (Pageau et al. 2006) and *Phaseolus vulgaris* (Tavernier et al. 2007) in response to viral and bacterial attack and during infection with pathogenic and non-pathogenic fungal strains. However, this induction did not depend on a hypersensitive response induction. Thus GS1 responded in the same manner as an early-response defence genes. The up-regulation of GS1 (*Gln-α*) in *P.vulgaris* was also accompanied by an increase in GS1 polypeptide abundance, but decline in overall activity, mainly resulting from decline in GS2 activity. Thus cytosolic GS regulated at transcript abundance level both by abiotic and biotic stress. However, it is unclear whether an increase in transcription occurs or mRNA transcript becomes more stable. Pathogen infection can also affect proline content and gene expression of proline metabolism. Fabro et al. (2004) found that inoculation of Arabidopsis with a virulent *P. syringae* produced a hypersensitive response led to increased proline synthesis around the inoculation site. The NH_4^+ imbalance thus created was compensated by plants assimilation of NH_4^+ into glutamine and glutamate.

1.3.7. Regulation of GS isoforms by senescence

Senescing leaves act as source of nitrogen for growing parts as their constituent proteins are hydrolysed and organic nitrogen is redistributed within the plant. The amides, glutamine and asparagines, are the major organic form for translocation of nitrogen in plants (Hayashi and Chino 1990). These amides are produced from glutamate and aspartate, respectively, using NH_4^+ released upon hydrolysis of Rubisco and other leaf proteins. Since GS is the main enzyme catalyzing the conversion glutamate and NH_4^+ to glutamine, a few studies have also been conducted on changes in GS activity in senescing leaves. GS activity is known to decrease rapidly during both natural and dark induced senescence (Kawakami and Watanabe 1988; Fischer and Feller 1994). The activities of GS1 and GS2 are differentially regulated by leaf age. The GS2 polypeptide level declined in parallel to Rubisco and other chloroplast enzymes during natural senescence in leaves of various plants. The decline in GS2 coincided with an increase in abundance of GS1 (Kamachi et al. 1992; Habash et al. 2001; Bernard et al. 2008). Similarly, in potato GS2 polypeptides and mRNAs were detected in leaves and their content decreased as leaves senesced, whereas GS1 was detected in non-photosynthetic tissues and in the later leaf senescing stages (Teixeira et al. 2005). Kichey et al (2005) studied the localization of GS1, GS2 and glutamate dehydrogenase (GDH) during natural senescence of the flag leaf and in the stem of wheat seedlings. In mature flag leaves, large amounts of GS1 were detected in the connections between the mestome sheath cells and the vascular cells, suggesting an active transfer of nitrogen organic molecules within the vascular system in the mature flag leaf. Parallel to leaf senescence, an increase of a GS1 polypeptide (GS1b) was detected in the mesophyll cytosol of senescing leaves, while the GS protein content represented by another polypetide (GS1a) in the phloem companion cells remained practically constant in both leaves and stems. Both GDH aminating activity and protein content were strongly induced in senescing flag leaves. The induction occurred both in the mitochondria and in the cytosol of phloem companion cells, suggesting that the shift in GDH cellular compartmentation is important during leaf nitrogen remobilization.

1.3.8. Physiological role of GS isoforms

Plants have evolved to capture and assimilate the available carbon and nitrogen from the environment and to store and relocate it efficiently. During the growth and

development of plants, nitrogen moves into and out of proteins in the different organs and transported between organs in a limited number of transport compounds that differ widely in their C/N ratio (Miflin and Habash 2002).

Plants have developed a variety of molecular strategies to use limiting nutrients with a maximum efficiency. Some of the organic nitrogen moves between compounds via the activity of transaminases and glutamine-amide transferases, but a significant portion is released as NH_4^+. For example, asparagine is a significant component of seed storage proteins in legumes and a major transport compound in cereals. It is metabolized to NH_4^+ and aspartate via the action of asparaginase. Similarly ureides, such as allantoin play an important role in nitrogen transport in legumes and their organic-nitrogen is released as NH_4^+ via the action of urease. Nitrogen containing into biomolecules also release NH_4^+ by plant metabolic activities in various physiological processes and such as photorespiration, the biosynthesis of phenylpropanoids. Thus over the life of a plant, nitrogen is released as NH_4^+ and refixed several times. Overall GS acts at the centre of nitrogen flow (*Figure 1.5*). As mentioned earlier, with a few exceptions leaves of most of higher plants contain cytosolic and chloroplastic GS though their relative proportion may vary markedly depending upon species and to some extent on the environmental factors.

1.3.8.1. Physiological role of cytosolic GS

In both C3 and C4 species, cytosolic GS is located in vascular bundle (Kichey et al. 2005; Martin et al. 2006). The promoter analysis of the GS3A gene of pea suggested that cytosolic GS is preferentially expressed in the vascular tissue of leaves (Edwards et al. 1990). Many subsequent studies have confirmed the importance of the location of GS1 in the phloem and related vascular tissues (Edwards et al. 1990). Furthermore, recent studies have shown that GS1 is located in intermediary cells, where nitrogen is exchanged between different cell types, such as the primary pit fields connecting the mestome sheath cells and the neighbouring parenchyma and vascular cells in wheat leaves (Kichey at al. 2005).

The localization of GS1 transcript and polypeptides in roots has led to suggestion that it is involved in assimilation of NH_4^+ from primary nitrogen uptake (Ishiyama et al.

2004a,b; Bernard et al. 2008). Furthermore, *in situ* hybridization study with rice has shown that the location of OsGS1;2 transcript is consistent with the site of NH_4^+ uptake (Sonoda et al. 2003; Ishiyama et al. 2004b; Tabuchi et al. 2007). A study of barley mutants deficient in GS2 showed a normal phenotype and no nitrogen deficiency when grown under nonphotorespiratory conditions (Wallsgrove et al. 1987; Leegood et al. 1995), suggesting that cytosolic GS is also involved in primary nitrogen assimilation. Recent study on quantitative comparative analyses between the metabolite profiles of a rice mutant lacking OsGS1;1 and its background wild type (WT). The mutant plants exhibited severe retardation of shoot growth in presence of NH_4^+ compared with the WT.

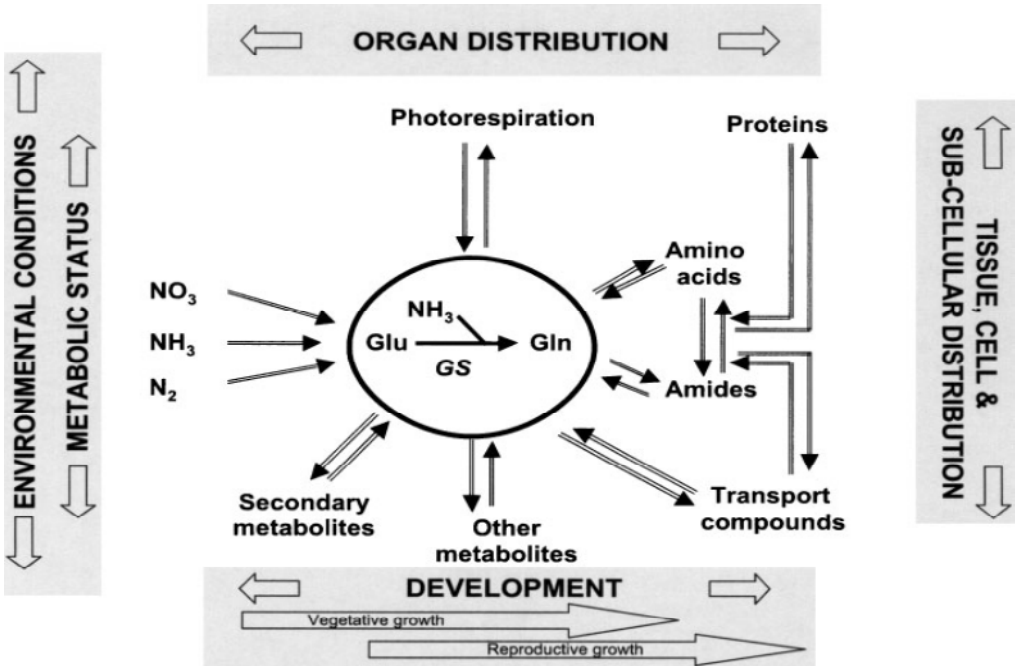

Figure 1.5. The central role of GS in the complex matrix of plant N metabolism. The central scheme encompasses the total role of GS. The boxes around the outside indicate the matrix of various locations and environments in which GS may be operating. Source: Mifilin and Habash 2002

Overaccumulation of free NH_4^+ in the leaf sheath and roots of the mutant indicated the importance of OsGS1;1 for NH_4^+ assimilation in both organs. The production of NH_4^+ that occurs either within the plants tissues or in nodules can significantly contribute to plant nitrogen nutrition. Cytosolic GS is a known to assimilate NH_4^+ from the three major types of nitrogen-fixing symbiotic association involving plant or either Rhizobium, actinomycetes or cyanobacteria (Rai et al. 2000). It also assimilates NH_4^+ produced by

endophytic diazotrophs colonizing the internal parts of some plants, such as sugarcane, without establishing symbiotic relationship (Nogueira et al. 2005). Cytosolic GS has also been implicated in remobilization of nitrogen for grain filling. Significant correlation were obtained between grain number/size and a locus of cytosolic GS protein (OsGS1;1) content in rice (Obara et al. 2004). In maize QTLs were found for GS activity, cytosolic GS locus (gln1) and grain yield (Hirel et al. 2001, 2007). These studies highlight the importance of cytosolic GS genes in determining several aspects of nitrogen use traits in the cereal crops with potential implications for breeding and agriculture (Hirel et al. 2007).

1.3.8.2. Physiological role of chloroplastic GS

The important function of GS2 is conceivably to assimilate NH_4^+ formed via NO_3^- reduction (Melo et al. 2003). Photorespiration, which is the side reaction of photosynthesis, has important implication in plant nitrogen metabolism since during the process substantial amounts of NH_4^+ is released in the reaction involving oxidative decarboxylation of glycine in mitochondria (Buchanan et al. 2002; Keys 2006). It has in fact estimated that in C3 plants the rate of ammonia evolution through photorespiratory cycle is about ten times the rate of NH_4^+ acquired through NO_3^- assimilation (Adriano et al. 2010). It has been unequivocally established that mitochondria do not contain GS. Since many higher plant species contain both chloroplastic and cytosolic GS isoforms in leaves, it was initially proposed that GS1 might be involved in reassimilation of photorespiratory NH_4^+. However, this contention was not supported by the subsequent studies. Barley mutant lacking chloroplastic GS accumulated ammonia in higher amounts only when grown under photorespiratory conditions (Wallsgrove et al. 1987; Blackwell et al. 1987), thereby indicating the importance of GS2 in reassimilation of photorespiratory NH_4^+. The role of GS2 was also supported by several other observations. In pea plants grown under conditions which favour higher photorespiratory activity (0.02 % CO_2), a four-fold increase in the level of GS2 mRNA was recorded whereas GS1 mRNA remained unaffected (Edward and Coruzzi 1989). Though the increase in GS2 mRNA was detectable after 14 days of the treatment, Woodall et al (1996) noted that activity of GS2 undergoes a much more rapid modulation even prior to the increase in level of transcript on altering the phorespiratory status of the plant. In their studies, during which the

photorespiratory rates were stimulated or depressed by lowering or increasing the growth temperature, caused much rapid response in activity of GS. The activity of GS2 in barley plant grown at 25^{o} C day temperature decreased by 75%, after three days at 15^{o} C with no effect on the cytosolic activity (Woodall et al. 1996).

1.3.9. The model plant, Rice (Oryza sativa L.)

Rice is one of the most important crops for mankind. It is the staple food of more than 60% of the world's population especially for most of the people of South-East Asia. It accounts for more than 50% of their daily calorie intake (Maclean et al. 2002). Among the rice growing countries in the world, India has the largest area under rice crop and ranks second in production next to China. Rice occupies about 23.3% of gross cropped area of India and plays vital role in the national food grain supply.

1.3.9.1. Taxonomy and Genome

The genus *Oryza* belongs to the tribe Oryzeae of the family Poaceae. There are 12 genera within the Oryzeae tribe (Vaughan 1994). The *Oryza* contains approximately 22 species, of which 20 are wild species and two; *O. sativa* and *O. glaberrima* are cultivated. *O. sativa* is most widely grown of the two cultivated species and is grown worldwide, including Asian, North and South American, European Union and African countries. However, *O. glaberrima* is grown solely in West African countries (Vaughan 1994). Among cereals, *O. sativa* has a relatively small (430 million base pairs) diploid genome (2n = 24) as compared to the significantly large genome sizes of sorghum, maize, barley, and wheat (about 750, 3000, 5000, and 16000 Mbp, respectively). Recent data place the number of genes in rice genome to about 50,000 (Goff et al 2002; Yu et al. 2002), reflecting even higher gene density. Moreover, rice contains relatively less repetitive DNA. Most other *Oryza* species are also diploid, however some are tetraploid (4n = 48) (Moore et al. 1995; Gale and Devos 1998).

1.3.9.2. Origin, Cultivation and Climate

O. sativa was cultivated in south-east Asia, India (*Figure 1.6*) and China between 8000 to 15000 years ago (Normile 2004). *O. glaberrima* was has been cultivated since approximately 1000 BC (Ahn et al. 1992; Murray 2005). Current cultivation of *O. sativa* is worldwide, over 110 countries.

Figure 1.6. Cultivation of *O. Sativa* in India.

Rice is grown from sea level to 3000 m and in both temperate and tropical climate. A varieties of water regimes are used, including unsubmerged upland rice (10% of total cultivation), moderately submerged lowland rice (irrigated, 45% or rainfed, 30%) and submerged rice (upto 6 m of water, 11% or floating 4%). Rice can grow in a wide range of soil types as well, including saline, alkaline and acid-sulfur soils (Ahn et al. 1992). The chemical properties of soils do not appear to be as important as physical ability of the soil to hold a flood (Scott et al. 2003). Proper growth and high production of rice crop is conditioned by temperature parameter at different growth stages. During flowering, it should range between 16º to 20 ºC, whereas during maturity 28º to 32 ºC temperature is optimum. The temperature beyond 35 ºC affects grain filling. During panicle initiation, 20º-22 ºC temperatures is ideal. The temperature below or above this range will adversely affect the growth and yield. Low temperature is one of the main limits on crop yield (McDonald 1994). Japonica cultivars are predominantly grown in temperate regions and can germinate and grow under low temperature (15º to 20 ºC) than the tropical and sub-tropical Indica cultivars. Temperature below 18 ºC at night during pollen formation results in sterile pollen in all cultivars (McDonald 1994).

Generally, rice plant requires about 14 hours of daylight every day during its growth period. When the rice plant is subjected to day length shorter than 12 hours, it will come to flowering. So, when rice is subjected to lower temperature and short duration of

sunshine, its vegetative phase will be reduced considerably. Too long or too short vegetative phase adversely affects the reproductive phase. In general, longer hours of sunshine with a temperature varying from 20º-35 ºC and abundant moisture supply are the ideal conditions for rice plant. Late maturing varieties are very sensitive to the day length and can be grown only during a specific season, while early maturing varieties can be grown at any time of the year but mostly during summer and kharif season (Vergara and Chang 1985).

1.3.9.3. O. sativa development

The life cycle of rice cultivars ranges from 110 to 150 days from germination to maturity, depending on the variety and the environment. *O. sativa* is an annual grass, bearing a fibrous root system as well as erect culms and developing long flat leaves (*Figure 1.7*). Rice plant forms multiple tillers, consisting of a culm and leaves, with or without panicle. The panicle emerges on the uppermost node of a culm, from within a flag-leaf sheath and bears the flowers in spikletes. Primary tillers emerge from nodes near the base of the main culm and secondary and tertiary tillers emerge sequentially from these.

Figure 1.7. Illustrating different parts of *O. sativa* plants

Single leaves develop alternatively on the culm, consisting of a sheath, which enclose the culm and a flat leaf blade. Rice cultivars can vary widely in the length, width, colour and pubescence of the leaves. Each spikelet has a single floret and two glumes. It is enclosed

by a rigid, keeled lemma which is sometimes extended to form an awn and partially envelops the smaller palea. The floret contains six stamens and a single plumose ovary with two branches. At anthesis, two lodules at the base of floret swell and force the lemma and palea apart as the stamens elongate and emerge. The stigma is sometime exposed as well. The fertilized ovary is caryopsis, meaning a small, single-seeded dry fruit with the pericarp and seed coat fused. It is commonly called a grain. The grain consists of an embryo, endosperm, pericarp and testa, surrounded by hull (the lemma and palea). Grain length varies with cultivar between 5 and 7 mm, and grains can be round, bold or slender (OECD 1999; McDonald 1979).

1.3.10. The model plant, Selaginella bryopteris L.

1.3.10.1. Origin, taxonomy and habitat

Selaginella, called spike moss, is an enigma in the plant kingdom. It belongs to the lycophyte lineage of vascular plants that arose over 400 million years ago during the Silurian and dominated the Earth's flora from the Devonian through the Carboniferous to the end of the Permian (Friedman 2011). The evolution of lycophytes was accompanied by the acquisition of roots with root caps, and typically simple and single-veined leaves called microphylls (Friedman 2011). Lycophyte includes families, the Lycopodiaceae (club mosses), the Isoeteaceae (quillworts), and the Selaginellaceae (spike mosses). *Selaginella* (family Selaginellaceae, order Selaginellales, class Lycopsida) constitutes a monophyletic group of plants with microphylls and adaxial, reniform sporangia (Banks 2009). It is represented by about 750 species in the world (Jermy 1990) and 65 species in India (Nisha et al. 2010). It grows in diverse array of arctic, temperate, tropical, and semiarid habitats (Banks 2009; Yobi et al. 2013). Although most spike moss species are susceptible to desiccation, a few species have evolved the ability to survive vegetative tissue drying, defined as the near complete loss (80-95%) of protoplasmic water (Tuba et al. 1998), and referred to as desiccation tolerant (DT) (Oliver et al. 2000). Such species include *S. lepidophylla* (Iturriaga et al. 2006), *S. bryopteris* (Deeba et al. 2009), and *S. tamariscina* (Wang et al. 2010). These DT species grow luxuriantly during rains exhibiting a lush green velvety landscape (*Figure 1.8*). During summer the plants undergo extreme desiccation. The fronds curl, become dry and virtually dead. The dry plants when left in water unfold their fronds, turn green and come back to active life (Sah et al. 2005).

1.3.10.2. Selaginella genome

The genome size of nine *Selaginella* species was determined by flow-cytometry analysis. The genome size estimates ranged from 84 - 110 Mb. Within the nine species the chromosome number varied between 2n = 16 to 2n = 27. Nuclear genome size appeared to be strongly correlated with chromosome number (Little et al. 2007).

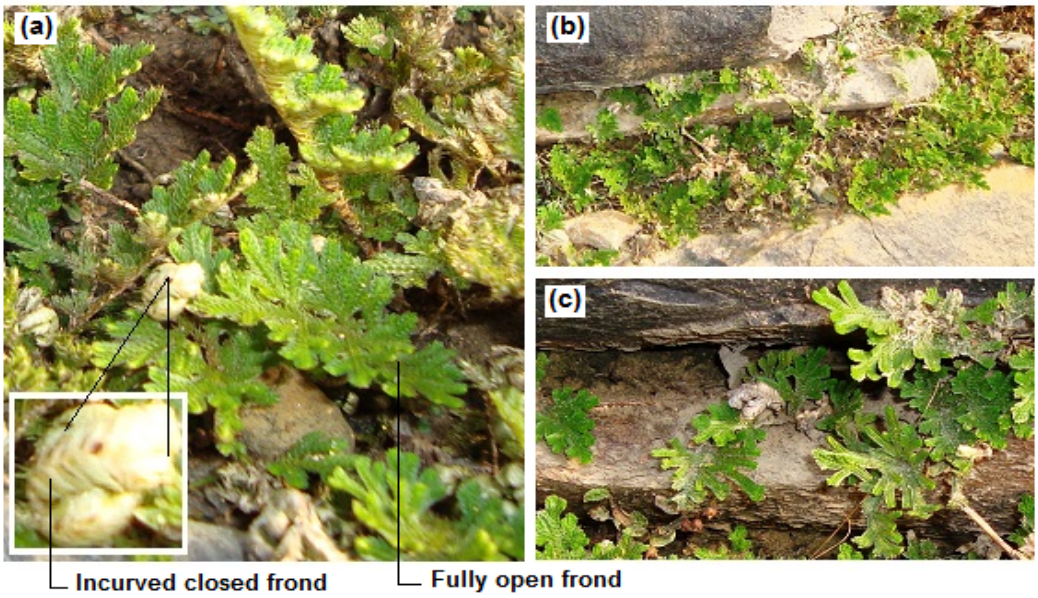

Figure 1.8. *Selaginella bryopteris* in natural habitat

The *Selaginella* genome was sequenced using whole-genome shotgun sequencing. The assembled genome size (212.60 Mbp) is twice that determined by flow cytometry (Wang et al. 2005; Little et al. 2007), indicating that the assembled genome includes two haplotypes of ~106 Mbp that are 98.50% identical at the nucleotide level. A deduced haplotype has 22,285 predicted protein-coding genes, of which 37% are supported by EST sequences (Axtell et al. 2007). Recently, Bank et al (2011) reported the genome sequence of *S. moellendprffti*. By comparing gene content in evolutionary diverse taxa, they found that the transition from a gametophyte to sporophyte - dominated life cycle required far fewer new genes than the transition from a non-seed vascular to a flowering plant. The secondary metabolic genes expanded extensively and in parallel in the lycophyte and angiosperm lineages. *Selaginella* differed in post-transcriptional gene regulation,

including small RNA regulation of repetitive elements, an absence of the tasiRNA pathway and extensive RNA editing of organellar genes.

1.3.10.3. Morphology and life cycle

Selaginella plants are 10-14 cm in size. Stem is erect, creeping and dichotomous branch with or without certain pattern, from which roots also arise (*Figure 1.8*). The dichotomous branches have two different sizes of leaves; where median is smaller than lateral ones (Jermy 1990). Microspore is much smaller than megaspore and usually has different colour (*Figure 1.9*).

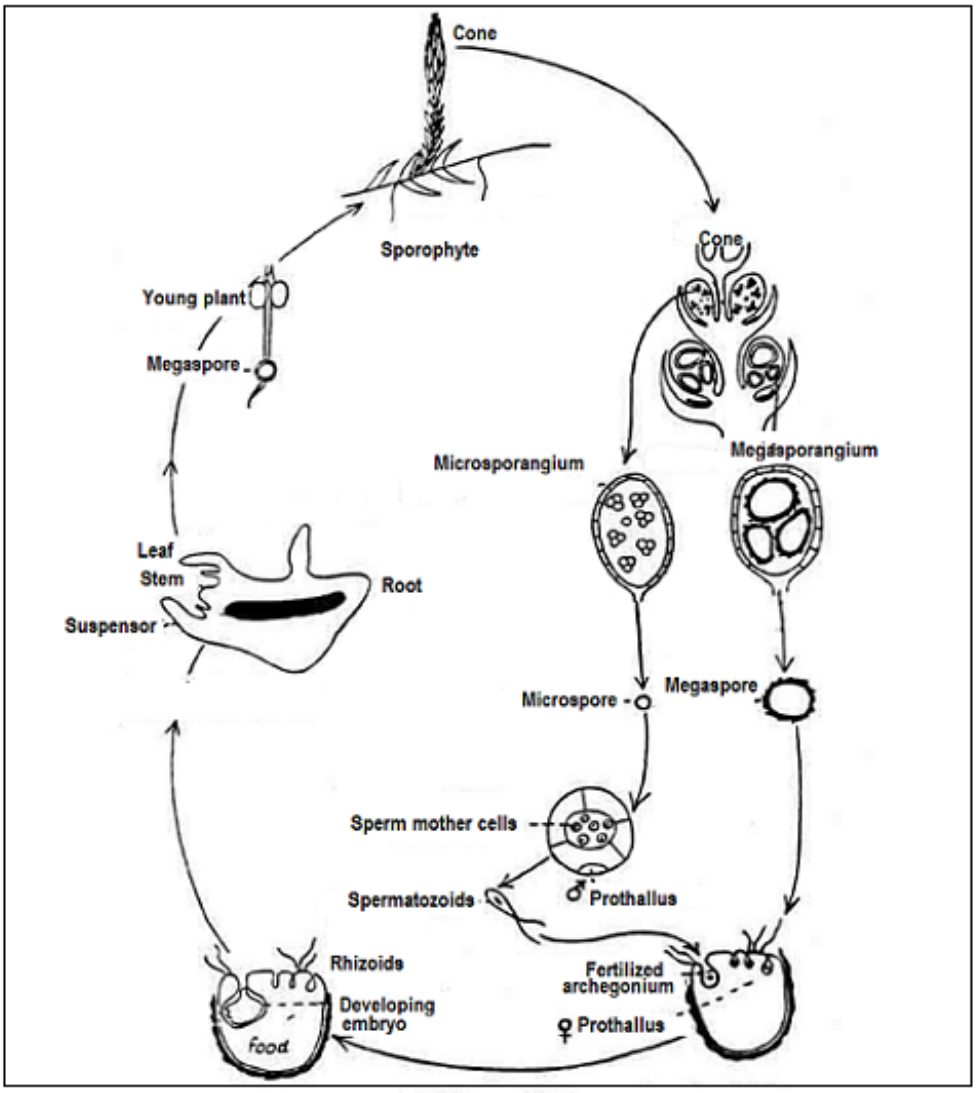

Figure 1.9. *Selaginella* life cycle.

The megaspores split open to reveal the female gametophyte. The Microspores develop many 100's of motile sperm, and when the spore bursts in contact with water these swim to the female gametophytes. This is a parallel to the situation with seed plants, in which the gametophyte is retained within the sporophyte.

1.3.10.4. Molecular properties

Biflavanoid is the major secondary metabolite of *Selaginella* species. *Selaginella* contains several secondary metabolites such as, alkaloids, phenolics (Flavanoid, tannin, saponin) and terpenoids (Chikmawati 2008). The different species of *Selaginella* varies in their secondary metabolite composition. The plant samples of the same species collected in different period, different environment or different locations, also shows variation in HPLC fingerprint. The therapeutic potential of *Selaginella* secondary metabolites has been well documented. *S. lepidophylla* has been used as a diuretic for uninary and kidney infections and in treatment of chronic gastritis and gastric cancer (Robles-Zepeda et al. 2011). Mishra et al (2011) have reported the anticancer properties of *S. bryopteris*. Total flavonoids from *S. tamariscina* have also been employed as an anti-diabetic treatment (Zheng et al. 2011). *Selaginella* species have also been studied for their therapeutic potential as anti-viral, anti-microbial, anti-inflammatory and anti-oxidant activities (Swamy et al. 2006; Aguilar et al. 2008; Tan et al. 2009; Liu et al. 2010; Wang et al. 2011).

1.3.10.5. Drought tolerance

Drought tolerance is extremely rare in vascular plants and only about 0.15% of species being known as resurrection plants (Oliver et al. 2000; Proctor and Tuba 2002). Although most spike moss species are susceptible to desiccation, a few species have evolved the ability to survive vegetative tissue drying, defined as the near complete loss (80-95%) of protoplasmic water (Tuba et al. 1998), and referred to as desiccation tolerant (DT) (Oliver et al. 2000). Such species include *S. lepidophylla* (Iturriaga et al. 2006), *S. bryopteris* (Deeba et al. 2009), and *S. tamariscina* (Wang et al. 2010).

The response of resurrection mosses to desiccation has been shown to rely on a combination of constitutive protection and repair mechanism (Oliver et al. 2005). In

contrast, DT angiosperm require a much longer time to dehydrate, presumably to allow sufficient time for developing adaptive responses and accumulation of key metabolite, such as sucrose, proline, to survive in the desiccation state (Oliver et al. 2000; Suzuki et al 2006). Since, lycophytes represents a plant lineage between mosses and angiosperms, they might be expected to exhibit both constitutive and inducible adaptive mechanism of drought tolerance (Banks et al. 2011). Several *Selaginella* species are able to survive on long drought and recover through rehydration (van Dijck et al. 2002). DT *Selaginella* species have high content of trehalose, a simple sugar responsible for endurance of heat and drought stress (Adams et al. 1990; Setyawan, 2011). The trehalose-6-phosphate synthase (TPS) gene of *S. lepidophylla* was homologous to that of yeast and bacteria (van Dijck et al. 2002). Several sugars (e.g., glucose, sucrose), sugar alcohols (e.g., inositol-1-phosphate, myoinositol, mannitol), and betaine, which act as osmoprotectants and/or hydroxyl radical scavengers were more abundant in *S. lepidophylla* at 100% and 50% relative water contents (Yobi et al. 2012).

A previous study by Harten and Eickmeier (1986) showed significant increase in activity of photosynthetic enzymes during hydration of DT *S. lepidophylla* fronds. However, a recent study has reported DT *Selaginella* species to retain their chlorophyll (and presumably their photosynthetic structures) during desiccation (Pandey et al. 2010). To understand the mechanisms of desiccation tolerance, Deeba et al (2009) carried out proteome based studies on detached *S. bryopteris* fronds to reveal proteins that were differentially expressed in response to dehydration and rehydration. It was observed that proteins involved in transport, targeting and degradation were expressed more in the desiccated fronds. The research work by Pandey et al (2010) indicated several desiccation induced changes in *S. bryopteris* fronds. The desiccated frond had the ability to recover complete physiological activities following rehydration, as all the measured traits returned to the control level. It is proposed that tolerance in *S. bryopteris* is likely to be a combination of (i) inward curling of the dehydrating fronds to minimize photo-oxidative damage (ii) complete recovery of photosynthetic activities (iii) high levels of antioxidative enzymes together with an osmoprotectant-proline. Thus the interplay of several mechanisms ensures optimal survival of *S. bryopteris*. Recent research on metabolic profiling of desiccation tolerant *S. lepidophylla* and desiccation sensitive *S. moellendorffi*

was compared at various hydration states using non-biased, global metabolomic profiling technology, based on GC/MS platform. *S. lepidophylla* retained significantly higher abundances of sucrose, mono- and polysaccharides and sugar alcohols than did *S. moellendorffi*. Aromatic amino acids and the well known osmoprotectant betain and flavanoids were also more abundant in *S. lepidophylla*. *Notably*, levels of γ-glutamyl amino acid, linked with glutathione metabolism in the detoxification of reactive oxygen species, and woth possible nitrogen remobilization following rehydration, were markedly higher in *S. lepidophylla*. Overall, *S. lepidophylla* appeared to tolerate desiccation in a constitutive manner using a wide range of metabolites with some inducible components (Yobi et al. 2012).

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CHAPTER 2

Regulation of glutamine synthetase isoforms in two differentially drought tolerant rice (*Oryza sativa* L.) cultivars under water deficit conditions

2.1. INTRODUCTION

Water deficit (WD) or dehydration is the most crucial environmental factor that limits crop productivity and the geographic distribution of several important crops such as rice, wheat and maize. The specific plant responses to WD are dependent on the rate and amount of water loss, the duration of the stress and the stage of plant development. Adaptation to WD at biochemical and molecular levels involves the activation/increased expression or induction of genes, transient increase in ABA level, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways. During prolonged period of WD, decrease in water availability for transport associated process also leads to limited uptake of nitrogen and reduced availability of CO_2 for photosynthesis as stomata are induced to close followed by disturbances in carbon and nitrogen metabolism (Foyer et al.1998; Xu and Zhou 2006). Acclimation to WD requires responses that allow essential reaction of primary metabolism to continue. Recent studies have shown nitrogen assimilation to be critical for plant acclimation to stress conditions. GS transcript and protein are shown to be regulated in response to both the plant status and environmental cues and hence, the enzyme constitutes a regulatory point at which environmental signals are integrated and translated into a plant response in terms of growth and seed production (Swarbreck et al. 2011).

WD is one of the major constraint depressing rice (*Oryza sativa* L.) production. The effect of WD varies with the variety, degree, growth stage and duration of stress. NH_4^+ is the main form of nitrogen available to the young rice plants, which is assimilated by GS to glutamine. Glutamine serves as the main form of organic nitrogen for transport through vascular tissues (Ishiyama et al. 2004b). Rice plants possess one gene (OsGS*2*) for GS2. The GS1 gene family consists of three isoforms encoded by OsGS1;1, OsGS1;2 and OsGS1;3. OsGS1:1 and OsGS1;2 are expressed in all organs with higher expression in leaf blades. They are present as minor form as compared to GS2. OsGS1:3 is expressed mainly in roots and spikelets, respectively (Tabuchi et al. 2005). These isoforms have been shown to be regulated by a developmentally controlled manner as well as by light and nitrogen nutrition (Kamachi et al. 1991; Kusano et al. 2011; Tabuchi et al. 2007). The role of GS isoforms in controlling N-metabolism during WD can be understood by studying their regulation in differentially drought tolerant rice varieties. Present study describes the regulation of GS isoforms in various organs of drought sensitive and tolerant cultivars of rice in response to WD.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals and reagents

All the chemicals used in this investigation were purchased from Sigma-Aldrich, USA; E. Merck, Germany; Sisco Research Laboratory, India and HiMedia Laboratory, India. DEAE-Sephacel was purchased from Sigma-Aldrich, USA. Biogel P-2 and P-100 were from Bio-Rad, USA. Molecular biology kits were from Promega, USA; QIAGEN, Germany and Invitrogen, USA. GS-antibody was purchased from Agrisera, Sweden.

2.2.2. Plant materials and growth conditions

Rice (*Oryza sativa* L.) seeds (cv. Khitish, Pokkali, Triguna, Satabdi, IR-64, IR-8, PNR-519) were obtained from Rice Research Station, Chinsurah, West Bengal. Seeds were germinated in moist cotton bed at 30 $^{\circ}$C for 2-3 days under dark conditions. About 50 germinated seedlings were transferred to each pot containing a mixture of soil: soilrite (3:1 v/v). Seedlings were grown under 250 µmol m^{-2} s^{-1} photon flux density (16 h/8 h day/night regime) at 27±2 ^{o}C and 70-80 % relative humidity in a Plant Growth Chamber (Conviron, Canada). After 3 weeks of sowing, WD was imposed by withholding water. Seedlings

were harvested from individual pots at indicated days of stress treatment till day12, frozen in liquid nitrogen, and stored at -80 ºC for further analysis. Fresh leaf tissue immediately after harvest was used for determination of RWC, electrolyte leakage, proline and protein contents.

2.2.3. Determination of RWC

The relative water content (RWC) of leaves was measured according to Barrs and Weatherley (1962). 1 g of leaf tissue was weighed immediately after sampling to determine fresh weight (FW) and then rehydrated in water at 4 ^{o}C for 24 h and blotted dry and turgid weight (TW) was recorded. Finally the sample was dried in an oven at 80 ^{o}C for 48 h and dry weight (DW) was recorded. The leaf relative content was calculated using the following formula: RWC = [(FW – DW)/ (TW – DW)] x 100. The experiment was carried out in triplicates.

2.2.4. Electrolyte leakage assay

Electrolyte leakage was assayed by (Bhusan et al. 2007) estimating the ions leaching from the leaf into Milli-Q water. Leaf tissue was placed in 20 ml of Milli-Q water in two sets. The first set was kept at room temperature for 4 h, and its conductivity (C1) was recorded using a conductivity meter. The second set was autoclaved and its conductivity was also recorded (C2). Electrolyte leakage (1- C1/C2) x 100 was calculated. The experiment was carried out in triplicates.

2.2.5. Estimation of proline

Free proline content was estimated following the method of Bates et al (1973). The leaf tissue (100 mg) was powdered with the help of liquid nitrogen and extracted in 3% aqueous sulphosalicylic acid. The homogenate was centrifuged at 10,000 rpm for 10 min. and the supernatant was collected in a fresh vial. 2 ml of the supernatant was reacted with 2 ml of acid ninhydrin reagent (1.25 g ninhydrin dissolved in 30 ml of acetic acid at boiling temperature to which 20 ml of ortho-phosphoric acid was added) and 2 ml of glacial acetic acid and boiled at 100 ºC for 1 h. After termination of reaction on ice, the reaction mixture was extracted with 4 ml of toluene. The chromophore containing aqueous phase was aspirated, warmed to room temperature (RT) and the absorbance was recorded

at 520 nm. Proline concentration was calculated from a standard curve using 0-100 µg L-proline (*Figure 2.1*). The assay was done in triplicates. Amount of proline was expressed as µmole proline g^{-1} dry wt.

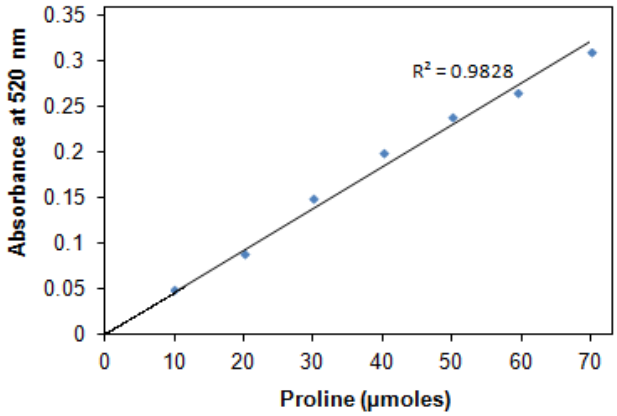

Figure 2.1. Standard curve for proline.

2.2.6. Protein estimation

One gram frozen tissue of rice (leaves, stems and roots) was homogenized in 5 ml of extraction buffer containing 50 mM Tris HCl (pH 8.0), 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15 % glycerol. The homogenate was filtered through four layers of muslin. The filtered homogenate was centrifuged at 10,000 rpm for 15 min at 4 ºC. Quantitative estimation of protein was carried out by following the method of Bradford (1976) using bovine serum albumin (BSA) as standard (*Figure 2.2*).

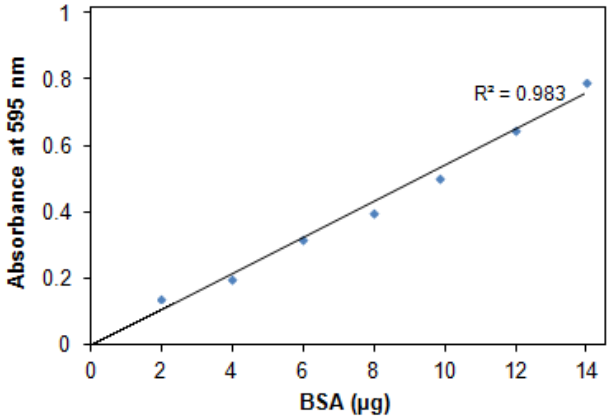

Figure 2.2. Standard curve for bovine serum albumin (BSA)

To 100 µl of protein sample, 3.0 ml of Bradford reagent [100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml 95% ethanol; to the solution100 ml 85% (w/v) phosphoric acid was added and diluted to 1 litre] was added. The reaction mixture was incubated at RT for 10 min. followed by determination of absorbance at 595 nm. Amount of protein was expressed as mg protein g^{-1} dry wt.

2.2.7. GS extraction and assay

Frozen rice tissue (leaf, stem or root) was homogenized in liquid nitrogen and suspended in GS Extraction Buffer (5 ml g^{-1} fresh wt.) containing 50 mM Tris HCl (pH 8.0), 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15% glycerol. After filtering through four layers of muslin the extract was centrifuged at 10,000 rpm for 15 min at 4 ^{o}C. The supernatant was desalted on pre-equilibrated Biogel P-2 column. GS activity in the supernatant was determined by either transferase or semisynthetase reaction (Washitani and Sato 1977). For semisynthetase reaction, 1 ml reaction mixture contained 25 µmol Tris HCl (pH 7.5), 200 µmol glutamate, 10 µmol ATP, 5 mM hydroxylamine hydrochloride, 20 µmol $MgCl_2$, and 100 µl enzyme preparation. For transferase activity, the reaction mixture in a final volume of 1 ml contained 100 µmol Tris HCl (pH 7.5), 100 µmol glutamine, 60 µmol hydroxylamine hydrochloride, 20µmol sodium hydrogen arsenate, 1 µmol $MnCl_2$, and 10 µl of the enzyme preparation.

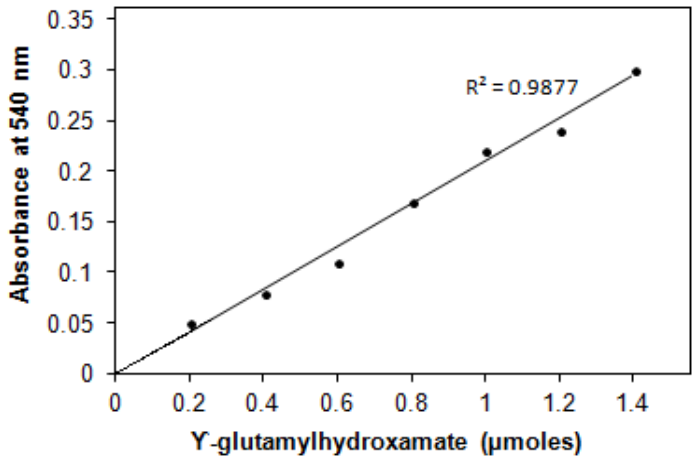

Figure 2.3. Standard curve for γ-glutamylhydroxamate.

The reaction was started by adding enzyme preparation and both the assays were carried out at 37 ^{o}C for 30 min. The reaction was terminated by adding 2 ml of $FeCl_3$ reagent (0.67 M $FeCl_3$, 0.37 M HCl and 20% (w/v) Tri-chloroacetic acid). After 20 min. the amount of γ-glutamylhydroxamate produced was determined spectrophotometrically by measuring the absorbance at 540 nm. γ-glutamylhydroxamate concentration was determined from a reference curve prepared with 0 - 2 µmole γ-glutamylhydroxamate (*Figure 2.3*). In blank, $FeCl_3$ reagent was added prior to the addition of enzyme preparation. One unit of GS activity represents 1.0 µmole of γ-glutamylhydroxamate produced 30 min^{-1}.

2.2.8. Separation of GS isoforms from leaf, stem and root of rice seedlings

GS isoforms in rice leaf, stem and root were separated by anion-exchange chromatography in a diethylaminoethyl (DEAE)-Sephacel column.

2.2.8.1. Preparation of DEAE-Sephacel

The pre-swollen DEAE-Sephacel slurry (Sigma-Aldrich, USA) was treated with 0.50 N HCl with slow stirring. The acid treated slurry was washed with distilled water till attaining pH 4. After discarding the supernatant, 0.50 N NaOH was added to the slurry followed by washing with distilled water till pH 6 to 7. Finally, the ion-exchange material was equilibrated with the GS Extraction Buffer, pH 8.0. About 10 ml of equilibrated slurry was packed into the column (10 x 2 cm) for separation of GS isoforms.

2.2.8.2. Separation of GS isoforms

All the steps of GS isoforms separation were performed at 4 ^{o}C. One gram of frozen rice tissue (leaf, stem or root) was homogenized in liquid nitrogen and suspended in 5 ml of GS extraction buffer. After filtering through four layers of muslin the extract was centrifuged at 10,000 rpm for 15 min at 4 ^{o}C. The homogenate was centrifuged at 10,000 rpm for 15 min. One ml of desalted supernatant was loaded onto a DEAE-Sephacel column (5x2 cm) pre-equilibrated with the GS extraction buffer. The column was washed with the same buffer until no protein was detectable in the eluate. Gradient elution of the adsorbed proteins was carried out by buffer containing, 0 - 0.50 M KCl. The flow rate was

maintained at 20 ml h^{-1}. Two ml fractions were collected and assayed for GS activity. The activities of chloroplastic and cytosolic isoforms were estimated from the area of the corresponding elution profile after fractionation. About 80-90 % of the total GS activity present in the crude extract was recovered after chromatographic separation.

2.2.9. RT- PCR analysis of GS genes

2.2.9.1. Isolation of total RNA from leaf, stem and root of rice seedlings

Total RNA was isolated from rice tissues by using TRIZOL reagent (Invitrogen, USA) following the manufacturer's instruction. 100 mg of plant tissue was homogenized in liquid nitrogen to fine powder and suspended in 1 ml of TRIZOL reagent. The homogenized sample was incubated at RT for 5 min to permit complete dissociation of the nucleoprotein complex. To the sample 0.20 ml of chloroform was added followed by vigorous shaking and incubation at RT for 2-3 min. The sample was centrifuged at 10,000 rpm for 15 min at 4 ºC. The aqueous phase was taken in a fresh tube and 0.50 ml of 100 % isopropanol was added. The tube was incubated at RT for 10 min followed by centrifugation at 10,000 rpm for 10 min at 4 ºC. The RNA pellet obtained after centrifugation was washed with 75% ethanol, air dried and dissolved in 25 µl of RNase free water.

2.2.9.2. Quantification of RNA

The purity and concentration of RNA were determined spectrophotometrically by measuring absorbance at 260 and 280 nm using a UV-spectrophotometer (Thermo, USA). The RNA concentration was calculated using the following formula:

$$\text{Concentration of RNA (µg /ml)} = A_{260} \text{ x dilution factor x } 40$$

The ratio of A_{260} and A_{280} determined the purity of the RNA preparation.

2.2.9.3. Agarose gel electrophoresis of RNA

1 % agarose in 1X TAE (Tris-acetate EDTA) buffer was melted in a microwave and then cooled to 50-60 ºC. It was then supplemented with 5µg ml^{-1} ethidium bromide. The melted agarose was then poured in a casting tray fitted with a teflon comb forming wells. RNA sample was mixed with RNA loading dye (1X) prior to loading in the wells. Electrophoresis was performed in a horizontal electrophoresis tank using 1X TAE buffer. RNA bands were visualized on a UV-transilluminator (Genei, India) (*Figure 2.4*).

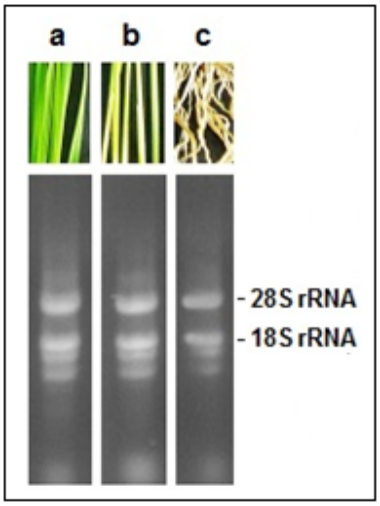

Figure 2.4. Agarose gel electrophoresis of total RNA isolated from (a) leaf, (b) stem and (c) root by using TRIZOL reagent

2.2.9.4. PCR Cloning of OsGS1;1, OsGS1;2 and OsGS2 ORF and sequence analysis

GS isoform genes were isolated by RT-PCR. The ImProm-II™ Reverse Transcription System (Promega, USA) was used for synthesis of first-strand cDNA in preparation for PCR amplification. The experimental RNA (1µg) and Oligo (dT) primer (0.50 µg) were combined in nuclease free water to a final volume of 5 µl. The tube was closed tightly and placed into a preheated 70 °C heat block for 5 min followed by immediate chilling in ice water for 5 min. To the tube other components of reverse transcriptase reaction were added. The reaction mixture in a final volume of 15 µl contained ImProm-II™ 5X Reaction Buffer, 4 µl; 25 mM $MgCl_2$, 1.20 µl; 10 mM dNTP mix, 1µl; Recombinant RNasin® Ribonuclease inhibitor, 20 units and 1µl ImProm-II™ reverse transcriptase. The reaction mix was incubated at 37 ^oC for 60 min. The reaction was terminated by incubation of reaction mix at 90 ^oC for 10 min. The cDNA was used as a template for PCR amplification in a 25 µl reaction mixture. Reaction contained selected couples of the following gene-specific primers: OsGS1;1-F (5′AGTATGGCT TCTCTCACCGATCTCGTC3’) and OsGS1;1-R (5′-GTACCTCGAGGGGCTTCCA GATGATGGTGGTC T-3′) for OsGS1;1; OsGS1;2-F (5′-GACTCATATGGCCAAC CTCACCGACCTCGTT-3′) and OsGS1;2-R (5′-TAGCGGCCGCGTTCTGCTTCCA CA GCAGCGTG-3′) for OsGS1;2 PCR was performed for 35 cycles. The PCR products were loaded and separated on 1% agarose TAE gel. The PCR products were then cloned

into pGEMT-Easy vector. For cloning the PCR product was extracted from the agarose gel by using Gel Extraction Kit (QIAGEN, Germany). The ligation reaction mixture in a total volume of 10 µl contained: 2X rapid ligation buffer, 5µl; pGEM-T-Easy vector, 50 ng (1µl); PCR product, 2µl; T4 DNA ligase (3weiss unit/µl), 1µl and 1µl H_2O. The reaction mixture incubated over night at 4 ºC was transformed into competent *E. coli* JM109 cells by heat shock at 42 ºC for 90 sec. The transformed cells were revived for 1.5 hours at 37 ºC in a shaking incubator. The transformants were selected in Ampicillin (50 µg ml^{-1}) agar plate supplemented with 20% IPTG and 2% (w/v) X-gal by incubating over night at 37 ºC. Plasmid was isolated from the recombinant colonies by Alkaline lysis method (Birnboirm and Dolly, 1979). The recombinant plasmids were analysed for the presence of DNA inserts by restriction digestion. The cloned PCR products were subjected to sequence analysis.

2.2.9.5. Quantative RT-PCR analysis of GS gene expression under WD

Semiquantitative RT-PCR was performed by using 5 µl of cDNA as template in 25 µl reaction mixture. Reaction contained selected couples of the following gene-specific primers: OsGS1;1-F (5′-AGTATGGCTTCTCTCACCGATCTCGTC-3') and OsGS1;1-R (5′-GTACCTCGAGGGGCTTCCAGATGATGGTGGTCT-3′) for OsGS1;1, OsGS1;2-F (5′-GACTCATATGGCCAACCTCACCGACCTCGTT-3′) and OsGS1;2-R (5′-TAGCGGCCGCGTTCTGCTTCCACAGCAGCGTG-3′) for OsGS1;2, OsGS2-F (5'-AGAACTTGGACGATGAATCGG-3') and OsGS2-R (5'-CATTTTATTTCGAG GGAAGG-3') for OsGS2 and OsActin-F (5'-GTCAGAATG GGATGATATGG-3') and OsActin-R (5'-TCTCCTTGCTCATCCTGTCAG-3') for actin. GS specific primers were designed according to the sequences of BAC clones represented in *Table 2.1*. PCR was performed for 27 to 29 cycles within a linear range of amplification of these genes. Expression of actin gene was used as a control to equalize cDNA quantity in different reactions. Seven microliters of the PCR products were loaded and separated on 1% agarose TAE gels. Gel was scanned using a gel documentation system (Spectronics, USA). The relative expression level of target genes under different experimental conditions were analysed using the software ImageAide version 3.06.04. The values were expressed relative to the standard. Results were repeated three times and representative one time gel pictures are shown.

Table 2.1. Detail of the BAC cloned genes of glutamine synthetase isoforms.

Gene Name	Product	BAC clone accession numbers	Chromosome location
OsGS1;1	GS1	AP004880 (P0487D09)	chr02
OsGS1;2	GS1	AC105364 (OJ1743A09)	chr03
OsGS2	GS2	AL662953(OSJNBa0011F23)	chr04

2.2.10. Immunoblot analysis of GS isoforms

2.2.10.1. Extraction of total soluble protein and SDS-PAGE

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out following the discontinuous method described by Laemmli (1970). Total soluble protein was resolved by 12.50 % SDS-PAGE (*Figure 2.5*). Resolving gel [30% acrylamide, 4 ml; 1.50 M Tris-Cl (pH8.8), 2.50 ml; 10 % SDS, 0.10 ml; 10% APS, 0.10 ml; TEMED 0.004 ml and 3.30 ml water] of 5 cm length was poured between two glass plates which were clamped together but held apart by plastic spacers and was allowed to set.

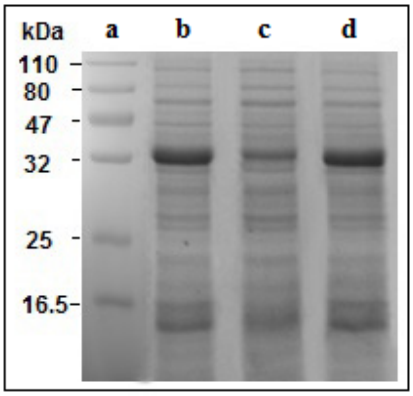

Figure 2.5. SDS-PAGE of total protein isolated from (a) protein marker, (b) leaf, (c) root, and (d) stem of rice seedlings.

The stacking gel (0.80 cm) [1M Tris HCl (pH 6.8), 0.38 ml; 10 % SDS, 0.03 ml; 10 % APS, 0.03 ml; TEMED, 0.003 ml; 2.10 ml water] was poured on the top of resolving gel and a plastic comb was placed on the stacking gel. After polymerization the comb was removed to provide loading wells. Glass plates with gel were placed in vertical electrophoresis system with running buffer tank containing running buffer [25 mM

TrisHCl (pH 8.0), 250 mM glycine, 0.10 % (w/v) SDS]. The protein sample was mixed with SDS gel loading buffer [50 mM TrisHCl (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.10 % bromophenol blue, 10 % glycerol] and heated in a boiling water bath for 10 min. Protein samples and prestained protein molecular weight marker were loaded in the wells and electric field was applied. When dye reached at the bottom of the tank, power was turned off. Gel was removed carefully from the glass plates and subjected to immunoblot analysis of GS isoforms.

2.2.10.2. Immunoblot analysis of GS isoforms

Proteins separated by 12.50 % SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane by semi-dry method using Electroblotting apparatus (Atto, Japan). The membrane was incubated with the anti-GS antibody (Agrisera, Sweden) raised against synthetic peptide from conserved region of GS1 and GS2. The reacted polypeptide was visualised with a secondary antibody, goat anti-rabbit IgG-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-p-toluidine (NBT/BCIP) detection kit (Invitrogen, USA). Broad range pre-stained standards were used as markers. Gel was scanned using a gel documentation system (Spectronics, USA). The relative expression level of target genes under different experimental conditions were analysed using the software ImageAide version 3.06.04. The values were expressed relative to the standard. Results were repeated three times and representative one time gel pictures are shown.

2.3. RESULTS

2.3.1. Screening of rice cultivars for tolerance to WD stress

Seven different varieties of rice (cv. IR-64, Khitish, Triguna, IR-8, Pokkali, PNR-519 and Satabadi) were screened for their drought tolerance characteristics. For this rice seedlings were grown for 3 weeks under controlled conditions and WD was imposed thereafter by withholding watering. Drought tolerance characteristic of rice cultivars was evaluated by monitoring changes in leaf relative water content (RWC), electrolyte leakage, proline and protein contents at indicated days of WD treatment.

2.3.1.1. Effect of WD on RWC in leaves of rice cultivars

The results of the effect of WD on RWC in leaves of different cultivars of rice are shown in *Figure 2.6*. The RWC measured at 3 h photoperiod was almost constant in well watered control plants.

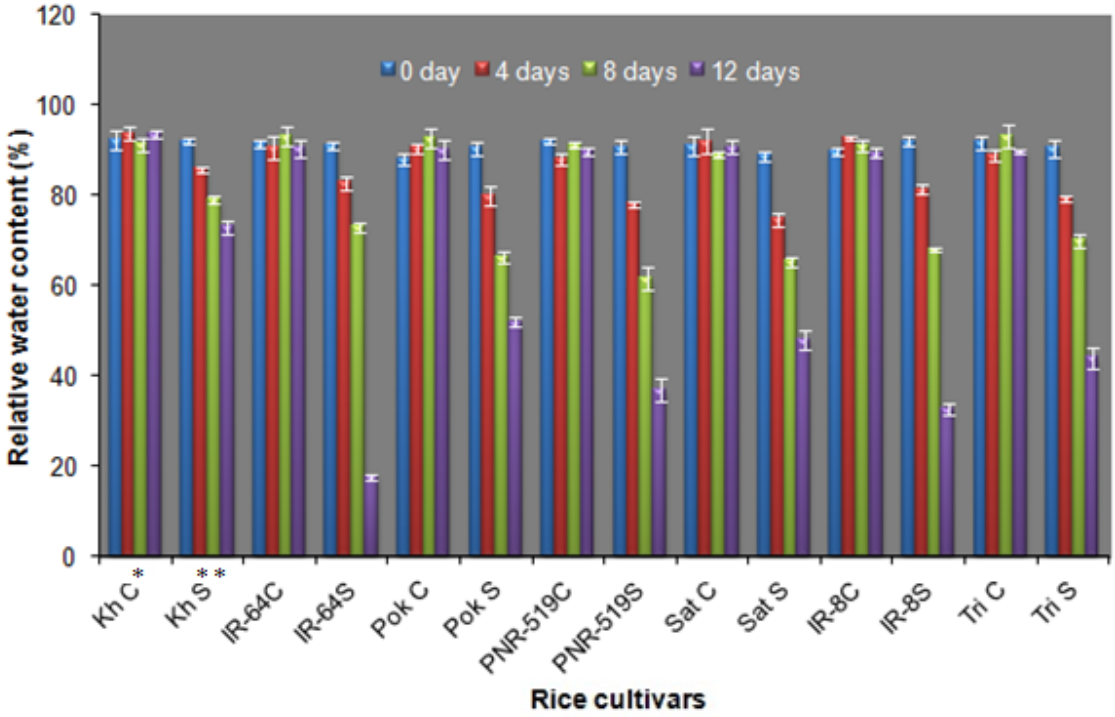

Figure 2.6. RWC in leaves of different rice (*O. sativa*) cultivars at 0, 4, 8 and 12 days of WD. All experiments were done in triplicates (n = 3), and average mean values of RWC were plotted against duration of WD. *C = Control, **S = WD treated

A differential effect of WD on rice cultivars was noted from day 8 of WD and became more significant on further treatment. Consequently, at 12 days of WD the decline was maximum in IR-64 (82 %); moderate in Pokkali (48 %), Satabadi (52 %), Triguna (56 %), PNR-519 (63 %), IR-8 (67 %); and least in Khitish (31 %).

2.3.1.2. Effect of WD on proline content in leaves of rice cultivars

The WD condition resulted in an increase in proline content in leaves of all the rice varieties. As can be seen from result in *Figure 2.7* that leaves of Khitish, Satabadi, Pokkali and Triguna cultivars had greater level of proline from the beginning of stress treatment. In these seedlings the accumulation of proline kept on increasing continuously throughout the duration of WD. However, in rice cultivars IR-8, PNR-519, and IR-64, proline content was maximum at day 12 of WD, followed by a severe decline on further dehydration.

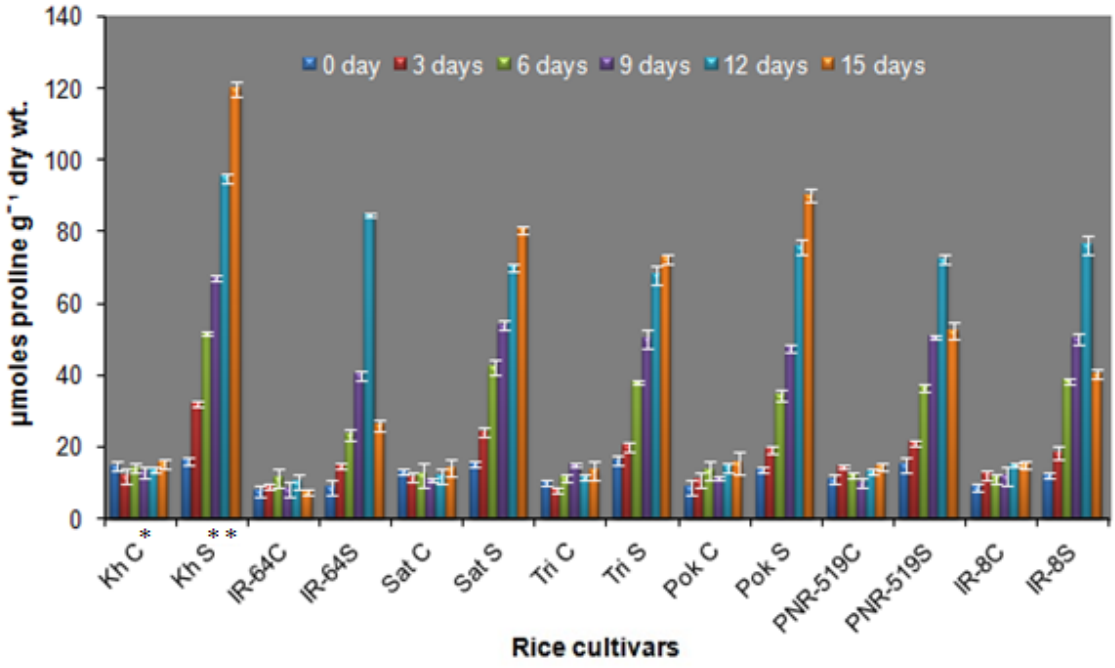

Figure 2.7. Proline content in leaves of different rice (*O. sativa*) cultivars at 0, 3, 6, 9, 12 and 15 days of WD. All experiments were done in triplicates (n = 3), and average mean values were plotted against duration of WD. *C = Control, **S = WD treated

At 15 days of WD, proline content increased to about 120, 90, 80 and 72 µmole g^{-1} dry wt. in Khitish, Pokkali, Satabadi and Triguna cultivars, respectively. Under similar condition

proline accumulation of about 52, 40 and 26 µmole g^{-1} dry wt. was noted in PNR-519, IR-8 and IR-64, respectively.

2.3.1.3. Effect of WD on protein content in leaves of rice cultivars

Influence of WD conditions on protein level in leaves of various rice cultivars is shown in *Figure 2.8.* As can be seen that total soluble protein increased throughout the experimental period in leaves of well watered control seedlings. WD treatment resulted in significant decline in protein level of all the varieties, however, the effect was variable. A comparison of protein content at day 12 of WD indicates that its decline was minimum in Khitish (15 %), moderate in Pokkali (33 %), Satabadi (35 %), Triguna (43 %), IR-8 (48 %), PNR-519 (48 %) and was highest in IR-64 (58 %).

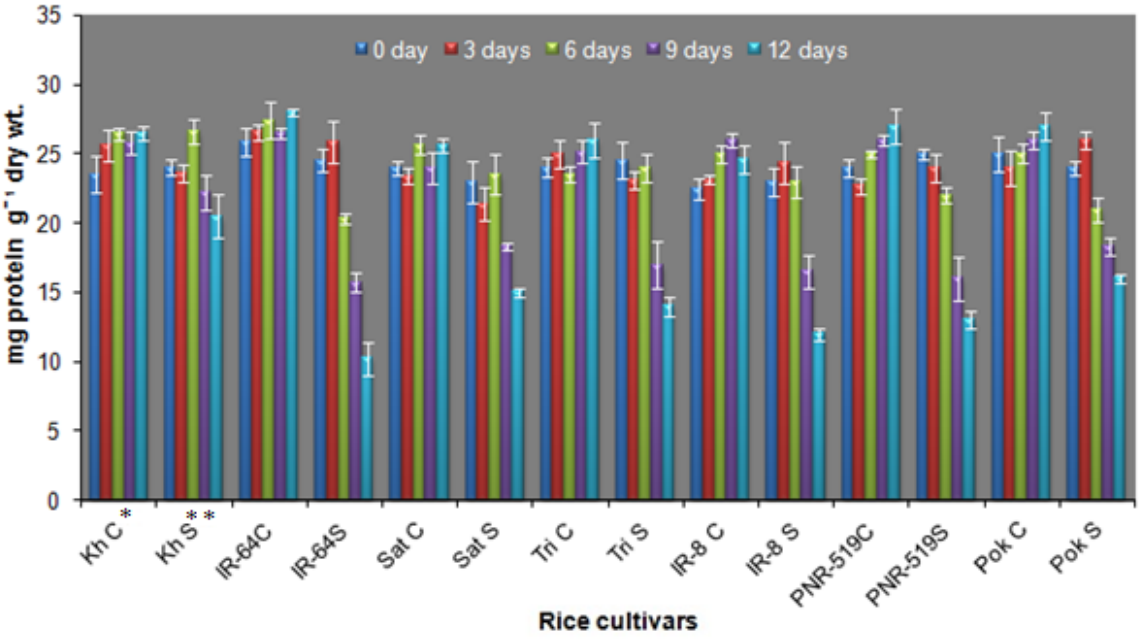

Figure 2.8. Protein content in leaves of different rice (*O. sativa*) cultivars at 0, 3, 6, 9, and 12 days of WD. All experiments were done in triplicates (n = 3), and average mean values were plotted against duration of WD. *C = Control, **S = WD treated

2.3.1.4. Effect of water-deficit on electrolyte leakage

The effect of WD on cell membrane integrity was evaluated by measuring electrolyte leakage from leaf. It is evident from the results in *Figure 2.9* that the electrolyte leakage was almost constant in well-watered control rice plants. Although, WD

treatment caused increase in electrolyte leakage from leaves, the response was variable in different rice cultivars. The effect of WD on leakage of electrolyte was more pronounced in IR-64, PNR-8 and IR-8 varieties. At 12 days of WD electrolyte leakage was enhanced by 3.6 and 2.6 folds in IR-64 and IR-8 cultivars, respectively. Electrolyte leakage was least in case of Khitish cultivar. Results of above studies indicated that *Oryza sativa* cv. IR-64 was the least tolerant, whereas *Oryza sativa* cv. Khitish was most tolerant to WD. Hence, IR-64 and Khitish cultivars were designated as drought-sensitive and drought-tolerant rice cultivar, respectively, and were selected for further studies.

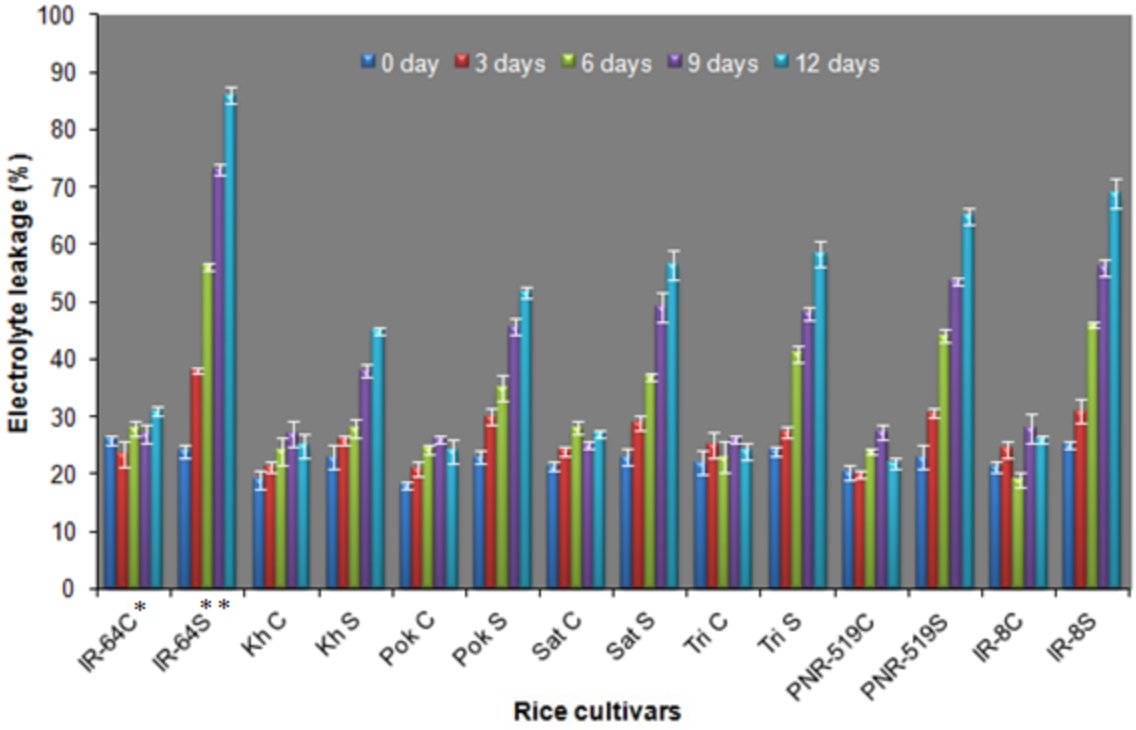

Figure 2.9. Electrolyte leakage from leaves of different rice (*O. sativa*) cultivars during 0, 3, 6, 9 and 12 days of WD. All experiments were done in triplicates (n = 3), and average mean values were plotted against duration of WD. *C = Control, **S = WD treated.

Ready comparisons of the effect of WD on morphological and biochemical changes in seedlings of these two varieties are shown in *Figure 2.10*, *2.11* and *2.12*. IR-64 and Khitish seedlings were grown under similar conditions with same level of irrigation for three weeks and then subjected to WD for 12 days. WD treatment caused rapid decrease in the water content of IR-64 as compared to Khitish. The susceptibility of IR-64 to WD is

clearly visible in *Figure 10a*. At 12 days of WD, morphology of IR-64 seedlings altered significantly with drying of shoot and rolling of leaves, however, such features were not observable in Khitish seedlings throughout the experimental duration.

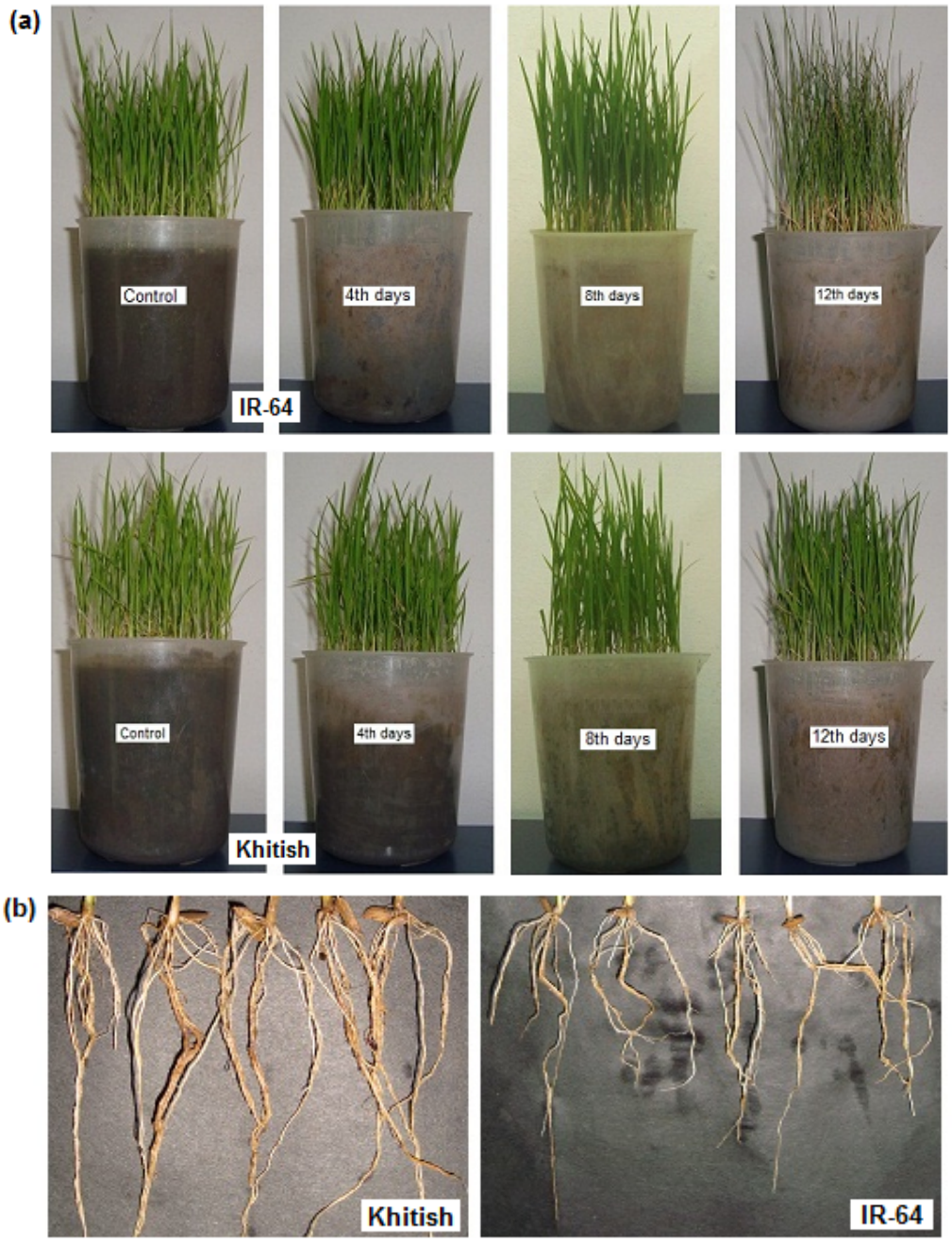

Figure 2.10. Effect of WD on IR-64 and Khitish cultivars of rice. Comparison of morphological characteristics of (a) shoot and (b) root of IR-64 and Khitish seedlings at 0, 4, 8 and 12 days of WD.

Drought tolerant and sensitive nature of Khitish and IR-64 are also evident from their root architectures. The root system of Khitish seedlings were more branched, longer and thicker as compared to that of IR-64 (*Figure 2.10 b*). The dry weight of leaves and roots of these seedlings were measured various days of WD treatment. The dry weights increased more significantly in IR-64 in comparison to Khitish. For example WD treatment for 12 days raised the dry wt. g^{-1} of leaves from 112 mg to 294 mg in IR-64 and from 115 mg to 151 mg in Khitish cultivar (*Figure 2.11a*). Under same condition, the dry wt. g^{-1} of root enhanced from 140 mg to 190 mg in IR-64, whereas it increased only marginally in Khitish cultivar (*Figure 2.11 b*). As a result RWC in leaf declined at a faster rate in IR-64 as compared to Khitish.

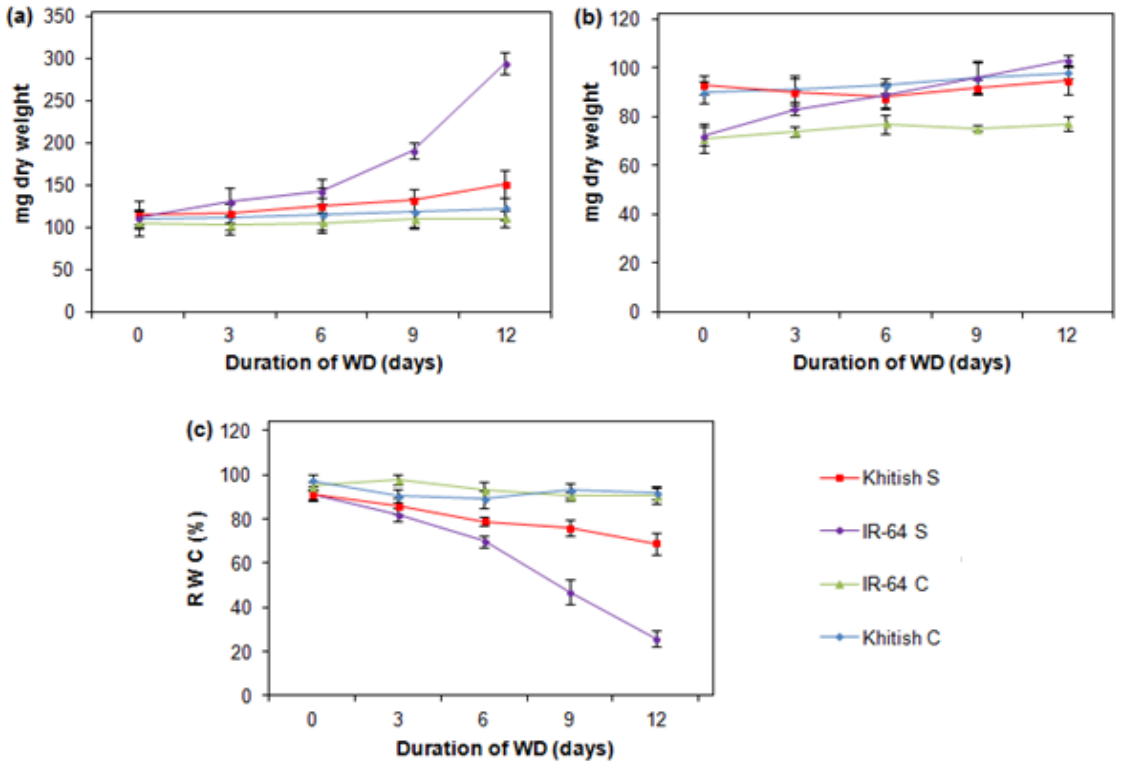

Figure 2.11. Effect of WD on dry weight and relative water content. Comparative analysis of dry weights of (a) leaf, and (b) root and (c) RWC of leaf between IR-64 and Khitish varieties of *O. sativa* in a time-dependent manner under WD conditions. Three week old seedlings were subjected to water-deficit and dry weight and RWC were determined at 0, 3, 6, 9 and 12 days of treatment. For determination of dry weight 0.50 g of fresh tissue was dried at 80 ºC for 48 h. All experiments were done in triplicates (n = 3), and average mean values were plotted against duration of WD.

WD treatment for 12 days reduced leaf RWC to 26 and 69 % in IR-64 and Khitish varieties, respectively (*Figure 2.11 c*). The protein contents in leaves and roots declined more rapidly in IR-64 seedlings. At the beginning of WD treatment protein content in leaves of both the cultivars was about 25 mg g^{-1} dry wt., which declined to 15 and 22 mg in IR-64 and Khitish, respectively, at day 9 of WD. At the end of WD treatment the soluble protein content in leaf and root was decreased by 58 % and 40 % in IR-64 and by 17 and 10 % in Khitish, respectively (*Figure 2.12 a, b*).

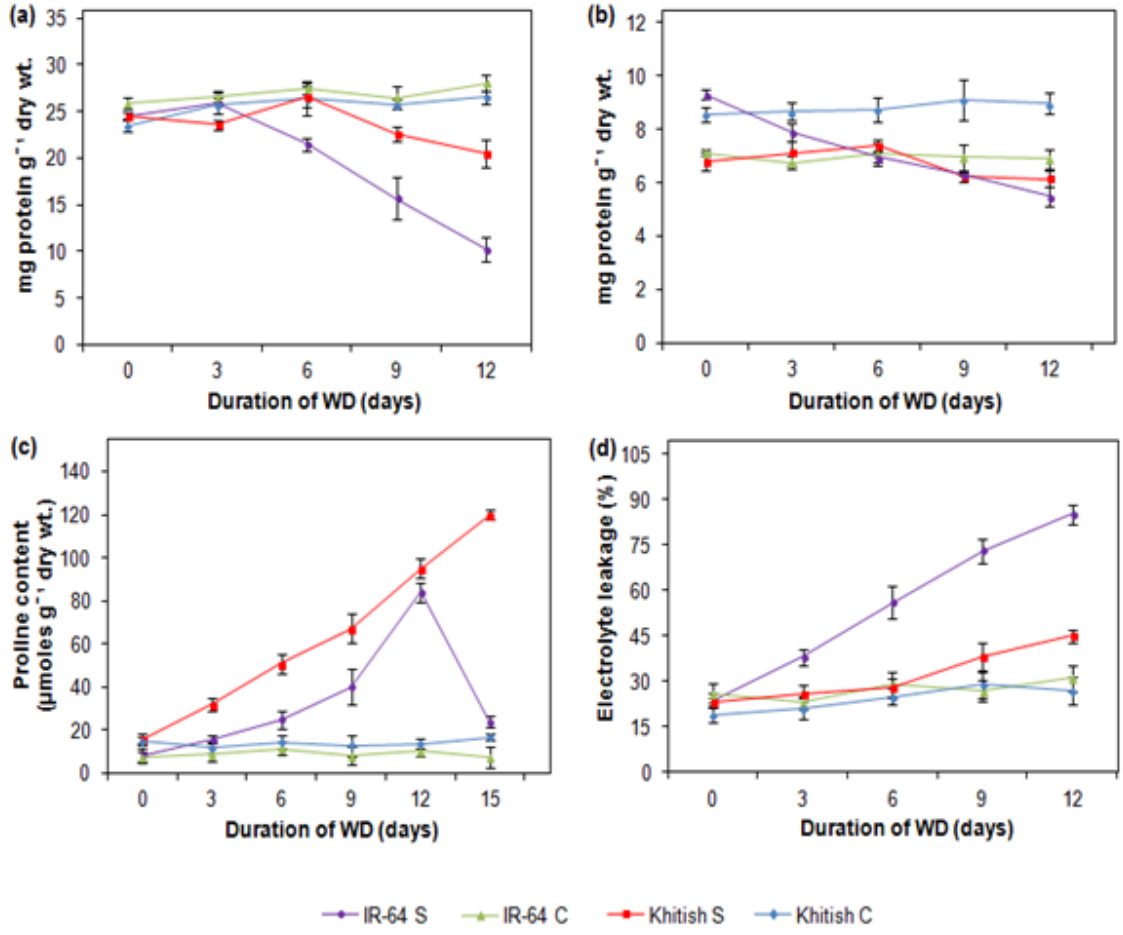

Figure 2.12. Effect of WD on protein content, proline content and electrolyte leakage. Comparative analysis of protein contents: (a) leaf, and (b) root; (c) proline content and (d) electrolyte leakage of leaf between IR-64 and Khitish varieties of *O. sativa* in a time-dependent manner under WD conditions. Three week old seedlings were subjected to water-deficit. All experiments were done in triplicates (*n*=3), and average mean values were plotted against duration of WD.

Although, the level of proline was increased during WD in leaves of both the varieties, its accumulation was greater in Khitish from the beginning of stress treatment (*Figure 2.12*

c). In IR-64 seedlings, proline content was maximum at day 12 of WD, followed by its severe decline on further dehydration. On the other hand, proline accumulation kept on increasing with WD in Khitish leaf. The electrolyte leakage was maintained at an almost constant level in Khitish until day 6 and increased marginally during later stages of WD. In contrast, a sharp rise in electrolyte leakage was noted in IR-64 cultivar (*Figure 2.12 d*).

2.3.2. Optimization of GS extraction and assay conditions

GS has been studied in many higher plants. However, the optimal conditions for its extraction vary with tissue as well as the plant species (Lea et al. 1990). Hence, the optimal conditions for extraction of the enzyme with respect to pH of the buffer and concentration of protective/stabilizing agents were established in order to ensure maximal extraction and recovery of the enzyme. Maximum recovery of the enzyme was obtained when 50 mM Tris HCl (pH 8.0) supplemented with 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15% glycerol was used as extraction buffer. The optimum pH of assay media for GS activity of was 7.5. The rate of semisynthetase activity rose proportionately upto 300 µl of enzyme extract. The amount of γ-glutamylhydroxamate produced increased proportionately with the reaction period upto 30 min when 300 µl of enzyme extract was used for assay. Hence in all the subsequent experiments GS activity was routinely determined over a period of 30 min with 300 µl of enzyme preparation.

2.3.3. GS isoforms in leaf, stem and root of rice varieties

GS was extracted from root, stem and leaf of three weeks old rice seedlings raised in 3:1mixture of soil and soilrite, under 250 µmol m^{-2} s^{-1} photon flux density (16h/8h day/night regime) at 27 ± 2 ^{o}C and 70-80 % relative humidity in a plant growth chamber.

The activity of GS in crude extract was determined by semisynthetase assay based on rate of formation of γ-glutamylhydroxamate. Anion-exchange chromatography was used to separate the isoforms of GS in leaf, stem and root extracts. As can be seen from result in *Figure 2.13a*, on subjecting the leaf extract to chromatography in DEAE-Sephacel column, the enzyme was resolved in two distinct peaks which were recovered with gradient elution buffer containing 0 - 0.50 M KCl. These two enzyme forms were

designated as GS1 and GS2, respectively. In case of the stem extract (*Figure 2.13 b*) also the enzyme activity was resolved into two peaks at the same salt concentration as GS1 and GS2 in the leaf extract. However, ion-exchange chromatography of root extract eluted almost the entire activity as a single peak at the same salt concentration as GS1 in leaf and stem extracts and no peak corresponding to GS2 was detectable (*Figure 2.13 c*). From the results in *Table-2.2* it is apparent that rice leaves contained 25 and 75 % of activity as GS1 and GS2, respectively. In stem most of the GS activity (70 %) was present as GS1 and rest 30 % activity represented as GS2.

2.3.4.Detection and quantization of GS isoforms mRNA and protein

2.3.4.1. Standardization of RT-PCR amplification of GS isoforms

Before performing the experiments on quantitative GS gene expression analysis, the reaction conditions for RT-PCR of full length GS isogenes were established. Total RNA was isolated from leaf, stem and root tissues of three week old rice seedlings. 1 µg of total RNA was used for synthesis of first strand cDNA by reverse transcriptase (RT) followed by PCR using gene specific forward and reverse primers. The number of cycles and annealing temperature were optimized for each gene specific primer pairs. To ascertain the PCR products as OsGS1;1, OsGS1;2 and OsGS2, the amplified PCR products (*Figure 2.14*) were cloned in pGEMT vector and sequenced. The sequence showed homology with OsGS sequences in the database.

2.3.4.2. Quantification of GS isoforms transcripts in rice seedlings

The transcript levels of GS isoform in various organs of rice plant were determined by RT-PCR. The PCR amplified full length OsGS1;1, OsGS1;2 and OsGS2 ORFs were resolved by agarose gel electrophoresis and quantified by ImageAide version 3.06.04, to calculate the level of gene expression. From the results in *Figure 2.15* it is apparent that among GS1 gene family, OsGS1;1 and OsGS1;2 transcripts were present in all the three organs, whereas OsGS2 was expressed in leaf and stem but not in root. In leaf tissue the OsGS2 mRNA level was considerably higher than that of cytosolic isoforms and its accumulation was found to be about 4.20 folds greater than OsGS1;1. OsGS2 was present as minor form in stem.

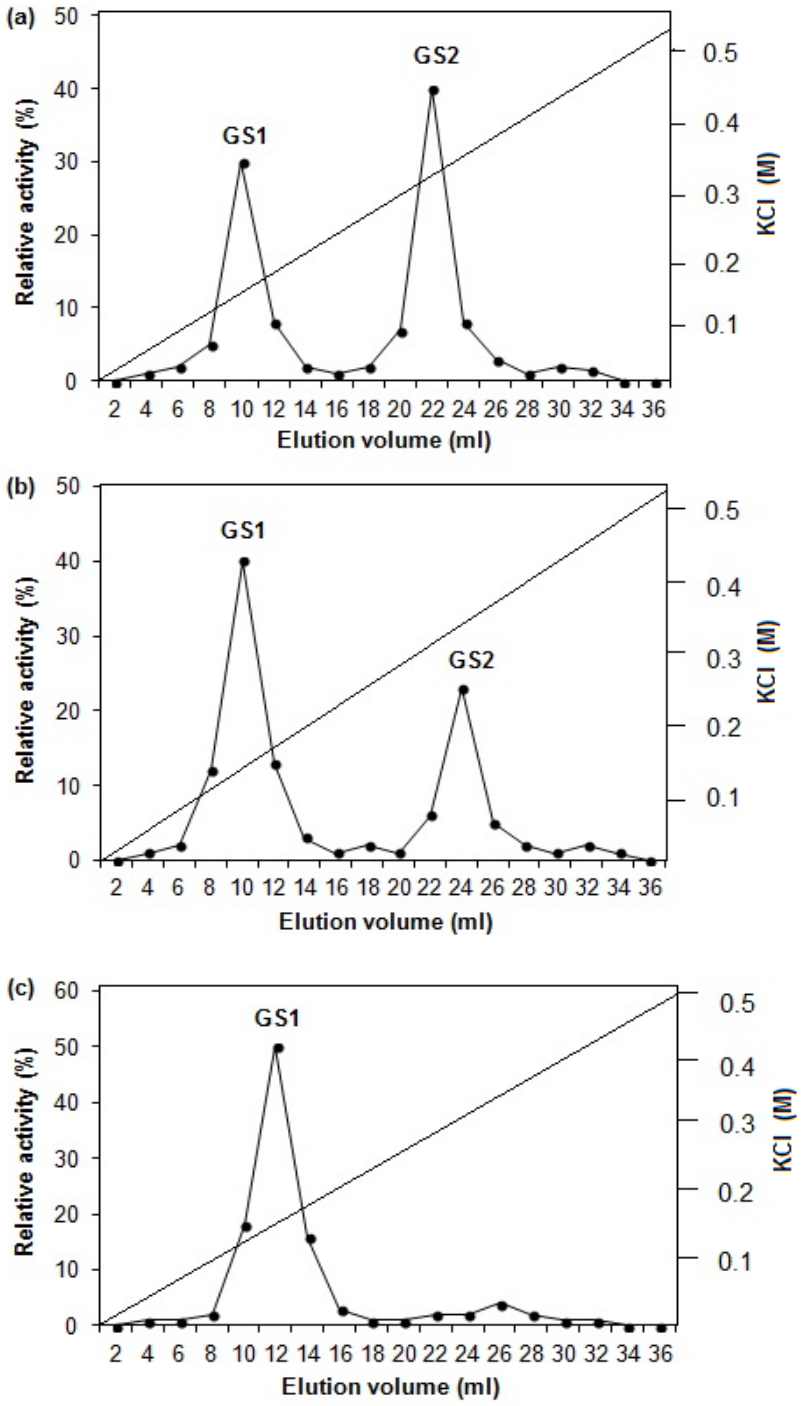

Figure 2.13. Elution profile during anion-exchange chromatography of GS isoforms (a) leaf, (b) stem and (c) root tissues of 3 weeks old rice seedlings. The enzyme activity in different fractions was assayed by the semisynthetase assay. One unit of GS activity represents 1.0 μmole of γ-glutamylhydroxamate produced 30 min^{-1}.

Table 2.2. Relative proportion of GS1 and GS2 in leaf, stem and root of rice seedlings.

Tissue	GS activity *				
	Total GS	GS1	GS2	Ratio GS2/GS1	Recovery
Leaf	31.25	6.10	22.70	3.72	92.16
Stem	13.20	8.21	3.80	0.46	90.90
Root	20.00	18.5	N.D.		92.50

N.D.: Not detectable

GS1 and GS2 were isolated by anion-exchange chromatography (Section 2.2.8.2) from leaf, stem and root tissues of 3 weeks old rice (*Oryza sativa* cv. IR-64) seedlings raised in plant growth chamber. The enzyme activity was assayed by the semisynthetase assay. *One unit of GS activity represents 1.0 µmole of γ-glutamylhydroxamate produced 30 min^{-1}.

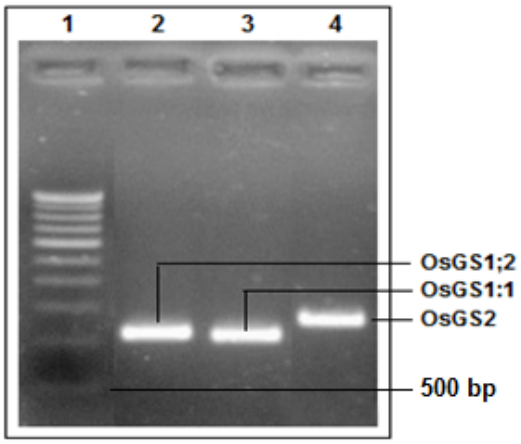

Figure 2.14. RT-PCR amplification of GS isoforms and agarose gel electrophoresis analysis of PCR products. Lane1- 500bp ladder, Lane2- OsGS1;2 (1171 bp), Lane3- OsGS1;1 (1039 bp) and Lane 4: OsGS2 (1255bp)

Among the GS1 isoforms OsGS1;1 was more abundant than OsGS1;2 in leaf. OsGS1;2 was the predominant form in stem and root of both IR-64 and Khitish cultivars. However, OsGS1;1 mRNA level was significantly greater in root and stem of IR-64 seedlings than that of Khitish. OsGS1;3 gene expression was not studied, as previous reports indicate its absence in vegetative stage of growth of rice seedlings (Ishiyama et al.2004b; Tabuchi et al. 2007).

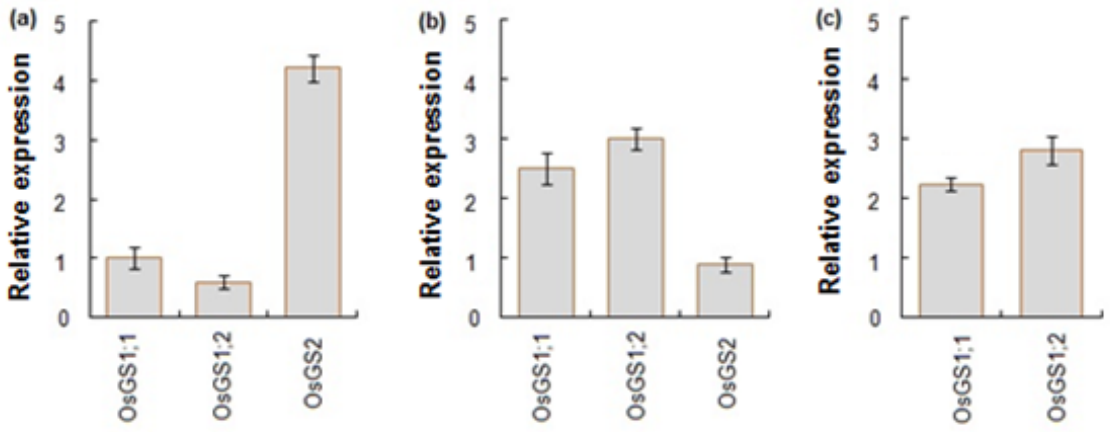

Figure 2.15. Quantitative analysis of OsGS1;1, OsGS1;2 and OsGS2 mRNA in (a) leaf, (b) stem and (c) root of rice (*Oryza sativa* cv. IR-64) seedlings.

2.3.4.3. Quantification of GS isoforms polypeptides in rice seedlings

The polypeptide levels of GS1 and GS2 isoforms in leaves, stems and roots of rice seedlings were determined by immunoblotting. Total soluble proteins (10 µg) of leaf, stem and root tissues were resolved by 12.50 % SDS-PAGE and transferred to PVDF membrane followed by probing the membrane with the anti-GS antibody raised against synthetic peptide from conserved region of GS1 and GS2. The reacted polypeptides were visualised with secondary antibody-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-p-toluidine (NBT/BCIP).

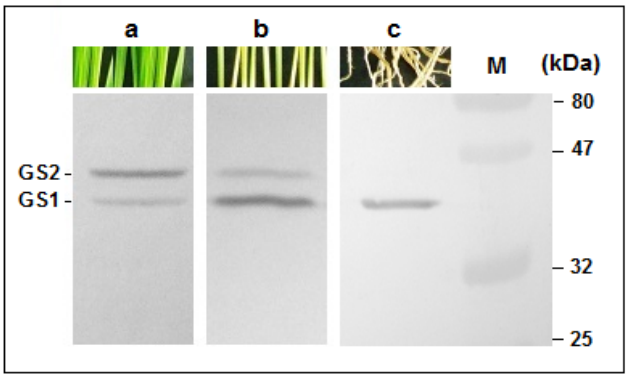

Figure 2.16. GS polypeptide in (a) leaf, (b) stem and (c) root of rice (*O. sativa* cv. IR-64) seedlings under normal conditions. GS polypeptides were detected by Immunoblotting using the anti-GS antibody against synthetic peptide from conserved region of GS1 and GS2. Immunoblot analysis was carried out with 10 µg of total soluble protein.

The result in *Figure 2.16* indicates the presence of two protein bands of approximately 39 and 43 kDa in leaf and stem that correspond to the molecular size of GS1 and GS2, respectively. The immunoblot analysis of root protein highlighted only one protein band corresponding to GS isoform. As the GS antibody was developed against conserved GS polypeptide, cytosolic isoforms could not be distinguished in immunoblot.

2.3.5. Effect of WD on activity and expression of GS isoforms in leaf, stem and root of IR-64 and Khitish seedlings

Rice seedlings of IR-64 and Khitish cultivars grown for 3 weeks under controlled conditions were subjected to WD by withholding watering and activity of GS1 and GS2 and the corresponding mRNA and polypeptide contents were simultaneously determined in leaf, stem and root tissue at 0, 4, 8 and 12 days of the treatment.

2.3.5.1. Effect of WD on total GS, GS1 and GS2 activities in leaf, stem and root of IR-64 and Khitish seedlings

Results of the influence of WD on total GS, GS1 and GS2 activities in leaves, stem and roots of IR-64 and Khitish cultivars are shown in Table 2.3. At the beginning of WD treatment the total GS activity was almost similar in both the cultivars. WD treatment caused rapid decline of GS activity in leaves and roots of IR-64 seedlings as compare to that of Khitish. In IR-64 leaf total GS activity decreased by 28 and 52 % at 8 and 12 days of WD, whereas in Khitish leaf a significant decrease of only 10 % was noted at day 12 of treatment. The rapid reduction in GS activity of IR-64 leaf was mainly due to disappearance of GS2 activity. For example, WD condition for 12 days reduced the GS2 activity from 23 to 11 in IR-64 and from 21 to 19 in Khitish cultivar. The differential rate of decline in GS2 activity in the two varieties decreased the ratio of GS2 to GS1 from 4.18 to 2.28 in IR-64 and from 4.00 to 3.75 in Khitish.

Total GS activity in stem of IR-64 and Khitish seedlings was almost unaffected by WD. However, alteration in both GS1 and GS2 activities was noted in stem of IR-64 seedling. In this seedling GS1 activity was reduced by 16, 36 and 41% on 4, 8 and 12 days of WD. On the other hand, the activity of GS2 was enhanced by 40 % at 12 days of stress

application. Thus the decline in GS1 activity was compensated by increase in GS2 activity, thus maintaining almost unchanged total GS activity. Alterations in GS1 and GS2 activities raised the ratio of GS2 to GS1 from 0.46 to 1.20 in IR-64 stem. Such change in ratio was not noticeable in Khitish stem as both GS1 and GS2 activities were almost constant throughout the WD treatment. The cytosolic GS1 was the only GS isoform in rice root. WD treatment had not much effect on GS activity in Khitish root. In contrast, the GS activity in roots of IR-64 cultivar was quite sensitive to WD. A rapid reduction in activity of about 50 and 60 % was noted at 8 and 12 days of WD treatment, respectively.

Table 2.3. Effect of WD on total GS, GS1 and GS2 activities in leaves, stem and roots of *O. sativa* cv. IR-64 and Khitish.

Rice Varieties	Tissues	Days of WD	GS activity*			Ratio
			Total GS	GS1	GS2	GS2:GS1
Khitish	Leaf	0	28.50 ± 1.51	5.28 ± 0.10	21.12 ± 2.42	4.00
		4	30.00 ± 2.12	5.45 ± 0.25	21.52 ± 1.84	3.95
		8	27.20 ± 2.40	5.25 ± 0.15	19.68 ± 2.80	3.75
		12	25.80 ± 3.21	5.11 ± 0.30	19.16 ± 3.41	3.75
	Stem	0	12.10 ± 1.10	7.89 ± 0.25	4.10 ± 0.02	0.52
		4	10.92 ± 1.25	6.77 ± 0.31	3.84 ± 0.03	0.56
		8	11.50 ± 2.50	7.10 ± 0.15	3.82 ± 0.10	0.53
		12	12.20 ± 1.80	7.00 ± 0.10	4.05 ± 0.02	0.57
	Root	0	17.00 ± 2.10	Total activity was present as GS1		
		4	16.70 ± 2.41			
		8	16.68 ± 2.15			
		12	15.69 ± 2.80			
IR-64	Leaf	0	32.06 ± 3.21	5.45 ± 0.02	22.78 ± 3.10	4.18
		4	34.00 ± 3.80	5.86 ± 0.14	22.45 ± 2.50	4.00
		8	23.00 ± 4.25	5.86 ± 0.14	15.88 ± 3.12	3.00
		12	17.22 ± 5.50	5.86 ± 0.14	11.69 ± 3.12	2.25
	Stem	0	12.40 ± 2.10	8.21 ± 2.11	3.80 ± 1.45	0.46
		4	11.95 ± 3.20	6.95 ± 1.61	4.32 ± 3.12	0.62
		8	11.38 ± 1.26	5.42 ± 2.23	5.35 ± 2.54	0.98
		12	11.60 ± 3.24	4.95 ± 3.41	6.00 ± 3.61	1.21
	Root	0	20.00 ± 3.22	Total activity was present as GS1		
		4	16.00 ± 2.65			
		8	10.00 ± 2.65			
		12	08.40 ± 1.82			

*Total GS activity was resolved into GS1 and GS2 by anion-exchange chromatography. Enzyme activity in different fractions was assayed by semisynthetase reaction. One unit of GS activity represents 1.0 μmole of γ-glutamylhydroxamate produced 30 min^{-1}.

2.3.5.2. Effect of WD on expression of GS1 and GS2 mRNA in leaf, stem and root of IR-64 and Khitish seedlings

Total RNA was isolated from various tissues of rice seedlings at indicated days of WD and mRNA levels of OsGS1;1, OsGS1;2 and OsGS2 were determined by semi quantitative RT-PCR .

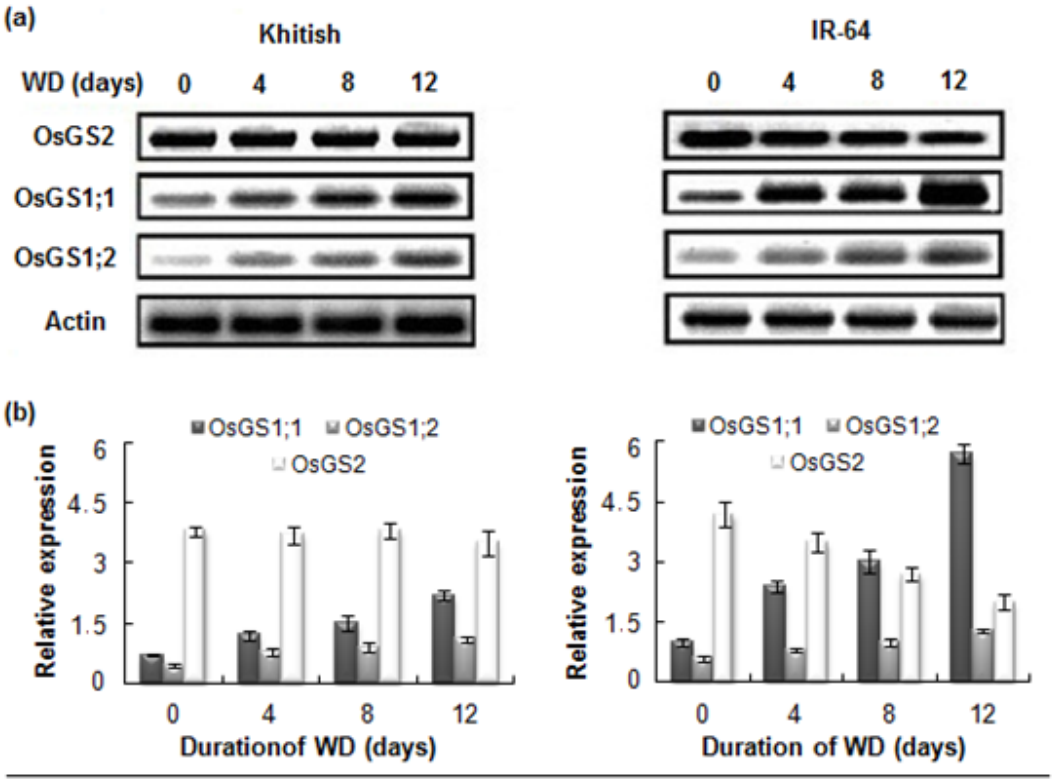

Figure 2.17. Effect of WD on expression of GS mRNA in leaves of *O. sativa* cv. IR-64 and Khitish. (a) Analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR. (b) Bar diagram of GS mRNA level

The results of WD mediated alteration in GS transcripts expression in leaf tissue are shown in *Figure 2.17*. Though, OsGS1;1 and OsGS1;2 mRNA levels increased in both IR-64 and Khitish cultivars in response to WD, expression level of OsGS1;1 mRNA was considerably higher in IR-64 leaf. OsGS1;1 mRNA level was increased by about 2.5 and 5 folds in IR-64 leaf and by 2 and 2.5 folds in Khitish leaf at 8 and 12 days of WD, respectively. On the other hand, OsGS1;2 mRNA content enhanced almost equally in both the varieties. It is also noticeable that OsGS2 mRNA level was significantly affected by WD in IR-64 cultivar. Its level declined by about 40 % at 8 days of WD. A further

reduction of upto 50 % was observed at 12 days of treatment. Under similar conditions no such change in OsGS2 expression was noticed in Khitish leaf.

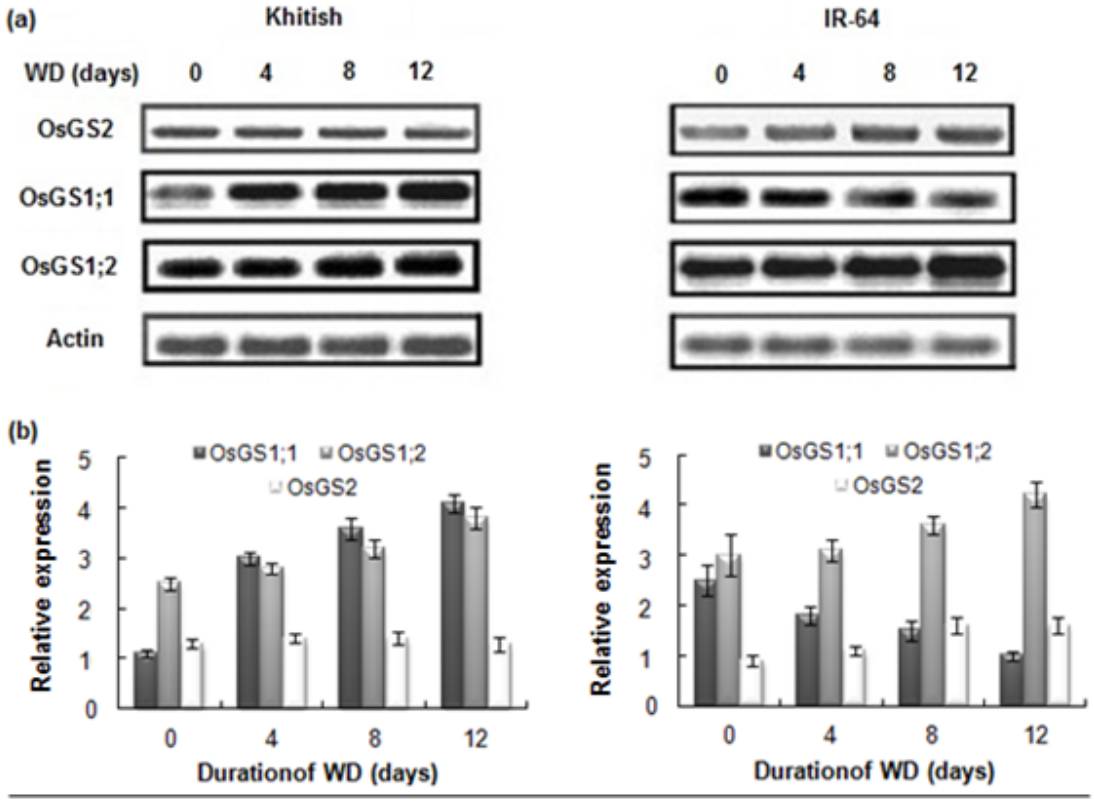

Figure 2.18. Effect of WD on expression of GS mRNA in stem of *O. sativa* cv. IR-64 and Khitish. (a) Analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR. (b) Bar diagram of GS mRNA level

The influence of WD on expression of GS mRNAs in stem of was significantly different from that of leaf. The results in *Figure 2.18* indicate a varietal variation in expression of OsGS1;1 in stem of IR-64 and Khitish seedling in response of WD. In stem, OsGS1;1 transcript content declined with the intensification of stress. Its level decreased to about 50 % at 8 days of WD followed by a further decline of upto 60% at 12 days of treatment. In contrast, OsGS1;1 mRNA was found to increase in Khitish stem with about 3 and 4 folds enhancement at 4 and 12 days of WD, respectively. The OsGS1;2 transcript responded almost equally in both the varieties, with almost 2 fold increase in its abundance at 12 days of stress treatment.

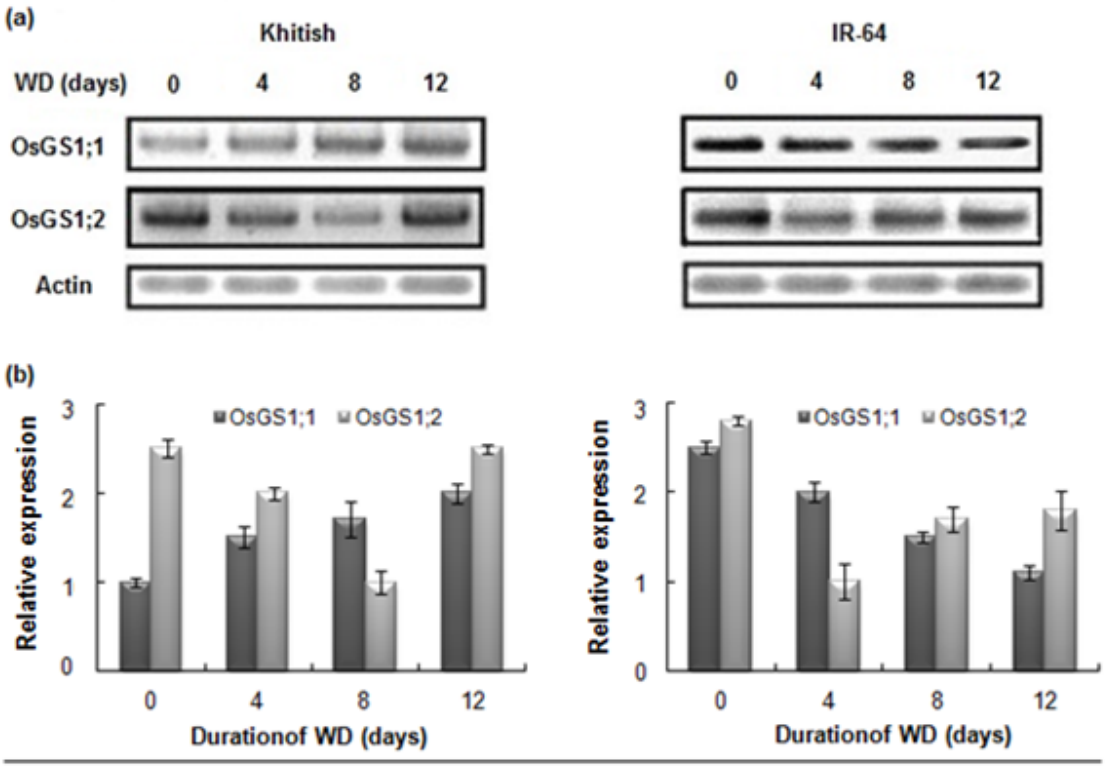

Figure 2.19. Effect of WD on expression of GS mRNA in root of *O. sativa* cv. IR-64 and Khitish (a) Analysis of OsGS1;1 and OsGS1;2 transcripts by RT-PCR. (b) Bar diagram of GS mRNA level

The chloroplastic GS2 mRNA was found to express in stem, but its mRNA level was significantly lower than that of cytosolic GS isoforms. A differential response of WD on GS2 mRNA expression was also noted. WD treatment for 12 days resulted in enhancement of OsGS2 transcript content by about 2 fold in IR-64 stem. However, such alteration in OsGS2 mRNA quantity was undetectable in Khitish stem, which maintained an almost constant level of the transcript throughout the treatment period.

Among GS transcripts only GS1 isoforms were expressed in root. As can be seen from the results in *Figure 2.19*, the expression of OsGS1;1 mRNA differed in root of the two cultivars. Its level was initially higher in IR-64 that declined with WD. At 8 days of WD the OsGS1;1 mRNA content was reduced to less than half in IR-64 root and increased to almost twice in Khitish root. Although, WD treatment resulted in initial decline of OsGS1;2 transcript content in roots of both the cultivar, the rate of decline was faster in IR-64, in comparison to Khitish. As a result, OsGS1;2 level fell to a minimum at day 4

and day 8 of WD in IR-64 and Khitish, respectively and then increased on further treatment.

2.3.5.3. Effect of WD on expression of GS1 and GS2 polypeptide in leaf, stem and root of IR-64 and Khitish seedlings

To ascertain whether the WD induced alterations in GS mRNA expression was reflected in corresponding polypeptides, immunoblot analysis of GS isoforms was carried out. The result of the effect of WD on expression of GS polypeptides in leaf tissue is shown in *Figure 2.20*. As can be seen that GS1 isoforms i.e. OsGS1;1 and OsGS1;2, couldn't be distinguished in immunoblot and is represented as single band. This is because of the development of GS antibody against conserved region of the GS polypeptide. As shown in *Figure 2.20a,* GS1 polypeptide accumulated in leaf of both IR-64 and Khitish seedlings with progress of WD.

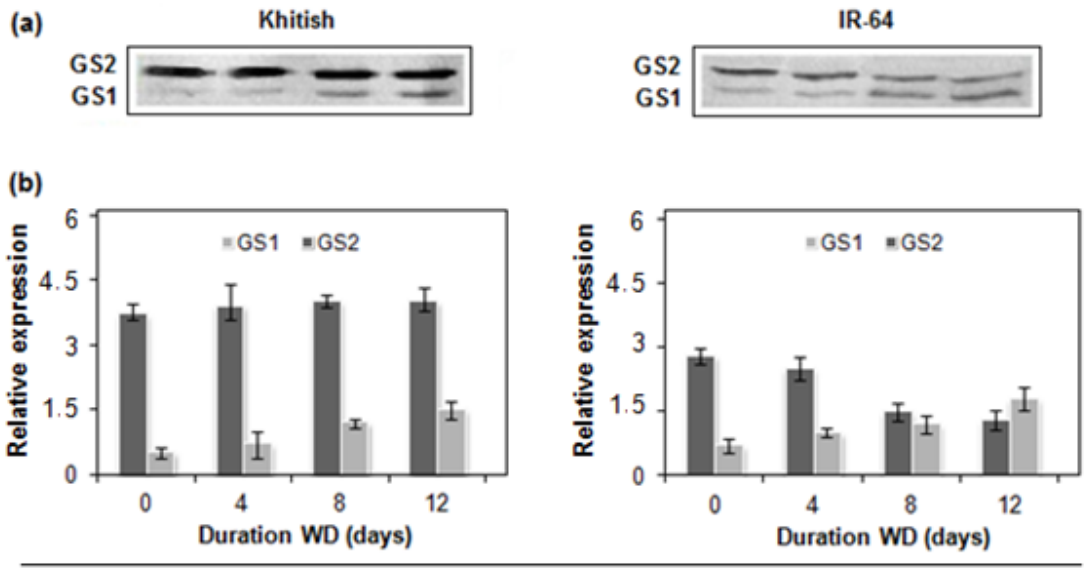

Figure 2.20. Effect of WD on expression of GS polypeptides in leaf of *O. sativa* cv. IR-64 and Khitish (a) Immunoblot analysis of GS1 and GS2 polypeptides (b) Bar diagram of GS polypeptide levels.

Its level increased by more than 2 fold by the end of the treatment. The GS2 polypeptide content of IR-64 leaf was reduced by about 50% at day 12 of WD, however, such change was not noticeable in Khitish leaf (*Figure 2.20b*).

The results in *Figure 2.21* show the effect of WD on expression of GS polypeptides in the stem of rice seedlings. WD significantly affected GS2 polypeptide expression in stem of IR-64 cultivar than that of Khitish cultivar. At 12 days of WD, GS2 polypeptide level rose by about 2 fold in IR-64 stem but remained almost unaltered in Khitish stem.

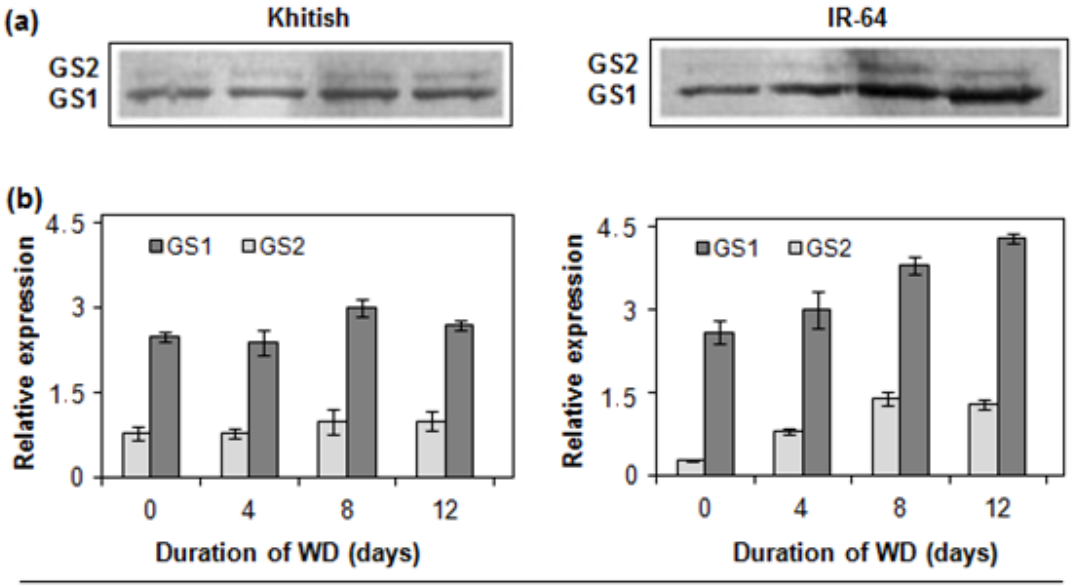

Figure 2.21. Effect of WD on expression of GS polypeptides in stem of *O. sativa* cv. IR-64 and Khitish; (a) Immunoblot analysis of GS1 and GS2 polypeptides (b) Bar diagram of GS polypeptide levels.

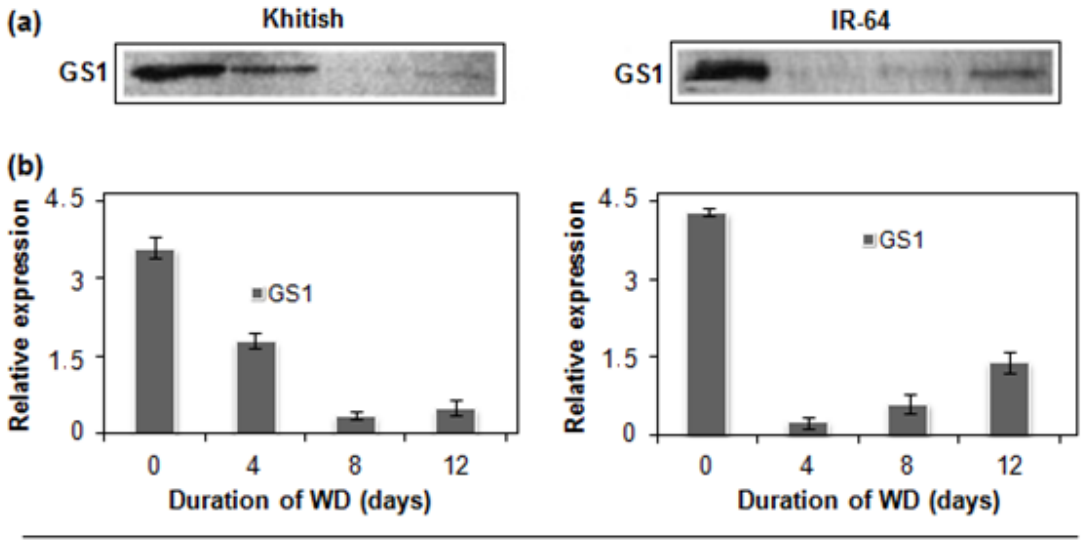

Figure 2.22. Effect of WD on expression of GS polypeptide in root of *O. sativa* cv. IR-64 and Khitish; (a) Immunoblot analysis of GS1 and GS2 polypeptide (b) Bar diagram of GS polypeptide levels.

Similarly the abundance of GS1 polypeptide was enhanced by 1.5 fold in IR-64 and didn't change significantly in Khitish. The immunoblot analysis of root proteins highlighted a single protein band of molecular size 39 kDa, which corresponds to the cytosolic GS1 isoform. In both IR-64 and Khitish cultivars, initial level of GS1 polypeptide was significantly higher. WD treatment resulted in drastic decline GS1 protein content. The rate of decline was faster in drought sensitive IR-64 as compared to Khitish. As a consequence its level was reached to a minimum on day 4 in IR-64 and on day 8 in Khitish. These declines began to reverse on further WD treatment in both the seedlings (*Figure 2.22*).

2.4. DISCUSSION

Environmental factors that impose water deficit (WD) stress, such as drought, salinity and temperature extremes, place major limits on plant productivity (Cushman and Bohnert 2000). The specific plant responses to WD are dependent on the amount and rate of water loss, duration of the stress and stages of plant development. Adaptation to WD at biochemical and molecular levels involves the activation or increased expression of several genes, transient increases in ABA levels, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways (Xiong et al. 2002; Waseem et al. 2011). During prolonged periods of WD, the decrease in water availability for transport-associated processes leads to changes in the concentrations of many metabolites, followed by disturbances in amino acid and carbohydrate metabolism. Acclimation to WD requires responses that allow essential reactions of primary metabolism to continue and enable the plant to tolerate WD (Foyer et al. 1998). Recent studies have indicated that nitrogen assimilation to be critical to plant acclimation to fluctuating environmental conditions (Swarbrek et al. 2011). However, the effect of WD on nitrogen metabolism remains relatively unexplored.

Rice is an important crop worldwide. It is also considered to be a model plant for monocots because of its relatively small genome size. WD is one of the major constraints depressing rice production (Jonaliza et al. 2004). Rice plants in paddy fields prefer to utilize ammonium as a major nitrogen source. GS serves for assimilation of ammonia to glutamine, which is the main form of organic nitrogen for transport through vascular tissues (Ishiyama et al. 2004b; Tabuchi et al. 2007). Present research work describes the effect of WD conditions on regulation of activity and expression of GS isoforms in leaf, stem and root of seedlings of two rice cultivars differently tolerant to WD conditions.

Dehydration tolerance in plants is attained by the maintenance of metabolic and physiological functions at low water status, which serve as the driving force for plant productivity. A few characteristics such as maintenance of RWC, osmotic adjustment and cell membrane stability are recognized as effective components of dehydration tolerance in many crops (Bhushan et al. 2007). RWC is considered to be the best integrated measure of plant water status, which represents variations in water potential (WP), turgor potential

(TP), and osmotic potential (OP). The choice of RWC as the best representation of plant water status in terms of genetic variation is also supported by genetic association between RWC and plant production under dehydration (Bhusan et al. 2007). Plants accumulate metabolites under stress conditions, which have been proposed as one of the mechanism of stress tolerance. Plant cells accumulate some kind of compatible solutes, such as proline, betaine, polyols, polyamines and ions (i.e. potassium) when subjected to abiotic and biotic stress. They act both by contributing to osmotic adjustment and by protecting proteins and cellular membranes. The beneficial roles of proline in conferring osmotolerance have been widely reported (Kishor et al. 1995; Bajji et al. 2000). It is shown to be involved in tolerance mechanisms against oxidative stress, which is the main strategy of plants to avoid detrimental effects of water stress (Vendruscolo et al. 2007). The WD stress on plant also results in inhibition of protein synthesis, increased protein degradation and accumulation or depletion of protein and non-protein amino acids in a variety of monocots and dicots (Gilbert et al. 1998). Moreover, the capacity to avoid or repair membrane damage during dehydration processes is also pivotal for the maintenance of membrane integrity, especially for those membranes in which functional proteins are embedded.

In the present study, rice varieties were initially screened for their dehydration tolerance characteristics by measuring RWC, accumulation of compatible solute like proline, and membrane permeability of ions and electrolytes. During WD, the dry wt. g^{-1} fresh wt. of root and leaf tissue of IR-64 seedling was increased significantly; however, no such change in dry weight was noted in Khitish. The RWC, protein and proline level declined markedly and the electrolyte leakage was increased sharply in IR-64 cultivar in response to WD treatment. Under similar condition, Khitish variety maintained relatively higher RWC, protein and proline level. Proline level kept on increasing continuously throughout the treatment period. Moreover, electrolyte leakage from Khitish leaf increased only marginally during WD. The results thus indicated more susceptibility of IR-64 to WD in comparison to Khitish and hence, were designated as drought- sensitive and tolerant- cultivar, respectively. These two cultivars were used for studying the effect of WD on regulation of GS isoforms.

GS isoforms in rice seedlings was determined by Anion exchange chromatography. GS activity in leaves and stem were resolved into two distinct peaks eluting at 0.15 M and 0.30 M and were designated as GS1 and GS2, respectively.GS activities in soluble fraction and isolated chloroplast were eluted from the column of DEAE-Sephacel at the salt concentrations corresponding to that of GS1 and GS2 thereby indicating their localization in cytosol and chloroplast, respectively. In rice root GS activity was localized only in cytosol. Anion exchange chromatography has commonly been used for separation of isoforms of GS from various plant tissues. This technique has been successfully employed for resolution of isoforms of GS from leaves of rice (Hirel and Gadal 1980), sorghum (Hirel and Gadal 1982), wheat (Tobin et al. 1985), maize (Becker et al. 1993), sunflower (Cabello et al. 1994, Larios et al. 2004), *Pennisetum glaucum* (Ghosh 2004), tobacco (Pageau et al. 2005) and potato (Teixeira et al. 2005); roots of rice, bean, maize (Suzuki et al.1981), pea and alfalfa (Vezina et al. 1987) and nodules of *Phaseolus vulgaris* (Robert and Wong 1986). As in the present study, leaves of several C_3 plants were found to contain most of the GS activity as GS2 (Mc Nally et al. 1983). Majority of studies conducted on roots indicate the presence only cytosolic isoform of GS in the tissue (Suzuki et al. 1981; Mack 1995; Ishiyama et al. 2004; Bernard et al. 2008). In few studies, substantial activity of GS has also been shown to be associated with plastid fraction of the root cells, such as in roots of pea and alfalfa (Vezina et al. 1987). Brugiere et al (1999) reported the occurrence of both GS1 and GS2 in phloem. They showed the major role of GS2 in stem in controlling proline production. In leaves of sorghum (Hirel and Gadal 1982) and soybean (Kang and Hymowitz 1988) GS activities in soluble fraction and isolated chloroplast were eluted from the column of DEAE-Sephacel at the salt concentrations corresponding to that of GS1 and GS2 thereby indicating their cytosolic and chloroplastic localization, respectively. The non-overlapping localization of GS1 and GS2 was further confirmed by subcellular and immunocytochemical studies. Immunolocalization studies in tobacco (Brugiere et al. 1999), pine (Canovas et al. 2007), potato (Pereira et al. 1995), rice (Tabuchi 2005) have shown predominant vascular location of GS1in different plant organs. A detailed immunolocalization study of mature flag leaf of wheat using anti-GS antibody showed the presence of GS2 label in the plastid of mesophyll parenchyma and in the plastid of parenchyma cells in the perifascicular sheath surrounding the vascular bundles (Bernard et al. 2008).

As in present study, a single chloroplastic GS2 is form has been reported in many higher plants. However, in soybean and alfalfa several GS2 isoforms have been identified (Zozaya-Garza and Sengupta Gopalan 1999). Two cytosolic GS isoforms, OsGS1;1 and OsGS1;2 were present in root, stem and leaf of IR-64 and Khitish seedling. Majority of studies indicated the presence of multiple homologous but distinct genes for cytosolic GS1 (Tingey and Corruzi 1987; Ireland and Lea 1999; Yamaya and Oak 2004; Canovas et al, 2007; Bernard et al. 2008). Earlier it was believed that GSr (OsGS1;2) is the only GS1 isoform expressed in rice root (Sakamoto et al. 1989). However, present investigation indicated the presence of both OsGS1;1 and OsGS1;2 in root of rice seedlings, as reported by Ishiyama et al (2004b). OsGS1;2 was the major cytosolic GS in root and stem, whereas OsGS1;1 was the major isoform of GS1 in leaf. Previous study (Ishiyama et al. 2004a,b) also showed approximately 2.5-fold greater abundance of OsGS1;2 as compared to OsGS1;1 in root. A varietal variation in expression of OsGS1;1 was noted in all the organs tested. At the beginning of WD treatment OsGS1;1 transcript level was significantly higher in IR-64 in comparison to Khitish. Genetic linkage studies using segregating mapping populations have implicated cytosolic GS genes with grain production in maize (Hirel et al. 2007; Fontaine et al. 2009), rice (Obara et al. 2004) and wheat (Habash et al. 2007). In rice crop significant correlations were obtained between grain number/ size and the locus for OsGS1;1 protein content (Obara et al. 2004). A higher level of OsGS1;1 in all the organs of IR-64 indicates that the cultivar can perform better under proper growth conditions.

To evaluate the effect of WD on GS isoform, activity of GS1 and GS2 and the corresponding mRNA and polypeptide contents were simultaneously monitored in different organs of the two cultivars, at various stages of WD. The WD mediated alteration in total GS activity in leaf and root was directly related to dehydration tolerance characteristics of rice varieties. The detailed view of regulation of GS isoforms in leaf of IR-64 and Khitish seedlings in response to WD has been depicted in *Figure 2.23*. Total GS activity declined significantly in IR-64 and didn't change markedly in Khitish cultivar. The decreased GS activity in IR-64 leaf was due to preferential reduction of GS2 activity and was correlated with decreased level of GS2 mRNA and protein. Under similar conditions, an almost constant GS2 mRNA and corresponding polypeptide maintained a

steady GS2 activity in Khitish leaf. The results suggest that WD mediated GS2 regulation resides mainly at the transcriptional and/or mRNA stability levels. As in the IR-64 seedlings, total GS activity declined to less than a quarter of its initial level during the natural senescence of rice leaves and this decline was mainly caused by a decrease in the GS2 level (Kamachi et al. 1991). Similarly, other studies have also shown the susceptibility of chloroplastic GS2 to other abiotic and biotic stresses as well as dark (Larios et al. 2004; Ghosh 2004; Santos et al. 2004; Pageau et al. 2005).

An important physiological function of GS2 is reassimilation of NH_4^+ produced during photorespiration (Wallsgrove et al. 1979). Photorespiration is a metabolic pathway in which CO_2 is released by light and is linked to Calvin-Benson cycle through the oxygenase activity of ribulose- bisphosphate carboxylase (Rubisco). Although photorespiration includes many metabolic steps which are performed across chloroplast, mitochondria and peroxisomes, several studies suggest that rate limiting step is the GS2 catalyzed reassimilation of ammonia (Hausler et al. 1994; Hosida et al. 2000). The phorespiration activity has been reported to be induced by abiotic stress and plays a protective role (Hoshida et al. 2000). The overexpression of GS2 in leaf of transgenic rice increased their photorespiration capacity and improved their salt tolerance. The transgenic rice line accumulating 1.5 fold more GS2 than the control plant, had an increased photorespiration capacity. They also retained more than 90% photosystem II activity when grown under osmotic stress treatment for two weeks indicating the physiological importance of GS2 in abiotic stress tolerance (Hoshida et al. 2000). Hence, in the present study a relatively unaltered GS2 expression in Khitish leaf could maintain the photorespiratory capacity of the plant at limited water availability that improves tolerance of the cultivar to WD.

WD treatment increased the expression of both OsGS1;1 and OsGS1;2 transcripts in leaf of IR-64 and Khitish cultivars. The time course of increase in GS1 transcripts corresponded with the accumulation of GS1 protein detected on Western blot. Although, the response of individual cytosolic GS genes to abiotic stress has not been studied earlier, the total GS1 transcript and polypeptide level have already been shown to accumulate in leaf during natural senescence and in response to biotic and abiotic stress (Kamachi et al.

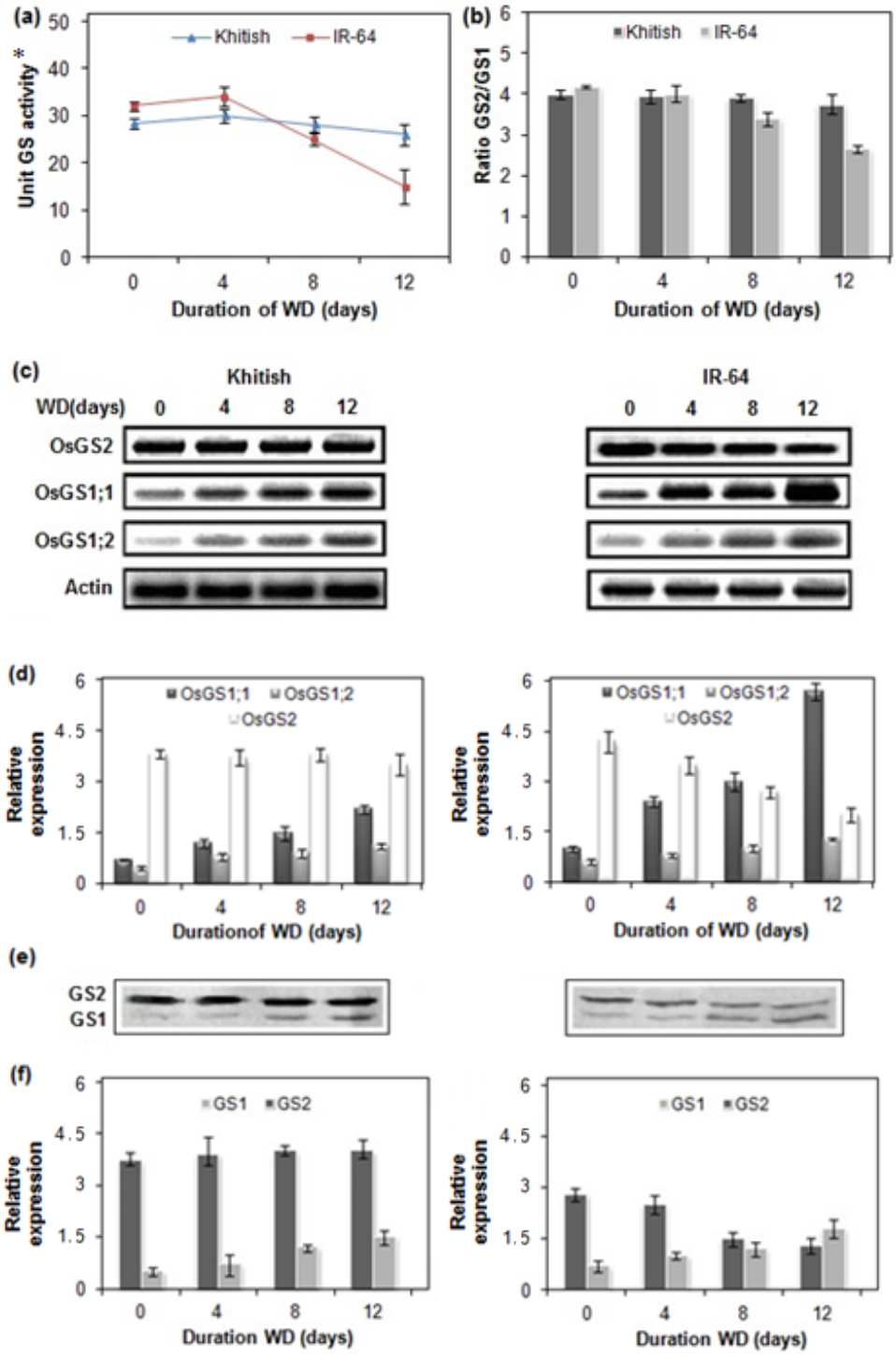

Figure 2.23. GS activity and expression in leaves of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment. (a) Relative change in total GS activity of IR-64 and Khitish, (b) Change in ratio of GS2/GS1 activity in IR-64 and Khitish seedlings , (c) Analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR,(d) Bar diagram for GS mRNA level (e)Western blot analysis of GS1 and GS2 polypeptide and (f) Bar diagram for GS polypeptide. Western blotting was carried out with 10µg of total soluble protein extracted from leaf. *One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1}.

1991; Pageau et al. 2005; Teixeira et al. 2005; Bernard and Habash 2009). In natural senescing potato plant GS1 transcript accumulation was coupled to increased synthesis of corresponding polypeptide (Teixeira et al. 2005). However, during natural senescence of rice leaves translatable GS1 mRNA level increased by four folds without affecting the corresponding polypeptide content (Kamachi et al. 1991). Similarly, the GS1 protein quantity has also been shown to increase in *Nicotiana tabaccum* leaf during *Potyvirus* infection (Pageau et al. 2005). In the present study, WD mediated increase in GS1 transcript and protein expression in rice leaf didn't correspond with GS1 activity. A similar type of response to GS1 mRNA overexpression was observed in *Arabidopsis* root. In this tissue the nitrogen nutrition mediated increase in GS1 transcript and polypeptide was related to the maintenance of GS1 activity rather than increase (Ishiyama et al. 2004a). A lack of correlation between constitutive overexpression of GS1 mRNA and abundance of corresponding polypeptide and activity has been reported earlier in leaf of Alfalfa (Ortega et al. 2001). WD treatment caused significantly greater OsGS1;1 accumulation in leaf of IR-64 as compared to that of Khitish. The increased OsGS1;1 in IR-64 leaf could be due to its higher rate of protein degradation (*Figure 2.12*), conforming to role of the isoform in reassimilation of nitrogen released from protein breakdown. The contention is supported by previous studies showing the localization of GS1 protein in companion cells and vascular parenchyma cells in senescing leaf blade of rice (Kamachi et al.1992) and wheat (Kichey et al. 2005) plants. The research work by Tabuchi et al (2005) with OsGS1;1 knockout mutant also showed the importance of OsGS1;1 in remobilization and reutilization of nitrogen in rice plant. Knockout mutants created by the insertion of Tos17 into the exon of OsGS1;1 were screened and characterized. Homozygously inserted mutants exhibited a severe retardation in growth rate and grain filling when grown under normal nitrogen fertilizer concentrations. Reintroduction of OsGS1;1 cDNA under the control of its own promoter into the mutant successfully complemented the slow growth phenotype.

In contrast to leaf, WD treatment reduced GS1 activity and OsGS1;1 transcript level in stem and root of IR-64 seedlings (*Figure 2.24 and 2.25*). Nitrogen remobilization from protein breakdown constitutes the major source of nitrogen in vascular tissue and glutamine is the most abundant free amino acid for transport in rice plant (Tabuchi et al.

2007). The repression in OsGS1;1 might result from remobilization and transport of high concentration of glutamine to stem and root from increased protein degradation in IR-64 leaf. The transcriptional down-regulation of OsGS1;1 has already been documented in presence of NH_4^+ in roots of *Arabidopsis* and rice seedlings (Ishiyama et al.2004a; Ishiyama et al. 2004b; Kusano et al. 2011). Several other rice genes associated with N-uptake and metabolism, such as, OsGS1;2, OsNADH-GOGAT1, OsAMT1;1 and OsAMT1;2, are also regulated by exogenous NH_4^+ ions (Ishiyama et al. 2004; Sonoda et al. 2003; Tabuchi et al.2007). However, pharmacological studies have suggested glutamine rather than NH_4^+ ions, being the real signaling molecule in regulation of expression of these genes (Oliveira and Coruzzi 1999; Tabuchi et al.2007). Oliveira and Coruzzi (1999) reported the association of metabolic regulation of GS1with relative abundance of carbon skeleton verses amino acids accumulated in the root tissue. Their results suggested a negative feedback regulation of GS1 by glutamine or the downstream nitrogen metabolites.

A comparison of kinetic properties of OsGS1;1 and OsGS1;2 encoded GS isoforms in rice root was carried out by Ishiyama et al. (2004b). The Vmax values were approximately 2-fold higher with OsGS1;1 than with OsGS1;2. In addition, OsGS1;1 exhibited extremely high substrate affinity for ammonium, as indicated from its Km value: the Km for ammonium was 2.7-fold lower in OsGS1;1 than in OsGS1;2. The result supported the importance of OsGS1;1 in promoting the rapid conversion of ammonium to glutamine even under low ammonium conditions (Ishiyama et al. 2004b). Similarly, in *Arabidopsis* root, GLN1;1 exhibited an extremely high affinity for ammonium (Km < 10 µM) as compared to GLN1;2 (Km = 2450 µM) (Ishiyama et al. 2004a). The implication of OsGS1;1 in NH_4^+ assimilation has been further indicated by the over accumulation of free ammonium in the leaf sheath and roots of the rice mutant lacking OsGS1;1(Kusano et al. 2011). In the present investigations WD mediated decrease in GS1 activity in stem and root of IR-64 seedling might correlate with reduction in OsGS1;1 mRNA level. Hence, OsGS1;1 seems to play significant role in performance of plant under stress condition.

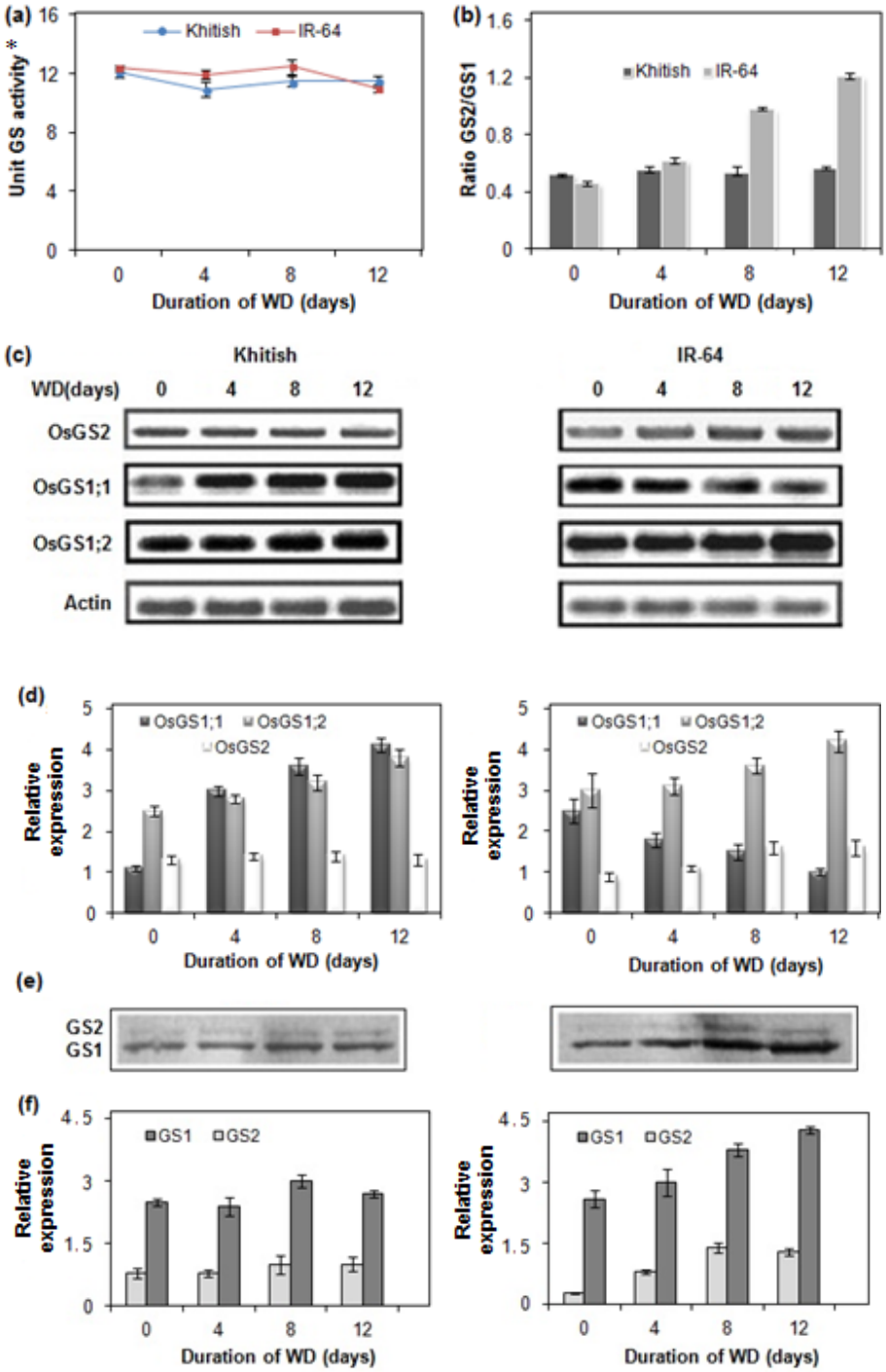

Figure 2.24. GS activity and expression in stem of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment. (a) Relative change in total GS activity of IR-64 and Khitish, (b) Change in ratio of GS2/GS1 activity in IR-64 and Khitish seedlings , (c) Analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR,(d) Bar diagram for GS mRNA level (e)Western blot analysis of GS1 and GS2 polypeptide and (f) Bar diagram for GS polypeptide. Western blotting was carried out with 10µg of total soluble protein extracted from leaf. *One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1}.

The decline in GS1 activity in IR-64 stem was compensated by increment in GS2 protein and activity, maintaining almost unchanged total GS activity. The increased GS2 protein can be due to observed increase in total protein content of stem during WD. As in the present study, a tissue specific response of WD stress was noted in *Lupinus albus* with strikingly increase in concentration of N and S in stem with intensification of water stress. At 13 days of WD, the ratio of stem protein concentration of WD to control plant increased from 0.8 to 1.3 (Pinheiro et al. 2001). Translation of some proteins has also been found to increase in stem in other type of stress conditions as well. Recently, Moller et al (2011) in their proteomic based study indicated an increase in synthesis of some proteins of chloroplastic transcription and translation machinery under N-starvation condition. The condition is quite similar to that encountered by rice plant under WD leading to limited uptake of nutrient from soil. Thus, a different metabolic status may contribute to the maintenance of GS protein and activity in stem during WD.

WD treatment had no differential effect on expression of OsGS1;2. The transcript level of OsGS1;2 was found to enhance progressively and almost equally in leaf and stem of IR-64 and Khitish seedlings. However, in roots of both the cultivars the highly expressed OsGS1;2 was found to decline initially followed by an increased accumulation on further dehydration. The rate of decline was faster in IR-64 root in comparison to Khitish root. The time course of OsGS1;2 mRNA was reflected in GS1 polypeptide abundance in all the three tissues. The result is supported by study of Ishiyama and co-worker (2004a) on effect of ammonium nutrition in *Arabidopsis* root GS isoforms. Arabidopsis root contained four different isoforms of GS1, among them amount of GS1;1, GS1;3, and GS1;4 mRNA decreased and GS1;2 mRNA was increased by ammonium nutrition. The time course increase in GS1;2 mRNA corresponded with the accumulation of GS1 protein detected on the western blot. However, the increase in GS1;2 mRNA and protein was not correlated with total GS1 activity. The lack of correlation was due to lower affinity of GS1;2 for ammonium as compared to other isoform. Furthermore, GS1;2 has been reported to overexpress in leaves and roots by ammonium supply. GS1;2 knockout mutants of *Arabidopsis* displayed lower GS activity, higher ammonium concentration, and reduced rosette biomass compared with the wild type (WT) under ample nitrogen supply only.

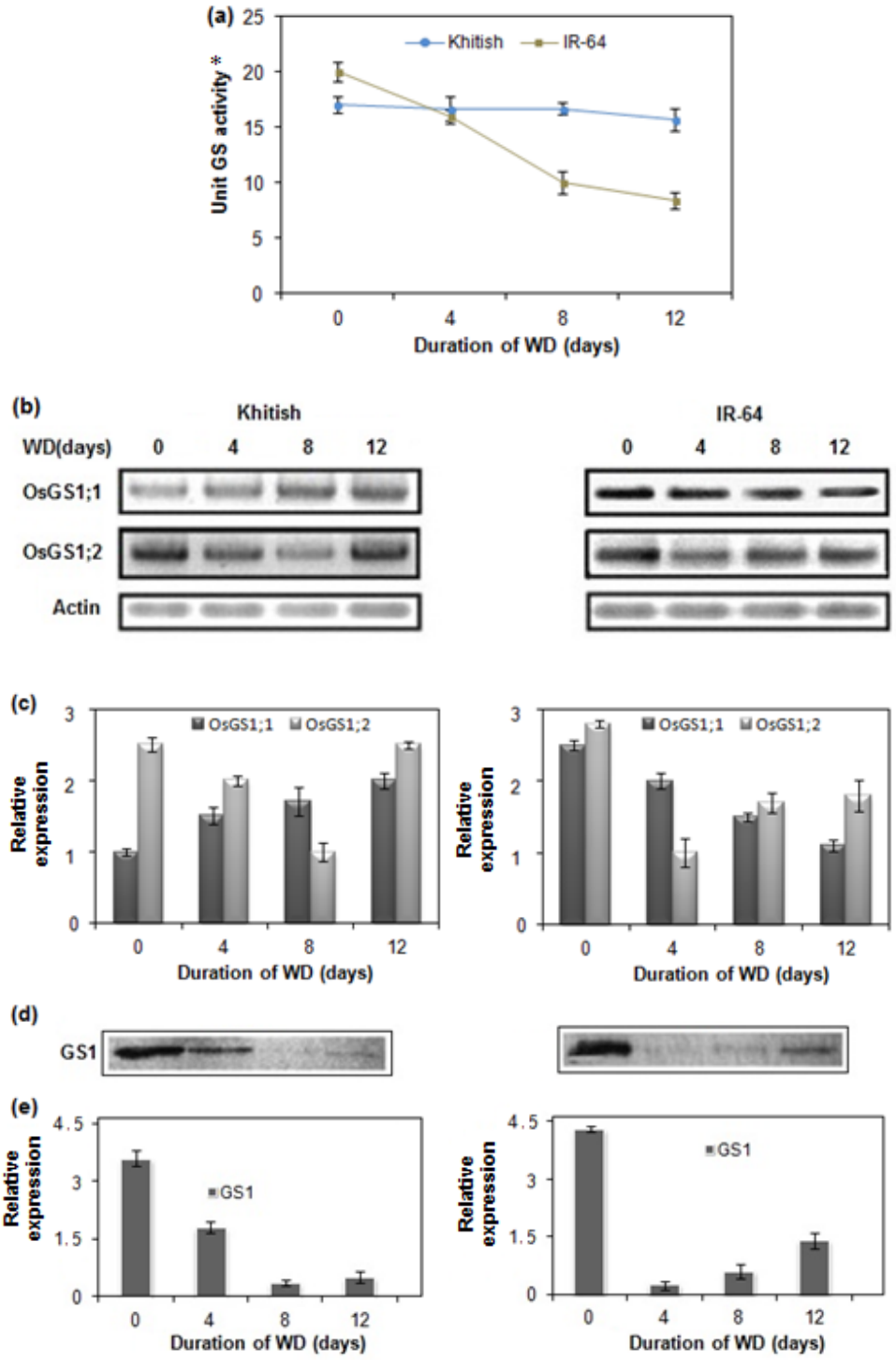

Figure 2.25. GS activity and expression in root of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment; (a) Relative change in total GS activity of IR-64 and Khitish, (b) Analysis of OsGS1;1 and OsGS1;2 OsGS2 transcripts by RT-PCR, (c) Bar diagram for GS mRNA level, (d) Western blot analysis of GS1 and GS2 polypeptide and (e) Bar diagram for GS polypeptide. Western blotting was carried out with 10µg of total soluble protein extracted from leaf. *One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1} .

However, it showed no significant difference from the wild type under nitrogen limiting conditions (Lothier et al. 2010). A similar nitrogen limiting condition prevailed during WD. IR-64 seedlings being more susceptible to WD showed faster decline in OsGS1;2 mRNA and protein in root with minimum expression levels at day 4 of treatment due to limited uptake of nitrogen from soil. The enhancement in OsGS1;2 expression on further intensification of WD might be related to increased accumulation of ammonium ion due to observed increased protein degradation.

In conclusion, the regulation of GS isoforms by WD was organ specific. Two GS isoforms i.e. GS1;1 and GS2 were differentially regulated in drought sensitive IR-64 and drought tolerant Khitish cultivars of rice. GS2 was the major GS isoform in leaf. GS2 expression in leaves decreased in IR-64 seedlings and remained almost unaltered in Khitish seedlings in response to WD. The maintenance of GS2 expression in leaf might be associated with the maintenance of photosynthetic and phorespiratory capacity of the Khitish and hence, with WD tolerance characteristics of the cultivar. The GS1;1 isoform has been shown to be involved in remobilization and reutilization of nitrogen during senescence and other stress conditions characterized by high rate of protein degradation. A higher substrate affinity of the enzyme for ammonium signifies its promoting the rapid conversion of ammonium to glutamine even under low ammonium conditions. Hence, from the results it can be inferred that a relatively maintained OsGS2 and the over-expression of OsGS1;1 in might contribute to improved drought tolerance characteristics of *Oryza sativa* cv. Khitish.

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CHAPTER 3

Studies on GS isoform in *Selaginella bryopteris* L. and its regulation during water deficit stress

3.1. INTRODUCTION

Plant growth is greatly affected by drought stress. It is a composite stress condition that includes soil WD, increased daytime temperature, and reduced nutrient availability. However, the most important factor limiting growth and impairing plant productivity is the drop in water availability to the plant. Plants survive the extreme water deficit (WD) conditions either by avoiding stressful events or by activating adaptive stress mechanisms. Most crop species are very sensitive to soil water potential and only rarely survive soil water deficit that drive leaf water potential to a lower value (Proctor and Pence 2002). However, resurrection plants can survive with less than 5% of their total water in the vegetative tissues and are able to regain normal metabolism and growth within several hours of rewatering (Ramanjulu and Bartels 2002; Jiang et al. 2007; Deeba et al. 2009).

In plants, WD induces complex change in nitrogen metabolism due to decreased water availability for transport leading to limited uptake of nitrogen nutrients. The effect of WD on nitrogen metabolism of resurrection plants has been studied with inhibition of protein synthesis, increased protein degradation and accumulation or depletion of protein and non-protein amino acids (Martinelli et al. 2007; Whittaker et al. 2007; Yobi et al. 2013). Nitrogen-rich amino acids such as glutamine, glutamate, arginine, aspartate, citrulline, asparagine, 3-(3-hydroxyphenyl) propionate, N-6-trimethyllysine, trans-4-hydroxyproline, and ophthalmate have been shown to accumulate in dehydrated state (Martinelli et al. 2007; Oliver et al. 2011; Yobi et al. 2013). Resurrection plants are also able to synthesize new proteins even at very low RWC (Bartels and Sunkar 2005). In

resurrection angiosperm *Craterostigma plantagineum*, protective proteins, such as hydrophilins, detoxifying enzymes**,** degradation and transport proteins, were overexpressed under dehydration (Bartels and Sunkar 2005; Deeba et al. 2009).

GS is the key enzyme of nitrogen assimilation and remobilization. GS together with GOGAT forms the GS-GOGAT cycle. Glutamate produced by the cycle is the preferential amino-donor for the different aminotransferase reactions for subsequent amino acid inter-conversions (Ford and Lea 2007). In most of the higher plants, GS and GOGAT exist as multiple isoforms. The cytosolic GS1 is considered to be operational along with the NADH-dependent isoform of GOGAT (NADH-GOGAT, EC 1.4.1.14) in the cytosol. On the other hand, chloroplastic GS2 together with the ferredoxin-dependent isoform of glutamate synthase (Fd-GOGAT, EC 1.4.7.1), is reported to be responsible for the chloroplastic GS/GOGAT cycle (Lea and Ireland 1999; Skopelitis et al. 2006). Finally, glutamate dehydrogenase (GDH) is also directly involved in glutamate metabolism and it's *in vivo* role is still under debate. In drought sensitive higher plants, GS is highly susceptible to WD conditions. Both the cytosolic and chloroplastic GS activities decline during WD (present study, Rice paper). Present study was undertaken with the aim to search dehydration tolerant isoforms of GS. Since resurrection plants have the ability to survive long dry period, they could be an excellent system for searching dehydration tolerant GS.

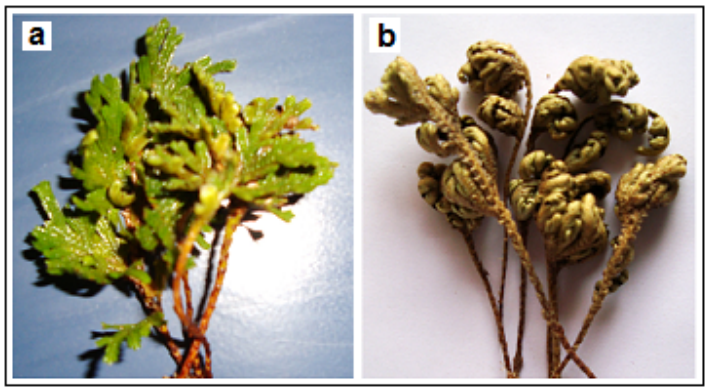

Figure3.1. *S. bryopteris* fronds; (a) hydrated, and (b) rehydrated.

Selaginella bryopteris belonging to the family Selaginellaceae, is a resurrection lycophyte growing in the hilly area of tropical regions (Deeba et al. 2009). It is a creeping or

ascendant plant with simple, scale-like leaves on branching stems from which roots also arise. It is capable of surviving almost complete dehydration for prolonged period. Under dry conditions, fronds of *Selaginella* roll into brown balls and become green on rehydration. The plant has the unique feature with detached fronds possessing a similar level of desiccation tolerance as that of whole plant. Present investigation describes isolation and characterization of GS from S. *bryopteris*. Further studies were carried out to determine the regulation of GS expression during dehydration and rehydration of *Selaginella* fronds and its relationship with other enzymes of ammonium metabolism.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals and reagents

The required chemicals and reagents used in this investigation were purchased from different companies as described in section 2.2.1.

3.2.2. Plant material

Selaginella bryopteris plants were collected from natural habitat (Darjeeling District, West Bengal, India, *latitude* 26°31' and 27°13' N and *longitude* 87°59' and 88°53'E). There, the plants grow on rocky slope, on a thin layer of organo-mineral rich, dark mountain soil. Plants were harvested and kept in water until the beginning of experiment. For dehydration, freshly detached fronds from well hydrated plant were kept in a growth chamber (Conviron, Canada), at 25 ^{o}C and ambient photoperiod. Rehydration is achieved by keeping the dehydrated fronds in moist filter paper. The plants were sampled at various stages of dehydration and rehydration. Sampling times were determined by visual appraisal of the fronds using decolouration and folding as benchmarks, at which RWC of fronds was determined.

3.2.3. Determination of RWC

The relative water content (RWC) of *Selaginella* fronds was measured according to Barrs and Weatherley (1962) as described in section 2.2.3.

3.2.4. Pigments estimation

For pigments determination, fronds were extracted in 80% chilled acetone followed by centrifugation in 10,000 rpm for 10 minutes at 4 ºC. The supernatant was taken for determination of photosynthetic pigments. Pigment contents were calculated according to the formulas of Lichtentaler and Wellburnn (1985). Chlorophyll *a* (mg/g) = ((12.7 x A_{663} - 2.69 x A_{645}) v/w), chlorophyll *b* (mg/g) = ((22.9 x A_{645} - 4.68 x A_{663}) v/w), and carotenoid (mg/g) = ((1000 x A_{470}) - (3.27 x chlorophyll *a* + 1.04 x chlorophyll *b*)) /227 v/w). The experiments were done in triplicates.

3.2.5. Proline estimation

Free proline was quantified according to Bates et al (1973) as described in section 2.2.5.

3.2.6. Protein estimation

Quantitative estimation of protein was carried out by the method of Bradford (1976) as described in section 2.2.6.

3.2.7. Ammonium estimation

Ammonium was estimated by the phenol hypochlorite colorimetric method (Russell, 1944) using ammonium sulphate as standard (*Figure 3.2*). One gram of *Selaginella* fronds was homogenized in 5 ml of Selaginella extraction buffer (SE-Buffer) containing 50 mM phosphate buffer (pH 6.5), 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15 % glycerol. The homogenate was filtered through four layers of muslin and was centrifuged at 10,000 rpm for 15 min at 4 ºC. After centrifugation the supernatant was collected in fresh tube. For quantification of ammonia-nitrogen, the procedure involved consecutive addition of 2 ml of phenol solution (10 g of reagent grade phenol dissolved in 100 ml of 95% v/v ethyl alcohol), 2 ml of 0.5% sodium nitroprusside and 5 ml of oxidizing reagent (prepared by mixing of alkaline-sodium citrate and sodium hypochlorite) to 1 ml of the plant extract. The reaction contents were mixed thoroughly after each addition. The colour was allowed to develop at room temperature (22-25 ºC) for 1 h and the absorbance was recorded at 640 nm in a spectrophotometer. The assay was done in triplicates. Amount of ammonia was expressed as µg NH_4^+ g^{-1} dry wt.

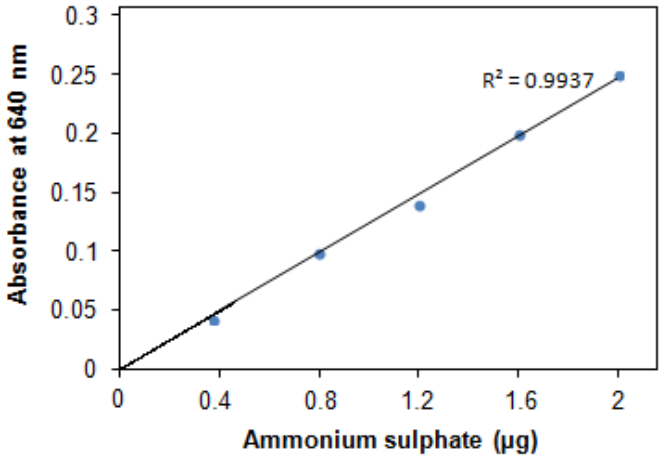

Figure 3.2. Standard curve for ammonium sulphate.

3.2.8. Separation of GS isoforms from S. bryopteris fronds

All the steps of isoenzyme separation were performed at 4 ^{o}C. One gram of *Selaginella* fronds was homogenized in 5 ml of SE-Buffer. The homogenate was centrifuged at 10,000 rpm for 15 min. One ml of desalted supernatant was loaded onto a DEAE-Sephacel column (5x1 cm) pre-equilibrated with the SE-Buffer. The column was washed with the same buffer until no protein was detectable in the eluate. The adsorbed proteins were eluted by gradient elution using 0 - 0.5 M KCl in SE-Buffer. The flow rate was maintained at 20 ml h^{-1}. 2 ml fractions were collected and assayed for GS activity. About 80-90 % of the total GS activity present in the crude extract was recovered after chromatographic separation.

3.2.9. Separation of cytosolic and chloroplastic fraction of S. bryopteris fronds

Chloroplasts were isolated from *Selaginella* fronds following the method of Rathnam and Edward (1976). Fronds were homogenised in a pre-chilled blender with approximately five volumes of chloroplast isolation buffer (300 mM sucrose, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1mM each of $MgCl_2$ and $MnCl_2$). The homogenate was sieved through four layers of muslin cloth and centrifuged at 1500 rpm for 10 min. The supernatant was centrifuged at 15,000 rpm (15K) for 20 min to obtain the crude chloroplast pellet. The 15K supernatant was fractionated by 40-70 % ammonium sulphate precipitation. The precipitated pellet was suspended in 2.5 ml of SE-Buffer, dialysed and used to measure cytosolic GS activity. The isolated chloroplast fraction was homogenized

in SE-Buffer and centrifuged at 15000 rpm for 40 min. The dialysed supernatant was used as a source of chloroplastic GS.

3.2.10. Enzyme assays

One gram *Selaginella* fronds were homogenized in 5ml of SE-Buffer. The homogenate was centrifuged at 10,000 rpm for 15 min at 4^{o}C. The supernatant was desalted on pre-equilibrated Biogel P-2 column and used for determination of GS, NADH-GOGAT and GDH activity.

3.2.10.1. Glutamine synthetase

GS activity was determined by semisynthetase reaction (Washitani and Sato 1977). A 1 ml reaction mixture contained 50 µmol acetate buffer (pH 5.5), 200 µmol glutamate, 10 µmol ATP, 5 µmol hydroxylamine hydrochloride, 20 µmol $MgCl_2$, and 100 µl enzyme preparation. The reaction mixture was incubated at 37 ^{o}C for 30 min. and terminated by adding 2 ml of $FeCl_3$ reagent (0.67 M $FeCl_3$, 0.37 M HCl and 20% (w/v) Tri-chloroacetic acid). After 20 min, the amount of γ-glutamylhydroxamate produced was determined spectrophotometrically by measuring the absorbance at 540 nm. One unit of enzyme activity is defined as the amount catalyzing the formation of 1.0 µmol of γ-glutamylhydroxamate 30 min^{-1} at 37^{o}C.

3.2.10.2. Glutamate oxoglutarate amino transferase (GOGAT)

GOGAT (EC 2.6.1.53) was assayed by determining the rate of glutamine-dependent NADH oxidation (Turano et al. 1996). The reaction mixture contained 5 µmol L-glutamine, 5 µmol 2-oxoglutarate, 0.08 µmol NADH, 5 µmol EDTA, 50 µmol Tris buffer (final pH 7.5) and 100 µl of enzyme preparation in final volume of 1 ml. One unit of enzyme activity is defined as the amount catalyzing the formation of 1.0 µmol of glutamate min^{-1} at 37^{o}C.

3.2.10.3. Glutamate dehydrogenase (GDH Aminating)

GDH (EC 1.4.1.2) was assayed by determining the rate of 2-oxoglutarate-dependent NADH oxidation (Suzuki et al. 1994). The reaction mixture in final volume 1 ml, contained 150 µmol NH_4Cl, 1 µmol $CaCl_2$, 0.3 µmol NADH, 20 µmol 2-oxoglutarate,

and 100 µmol Tris buffer (final pH 7.5) and 100 µl enzyme preparation. One unit of GDH represents the reduction of 1 µmol NADH min^{-1} at 30°C.

3.2.11. Partial purification of GS from S. bryopteris fronds

All steps of purification were carried out at 4 ºC. Five gram of *Selaginella* fronds were homogenized in 25 ml of SE-Buffer, pH 6.5. The homogenate was filtered through four layers of muslin and was centrifuged at 12,000 rpm for 20 min. The supernatant so obtained, was designated as crude extract. Solid ammonium sulphate was added to it gradually with continuous stirring to bring the final concentration to 30% saturation. After 30 min, the precipitated proteins were removed by centrifugation at 10,000 rpm for 20 min. The supernatant was decanted and further addition of ammonium sulphate was added to it to achieve 70% saturation. After allowing it to stand for 30 min, it was centrifuged at 10,000 rpm for 20 min. The pellet was dissolved in 1 ml of SE-Buffer and loaded onto gel-filtration column (Biogel P-100, Bio-Rad, 1 × 25 cm) pre-equilibrated with SE-buffer and eluted with the same buffer at a flow rate of 10 ml h^{-1}. The fractions thus obtained were checked for GS activity. The active fractions were pooled and subjected to ion-exchange chromatography onto a DEAE-sephacel column (2 × 15 cm) pre-equilibrated with SE-buffer. The column was initially washed with the 100mM SE-Buffer, pH 6.5 to remove unbound proteins. Thereafter, elution of the bound protein was carried out by gradient elution using 0-0.50 M KCl in the buffer. The flow rate was maintained at 20 ml h^{-1}. Fractions of 2 ml were collected and analyzed for protein and GS activity. The active fractions eluted as a single peak were pooled and stored at 4°C.

3.2.12. Characterization of GS

The kinetic properties of the partially purified enzyme were determined with respect to pH optima, temperature optima, *Km* for glutamate and thermostability. The pH optimum was determined by measuring the enzyme activity at pH 2.5-8.5 in the following buffers: 100 mM glycine (pH 2.5), 100 mM sodium acetate (pH 4.5, 5.5), 100 mM phosphate buffer (pH 6.5), and 100 mM Tris-HCl (pH 7.5 and 8.5). For determination of Km for glutamate, substrate concentrations were varied in the range of 12.5-300 µM followed by measuring the activity under standard conditions. Km value for glutamate was determined by Lineweaver-Burk plot. The optimum temperature for activity was

determined at optimum pH and at temperatures ranging from 15 to 80 ºC. For determination of thermal stability, the enzyme was pre-incubated at 15-80 ºC for 30 min, followed by measurement of activity at standard temperature and pH.

3.2.13. SDS-PAGE analysis

Total soluble proteins were resolved by SDS-PAGE (Laemmli 1970) as described in section 2.2.10.1.

3.2.14. Immunoblot analysis of GS and GDH protein

Total soluble proteins (10 µg) were resolved by 12 % SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Polypeptide detection was performed using polyclonal antibody raised against synthetic peptide from conserved region of GS and tobacco GDH (Agrisera, Sweden). The reacted polypeptide was visualised with a goat anti-rabbit IgG-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-p-toluidine (NBT/BCIP) detection kit (Invitrogen, USA). Broad range pre-stained standards were used as markers.

3.2.15. Bioinformatic analysis

Amino acid sequences of plant GS isoforms were retrieved from GenBank databases (*Table 3.2*). Alignment of the coding regions was performed by the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) program (Robert 2004; http://www.ebi.ac.uk) and multiple align show (http:/bioinformatics.org/sms/).

3.3. RESULTS

3.3.1. Determination of dehydration-rehydration kinetics of S. bryopteris fronds

Preliminary experiment was carried out to determine the dehydration-rehydration kinetics of *S. bryopteris* fronds by measuring relative water content (RWC). As shown in *Figure 3.3a*, RWC decreased from 95 % to 70 % at 3h of dehydration and further decreased to 5% at 24 h of treatment. However, fronds were able to recover completely from dehydration by massive uptake of water within a much shorter duration of rewatering.

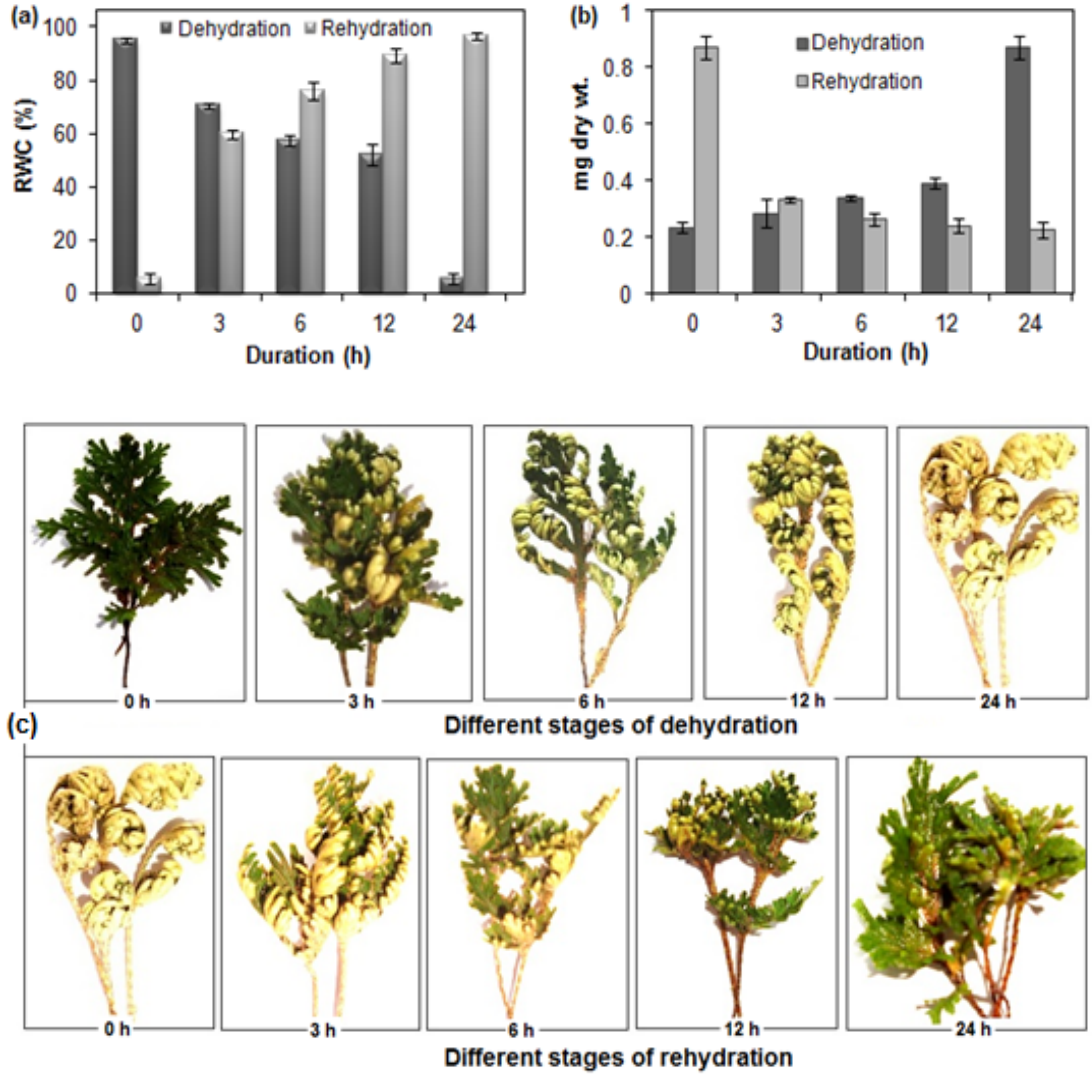

Figure 3.3. Effect of dehydration-rehydration on (a) RWC, (b) dry weight, and (c) morphology of *S. byopteris* fronds. Each point is the mean of three samples ±SE.

Consequently, RWC increased to 60 % at 3h, 76 % at 6 h and then gradually to 96 % at 24 h of rehydration. The dry weight (mg dry wt. g^{-1} tissue) of fronds altered considerably during various stages of dehydration-rehydration. Its value increased steadily till 12 h followed by a sharper increase at 24h of treatment. Similarly, rehydration of dried fronds for 3h reduced dry weight of fronds by about 60% and followed by a gradual decrease till the end of the treatment (*Figure 3.3b*). The phenotype of the dehydrated fronds was different from hydrated one. The dehydrated fronds showed inward curling and brownish appearance. It regained original morphology with opening of adaxial surface and greening on rehydration. Greening of fronds was clearly visualized at 3 h of hydration with complete green appearance at 24 h of hydration (*Figure 3.3c*). To ascertain whether the observed colour transition was correlated with changes in plant pigment levels, chlorophyll a (chl a), chlorophyll b (chl b) and carotenoid contents were monitored during various stages of dehydration-rehydration.

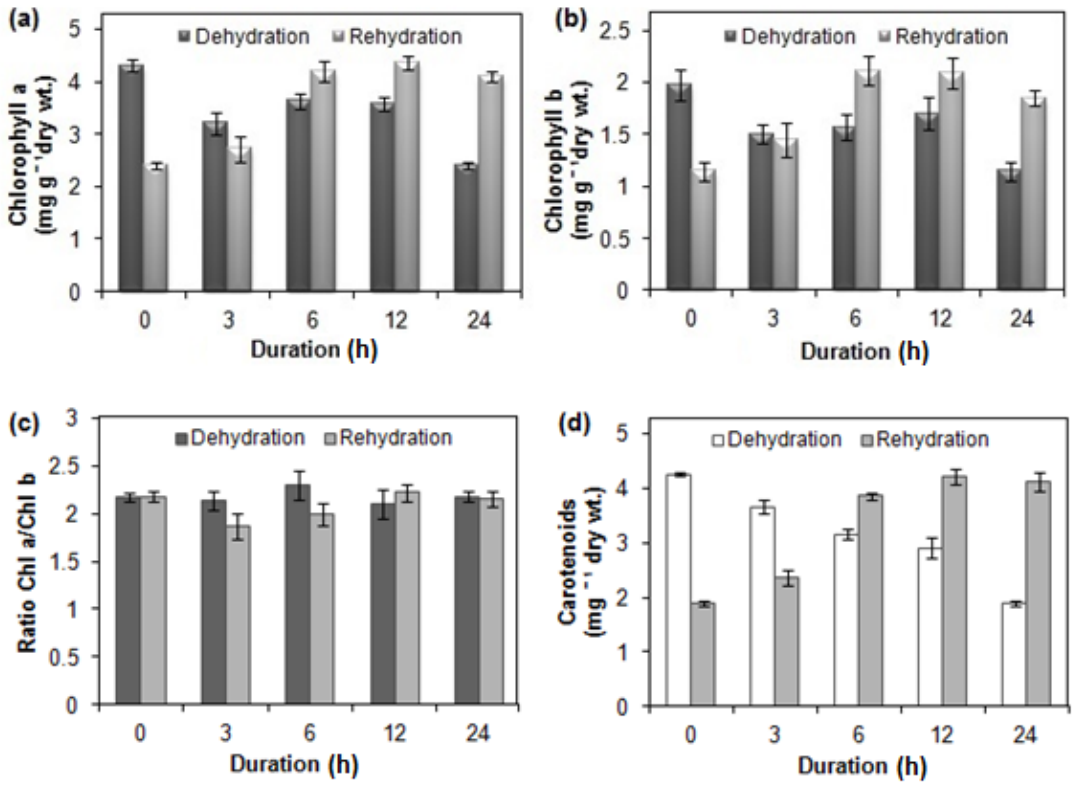

Figure 3.4. Effect of dehydration-rehydration on (a) Chlorophyll a, (b) Chlorophyll b, (c) Ratio of Chlorophyll a and Chlorophyll b, (d) Carotenoid contents of *S. bryopteris* fronds. Each point is the mean of three samples ±SE.

Although, chl a and chl b contents didn't alter significantly during 12 h of dehydration, however, their levels declined by about 40 % at 24 h of treatment. Rehydration of fronds restored chl a and chl b contents to a value at the beginning of dehydration treatment (*Figure 3.4 a, b*). However, the ratio of Chl a to chl b remained unchanged throughout the hydration cycle (*Figure 3.4 c*). In contrast to chlorophyll, a noticeable alteration in carotenoid level was detected. The carotenoid content gradually reduced on dehydration with about 50% decrease at 24 h of treatment. The carotenoid content was regained on rehydration of frond (*Figure 3.4 d*).

3.3.2. Effect of dehydration-rehydration of S. bryopteris fronds on protein, proline and ammonium contents

The change in total protein, free ammonium and proline contents were monitored at various stages of dehydration-rehydration treatment. The total protein content decreased by about 30 % and 50 % at 3 h and 24 h of dehydration, respectively and the decrease was reversed on subsequent rehydration of the fronds (*Figure 3.5 a*).

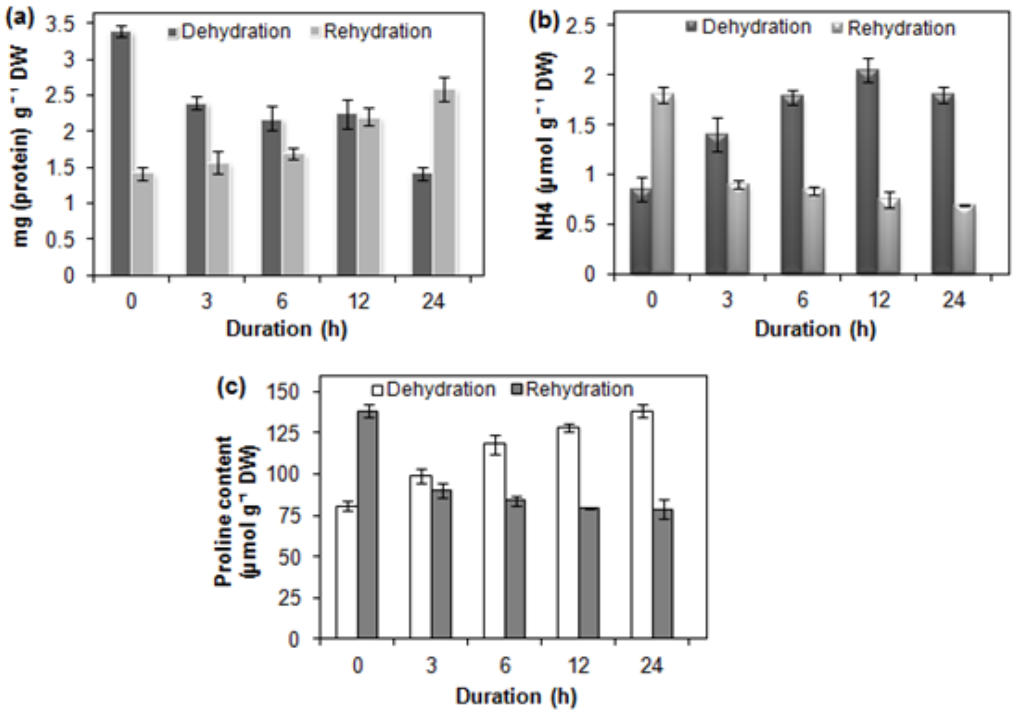

Figure 3.5. Effect of dehydration-rehydration on (a) total soluble protein, (b) ammonium, and (c) proline contents of *S. byopteris* fronds. Each point is the mean of three samples ±SE.

Present study showed a close relationship between the alteration in protein content and accumulation of ammonium. Dehydration mediated decline in protein level paralleled a significant increase in free ammonium content. Ammonium level rose by almost 2.5 folds at 12 h of dehydration, but didn't change markedly on further intensification of stress. Subsequent rehydration of the fronds for 3 h lowered the ammonium content sharply by about 50 %, followed by a steady decrease on further rehydration (*Figure 3.5 b*). The change in proline content during dehydration-rehydration of *Selaginella* fronds was also examined. Proline content increased progressively from the beginning with almost two folds accumulation at 24 h of dehydration treatment. Its level fell sharply to about 40 % on subsequent rehydration for 3 h and didn't alter significantly on further rehydration (*Figure 3.5c*).

3.3.3. Optimization of GS extraction and assay conditions

The optimal conditions for extraction of the enzyme with respect to molarity and pH of the extraction buffer and concentration of protective/ stabilizing agent was established in order to ensure maximal extraction and recovery of the enzyme from *Selaginella* fronds. Maximum recovery of the enzyme obtained when 50 mM Phosphate buffer (pH 6.5) buffer containing 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 10% glycerol was used as extraction buffer. The reaction conditions for GS activity assay were standardized by varying the pH of assay buffer as well as concentrations of other reaction components such as glutamate, ATP and $MgCl_2$. Maximum activity of the enzyme was obtained in the reaction mix containing 50 µmol acetate buffer (pH 5.5), 200 µmol glutamate, 10 µmol ATP, 5 µmol hydroxylamine hydrochloride, 20 µmol $MgCl_2$, and 100 µl enzyme preparation.

3.3.4. GS isoforms in S. bryopteris fronds

Selaginella fronds were extracted in SE-Buffer and subjected to anion-exchange chromatography in DEAE-Sephacel column. The elution of adsorbed protein by 0 - 0.5M KCl gradient revealed only one peak of GS activity (*Figure 3.6 a*). To ascertain the subcellular localization of the enzyme, GS activity was determined in chloroplastic and cytosolic fractions of the frond. GS activity was only present in the cytosolic fraction and was completely undetectable in the chloroplastic fraction. Anion-exchange

chromatography of the cytosolic fraction showed peak of GS activity eluting from the column at the same salt concentration as in the total *Selaginella* frond extract. The chloroplastic fraction did't reveals any GS activity peak. Immunoblot analysis of total soluble protein of the frond using GS antibody also indicated the presence of single immunoreactive band of molecular weight about 39 kDa (*Figure 3.6 b*).

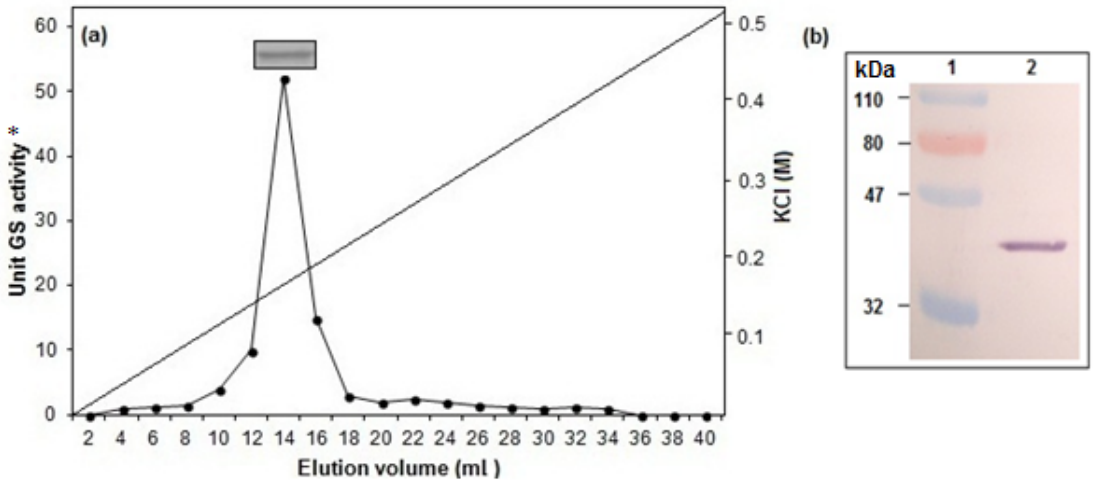

Figure3.6. GS in *S. bryopteris* fronds (a) Anion-exchange chromatography of *Selaginella* frond extract for GS activity in DEAE-sephacel column. Immunoblot analysis of peak fraction showing the one immunoreactive GS band (inset). (b) Approx. 20 µg of protein resolved on a 10 % SDS-PAGE gel and immunoblotted on a PVDF membrane and probed with GS antibody. *One unit of GS activity represents 1 µmole of γ-glutamylhydroxamate produced 30 min^{-1}.

3.3.5. Partial purification of GS from S. bryopteris fronds

GS was partially purified from *S. bryopteris* fronds by $(NH_4)_2SO_4$ precipitation, gel-filtration and anion-exchange chromatography as described in method section and the results are summarized in *Table-3.1*. The crude extract was subjected to ammonium sulphate fractionation and about 82 % of enzyme was recovered in 30-70 % saturated ammonium sulphate fraction. The 30-70 % pellet dissolved in 1ml of GS extraction buffer and subjected to gel-filtration chromatography in Biogel P-100 column. It resulted in purification of enzyme by 15.67 fold with specific activity of 359.40 U mg^{-1} protein and recovery of enzyme was 33.60 %**.** The active fractions were pooled, loaded onto DEAE-sephacel column and proteins bound to the column matrix were eluted by using elution buffer containing 0 to 0.50 M KCl. Anion-exchange chromatography resulted in

enhancement of specific activity to 662.50. The yield and purification fold of the enzyme purified were found to be 15.40 % and 28.89, respectively.

Table 3.1. Purification of GS from *S. bryopteris* fronds.

Purification steps	Total Activity (U*)	Total Protein (mg)	Specific Activity U/mg protein	Purification fold	Yield (%)
Crude	344.00	15.0	22.93	1.00	100
$(NH_4)_2SO_4$ (30-70%)	280.80	6.20	45.16	1.96	82
Biogel P-100	115.60	0.32	359.37	15.67	33.60
DEAE-Sephacel	53.00	0.08	662.50	28.89	15.40

*1U=1.0 µmol of γ-glutamylhydroxamate min^{-1} at 37°C.

3.3.6. Kinetic properties of S. bryopteris GS

The optimum pH for semisynthetase activity of GS was determined over a range of pH 2.5 to 8.5. The enzyme had pH optimum of 5.5. The enzyme exhibited 88.60 % and 66 % activity at pH 4.5 and 6.5, respectively, as compared to that at its optimum pH 5.5 (*Figure 3.7 a*). Effect of temperature on GS activity was also studied. For determining the effect of temperature, the GS reaction was carried out at different temperature ranging from 15 to 80 °C. The rate of reaction increased with increasing temperature and the calculated Q_{10} value (between 50 and 60 °C) was 1.13. The result in *Figure 3.7b* indicate that the optimum temperature for *Selaginella* GS was 60°C. At 70°C, GS gave 35% lower activity than that at its optimum temperature. Thermal stability of GS was determined by pre-incubating the partially purified enzyme preparation for 30 min. at the specified temperatures. The result in *Figure 3.7 c* reveals that activity of GS remains unaffected on pre-incubation of enzyme till 60 °C. Further increase in pre-incubating temperature to 70 °C resulted in significant loss in GS activity by about 80%. The Km value for glutamate as determined by Lineweaver-Burk plot was 2.4 mM (*Figure 3.7d*).

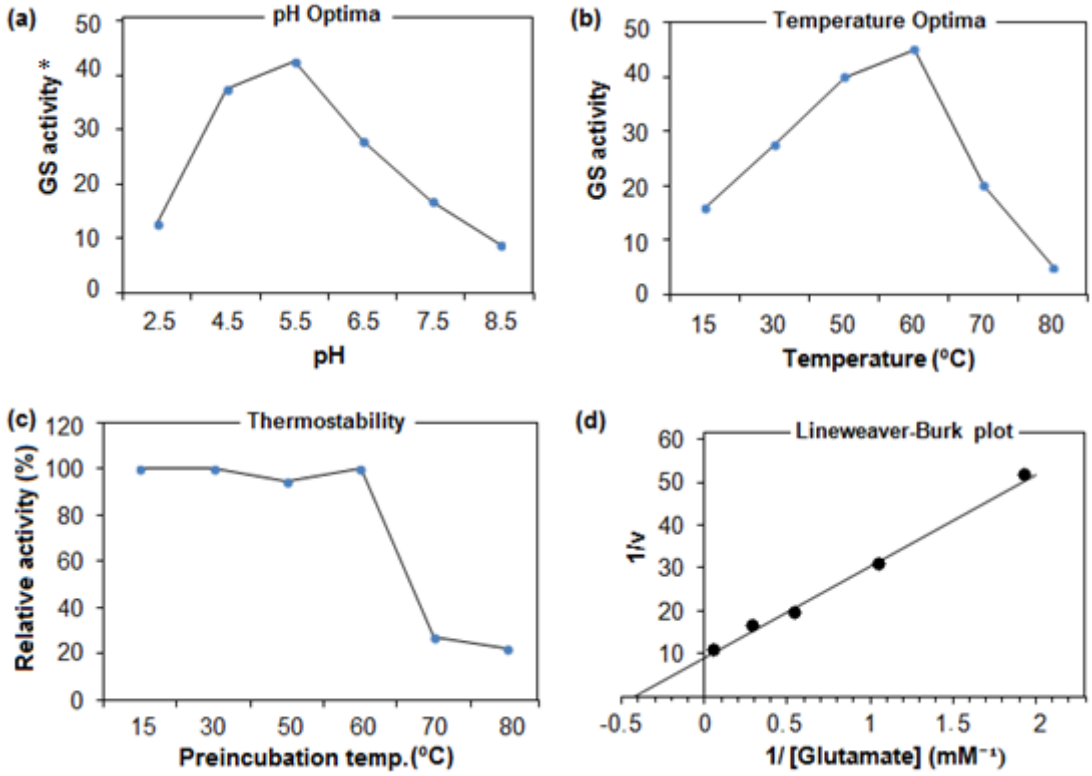

Figure 3.7. Kinetic properties of GS from *S. bryopteris* (a) Effect of pH on GS activity, (b) Effect of temperature on GS activity, (c) Effect of temperature on stability of GS, and (d) Km value for glutamate (Lineweaver-Burk plot). * One unit of GS activity represents 1.0 µmole of γ-glutamylhydroxamate produced 30 min^{-1}.

3.3.7. Sequence homology between GS of Selaginella and other angiosperms

Genome sequence analysis of *S. moellendorffii* has identified only ORF encoding GS (SmGS) (Grigoriev et al. 2011). Amino acid sequence of the ORF was aligned with the coding region of cytosolic and chloroplastic GS sequence in the GenBank database (*Table 3.2*) by using MUSCLE program (Robert 2004). From the results in *Figure 3.8* it is evident that SmGS sequence alignment highlighted amino acid residues His-249, Asp-56, Glu-297, Cys-92, Cys-303 and Cys-368, which are known to be highly conserved across all GS sequences. The polypeptide length and molecular weight were similar to that of cytosolic GS. In addition, SmGS lacked transit peptide sequence, the highly conserved motif for cleavage of transit peptide and lacked the highly conserved cysteine residues of chloroplastic GS2. Hence, SmGS encodes cytosolic GS isoform.

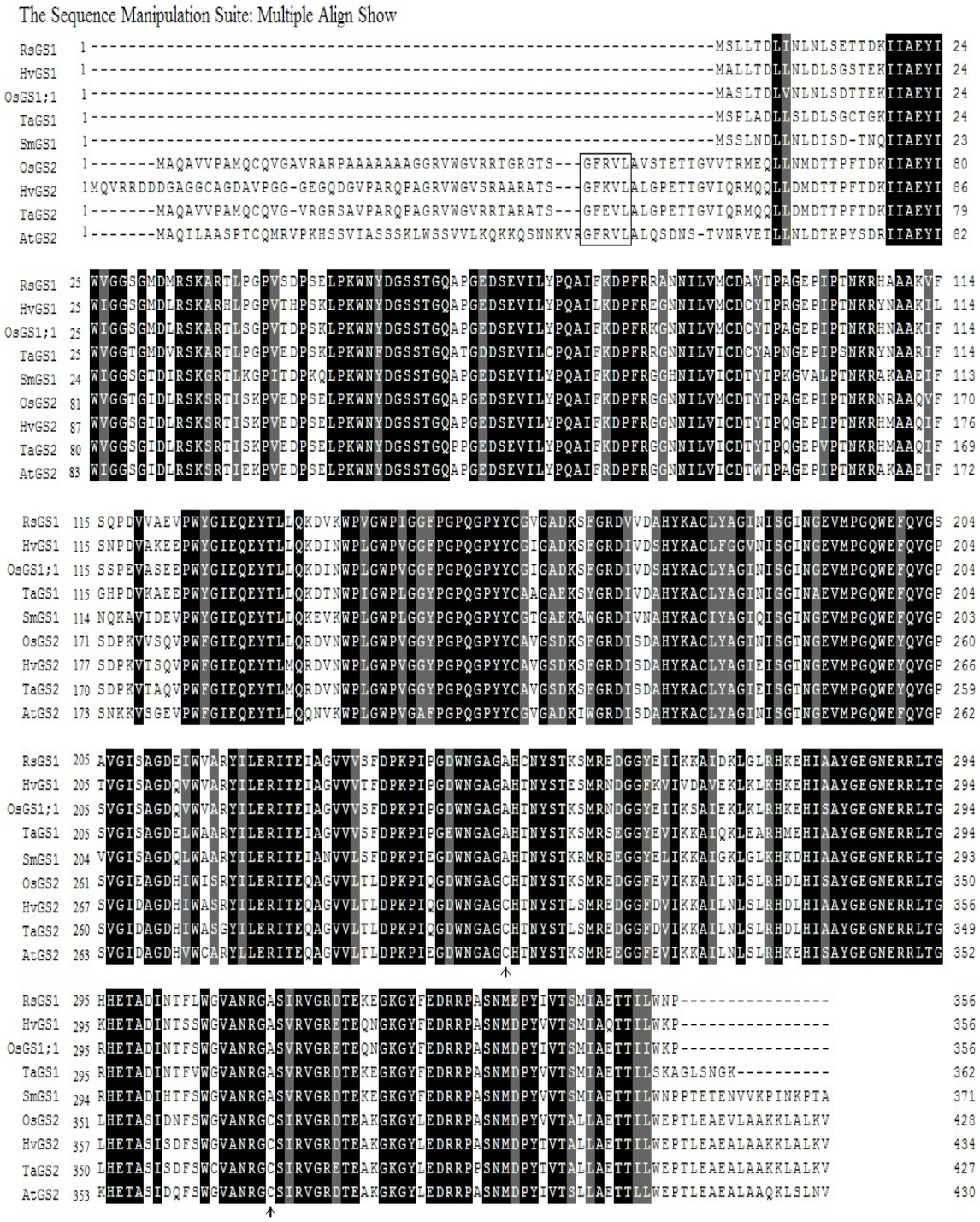

Figure 3.8. Alignment of SmGS1 of *Selaginella moellendorffii* with GS1 and GS2 proteins from different plants. The conserved motif indicating the position of cleavage site for the transit peptide are encircled by a box on GS sequences and the two cysteine residue conserved only in GS2 (Choi et al. 1999), is indicated by the two small arrows.

Table 3.2. Details of plants and accession numbers of GS amino acid sequences.

Species	Accession No.	Localization	References
Oryza sativa	P1465	Shoot	Sakomato et al. 1989
Selaginella moellendorffii	EFJ27072	Shoot	Grigoriev et al. 2011
Hordeum vulgare	Q06378	Shoot	Marigo et al. 1993
Triticum aestivum	AAR84349	Shoot	Bernard et al. 2008
Raphanus sativus	BAA04996	Shoot	Watanabe et al. 1994
Oryza sativa	P14655	Chloroplast	Sakomato et al. 1989
Hordeum vulgare	P13564	Chloroplast	Stroman et al. 1990
Triticum aestivum	AAZ30060	Chloroplast	Bernard et al. 2008
Arabidopsis thaliana	Q43127	Chloroplast	Peterman et al. 1991

3.3.8. Effect of dehydration-rehydration on GS/GOGAT activity and expression of GS

To examine the effect of hydration cycle on GS, *S. bryopteris* fronds were subjected to dehydration for 24 h (5% RWC) followed by rehydration for the same duration (95 % RWC) and GS activity was monitored at various stages of treatment. As can be seen from the result in *Figure 3.9 a*. the activity of GS didn't change markedly except at 24 h when it decreased to about 25 %. Its activity remained unchanged throughout the rehydration period. As the cytosolic GS has been shown to be operational along with NADH-dependent isoform of GOGAT and GS activity in *Selaginella* was localized in the cytosol, the activity profile of only NADH-GOGAT was examined. The NADH-GOGAT activity didn't alter significantly during dehydration-rehydration cycle (*Figure 3.9 b*). Immunoblot analysis of GS protein at various stage of dehydration-rehydration reveals that GS protein level increased marginally during dehydration with maximum 2 fold accumulation at 24 h treatment. Rehydration of frond for 3 h lowered of GS polypeptide content by 2 fold and the level remain maintained till the end of the treatment (*Figure 3.9 c, d*).

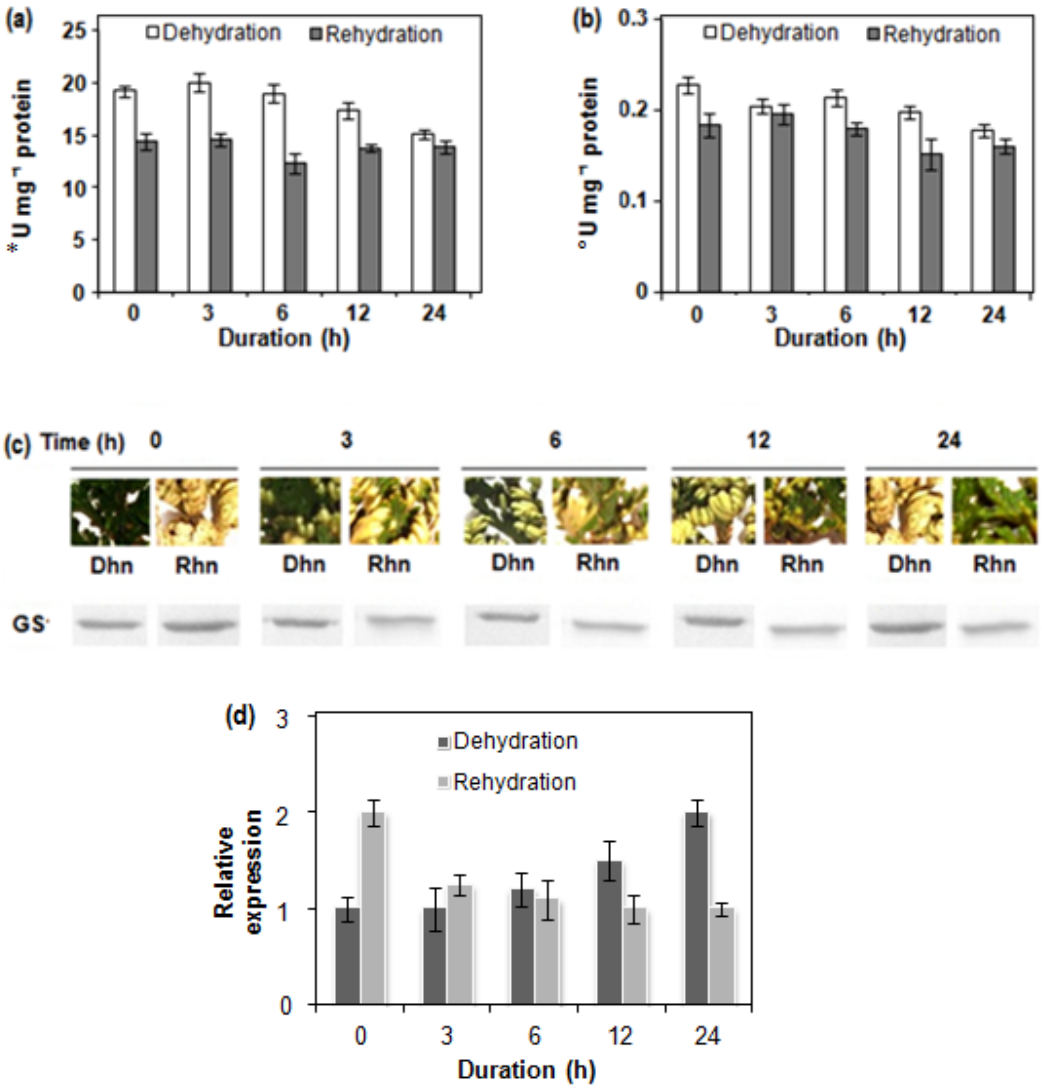

Figure 3.9. GS/GOGAT activities and GS expression during dehydration-rehydration of *S. bryopteris* fronds; (a) Total GS activity, (b) NADH-GOGAT activity, (c) Immunoblot analysis of GS, and (d) Bar diagram for GS polypeptide content. Each point is the mean of three samples ±SE. *One unit of GS activity represents 1.0 µmole of γ-glutamylhydroxamate produced 30 min^{-1} and °one unit of NADH-GOGAT activity represents 1.0 µmol of glutamate produced min^{-1} at 37 °C.

3.3.9. Effect of dehydration-rehydration of S. bryopteris fronds *on activity and expression of GDH*

The influence of dehydration-rehydration of fronds on aminating and deaminating activities of GDH was determined The aminating GDH activity was found to increase on dehydration and at 12 h became almost twice with no considerable alteration thereafter. Dehydration mediated enhancement in GDH activity was reversed on rehydration.

Rehydration of dehydrated fronds for 3h lowered the GDH activity by 50 % and the activity further reduced to 33 % at 24h of treatment (*Figure 3.10a*).

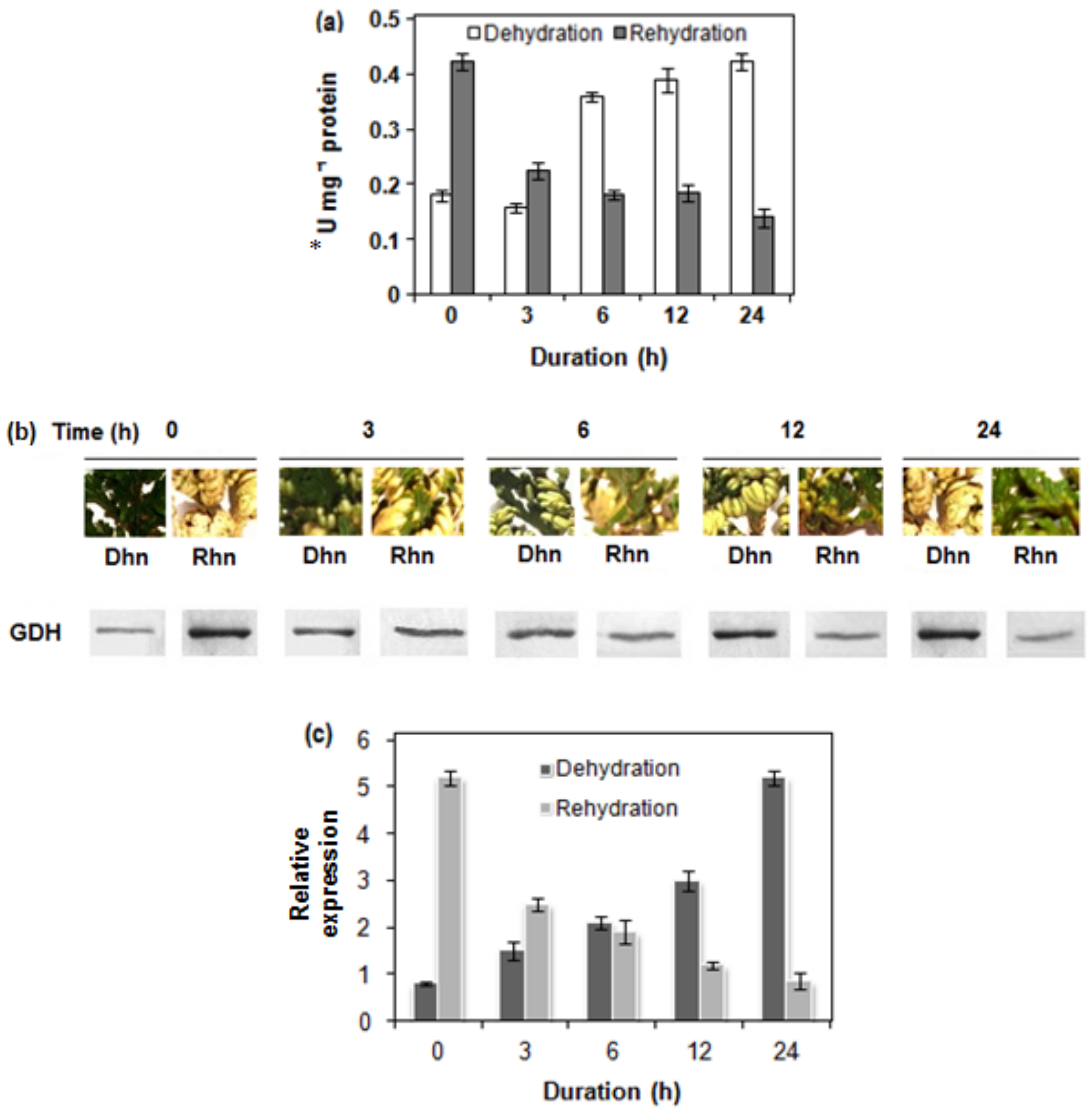

Figure 3.10. GDH activity and GDH expression during dehydration-rehydration of *S. bryopteris* fronds; (a) aminating GDH, (b) Immunoblot analysis of GDH, and (c) Bar diagram for GS polypeptide content. Each point is the mean of three samples ±SE. *One unit of GDH represents the reduction of 1 µmol NADH min^{-1} at 30^{o}C.

A comparison of GDH activity with corresponding protein content indicates that the time course of GDH protein correlated with GDH activity. Dehydration treatment for 12 h induced the GDH polypeptide expression by almost 2.5 folds, which again declined on rehydration (*Figure 3.10b*). The aminating GDH activity remained unaltered during entire dehydration-rehydration cycle.

3.4. DISCUSSION

Resurrection plants have evolved a wide spectrum of adaptations to cope with the challenges of environmental stress. They have unusual capability to survive long dry period and hence, can serve as a system for studying metabolic events during dehydration and rehydration. Several species of the *Selaginella* genus, including *S. lepidophylla, S. bryopteris and S. tamariscina,* have evolved the desiccation tolerance or ability to survive dry conditions (Yobi et al. 2012). They are able to recover complete physiological activity following repeated protoplasmic dehydration of fully differentiated tissues. Present study describes partial purification and properties of glutamine synthetase isolated from *S. bryopteris* fronds. Further studies were carried out to determine the regulation of GS expression during dehydration and rehydration of *Selaginella* fronds and its relationship with other enzymes of ammonia metabolism.

The dehydration-rehydration kinetics of *Selaginella* frond was determined by monitoring changes in RWC. The RWC decreased from 95 to 5 % within 24 h of dehydration treatment. However, fronds were able to recover completely from dehydration status by massive uptake of water within a much shorter duration of rewatering driven by the extreme osmotic gradient between the dried fronds and the environment. The results dehydration- rehydration kinetics was significantly different from that reported by Deeba et al (2009). They showed a relatively rapid rate of dehydration of the fronds in comparison to the present study. The deviation in the rate of RWC decline could be due to the differences in plant habitat or humidity level during sampling. Several resurrection plants, such as *Craterostigma plantagineum* (Norwood et al. 2000), *Xerophyta viscose* (Peters et al. 2007), *Sporobolus stapfianus* (Oliver et al. 2011) have the ability to survive almost complete (< 5%) tissue dehydration. However, most of the angiosperms cannot survive dehydration of their vegetative tissue to 20-30 % RWC, which translates to between -5 and -10 MPa (Proctor and Pence, 2002). Dehydration treatment caused significant changes in morphology of fronds, like, inward curling, disappearance of green colour and brownish appearance. The original morphology was regained on rehydration, with opening of adaxial surface and greening of the desiccated fronds. The folding of fronds during drying of plants has been proposed to prevent light-chlorophyll interaction and light-inducd damage (Farrant and Sherwin 1998). Hydration cycle mediated

phenotypic changes in detached fronds were not completely correlated to chl a and chl b contents. Although, the fronds were almost brown at 24 h of dehydration, they contained significantly high level of chl a and chl b indicating constitutive presence of the pigment molecules. As in the present investigations, constitutive presence of chl a and chl b and maintenance of their ratio during dehydration were also noted by Pandey et al (2010). Chlorophyll content has been shown to decline rapidly in plants under stress and during leaf senescence (Sims and Gamon 2010). The insensitivity of chlorophyll contents to dehydration might serve as one of the factor for dehydration tolerant characteristics of *S. bryopteris* plant. In contrast to chlorophyll pigments, dehydration resulted in severe decline in the level of carotenoid, which can be associated with browning of the fronds. The protein content of fronds reduced considerably during dehydration treatment. The breakdown of soluble and insoluble proteins and accumulation of amino acids during dehydration are features of many plants (Bernacchia 1996; Martinelli et al. 2007). However in resurrection plant *Sporobolus stapfianus*, the total soluble protein content declined at the beginning of desiccation and the decrease was reversed in the remaining step of dehydration (Martinelli et al. 2007). Present study showed a close relationship between the alteration in protein content and accumulation of ammonium. The dehydration mediated decline in protein level paralleled a significant increase in free ammonium content. Subsequent rehydration of the fronds lowered the ammonium content. Previous research work by Martinelli et al (2007) also showed dehydration mediated accumulation of ammonium in leaves of *Sporobolus stapfianus*, however, the fate of accumulated ammonium ions on rehydration of resurrection plants has not been studied so far. It has been well proposed that metabolite accumulation is one of the mechanisms for stress tolerance. Several solutes, such as, proline, glycine betaine, polyols, polyamines and ions (i.e. potassium), can accumulate during WD conditions. They act both by contributing to osmotic adjustment and by protecting proteins and cellular membranes. The beneficial roles of proline in conferring osmotolerance have been widely reported (Kishor et al. 1995; Bajji et al. 2000). *Selaginella* fronds were found to accumulate proline in markedly high quantity on dehydration. Recent study on metabolic profiling of *S. lepidophylla* during dehydration-rehydration cycle indicated the accumulation of several nitrogen rich amino acids including proline in the dehydrated fronds (Yobi et al. 2012). The increased accumulation of nitrogen-rich amino acids might reflect an adaptation to the

nitrogen-limiting conditions typically encountered by *Selaginella* and other dehydration tolerant species in their natural habitat of rocky outcrops with nitrogen-poor soils (Burke, 2002). Alternatively, these amino acids might provide a nitrogen reservoir useful during the early stages of rehydration before the recovery of photosynthetic activity (Martinelli et al. 2007).

The regulation of nitrogen metabolism enzymes was studied during dehydration and rehydration of *S. bryopteris* fronds. Initial screening of *S. bryopteris* frond extract by anion exchange chromatography indicated the presence of only one GS isoform. The cytosolic localization of the isoform was confirmed by the presence of GS activity only in the soluble fraction but not in the chloroplastic fraction of the fronds. Furthermore, immonoblot analysis of the total soluble protein as well as the GS activity fraction showed the presence of single band of molecular size about 39 kDa. GS is present as cytosolic (GS1) and chloroplastic (GS2) isoforms in most of the higher plant. GS1 is encoded by two to five genes, whereas there is a single nuclear gene encoding GS2 (Bernard et al. 2008; Bernard and Habash 2009). The multiplicity of the cytosolic isoforms couldn't be established in the present study. The information on isoforms of GS in resurrection lycophytes is limited. The complete genome sequence of *S. moellendorffi* has shown the presence of an ORF encoding GS (SmGS). Amino acid sequence corresponding to the ORF was aligned with other higher plant cytosolic and chloroplastic GS. The size of ORF was similar to cytosolic GS of higher plants. Moreover, the lack of chloroplastic GS2 specific transit peptide sequence, conserved cleavage site for transit peptide and other conserved amino acids, suggested cytosolic origin of SmGS.

The GS enzyme was partially purified from *S. bryopteris* fronds by ammonium sulphate precipitation, gel-filtration and ion-exchange chromatography. The kinetic properties of GS from *S. bryopteris* were considerably different from that of higher plants. The enzyme showed pH optima of around 5.5, which is slightly more acidic in comparison to cytosolic and chloroplastic GS from higher plants. The enzyme was remarkably thermostable as it didn't lose activity on pre-incubation at temperature upto 60 ^{o}C. At this temperature GS from most of the higher plants underwent almost complete denaturation (Lea et al. 1990). For example, rice leaves GS2 was highly heat labile and its activity

diminished by 80% of the initial activity after 15 min of treatment at 40 ^{o}C and it lost activity completely when kept for 60 min at this temperature (Hirel and Gadal 1980). GS from *S. bryopteris* showed higher considerably temperature optima of about 60 ^{o}C. The semi-synthetase activity of the enzyme showed hyperbolic response with increasing concentration of glutamate. The Km value for glutamate was lower than that reported for enzymes from leaves of higher plants (Hirel and Gadal 1980; Hirel and Gadal 1982; Cullimore et al. 1983; Ericson et al. 1985; Bedell et al. 1995) indicating higher affinity of the *Selaginella* GS for glutamate. Hence, GS from *S. bryopteris* was superior in kinetic properties as compared to that of higher plants. Purification and kinetic properties of GS from resurrection plants have not been reported earlier.

The effect of dehydration-rehydration cycle on activity and expression of GS was examined. The activity of GS did not alter markedly indicating its lower sensitivity to dehydration stress. The information on response of resurrection plant GS to dehydration is limited. In resurrection angiosperm *Sporobolus stafianus*, desiccation tolerant young leaves had higher total GS activity at the end of dehydration stress and were shown to maintain high chloroplastic and cytosolic GS protein content during the entire stress period (Martinelli et al. 2007). In contrast, GS from some higher plants has shown susceptibility to abiotic stress (Teixeira and Pereira 2007; present study). Total GS activity decreased during drought and salt stress and most of the decline was in GS2 activity. The reduction in GS2 activity correlated with corresponding polypeptide (Lutts et al. 1999; Santos et al. 2004). Immonoblot analysis of *Selaginella* GS at various stages of dehydration-rehydration indicated its increased accumulation during dehydration and reduction in level on subsequent rehydration. However, GS polypeptide content reduced on subsequent rehydration. As in the present study, increased expression of cytosolic GS transcript and polypeptide has been reported in higher plants during natural senescence and in response to biotic and abiotic stress (Bernard and Habash 2009). However, the enhanced expression didn't correlate with GS activity (Kamachi et al. 1991; Ishiyama *et al.* 2004a). As the cytosolic GS has been shown to be operational along with NADH-dependent isoform of GOGAT and in *Selaginella* fronds GS activity was present only in the cytosol, the activity profile of only NADH-GOGAT was examined. The NADH-GOGAT activity didn't alter significantly during dehydration-rehydration cycle. In

contrast, GOGAT activity was found to decline during dehydration in both desiccation tolerant young leaves and desiccation sensitive old leaves of *Sporobolus stapfianus* (Martinelli et al. 2007).

Since ammonium level increased during dehydration and both GS and GOGAT activity remained unaltered, it was likely that senescence-like process was induced during dehydration. However, the conditions seem to be reversed by significant increase in immunoreactive α-GDH polypeptide and aminating GDH activity. The aminating GDH activity increased progressively from the beginning of dehydration and became about 2 fold by the end of dehydration. The dehydration mediated increase in GDH activity was reversed on rehydration. A comparison of GDH activity with corresponding protein content indicates that the time course of GDH protein correlated with GDH activity. The desiccation tolerant young leaves of *Sporobolous stapfianus* also showed higher activity of both aminating and deaminating GDH in comparison to desiccation sensitive older leaves (Martinelli et al. 2007). The dehydration mediated enhancement in aminating GDH activity in the present investigations could be related to increased protein degradation producing high concentration of ammonium and α-ketoglutarate from deamination of aminoacids. The contention is supported by research work of Yobi et al (2012) reporting increased accumulation of α-ketoglutarate during dehydration of desiccation tolerant *Selaginella lepidophylla*. There are reports on increased aminating activity and expression of GDH under intracellular hyperammonia conditions due to either exogenous ammonium, senescence induced high proteolytic activity or abiotic stress (Kumar et al. 2000; Hoai et al. 2003; Skopeltis et al. 2006). The aminating GDH activity increased with increasing salt stress in salt-tolerant rice (*Oryza sativa*) cultivar, whereas it decreased in the salt-sensitive one (Kumar et al. 2000). Furthermore, high NaCl has been shown to induce the formation of reactive oxygen species, which in turn induces the synthesis of GDH in tobacco and grapevine. The inhibition of GS in these plants resulted in the incorporation of ammonia via GDH into [^{15}N] glutamate and [^{15}N] proline in the presence of high salt (Skopelitis et al. 2006). Hence, the enhanced level of proline during dehydration of *S. bryopteris* fronds could also be correlated with enhanced GDH activity. Present study concludes that *S. bryopteris* frond contained only one isoform of GS localised in cytosol. The enzyme was superior in kinetic properties in comparison to that of higher plants. The enzyme showed

tolerance to dehydration. The activity GS /GOGAT remained largely unaltered in dehydration treated *Selaginella* fronds. Moreover, GDH aminating activity and expression were increased during dehydration. GDH thus seems to be involved in amelioration of toxicity of excess ammonia generated during dehydration. The relatively maintained GS/GOGAT activities and increased activity and expression of GDH also correlate with dehydration mediated increase in glutamate, the substrate for proline biosynthesis. Hence, GDH along with GS/GOGAT plays important role in regulation of ammonia assimilation during WD in *S. bryopteris*.

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General Discussion and Conclusion

Nitrogen is an essential nutrient for plant growth and productivity. In higher plants inorganic nitrogen is assimilated predominantly via Glutamine synthetase (GS; EC 6.3.1.2)/ Glutamate synthase (Glutamate oxo-glutarate ammonium transferase, GOGAT; EC 1.4.7.1) pathway. In higher plants, GS exists in multiple isoforms that are either cytosolic (GS1) or plastidic (GS2). GS isoforms are also differentially regulated in response to environmental signals, such as, nitrogen status, light, biotic and abiotic stress.

Drought is important environmental stress factors limiting plant productivity in cultivated areas worldwide. The stress condition is due to soil water deficit, increased daytime temperature and reduced nutrient availability. Among these water deficit (WD) is the most important factor for plant growth (Oliver et al. 2011). The prolonged period of WD leads to limited uptake of nitrogen from soil and reduced availability of CO_2 for photosynthesis as stomata are induced to close followed by disturbances in carbon and nitrogen metabolism (Foyer et al.1998; Xu and Zhou 2006). Acclimation to WD requires responses that allow the primary metabolism to continue. Recent studies have shown nitrogen assimilation to be critical for plant acclimation to stress conditions. The enzyme GS constitutes a regulatory point at which environmental signals are integrated and translated into a plant response in terms of growth and productivity (Swarbreck et al. 2011).

Rice (*Oryza sativa* L.) plant growth and productivity are adversely affected by WD. The effect of WD varies with the variety, degree, and duration of stress and its coincidence with different growth stages (Gao et al. 2007). NH_4^+ is the main form of nitrogen available to the young rice plants, which is assimilated by GS to glutamine. Glutamine serves as the main form of organic-nitrogen for transport through vascular tissues (Ishiyama et al. 2004b). Rice plants possess one gene (OsGS2) for chloroplastic GS2. The cytosolic GS1 gene family consists of three isoforms encoded by OsGS1;1, OsGS1;2 and OsGS1;3. These isoforms have been shown to be regulated in a developmentally controlled manner as well as by light and nitrogen nutrition (Kamachi et

al. 1991; Ishiyama et al. 2004a, b; Kusano et al. 2011). However, the regulation of these isoforms during WD has not been investigated in detail.

Resurrection plants have evolved a wide spectrum of adaptations to cope with the challenges of environmental stress (Oliver et al. 2011; Yobi et al. 2012). *Selaginella bryopteris* belonging to the family Selaginellaceae, is a resurrection lycophyte growing in the hilly area of tropical regions (Deeba et al. 2009). A useful insight into the role of GS isoforms under WD conditions can be obtained by studying the enzyme in *S. bryopteris*. Present investigations were carried out primarily to gain some insight into the regulation of glutamine synthetase isoforms during WD in two differentially drought tolerant rice (*Oryza sativa* L.) cultivars and in *S. bryopteris* (L.).

Rice cultivars were screened for their drought tolerance characteristics by monitoring changes in leaf relative water content (RWC), electrolyte leakage, proline and protein contents. During water deficit, RWC, protein and proline level declined markedly and the electrolyte leakage increased sharply in *O. sativa* cv. IR-64. Under similar condition, *O. sativa* cv. Khitish variety maintained relatively higher RWC, protein and proline level and showed least leakage of electrolyte. The morphological changes associated with WD were more prominent in IR-64 seedling in comparison to Khitish seedlings. Hence, IR-64 and Khitish cultivars were designated as drought-sensitive and tolerant-cultivar, respectively. These two cultivars were used further to study the effect of WD on regulation of GS isoforms.

GS was extracted from root, stem and leaf of three weeks old rice seedlings. The isoforms of GS in leaf, stem and root extracts were separated by anion-exchange chromatography on DEAE-Sephacel column using gradient elution technique. In leaf and stem extract, the enzyme was resolved into two peaks corresponding to cytosolic GS1 and chloroplastic GS2. In root extract entire activity was eluted as single peak at the same salt concentration as GS1. Rice leaves contained 25 and 75 % of activity as GS1 and GS2, respectively. In stem most of the GS activity (70 %) was present as GS1 and rest 30 % activity represented as GS2.

The transcript levels of GS isoform in various organs of rice plant were determined by RT-PCR. Among GS1 gene family, OsGS1;1 and OsGS1;2 transcripts were present in all the three organs, whereas OsGS2 was expressed in leaf and stem but not in root. In leaf tissue the OsGS2 mRNA level was considerably higher than that of cytosolic isoforms,

but it was present as minor form in stem. Among the GS1 isoforms OsGS1;1 was more abundant than OsGS1;2 in leaf. OsGS1;2 was the predominant form in stem and root. However, OsGS1;1 mRNA level was significantly greater in root and stem of IR-64 seedlings than that of Khitish.

The polypeptide levels of GS1 and GS2 isoforms in leaves, stems and roots of rice seedlings were determined by immunoblotting. Two GS protein bands of approximately 39 and 43 kDa that correspond to the molecular size of GS1 and GS2, respectively, were present in leaf and stem. The immunoblot analysis of root protein highlighted only one protein band corresponding to GS1 isoform. As the GS antibody was developed against conserved GS polypeptide, cytosolic isoforms could not be distinguished in immunoblot.

Effect of WD on total GS, GS1 and GS2 activities in leaf, stem and root of IR-64 and Khitish seedlings were determined. WD had a profound effect of total GS activity in leaves and roots of IR-64 seedlings as compare to that of Khitish. The rapid reduction in GS activity of IR-64 leaf was mainly due to disappearance of GS2 activity. The GS2 activity decrease in IR-64 leaf was correlated with decreased level of GS2 mRNA and protein. As for activity, the GS2 mRNA and protein remained almost unaltered in Khitish leaf. The results suggested WD mediated GS2 regulation mainly at the transcriptional and /or mRNA stability levels. GS2 is known to catalyse the rate limiting step of photorespiration i.e. reassimilation of ammonia. The photorespiration activity has been reported to be induced by abiotic stress and plays a protective role. Hence, a relatively unaltered activity and expression of GS2 in Khitish leaf could maintain the photorespiratory capacity of the plant at limited water availability that improves tolerance of the cultivar to WD.

Although, WD treatment increased the expression of both OsGS1;1 and OsGS1;2 transcripts in leaf of IR-64 and Khitish cultivars, expression level of OsGS1;1 mRNA was considerably higher in IR-64 leaf. The increased OsGS1;1 in IR-64 leaf could be due to its higher rate of protein degradation (*Figure 2.12*), conforming to role of the isoform in reassimilation of nitrogen released from protein breakdown. The time course of increase in GS1 transcripts corresponded with the accumulation of GS1 protein detected on Western blot. WD mediated increase in GS1 transcript and polypeptide was related to the maintenance of GS1 activity rather than increase. A similar type of response to GS1 mRNA over expression was observed in *Arabidopsis* root. In this tissue the nitrogen

nutrition mediated increase in GS1 transcript and polypeptide was related to the maintenance of GS1 activity rather than increase (Ishiyama et al. 2004a).

Total GS activity in stem of IR-64 and Khitish seedlings was almost unaffected by WD. However, alteration in both GS1 and GS2 activities was noted in stem of IR-64 seedling. GS1 activity was reduced and GS2 activity enhanced on stress application. The decline in GS1 activity was compensated by increase in GS2 activity, thus maintaining almost unchanged total GS activity. A varietal variation was noted in expression of OsGS1;1 and OsGS2 in response of WD. OsGS1;1 transcript content declined in IR-64 and enhanced in Khitish with the intensification of stress. The repression in OsGS1;1 in IR-64 stem might result from remobilization and transport of high concentration of glutamine to stem and root from increased protein degradation in IR-64 leaf. The reduction in OsGS1;1 mRNA in IR-64 stem was not reflected in GS1 polypepeptide abundance. The results thus indicated a different metabolic status of stem during WD maintaining a constant GS activity. A tissue specific response of WD has been noted earlier with role of stem in survival of plant under stress (Pinheiro et al. 2001).

The cytosolic GS1 was the only GS isoform in rice root. The GS activity in roots of IR-64 cultivar was quite sensitive to WD and reduced to more than half at the end of treatment. As in stem, the mRNA level of OsGS1;1 differed in root of the two cultivars. Its level was initially higher in IR-64 that declined with WD. WD mediated decrease in GS1 activity in stem and root of IR-64 seedling might correlate with reduction in OsGS1;1 mRNA level. The GS1;1 gene has been previously reported to be involved in remobilization and reutilization of nitrogen in rice plant during senescence (Tabuchi et al. 2007). Moreover, a comparison of kinetic properties of OsGS1;1 and OsGS1;2 encoded GS isoforms in rice root has shown that OsGS1;1 exhibit higher Vmax and lower Km value for substrate (Ishiyama et al. 2004b). Hence, OsGS1;1 seems to play significant role in performance of plant under stress condition.

In the present study, the two rice varieties did not differ much in expression pattern of OsGS1;2 during WD, despite difference in their drought tolerance properties. Its transcript level was found to enhance in leaf and stem. However, in root the highly expressed OsGS1;2 was found to decline initially followed by an increased accumulation on further dehydration. Although, the time course of OsGS1;2 mRNA was reflected in GS1 polypeptide abundance, it was not correlated with total GS activity in all the three

tissues. The result is supported by study of Ishiyama et al (2004b) on effect of ammonium nutrition in Arabidopsis root GS isoforms. The time course increase in GS1;2 mRNA corresponded with the accumulation of GS1 protein detected on the western blot. However, the increase in GS1;2 mRNA and protein was not correlated with total GS1 activity. The lack of correlation could be due to lower affinity of GS1;2 for ammonium as compared to other isoform.

The effect of WD in regulation of GS of *S. bryopteris* was also studied. Preliminary experiment was carried out to determine the dehydration-rehydration kinetics of *S. bryopteris* fronds by measuring relative water content (RWC). The RWC decreased from 95 to 5 % within 24 h of dehydration treatment. However, fronds were able to recover completely from dehydration status by massive uptake of water within a much shorter duration of rewatering driven by the extreme osmotic gradient between the dried fronds and the environment. Dehydration treatment caused significant changes in morphology of fronds, like, inward curling, disappearance of green colour and brownish appearance. The original morphology was regained on rehydration, with opening of adaxial surface and greening of the desiccated fronds. Dehydration mediated browning of fronds was not completely correlated to the change in chl a and chl b contents. As in the present investigations, constitutive presence of chl a and chl b and maintenance of their ratio during dehydration were also noted by Pandey et al (2010). The protein content of fronds reduced considerably during dehydration and this decline paralleled significant increase in free ammonium content. *Selaginella* fronds were found to accumulate proline in markedly high quantity on dehydration. Recent study on metabolic profiling of *S. lepidophylla* during dehydration-rehydration cycle indicated the accumulation of several nitrogen rich amino acids including proline in the dehydrated fronds (Yobi et al. 2012). The increased accumulation of nitrogen-rich amino acids might reflect an adaptation to the nitrogen-limiting conditions typically encountered by *Selaginella* and other dehydration tolerant species in their natural habitat of rocky outcrops with nitrogen-poor soils (Burke, 2002).

The regulation of GS was studied during dehydration and rehydration of *S. bryopteris* fronds. Anion-exchange chromatography followed by immunoblot analysis of GS indicated the cytosolic localization of GS. Alignment of GS amino acid sequence of *Selaginella* in database with that of higher plant cytosolic and chloroplastic GS suggested cytosolic origin of SmGS. GS was partially purified from *S. bryopteris* fronds by ammonium sulphate precipitation, gel-filtration and ion-exchange chromatography. The enzyme showed pH optima of around 5.5. The enzyme was remarkably thermostable as it

didn't lose activity on pre-incubation at temperature upto 60^{o}C. GS from *S. bryopteris* showed considerably higher temperature optima of about 60 ^{o}C. The Km value (2.4 mM) for glutamate was lower than that reported for enzymes from leaves of higher plants (Cullimore et al. 1983; Ericson et al. 1985; Bedell et al. 1995). The effect of dehydration-rehydration cycle on activity and expression of GS was examined. The activity of GS didn't alter markedly indicating its lower sensitivity to dehydration stress. Immonoblot analysis of *Selaginella* GS at various stages of dehydration-rehydration indicated its increased accumulation during dehydration and reduction in level on subsequent rehydration. A lack of correlation between GS expression and activity has been reported earlier (Kamachi et al. 1991; Ishiyama et al. 2004a; Bernard and Habash 2009). As in case of GS, NADH-GOGAT activity didn't alter significantly during dehydration-rehydration cycle.

Since GS and GOGAT activity remained unaltered and ammonia concentration was increased, it was likely that senescence-like process was induced during dehydration. In plants, glutamate dehydrogenase (GDH) constitutes an alternative route for ammonia assimilation. Both GDH aminating activity and polypeptides were found to increase during dehydration and thus seem to reverse the condition of ammonia toxicity. The dehydration mediated increase in GDH activity was reversed on rehydration. The dehydration mediated enhancement in aminating GDH activity in the present investigations could be related to increased protein degradation producing high concentration of ammonium and α-ketoglutarate from deamination of amino acids. The contention is supported by research work of Yobi et al (2012) reporting increased accumulation of α-ketoglutarate during dehydration of desiccation tolerant *Selaginella lepidophylla*. In another study, the inhibition of GS in grapevine plant resulted in the incorporation of ammonia via GDH into [^{15}N] glutamate and [^{15}N] proline in the presence of high salt (Skopelitis et al. 2006). Hence, the enhanced level of proline during dehydration of *S. bryopteris* fronds could also be correlated with enhanced GDH activity.

From the present investigations, it can be concluded that in rice plant the regulation of GS isoforms by WD was organ specific. Two GS isoforms i.e. GS1;1 and GS2 were differentially regulated in drought-sensitive and -tolerant rice cultivars. GS2 is the major GS isoform in leaf and its over-expression in leaf has been found to be associated with the maintenance of photosynthetic and photorespiratory capacity of the plant. The GS1;1 isoform has been shown to be involved in remobilization and reutilization of nitrogen. A higher substrate affinity of the enzyme for ammonium signifies its promotion of the rapid

conversion of ammonium to glutamine even under low ammonium conditions. Hence, it can be inferred that a relatively maintained OsGS2 and the over-expression of OsGS1;1 may contribute to improved drought tolerance characteristics of *Oryza sativa* cv. Khitish.

GS from *S. bryopteris* showed tolerance to dehydration. The activity GS /GOGAT remained largely unaltered in dehydration treated *Selaginella* fronds. Moreover, GDH aminating activity and expression were increased during dehydration. GDH thus seems to be involved in amelioration of toxicity of excess ammonia generated during dehydration. The relatively maintained GS/GOGAT activities and increased activity and expression of GDH also correlate with dehydration mediated increase in glutamate, the substrate for proline biosynthesis. Hence, GDH along with GS/GOGAT plays important role in regulation of ammonia assimilation during WD in *S. bryopteris.* A comparison of GS of *S. bryopteris* and *O. sativa* indicated that in *S. bryopteris* GS was only present in cytosol. The properties of cytosolic GS from the two sources also varied. GS from *S. bryopteris* was remarkably thermostable as it didn't lose activity on pre-incubation at temperature upto 60^{o}C. At this temperature GS from rice plants undergo almost complete denaturation (Lea et al. 1990). The temperature optima of *Selaginella* GS was also higher than that of rice. *Selaginella* enzyme showed more affinity for the substrate glutamate. The susceptibility of GS to WD in *O. sativa* was correlated to their drought tolerance properties. In contrast, the enzyme from *S. bryopteris* showed insensitivity to WD.

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Abstract

Water deficit (WD) has adverse effect on rice (*Oryza sativa* L.) and acclimation requires essential reactions of primary metabolism to continue. Rice plants utilize ammonium as major nitrogen source, which is assimilated into glutamine by the reaction of Glutamine synthetase (GS, EC 6.3.1.2). Rice plants possess one gene (OsGS2) for chloroplastic GS2 and three genes (OsGS1;1, OsGS1;2 and OsGS1;3) for cytosolic GS1.Here, we report the effect of WD on regulation of GS isoforms in drought-sensitive (cv.IR-64) and drought-tolerant (cv. Khitish) rice cultivars. Under WD, total GS activity in root and leaf decreased significantly in IR-64 seedlings in comparison to Khitish seedlings. The reduced GS activity in IR-64 leaf was mainly due to decrease in GS2 activity, which correlated with decrease in corresponding transcript and polypeptide contents. GS1 transcript and polypeptide accumulated in leaf during WD, however, GS1 activity was maintained at a constant level. Total GS activity in stem of both the varieties was insensitive to WD. Among GS1 genes, OsGS1;1 expression was differently regulated by WD in the two rice varieties. Its transcript accumulated more abundantly in IR-64 leaf than in Khitish leaf. Following WD, OsGS1;1 mRNA level in stem and root tissues declined in IR-64 and enhanced in Khitish. A steady OsGS1;2 expression patterns were noted in leaf, stem and root of both the cultivars. Results suggest that OsGS2 and OsGS1;1 expression may contribute to drought tolerance of Khitish cultivar under WD conditions.

Resurrection plants have evolved a wide spectrum of adaptations to cope with the challenges of environmental stress and can serve as a system for studying metabolic events during dehydration and rehydration. *Selaginella bryopteris* belonging to the family Selaginellaceae, is a resurrection lycophyte growing in the hilly area of tropical regions. In present investigation attempts were made to isolate and characterize GS from S. *bryopteris*. The regulation of GS expression during dehydration and rehydration of *Selaginella* fronds and its relationship with other enzymes of ammonium metabolism were also determined. The RWC of the *S. bryopteris* fronds decreased from 95 to 5 % within 24 h of dehydration treatment. Dehydration condition resulted in decrease in the total protein content. The decline in protein level paralleled a significant increase in free ammonium content. The level of proline also enhanced during dehydration. The effects

were reversible with concentrations returning to level comparable with that of full turgor state 24 h after the water deficit condition was alleviated. Anion-exchange chromatography of *S. bryopteris* frond extract revealed the presence of only cytosolic GS isoform. Furthermore, immunoblot analysis showed the presence of single band of molecular size about 39 kDa. The GS enzyme was partially purified from *S. bryopteris* fronds by ammonium sulphate precipitation, gel-filtration and ion-exchange chromatography. The partially purified enzyme showed pH optima of around 5.5. The enzyme was remarkably thermostable as it didn't lose activity on pre-incubation at temperature upto 60 ^{o}C. GS from *S. bryopteris* showed considerably higher temperature optima of about 60 ^{o}C. The Km value (2.4 mM) for glutamate was lower than that reported for enzymes from leaves of higher plants. In dehydration treated fronds, the activity of glutamine synthetase and glutamate synthase (GS /GOGAT), the main enzymes for ammonium assimilation remained largely unaltered. In contrast, glutamate dehydrogenase (GDH) aminating activity and polypeptide content increased from the beginning and became almost two fold at 24 h of dehydration treatment thus providing an alternative route for assimilation of ammonia. Results suggested that GS/GOGAT along with GDH may play important role in amelioration of ammonia toxicity and enhanced production of proline observed during dehydration.

Appendix A

LIST OF ABBREVIATIONS

ºC	Degree Celsius
µg	Microgram
µl	Micro liter
µM	Micro molar
µmol	Micromole
Å	Angstrom
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ABA	Abscisic acid
ADP	Adenosine-5'-diphosphate
APS	Ammonium per sulfate
ATP	Adenosine-5'-triphosphate
BAC	Bacterial artificial chromosome
BC	Before Christ
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine
BSA	Bovine serum albumin
C	Control
C/N	Carbon/Nitrogen
C1	Conductivity 1
C2	Conductivity 2
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
Chl a	Chlorophyll a
Chl b	Chlorophyll b
cm	Centimeter
CO_2	Carbon dioxide
cv	Cultivar
DEAE-Sephacel	Diethylaminoethyl-Sephacel
dry wt.	Dry weight
dT	deoxy-thymine nucleotide
Dhn	Dehydration
DT	Drought tolerance
EDTA	Ethylene diamine tetraacetic acid
Fd	Ferredoxins
$FeCl_3$	Iron (III) chloride
FW	Fresh weight
g	Gram
GDH	Glutamate dehydrogenase
GOGAT	Glutamate oxo-glutarate amino transferase
GS	Glutamine synthetase
GS1	Cytosolic glutamine synthetase

GS2	Chloroplastic glutamine synthetase
h	Hour
HCl	Hydrochloric acid
IPTG	Isopropyl thio-β-galactoside
KCl	Potassium chloride
kDa	Kilo Dalton
Kh C	Khitish control
Kh S	Khitish stress
K_m	Michaelis constant
m	Meter
M	Molar
Mbp	Mega base pairs
Me^{2+}	Magnesium/manganese ion
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mM	Millimolar
Mn^{2+}	Manganese ion
$MnCl_2$	Manganese chloride
MPa	Mega pascal
mRNA	Messenger RNA
N_2	Nitrogen
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NBT	Nitro-blue tetrazolium chloride
ng	Nano gram
NH_2OH	Hydroxylamine
NH_4^+	Ammonium
NH_4Cl	Ammonium chloride
nm	Nanometer
NO_3^-	Nitrate
OP	Osmotic potential
ORF	Open reading frame
OsGS1;1	Gene of cytosolic glutamine synthetase in *Oryza sativa*
OsGS1;2	Gene of cytosolic glutamine synthetase in *Oryza sativa*
OsGS1;3	Gene of cytosolic glutamine synthetase in *Oryza sativa*
OsGS2	Gene of chloroplastic glutamine synthetase in *Oryza sativa*
PCR	Polymerase chain reaction
Pi	Inorganic phosphate
Pok C	Pokkali control
Pok S	Pokkali stress
PVDF	Polyvinylidene difluoride
Q_{10}	Temperature coefficient
RNA	Ribonucleic acid

RNase	Ribonuclease
Rhn	Rehydration
rpm	Revolution per minute
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Reverse transcription- polymerase chain reaction
RuBisCo	Ribulose-1,5-bisphosphate carboxylase oxygenase
RWC	Relative water content
s	Second
S	Stress
Sat C	Satabdi control
Sat S	Satabdi stress
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SE	Standard error
SE-Buffer	*Sellaginella* extraction buffer
sec	Second
TAE	Tris-acetate-EDTA
TCA	Tri-carboxylic acid
TEMED	Tetramethylethylenediamine
TP	Turgor pressure
TPS	Trehalose-6-phosphate synthase
Tri C	Triguna control
Tri S	Triguna stress
Tris-HCl	tris (hydroxymethyl) amino methane
TW	Turgid weight
U	Unit
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
WD	Water-deficit
WP	Water potential
WT	Wild type
X	5-bromo-4-chloro-indolyl-β-D-galactopyranoside
γ	Gamma
α	Alpha

Appendix B

THESIS RELATED PUBLICATIONS/ ABTRACTS/ PROCEEDINGS

(A) Publications

1. **Kamal Krishna Singh** and Shilpi Ghosh (2013) Differential regulation of glutamine synthetase isoforms in drought tolerant and sensitive rice (Oryza sativa) cultivars under water deficit conditions. *Plant Cell Reports* 32: 183-193

2. Shilpi Ghosh and **Kamal Krishna Singh** (2013) Glutamate metabolism in fronds of resurrection plant *Selaginella bryopteris* in response to dehydration and rehydration. *Plant Biology* (***Communicated***)

(B) Abstracts/ proceedings

1. **K. K. Singh** and S. Ghosh (2010) Differential regulation of glutamine isoenzymes in *Oryza sativa* L. seedlings in relation to water-deficit stress. In abstract volume of National Symposium on "New perspective in Plant research" held on 29-31 January, 2010, organized by Centre for Applied Science and Technology with Indian Association of Biological Sciences, at the University College of Science, Kolkata, India. (Poster)

2. S. Ghosh and **K. K. Singh** (2011) Regulation of isoenzymes of glutamine synthetase during dehydration stress in rice seedlings: A comparison between drought sensitive and tolerant cultivar. In abstracts volume of National Symposium on "Advancement in plant pathology and stress management" held on 22-24 September, 2011, organized by Department of Botany, University of North Bengal, Siliguri, India. (Oral)

3. **K. K. Singh**, B. K. Pandey and S. Ghosh (2013) Metabolic shift of ammonium assimilation in resurrection plant *Selaginella bryopteris* in response to dehydration. In abstracts volume of National Seminar on "Micro and Macro Resources in Biomolecular Technology" held on 25-26 February, 2013, organized by Departments of Biotechnology & Microbiology, University of North Bengal, Siliguri, India. (Poster)

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PREFACE

I joined Dr. Ghosh's lab at Department of Biotechnology, University of North Bengal in August 2007. Here, I interacted routinely with lots of experiments and tried to take a tangential taste of intermingled problems of biological science, especially plants. Living beings are fast reactive because when a seed is left in the environment, it interacts with moisture, air, light and enzymatic thunderings inside it gives root and shoot outside. On the other hand, plants are authorized by nature in this universe to trap solar energy and supply it to us; so we can call them, Almighty in terms of energy. In spite of adverse conditions, they survive for years at the same place due to cellular brilliancy of stress management, unfortunately they are also caught by stresses at times. Similarly, the work was also perturbing and stressful for me, though I was not sessile like plants. Anyway, I enjoyed the job and it's my pleasure to have the opportunity to express my gratitude to all of them who assisted me.

I am highly grateful to my supervisor Dr. Shilpi Ghosh, Assistant Professor, Department of Biotechnology, North Bengal University, by whose grace I completed my research work successfully. It gives me immense pleasure to express my indebtedness to her for giving valuable suggestions and constant guidance during the entire course of study. It was not possible to complete this journey without her guidance. It is difficult for me to write in few words my gratitude towards her.

I would like to pay my sincere thanks to the H.O.D., Dr. Ranadhir Chakraborty, Department of Biotechnology, North Bengal University, who inspired me always to do best in life. And also, thanks to the faculty members; Dr. Dipanwita Saha and Dr. Anoop Kumar, Department of Biotechnology, University of North Bengal for their active support to carry out the research.

I express my sincere thanks to the Vice chancellor, Registrar, Science Dean, University of North Bengal, for granting me opportunity to carry out the research work in this university.

I am very grateful to Dr. Joydeb Pal, Dr. Tapas Kumar Chaudhuri and Dr. Min Bahadur, Department of Zoology, University of North Bengal; Dr. Arnab Sen, Department of Botany and Dr. Ashish Nanda, Department of Chemistry, University of North Bengal, for their kind support and infrastructural aid.

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Singh, Mr. Sunil Kumar, Mr. Kripa Shankar Singh and Dr. Chandramani Kumar, Mr. Chandan Kumar for being with me when nothing seemed to be going right.

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I sincerely thank Mrs. Sujata Singh, my wife, for her sincere encouragement. She had shouldered my errands and gave me her valuable company in all my dire times; all her direct and indirect cooperation helped me to perk up my research. And lots of love to my little confidence, Astha. Words cannot express my thanks to my father-in-law Sri Ashok Singh and my mother-in-law Smt. Barmati Singh for all their love and encouragement.

Heartiest thanks to my aunty Late Shanti Devi and Jiju, Late Shivvash Singh; your absence will always be a matter of immense regret for me. And I extend the hand of special thanks to my family members; Sri Shivpujan Singh, Sri Shambhu Nath Singh, Sri Prem Chand Singh, Sri Manik Chand Singh and rest of the family members for their munificent support during my nasty situation.

Finally, I would like to thank and express 'inexpressible' gratitude to my father Sri Arjun Singh and my mother Smt. Roopkali Devi for perpetual support in all directions.

The victory really becomes more pleasurable when you strike the target in very tough situations. Anyway, the moment becomes truly exceptional, when, imaginings turn into reality.

Kamal Krishna Singh

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ORIGINAL PAPER

Regulation of glutamine synthetase isoforms in two differentially drought-tolerant rice (*Oryza sativa* L.) cultivars under water deficit conditions

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Abstract
Key message **The regulation of GS isoforms by WD was organ specific. Two GS isoforms i.e. OsGS1;1 and OsGS2 were differentially regulated in IR-64 (drought-sensitive) and Khitish (drought-tolerant) cultivars of rice.**
Abstract Water deficit (WD) has adverse effect on rice (*Oryza sativa* L.) and acclimation requires essential reactions of primary metabolism to continue. Rice plants utilize ammonium as major nitrogen source, which is assimilated into glutamine by the reaction of Glutamine synthetase (GS, EC 6.3.1.2). Rice plants possess one gene (OsGS2) for chloroplastic GS2 and three genes (OsGS1;1, OsGS1;2 and OsGS1;3) for cytosolic GS1. Here, we report the effect of WD on regulation of GS isoforms in drought-sensitive (cv. IR-64) and drought-tolerant (cv. Khitish) rice cultivars. Under WD, total GS activity in root and leaf decreased significantly in IR-64 seedlings in comparison to Khitish seedlings. The reduced GS activity in IR-64 leaf was mainly due to decrease in GS2 activity, which correlated with decrease in corresponding transcript and polypeptide contents. GS1 transcript and polypeptide accumulated in leaf during WD, however, GS1 activity was maintained at a constant level. Total GS activity in stem of both the varieties was insensitive to WD. Among GS1 genes, OsGS1;1 expression was differently regulated by WD in the two rice varieties. Its transcript accumulated more abundantly in IR-64 leaf than in Khitish leaf. Following WD, OsGS1;1 mRNA level in stem and root tissues declined in IR-64 and enhanced in Khitish. A steady OsGS1;2 expression patterns were noted in leaf, stem and root of both the cultivars. Results suggest that OsGS2 and OsGS1;1 expression may contribute to drought tolerance of Khitish cultivar under WD conditions.

Keywords Rice · Water deficit · Glutamine synthetase · Drought tolerance

Communicated by P. Lakshmanan.

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Introduction

Nitrogen (N) is an essential nutrient for plant growth and productivity. In higher plants, the main pathway for assimilation of inorganic-N to organic form is the Glutamine synthetase (GS, EC 6.3.1.2)/Glutamate synthase (GOGAT, E.C.1.4.7.1) cycle. GS catalyzes the ATP-dependent assimilation of ammonium (NH_4^+) into glutamate to yield glutamine, which is then used for the biosynthesis of essentially all nitrogenous compounds. GS exists in multiple isoforms that are either cytosolic (GS1) or plastidic (GS2). GS2 is encoded by 1 gene and GS1 is encoded by 3–5 genes depending on the species (Forde and Cullimore 1989; Miflin and Habash 2002). During the vegetative stage, GS2 is the predominant isoform in the leaf, where it assimilates ammonia originating from nitrate reduction and photorespiration (Bernard et al. 2008; Wallsgrove et al. 1987). Immunocytochemical studies have demonstrated that GS1 protein is localized in the vascular tissue suggesting its metabolic role in assimilation of NH_4^+ to glutamine for transport and distribution throughout the plant (Bernard and Habash 2009; Pereira et al. 1996). In root, GS1 facilitates the assimilation of NH_4^+ taken from the soil or from symbiotic N-fixation (Hirel et al. 1987). GS

isoforms are differentially regulated in response to plant N-status as well as environmental signals (Oliveira and Coruzzi 1999). Although, transcriptional control of GS is important in determining polypeptide abundance and cellular localization, the enzyme is also regulated by post-transcriptional and post-translational mechanism or by protein turnover (Lima et al. 2006).

Drought, heat and salinity are the most important abiotic stresses that have adverse effect on plant growth and productivity. All these major stresses result in water deficit (WD) stress. During prolonged period of WD, decrease in water availability for transport-associated process leads to limited uptake of N and reduced availability of CO_2 for photosynthesis as stomata are induced to close followed by the disturbances in carbon (C) and N metabolism (Foyer et al. 1998; Xu and Zhou 2006). Acclimation to WD requires responses that allow essential reaction of primary metabolism to continue.

Rice (*Oryza sativa* L.) is adversely affected by WD stress. The effect of water deprivation varies with variety, degree and duration of stress (Blum 1982). NH_4^+ is the main form of N available to the young rice plants, which is assimilated by GS to glutamine. Glutamine serves as the main form of organic-N transported through vascular tissues (Ishiyama et al. 2004a). Rice plants possess one gene (OsGS*2*) for GS2. The GS1 gene family consists of three isoforms encoded by OsGS1;1, OsGS1;2 and OsGS1;3. These isoforms show organ and cell specific patterns of expression and are developmentally regulated. They are also differentially regulated at the level of gene expression by light, inorganic-N, soluble carbohydrate and amino acids (Kamachi et al. 1991; Kusano et al. 2011; Tabuchi et al. 2007). However, regulation of these isoforms during WD has not been investigated in detail. The role of GS isoforms in controlling N metabolism during WD can be understood by studying their regulation in differentially drought-tolerant rice varieties. The work presented here focuses on differential effect of WD on activity and expression of GS isoforms in leaf, stem and root of two differentially drought-tolerant rice cultivars.

Materials and methods

Plant material and growth conditions

Rice (*Oryza sativa* var. *indica*) seeds (cv. Khitish, Triguna, Satabdi, PNR-519, IR-8 and IR-64) were obtained from Rice Research Station, Chinsurah, West Bengal. Seeds were germinated in moist cotton bed at 30 °C for 2–3 days in dark. About 50 germinated seedlings were transplanted to each pot containing a mixture of soil: soilrite (3:1 v/v). Seedlings were grown under 250 μmol m^{-2} s^{-1} photon flux density (16 h/8 h day/night regime) at 27 ± 2 °C and 70–80 % relative humidity, in a Plant Growth Chamber (Conviron, Canada). After 3 weeks of sowing, WD was imposed by withholding water. Seedlings were harvested from individual pots at indicated days of stress treatment till day 12, frozen in liquid N_2, and stored at −80 °C for further analysis. Fresh plant tissue immediately after harvest was used for the determination of relative water content (RWC), electrolyte leakage, proline and protein contents. For determination of dry weight (DW), 1.0 g fresh leaf, stem and root tissues were dried in an oven at 80 °C for 48 h.

Determination of RWC

Leaf tissue was weighed to determine fresh weight (FW) and then rehydrated in water at 4 °C for 24 h and turgid weight (TW) was recorded. Finally, the sample was dried in an oven at 80 °C for 48 h and dry weight (DW) was recorded. The RWC was calculated by the following formula: RWC (%) = (FW − DW/TW − DW) × 100. The experiment was carried out in triplicates.

Proline estimation

Free proline was quantified according to Bates et al. (1973). The leaf tissue was homogenized in 3 % aqueous sulfosalicylic acid, and the homogenate was centrifuged at 10,000 rpm for 20 min. 2 ml of supernatant was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid and boiled at 100 °C for 1 h. After termination of the reaction on ice, the reaction mixture was extracted with 4 ml of toluene, and the absorbance of the aqueous phase was recorded at 520 nm. The assay was done in triplicates. Amount of proline was expressed as μmol proline g^{-1} dry wt.

Electrolyte leakage assay

Electrolyte leakage was assayed by estimating the ions leaching from the leaf into Milli-Q water. Leaf tissue was placed in 20 ml of Milli-Q water in two sets. The first set was kept at room temperature for 4 h, and its conductivity (C1) was recorded using a conductivity meter. The second set was autoclaved and its conductivity was also recorded (C2). Electrolyte leakage (1 − C1/C2) × 100 was calculated. The experiment was carried out in triplicates.

Protein estimation

Quantitative estimation of protein was carried out by following the method of Bradford (1976) using bovine serum albumin (BSA) as standard. Amount of protein was expressed as mg protein g^{-1} dry wt.

GS extraction and assay

Frozen rice tissue was homogenized in GS extraction buffer (5 ml g^{-1} fresh wt) containing 0.05 M Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15 % glycerol. The homogenate was centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was desalted on pre-equilibrated Biogel P-2 column. GS activity was determined by semisynthetase reaction (Washitani and Sato 1977). A 1 ml reaction mixture contained 25 mM Tris–HCl (pH 7.5), 200 mM glutamate, 10 mM ATP, 5 mM hydroxylamine hydrochloride, 20 mM $MgCl_2$, and 100 µl enzyme preparation. The reaction mixture was incubated at 37 °C for 30 min and terminated by adding 2 ml of $FeCl_3$ reagent (0.67 M $FeCl_3$, 0.37 M HCl and 20 % (w/v) tri-chloroacetic acid). After 20 min, the amount of γ-glutamylhydroxamate produced was determined spectrophotometrically by measuring the absorbance at 540 nm. One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1}.

Separation of GS isoforms

All the steps of GS isoforms separation were performed at 4 °C. 1 g of frozen rice tissue was homogenized in 5 ml GS extraction buffer. The homogenate was centrifuged at 10,000 rpm for 15 min. 1 ml of desalted supernatant was loaded onto a diethylaminoethyl (DEAE)-sephacel (Sigma, USA) column (5 × 1 cm) pre-equilibrated with the GS extraction buffer. The column was washed with the same buffer until no protein was detectable in the eluate. Elution of the adsorbed proteins was carried out by the buffer containing, first 0.15 M and then 0.30 M KCl. The flow rate was maintained at 20 ml h^{-1}. 2 ml fractions were collected and assayed for GS activity. The activities of chloroplastic and cytosolic isoforms were estimated from the area of the corresponding elution profile after fractionation. About 80–90 % of the total GS activity present in the crude extract was recovered after chromatographic separation.

RNA extraction and RT-PCR analysis

Total RNA was isolated from rice tissues using TRIZOL reagent (Invitrogen, USA) following the manufacturer's instruction and quantified spectrophotometrically. To ensure comparability of the resulting band intensity, spectrophotometric quantitation of RNA was confirmed by applying equal amount of total RNA to an agarose gel. First-strand cDNA was synthesised using 1 µg of total RNA, oligo (dT) primer and AMV reverse transcriptase (Promega) in 25 µl of reaction. Semiquantitative RT-PCR was performed using 5 µl of cDNA as template in 25 µl reaction mixture. Reaction contained selected couples of the following gene-specific primers: OsGS1;1-F (5′-AGT ATGGCTTCTCTCACCGATCTCGTC-3′) and OsGS1;1-R (5′-GTACCTCGAG GGGCT TCCAGATGATGGTGGTC T-3′) for OsGS1;1, OsGS1;2-F (5′-GACTCATATGGC CAACCTCACC GACCTCGTT-3′) and OsGS1;2-R (5′-T AGCGGCCGCGTTCTGCTTCCACAGCAGCGTG-3′) for OsGS1;2, OsGS2-F (5′-AGAACTTGGACGATGAATC GG-3′) and OsGS2-R (5′- CATTTTATTTCGAGGGAA GG-3′) for OsGS2 and OsActin-F (5′-GTCAGAATGGG ATGATATGG-3′) and OsActin-R (5′-TCTCCTTGCTCA TCCTGTCAG-3′) for actin. PCR was performed for 27–29 cycles within a linear range of amplification of these genes. Expression of actin gene was used as a control to equalize cDNA quantity in different reactions. 7 µl of the PCR product were loaded and separated on 1 % agarose Tris–acetate EDTA gel. Gels were scanned using a gel documentation system (Spectronics, USA) and quantified by ImageAide version 3.06.04, to calculate changes in gene expression. Results were repeated three times and representative one time gel pictures are shown.

Protein gel blot analysis of GS protein

Proteins were separated by 12.5 % SDS-PAGE (Laemmli 1970) and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with the anti-GS antibody raised against synthetic peptide from conserved region of GS1 and GS2. The reacted polypeptide was visualized with a goat anti-rabbit IgG-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine (NBT/BCIP) detection kit (Invitrogen, USA). Broad range pre-stained standards were used as markers.

Results

Screening of Rice varieties for WD tolerance

Preliminary experiments were carried out to evaluate the drought tolerance characteristics of rice cultivars. 3 weeks old rice seedlings of different varieties were subjected to WD and relative water content (RWC), proline and protein contents and electrolyte leakage of leaf tissues were monitored at various stages of WD. IR-64 and Khitish cultivars were finally selected for further studies due to their relative drought-sensitive and tolerant properties, respectively. The results of drought tolerance characteristics of these two cultivars are shown in Figs. 1 and 2. WD treatment resulted in rapid decrease in the water content of IR-64 seedlings in comparison to Khitish seedlings. Consequently, a greater increase in dry wt g^{-1} of leaf and root tissue was noted in

IR-64 seedlings. At 12 days of WD, soluble protein content in leaf and root was decreased by 58 % and 40 % in IR-64 and by 17 and 10 % in Khitish, respectively (Fig. 1). The RWC measured at 3 h photoperiod was almost constant in well-watered control plants. WD treatment for 12 days reduced RWC to 26 and 69 % in IR-64 and Khitish varieties, respectively (Fig. 2a). Although, proline content was increased during WD in both the cultivars, its accumulation was greater in Khitish from the beginning of stress treatment (Fig. 2b). In IR-64 leaf, proline content was maximum at day 12 of WD, followed by a severe decline on further dehydration. On the other hand, proline accumulation kept on increasing in Khitish leaf with intensification of WD. The effect of WD on membrane integrity was evaluated by measuring electrolyte leakage from leaf. The electrolyte leakage was maintained at an almost constant level in Khitish until day 6 and increased marginally during later stages of WD. In contrast, a sharp rise in electrolyte leakage was noted in IR-64 cultivar (Fig. 2c).

GS isoforms in leaf, stem and root of rice seedlings

The mRNA and protein contents of GS isoform in rice seedlings were determined by quantitative RT-PCR and Western blot analysis, respectively. The activity of GS1 and GS2 was determined by fractionating them from the total cell free extract by anion exchange chromatography followed by estimating the activity from the area of corresponding elution profile after fractionation. GS1 activity was present in all the organs. GS2 activity was present in leaf and stem but was not detectable in root. The contribution of GS2 to total GS activity was about 80 and 30 % in leaf and stem, respectively (Table 1). Among GS1 gene family, OsGS1;1 and OsGS1;2 transcripts were present in all the three organs. OsGS1;1 transcript was more abundant in leaf, whereas OsGS1;2 was the predominant form in stem and root. Moreover, OsGS1;1 mRNA level was significantly greater in root and stem of IR-64 seedlings than that of Khitish. In the present study, OsGS1;3 gene expression was not studied as the previous reports indicate its absence in vegetative stage of growth of rice seedlings (Ishiyama et al. 2004a; Tabuchi et al. 2007). Western blot analysis indicated the presence of two proteins of about 39 and 43 kDa in leaf and stem that correspond to the molecular size of GS1 and GS2, respectively. Only GS1 was found to express in root. As the GS antibody was developed against conserved GS polypeptide, cytosolic isoforms could not be distinguished in the immunoblot.

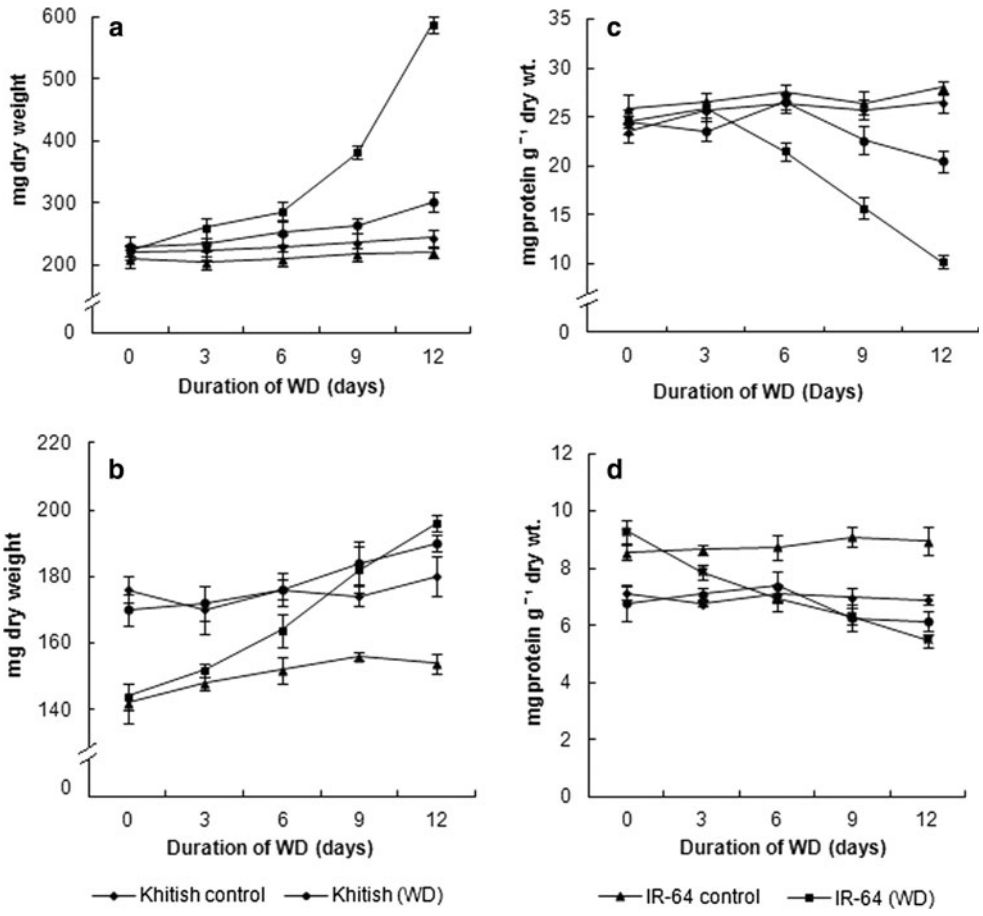

Fig. 1 Effect of WD on dry weight and protein content. Comparative analysis of dry weight of (**a**) leaf, (**b**) root and protein content of (**c**) leaf, (**d**) root between IR-64 and Khitish varieties of *O. sativa* in a time-dependent manner under WD conditions. For determination of dry weight, 1 g of fresh tissue was dried at 80 °C for 48 h. All experiments were done in triplicates ($n = 3$), and average mean values were plotted against duration of WD

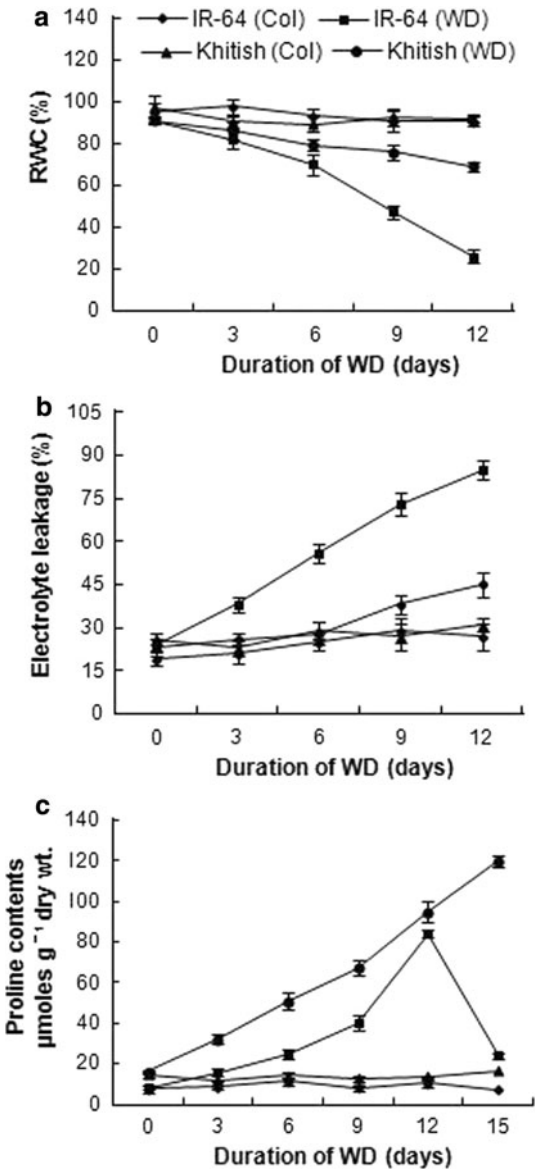

Fig. 2 Effect of WD on leaf RWC, proline content and electrolyte leakage. Comparative analysis of (**a**) RWC, (**b**) electrolyte leakage and (**c**) proline accumulation between IR-64 and Khitish varieties of *O. sativa* in a time-dependent manner under WD conditions. All experiments were done in triplicates ($n = 3$), and average mean values were plotted against duration of dehydration

Effect of WD on expression of GS isoforms in leaf

3 weeks old, IR-64 and Khitish seedlings were subjected to WD for 12 days and activity of GS1 and GS2 and the corresponding mRNA and polypeptide contents were simultaneously monitored in different organs, at various stages. The results in Fig. 3 show the effect of WD on activity and expression of leaf GS isoforms. Total GS activity was almost similar in leaf of both the varieties at the beginning of stress treatment. However, it decreased by 52 and 10 % in IR-64 and Khitish leaf, respectively, at 12 days of WD (Table 1; Fig. 3a). The rapid reduction in activity in IR-64 was mainly due to disappearance of GS2 activity. The differential rate of decline in GS2 activity decreased the GS2 to GS1 ratio from 4.18 to 2.28 in IR-64 and from 4.00 to 3.75 in Khitish (Fig. 3b). The OsGS1;1 mRNA accumulated differentially in the two varieties. Its level was increased by about 5 and 2.5-fold in IR-64 and Khitish cultivars, respectively. On the other hand, OsGS1;2 mRNA content enhanced almost equally in both the varieties. It is also noticeable that OsGS2 mRNA content was reduced to about half in IR-64 leaf by the end of WD treatment, but no such change was noticed in Khitish leaf (Fig. 3c, d). The results of immunoblot analysis indicate that the GS1 polypeptide content was increased in both the varieties. GS2 polypeptide level was decreased in IR-64 leaf and remained almost unaltered in Khitish leaf (Fig. 3e, f).

Effect of WD on expression of GS isoforms in stem

GS isoforms in stem responded differently to WD. Total GS activity did not alter significantly in both the cultivars. In IR-64 stem, GS1 activity was reduced with WD. The reduction in GS1 activity was compensated by increase in GS2 activity, thus maintaining almost unchanged total GS activity (Table 1; Fig. 4a). These changes in GS1and GS2 activities raised the ratio of GS2 to GS1 from 0.46 to 1.20. The activity of GS isoforms did not alter significantly in Khitish stem (Fig. 4b). A varietal variation was also noted in OsGS1;1 mRNA expression as its level declined to less than half in IR-64 and enhanced about fourfold in Khitish, by the end of stress treatment. The OsGS1;2 mRNA was almost equally increased in both the varieties and was reflected in the time course of GS1 polypeptide abundance (Fig. 4 c–f). During WD treatment, OsGS2 transcript level was enhanced about twofold in IR-64 and was almost maintained in Khitish (Fig. 4c, d). The increased level of OsGS2 mRNA in IR-64 stem corresponded with the abundance of GS2 polypeptide (Fig. 4e, f).

Effect of WD on expression of GS isoforms in root

The cytosolic GS1 was the only GS isoform in rice root. WD treatment reduced the total GS activity of root by almost 50 % in IR-64, but hadn't much effect on Khitish cultivar (Table 1; Fig. 5a). OsGS1;1 mRNA expression also differed in root of the two cultivars. The initial higher level of OsGS1;1 transcript declined with WD in IR-64. In contrast, its expression increased almost twice in Khitish. OsGS1;2 transcript content declined faster in IR-64 in comparison to Khitish. As a result, its level fell to a minimum at day 4 and day 8 of WD in IR-64 and Khitish, respectively and then increased on further WD treatment

Table 1 Total GS, GS1 and GS2 activities in leaf, stem and root of rice (cv. IR-64 & Khitish) seedlings at various stages of WD

Rice varieties	Tissue	Days of WD	GS activity*			Ratio GS2; GS1
			Total GS	GS1	GS2	
Khitish	Leaf	0	28.50 ± 1.51	5.28 ± 0.10	21.12 ± 2.42	4.00
		4	30.00 ± 2.12	5.45 ± 0.25	21.52 ± 1.84	3.95
		8	27.20 ± 2.40	5.25 ± 0.15	19.68 ± 2.80	3.75
		12	25.80 ± 3.21	5.11 ± 0.30	19.16 ± 3.41	3.75
	Stem	0	12.10 ± 1.10	7.89 ± 0.25	4.10 ± 0.02	0.52
		4	10.92 ± 1.25	6.77 ± 0.31	3.84 ± 0.03	0.56
		8	11.50 ± 2.50	7.10 ± 0.15	3.82 ± 0.10	0.53
		12	12.20 ± 1.80	7.00 ± 0.10	4.05 ± 0.02	0.57
	Root	0	17.00 ± 2.10	Total activity was present as GS1		
		4	16.70 ± 2.41			
		8	16.68 ± 2.15			
		12	15.69 ± 2.80			
IR-64	Leaf	0	32.06 ± 3.21	5.45 ± 0.02	22.78 ± 3.10	4.18
		4	34.00 ± 3.80	5.86 ± 0.14	22.45 ± 2.50	4.00
		8	23.00 ± 4.25	5.30 ± 0.04	15.88 ± 3.12	3.00
		12	17.22 ± 5.50	5.13 ± 0.10	11.69 ± 3.12	2.28
	Stem	0	12.40 ± 2.10	8.21 ± 2.11	3.80 ± 1.45	0.46
		4	11.95 ± 3.20	6.95 ± 1.61	4.32 ± 3.12	0.62
		8	11.38 ± 1.26	5.42 ± 2.23	5.35 ± 2.54	0.98
		12	11.60 ± 3.24	4.95 ± 3.41	6.00 ± 3.61	1.21
	Root	0	20.00 ± 3.22	Total activity was present as GS1		
		4	16.00 ± 2.65			
		8	10.00 ± 2.65			
		12	08.40 ± 1.82			

GS1 and GS2 from various tissues were isolated by anion exchange chromatography. The enzyme activity in different fractions was assayed by semisynthetase method as described in "GS extraction and assay"

*One unit of GS activity represents 1.0 μmol of γ-glutamylhydroxamate produced 30 min^{-1}

(Fig. 5b, c). The time course of GS1protein detected on Western blot corresponded with the OsGS1;2 transcript level (Fig. 5d, e).

Discussion

Drought induces complex change in N metabolism due to decreased water availability for transport, leading to limited uptake of N-nutrients. GS is a key enzyme of N metabolism in rice crop and has pivotal role in survival of plant during WD. Present study describes the effect of WD on expression of GS isoforms in leaf, stem and root of two rice cultivars differently tolerant to drought conditions.

Dehydration tolerance in plants is attained by the maintenance of metabolic and physiological functions at low water status, which serve as the driving force for plant productivity. A few characteristics such as osmotic adjustment and cell membrane stability are recognized as effective components of dehydration tolerance in many crops. These are expressed in terms of RWC of the plant, accumulation of compatible solutes like proline, and membrane permeability of ions and electrolytes (Blum 2005). In the present study, rice varieties were initially screened for drought tolerance characteristics. During WD, Khitish variety maintained high RWC and showed maximum accumulation of proline and marginal increase in electrolyte leakage. Under similar conditions, IR-64 cultivar showed the least RWC and proline accumulation and sharp rise in electrolyte leakage. As a consequence, dry wt g^{-1} of leaf and root tissues of IR-64 seedling was increased significantly. The results thus indicated more susceptibility of IR-64 to WD as compared to Khitish and hence were designated as drought-sensitive and -tolerant cultivar, respectively.

The activity of GS1 and GS2 and the corresponding mRNA and polypeptide contents were simultaneously monitored in different organs of the two cultivars, at various stages of WD. The WD mediated alteration in total GS activity in leaf and root was directly related to dehydration tolerance characteristics of rice varieties, as it declined

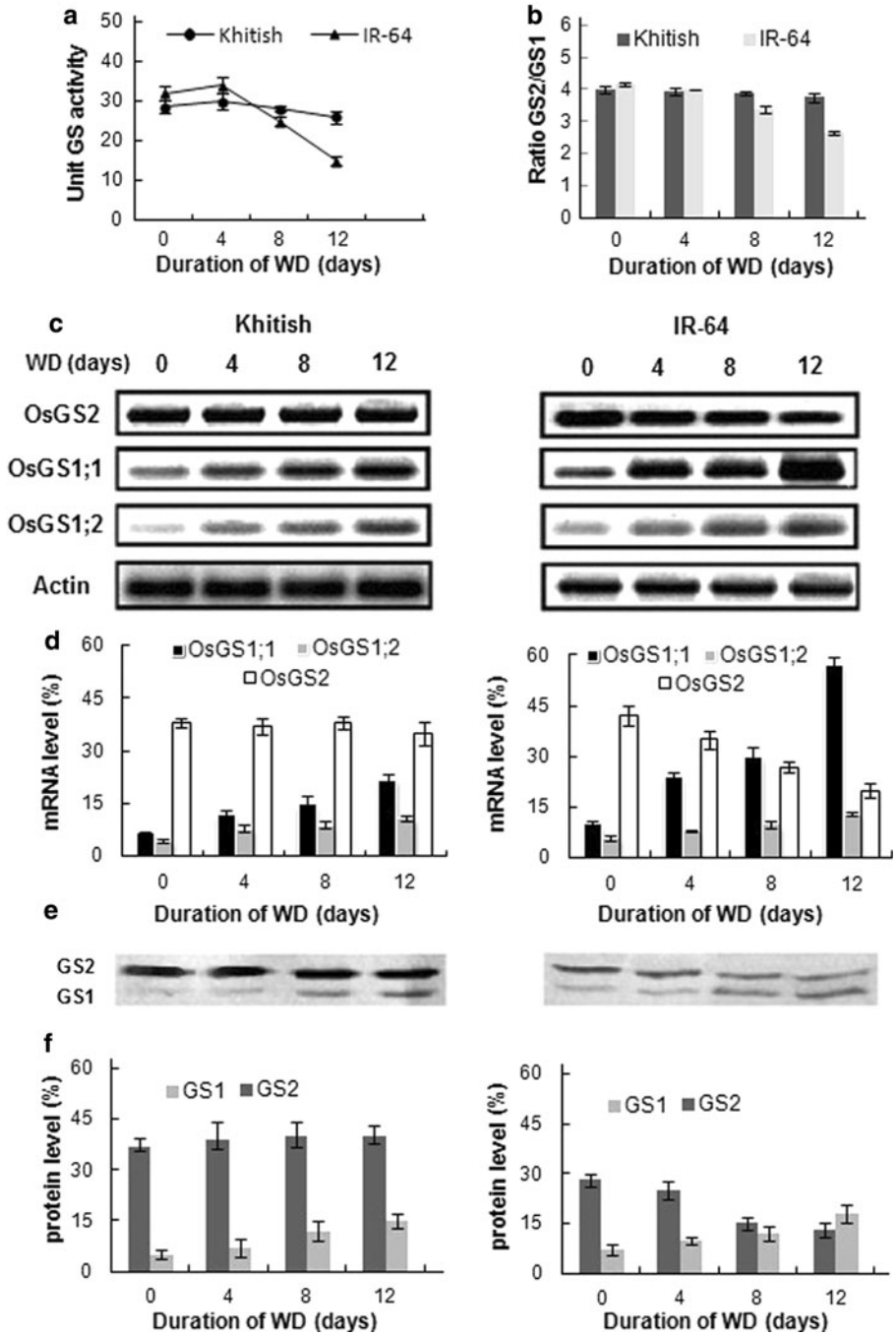

Fig. 3 GS activity and expression in leaves of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD. (**a**) Relative change in total GS activity of IR-64 (*black triangle*) and Khitish (*black circle*), (**b**) change in ratio of GS2/GS1 activity in IR-64 (*grey square*) and Khitish (*black square*) seedlings, (**c**) analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR, (**d**) *bar diagram* of GS mRNA level, (e) Western blot analysis of GS1 and GS2 polypeptides and (f) *bar diagram* of GS polypeptide level. Western blotting was carried out with 10 µg of total soluble protein extracted from leaf. One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1}

significantly in IR-64 and did not change markedly in Khitish cultivar. The decreased GS activity in IR-64 leaf was due to preferential reduction of GS2 activity and was correlated with decreased level of GS2 mRNA and protein. Under similar conditions, an almost constant GS2 mRNA and corresponding polypeptide maintained a steady GS2 activity in Khitish leaf. The results suggest that WD mediated GS2 regulation resides mainly at the transcriptional and/or mRNA stability levels. An important physiological function of GS2 is reassimilation of NH_4^+ produced during photorespiration, a rate limiting step of the pathway (Hausler et al. 1994; Hoshida et al. 2000). The physiological importance of GS2 has been demonstrated in transgenic plants overexpressing GS2 in the leaves. The transgenic plant line accumulating 1.5 fold more GS2 than the control plant had an increased photorespiration capacity. They also retained more than 90 % photosystem II activity when grown under osmotic stress treatment for 2 weeks (Hoshida et al. 2000). Hence in the present study, a relatively unaltered GS2 expression in Khitish leaf could

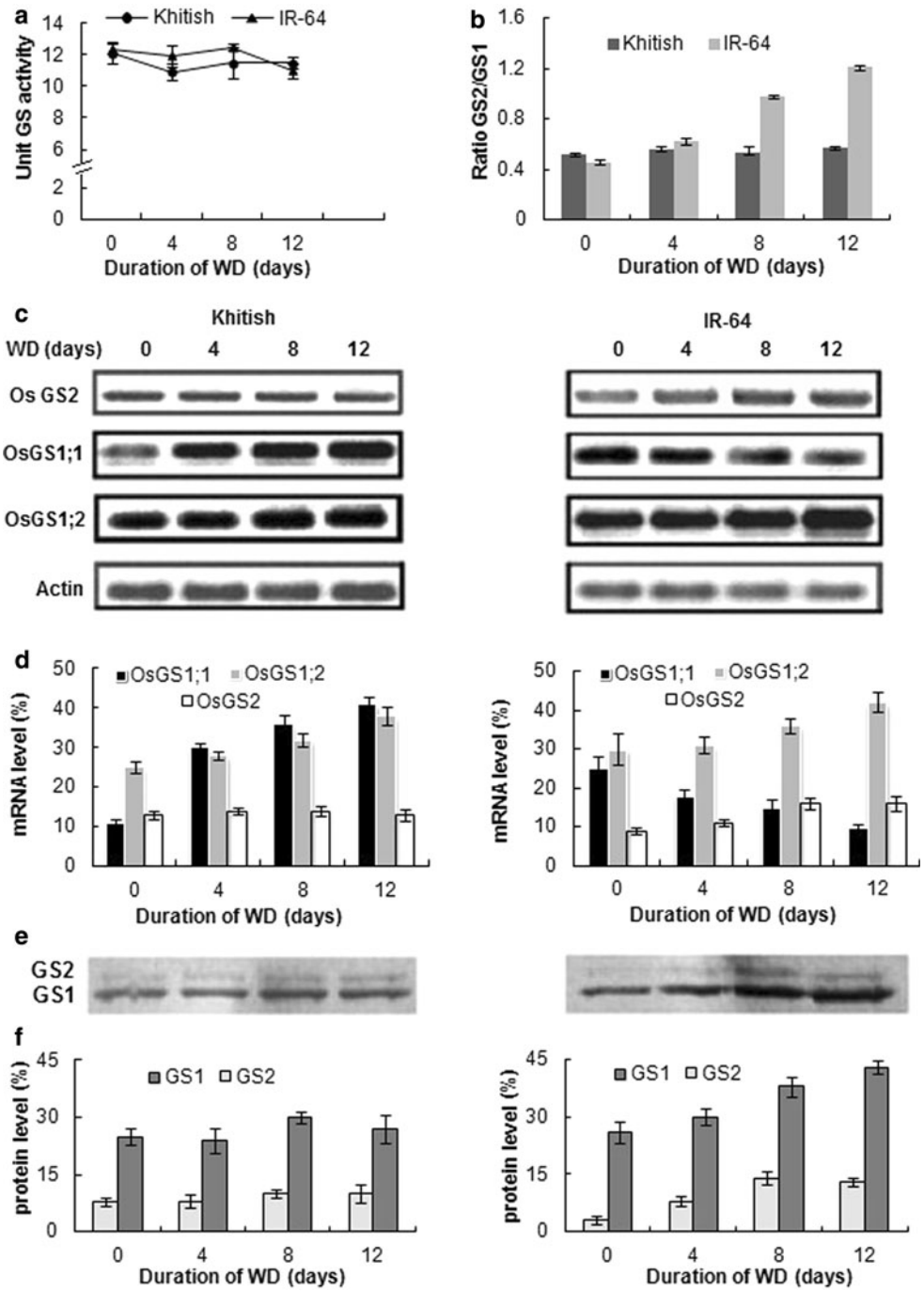

Fig. 4 GS activity and expression in stem of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment. (**a**) Relative change in total GS activity of IR-64 (*black triangle*) and Khitish (*black circle*), (**b**) changes ratio of GS2/GS1 activity in IR-64 (*grey square*) and Khitish (*black square*), (**c**) analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR, (**d**) *bar diagram* of GS gene expression, (**e**) Western blot analysis of GS1 and GS2 polypeptides and (**f**) *bar diagram* of GS polypeptide level. Western blotting was carried out with 10 µg of total soluble protein extracted from stem. One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1}

maintain the photorespiratory capacity at limited water availability that improves tolerance of the cultivar to WD.

The WD treatment increased the expression of GS1 transcripts and polypeptide in leaf of both IR-64 and Khitish. Although the total GS1 transcript level has already been reported to increase in leaf during natural senescence and in response to biotic and abiotic stress, the response of individual cytosolic GS gene to abiotic stress has not been studied. The observed increase in GS1 expression in rice leaf corresponded with an almost unchanged GS1 activity during WD. As in the present study, the nitrogen nutrition-mediated increase in GS1 transcript and polypeptide in *Arabidopsis* root was related to the maintenance of GS1 activity rather than increase (Ishiyama et al. 2004b). Moreover, a significantly greater expression of OsGS1;1 mRNA in IR-64 leaf could be due to its higher rate of protein degradation (Fig. 1c), conforming to role of the isoform in reassimilation of nitrogen released from protein breakdown as suggested earlier by Tabuchi et al. (2007).

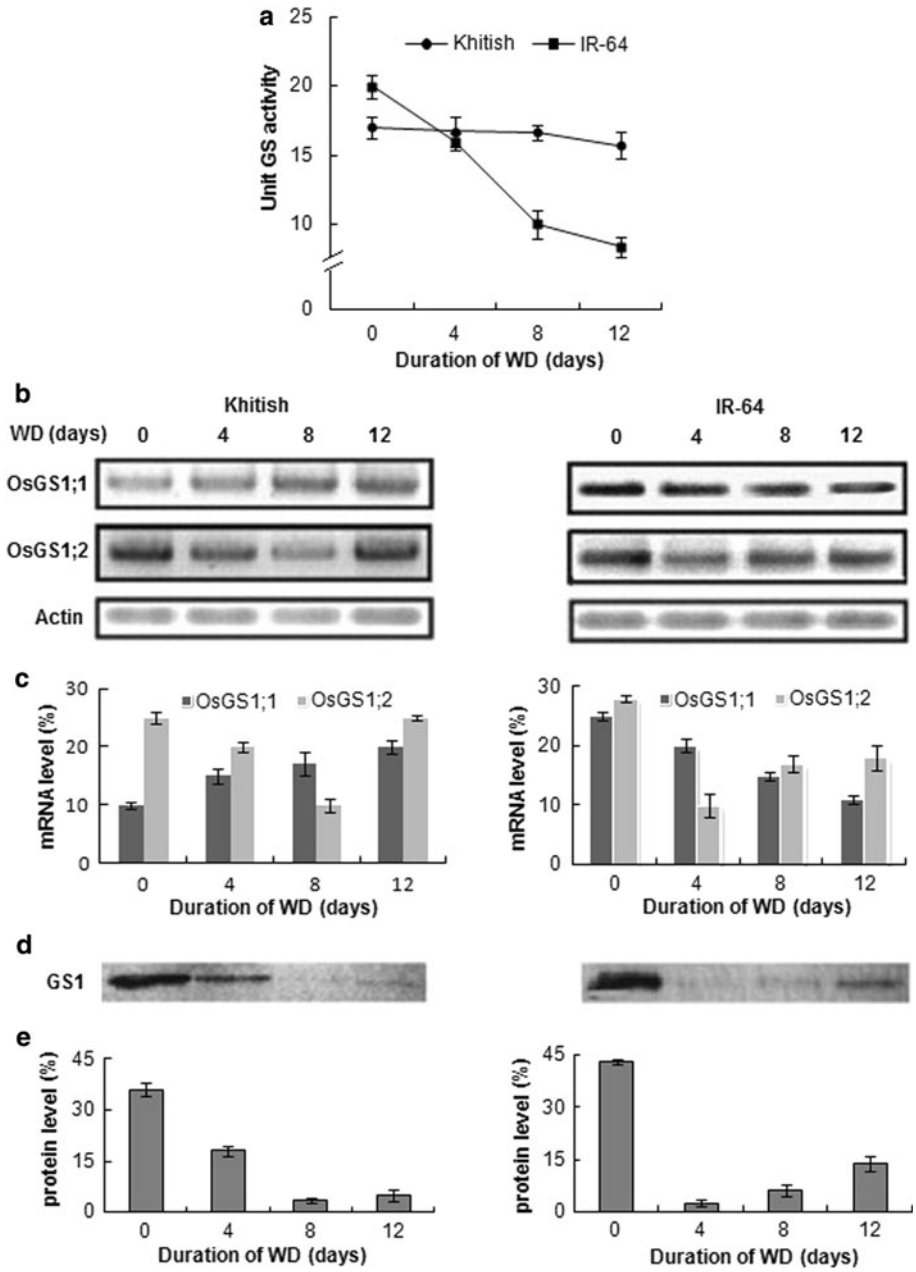

Fig. 5 GS activity and expression in roots of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment. (**a**) Relative change in total GS activity of IR-64 (*black square*) and Khitish (*black circle*), (**b**) analysis of OsGS1;1 and OsGS1;2 transcripts by RT-PCR, (**c**) *bar diagram* of GS gene expression, (**d**) Western blot analysis of GS1 polypeptide and (**e**) *bar diagram* of GS polypeptide level. Western blotting was carried out with 10 μg of total soluble protein extracted from root. One unit of GS activity represents 1.0 μmol of γ-glutamylhydroxamate produced 30 min^{-1}

In contrast to leaf, OsGS1;1 transcript level was found to decrease in IR-64 stem and root in response to WD. Nitrogen remobilization from protein breakdown constitutes the major source of nitrogen in vascular tissue and glutamine is the most abundant free amino acid for transport in rice plant (Tabuchi et al. 2007). The repression in OsGS1;1 might result from remobilization and transport of high concentration of glutamine to stem and root from increased protein degradation in leaf. The transcriptional down-regulation of OsGS1;1 has already been documented in presence of NH_4^+ in roots of *Arabidopsis* and rice seedlings (Ishiyama et al. 2004a, b; Kusano et al. 2011). Several other rice genes associated with N-uptake and metabolism, such as, OsGS1;2, OsNADH-GOGAT1, OsAMT1;1 and OsAMT1;2, are also regulated by exogenous NH_4^+ ions (Ishiyama et al. 2004a; Sonoda et al. 2003; Tabuchi et al. 2007). However, pharmacological studies have suggested glutamine rather than NH_4^+ ions, being the real signaling molecule in regulation of expression of these genes (Kamachi et al. 1991; Oliveira and Coruzzi 1999; Tabuchi et al. 2007). The study by Ishiyama et al. (2004b) also reported glutamine as negative feedback regulator of GS1 in *Arabidopsis*.

WD mediated decrease in GS1 activity in stem and root of IR-64 seedling might correlate with reduction in OsGS1;1 mRNA level. The GS1;1 gene has been

previously reported to be involved in remobilization and reutilization of nitrogen in rice plant during senescence (Tabuchi et al. 2007) and thus seems to play significant role in performance of plant under stress condition. The significance of OsGS1;1 in ammonium assimilation has also been studied earlier by Ishiyama and co-workers (2004a). A comparison of kinetic properties of OsGS1;1 and OsGS1;2 encoded GS isoforms in rice root indicated former having more substrate affinity for ammonium and approximately twofold higher V_{max} value. The result supported the importance of OsGS1;1 in promoting the rapid conversion of ammonium to glutamine even under low ammonium conditions (Ishiyama et al. 2004a). The implication of OsGS1;1 in NH_4^+ assimilation has been further indicated by the over accumulation of free ammonium in the leaf sheath and roots of the rice mutant lacking OsGS1;1(Kusano et al. 2011). The decline in GS1 activity in IR-64 stem was compensated by increment in GS2 protein and activity, maintaining almost unchanged total GS activity. The increased GS2 protein can be due to observed increase in total protein content of stem during WD (data not shown). As in the present study, a tissue-specific response of WD stress was noted in *Lupinus albus* with strikingly increase in concentration of N and S in stem with intensification of water stress (Pinheiro et al. 2001). Thus, a different metabolic status may contribute to the maintenance of GS protein and activity in stem during WD.

In the present study, the two rice varieties did not differ much in expression pattern of OsGS1;2 during WD, despite difference in their drought tolerance properties. Its transcript level was found to enhance in leaf and stem. However, in root the highly expressed OsGS1;2 was found to decline initially followed by an increased accumulation on further dehydration. Although, the time course of OsGS1;2 mRNA was reflected in GS1 polypeptide abundance, it was not correlated with total GS activity in all the three tissues. The result is supported by study of Ishiyama et al. (2004b) on effect of ammonium nutrition in Arabidopsis root GS isoforms. Arabidopsis root has four different isoforms of GS1, among them amount of GS1;1, GS1;3, and GS1;4 mRNA decreased and GS1;2 mRNA was increased by ammonium nutrition. The time course increase in GS1;2 mRNA corresponded with the accumulation of GS1 protein detected on the western blot. However, the increase in GS1;2 mRNA and protein was not correlated with total GS1 activity. The lack of correlation was due to lower affinity of GS1;2 for ammonium as compared to other isoform.

In conclusion, the regulation of GS isoforms by WD was organ specific. Two GS isoforms i.e. GS1;1 and GS2 were differentially regulated in drought-sensitive and -tolerant rice cultivars. GS2 is the major GS isoform in leaf and its over-expression in leaf has been found to be associated with the maintenance of photosynthetic and photorespiratory capacity of the plant. The GS1;1 isoform has been shown to be involved in remobilization and reutilization of nitrogen. A higher substrate affinity of the enzyme for ammonium signifies its promotion of the rapid conversion of ammonium to glutamine even under low ammonium conditions. Hence from the results, it can be inferred that a relatively maintained OsGS2 and the over-expression of OsGS1;1 may contribute to improved drought tolerance characteristics of *Oryza sativa* cv. Khitish.

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DECLARATION

I declare that the thesis entitled "**Studies on regulation of glutamine synthetase isoforms during water deficit in differentially drought tolerant rice (*Oryza sativa* L.) cultivars and in a resurrection plant, *Selaginella bryopteris* L.**" has been prepared by under the guidance of Dr. Shilpi Ghosh, Assistant Professor of Department of Biotechnology, University of North Bengal.
No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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Date: 06.08.2013

CERTIFICATE

I certify that **Mr. Kamal Krishna Singh** has prepared thesis entitled "**Studies on regulation of glutamine synthetase isoforms during water deficit in differentially drought tolerant rice (*Oryza sativa L.*) cultivars and in a resurrection plant, *Selaginella bryopteris L.***", for the award of Ph.D degree of the University of North Bengal, under my guidance. He has carried out the work at the Department of Biotechnology, University of North Bengal.

Dr. Shilpi Ghosh

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Date: 29.07.13

Abstract

Water deficit (WD) has adverse effect on rice (*Oryza sativa* L.) and acclimation requires essential reactions of primary metabolism to continue. Rice plants utilize ammonium as major nitrogen source, which is assimilated into glutamine by the reaction of Glutamine synthetase (GS, EC 6.3.1.2). Rice plants possess one gene (OsGS2) for chloroplastic GS2 and three genes (OsGS1;1, OsGS1;2 and OsGS1;3) for cytosolic GS1.Here, we report the effect of WD on regulation of GS isoforms in drought-sensitive (cv.IR-64) and drought-tolerant (cv. Khitish) rice cultivars. Under WD, total GS activity in root and leaf decreased significantly in IR-64 seedlings in comparison to Khitish seedlings. The reduced GS activity in IR-64 leaf was mainly due to decrease in GS2 activity, which correlated with decrease in corresponding transcript and polypeptide contents. GS1 transcript and polypeptide accumulated in leaf during WD, however, GS1 activity was maintained at a constant level. Total GS activity in stem of both the varieties was insensitive to WD. Among GS1 genes, OsGS1;1 expression was differently regulated by WD in the two rice varieties. Its transcript accumulated more abundantly in IR-64 leaf than in Khitish leaf. Following WD, OsGS1;1 mRNA level in stem and root tissues declined in IR-64 and enhanced in Khitish. A steady OsGS1;2 expression patterns were noted in leaf, stem and root of both the cultivars. Results suggest that OsGS2 and OsGS1;1 expression may contribute to drought tolerance of Khitish cultivar under WD conditions.

Resurrection plants have evolved a wide spectrum of adaptations to cope with the challenges of environmental stress and can serve as a system for studying metabolic events during dehydration and rehydration. *Selaginella bryopteris* belonging to the family Selaginellaceae, is a resurrection lycophyte growing in the hilly area of tropical regions. In present investigation attempts were made to isolate and characterize GS from S. *bryopteris*. The regulation of GS expression during dehydration and rehydration of *Selaginella* fronds and its relationship with other enzymes of ammonium metabolism were also determined. The RWC of the *S. bryopteris* fronds decreased from 95 to 5 % within 24 h of dehydration treatment. Dehydration condition resulted in decrease in the total protein content. The decline in protein level paralleled a significant increase in free ammonium content. The level of proline also enhanced during dehydration. The effects

were reversible with concentrations returning to level comparable with that of full turgor state 24 h after the water deficit condition was alleviated. Anion-exchange chromatography of *S. bryopteris* frond extract revealed the presence of only cytosolic GS isoform. Furthermore, immunoblot analysis showed the presence of single band of molecular size about 39 kDa. The GS enzyme was partially purified from *S. bryopteris* fronds by ammonium sulphate precipitation, gel-filtration and ion-exchange chromatography. The partially purified enzyme showed pH optima of around 5.5. The enzyme was remarkably thermostable as it didn't lose activity on pre-incubation at temperature upto 60 ^{o}C. GS from *S. bryopteris* showed considerably higher temperature optima of about 60 ^{o}C. The Km value (2.4 mM) for glutamate was lower than that reported for enzymes from leaves of higher plants. In dehydration treated fronds, the activity of glutamine synthetase and glutamate synthase (GS /GOGAT), the main enzymes for ammonium assimilation remained largely unaltered. In contrast, glutamate dehydrogenase (GDH) aminating activity and polypeptide content increased from the beginning and became almost two fold at 24 h of dehydration treatment thus providing an alternative route for assimilation of ammonia. Results suggested that GS/GOGAT along with GDH may play important role in amelioration of ammonia toxicity and enhanced production of proline observed during dehydration.

PREFACE

I joined Dr. Ghosh's lab at Department of Biotechnology, University of North Bengal in August 2007. Here, I interacted routinely with lots of experiments and tried to take a tangential taste of intermingled problems of biological science, especially plants. Living beings are fast reactive because when a seed is left in the environment, it interacts with moisture, air, light and enzymatic thunderings inside it gives root and shoot outside. On the other hand, plants are authorized by nature in this universe to trap solar energy and supply it to us; so we can call them, Almighty in terms of energy. In spite of adverse conditions, they survive for years at the same place due to cellular brilliancy of stress management, unfortunately they are also caught by stresses at times. Similarly, the work was also perturbing and stressful for me, though I was not sessile like plants. Anyway, I enjoyed the job and it's my pleasure to have the opportunity to express my gratitude to all of them who assisted me.

I am highly grateful to my supervisor Dr. Shilpi Ghosh, Assistant Professor, Department of Biotechnology, North Bengal University, by whose grace I completed my research work successfully. It gives me immense pleasure to express my indebtedness to her for giving valuable suggestions and constant guidance during the entire course of study. It was not possible to complete this journey without her guidance. It is difficult for me to write in few words my gratitude towards her.

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Heartiest thanks to my aunty Late Shanti Devi and Jiju, Late Shivvash Singh; your absence will always be a matter of immense regret for me. And I extend the hand of special thanks to my family members; Sri Shivpujan Singh, Sri Shambhu Nath Singh, Sri Prem Chand Singh, Sri Manik Chand Singh and rest of the family members for their munificent support during my nasty situation.

Finally, I would like to thank and express 'inexpressible' gratitude to my father Sri Arjun Singh and my mother Smt. Roopkali Devi for perpetual support in all directions.

The victory really becomes more pleasurable when you strike the target in very tough situations. Anyway, the moment becomes truly exceptional, when, imaginings turn into reality.

Kamal Krishna Singh

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CHAPTER 1

General introduction and literature review

1.1. INTRODUCTION

Nitrogen is an essential nutrient for plant growth and productivity. In higher plants inorganic nitrogen is assimilated predominantly via Glutamine synthetase (GS; EC 6.3.1.2)/ Glutamate synthase (Glutamate oxo-glutarate ammonium transferase, GOGAT; EC 1.4.7.1) pathway. GS catalyses the ATP-dependent condensation of ammonium (NH_4^+) with glutamate to yield glutamine. The enzyme GOGAT catalyses the conversion of glutamine and 2-oxoglutarate to two molecules of glutamate. The net outcome of the GS-GOGAT cycle is the production of glutamate, which can then be incorporated into other amino acids through the action of aminotransferases or transaminases (Forde and Lea 2007; Bernard and Habash 2009). Specific amino acids subsequently become precursors for all nitrogen-containing organic molecules, such as proteins, chlorophyll, cytochrome/phytochrome, secondary metabolites and nucleic acids. In higher plants, GS exists in multiple isoforms that are either cytosolic (GS1) or plastidic (GS2). The relative abundance of GS isoforms varies within different plant species and within different organs of the same plant, depending on their photosynthetic type (McNally and Hirel 1983; McNally et al. 1983; Bernard and Habash 2009). GS2 is encoded by one gene and GS1 is encoded by three to five genes depending on plant species (Forde and Cullimore1989; Miflin and Habash 2002). Phylogenetic studies on GS nucleotide or amino acid sequences have shown that chloroplastic and cytosolic GS form two sisters groups (Pesole et al. 1991) and have evolved through gene duplication (Doyle 1991; Swarbreck et al. 2011). These two major GS isoforms play primarily nonoverlapping roles in plant nitrogen assimilation. GS2 is the predominant isoform in the leaf, where it assimilates ammonia originating from

nitrate reduction and photorespiration (Wallsgrove et al. 1987; Bernard et al. 2008). Immunocytochemical studies have demonstrated that GS1 protein is localized in the vascular tissue, suggesting its metabolic role in assimilation of NH_4^+ to glutamine for transport and distribution throughout the plant (Pereira et al. 1996; Bernard and Habash 2009). In root, GS1 facilitates the assimilation of NH_4^+ taken from the soil or from symbiotic nitrogen fixation (Rai et al. 2000; Tobin and Yamaya 2001). GS has also been implicated in nitrogen remobilization during seed germination and regulating biosynthesis of proline, an amino acid with increased accumulation in response to osmotic stress (Brugiere et al. 1999; Silveira et al. 2003).

GS is regulated in a developmentally controlled manner during vegetative and reproductive plant growth stages and particularly through senescence. GS isoforms are also differentially regulated in response to environmental signals, such as, nitrogen status, light, biotic and abiotic stress (Oliveira and Coruzzi 1999; Bernard and Habash 2009). Although transcriptional control of GS is important in determining polypeptide abundance, the enzyme is also regulated by post-transcriptional and post-translational mechanism or by protein turnover (Bernard and Habash 2009). Moreover, phosphorylation and 14-3-3 interaction have been implied in the modulation of GS activity in plants (Man and Kaiser 2001; Riedel et al. 2001; Lima et al. 2006).

Drought, heat and salinity are the most important environmental stress factors that influence plant growth and development and place major limits on plant productivity in cultivated areas worldwide. Drought is a composite stress condition that includes soil water deficit, increased daytime temperature and reduced nutrient availability. However, the most important factor limiting growth and impairing plant productivity is the drop in water availability to the plant (Oliver et al. 2011). The specific plant responses to water deficit (WD) are dependent on the rate and amount of water loss, the duration of stress and the stage of plant development. Plants can perceive WD and elicit appropriate responses at the morphological, physiological, biochemical and molecular levels leading to altered metabolism, growth and development. Several features, like attenuated growth, stomatal closure, rolling of leaf, deep rooting, limited transpiration and reduced photosynthesis, and osmotic adjustments were found to be linked with drought tolerance (Ludlow and Muchow

1990). Adaptation to drought at biochemical and molecular levels undoubtedly involves the activation/increased expression of several genes, transient increases in ABA levels, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways. However, no consensus has been reached in defining the key processes determining tolerance and the secondary follow-up processes (Shinozaki and Yamaguchi-Shinozaki 2000; Bartel and Sunkar 2005).

During prolonged period of WD, decrease in water availability for transport associated process leads to limited uptake of nitrogen from soil and reduced availability of CO_2 for photosynthesis as stomata are induced to close followed by disturbances in carbon and nitrogen metabolism (Foyer et al.1998; Xu and Zhou 2006). Acclimation to WD requires responses that allow essential reaction of primary metabolism to continue. Recent studies have shown nitrogen assimilation to be critical for plant acclimation to stress conditions. It has been demonstrated that GS isoforms are regulated both at transcript and protein levels in response to plant status as well as environmental cues. The enzyme constitutes a regulatory point at which environmental signals are integrated and translated into a plant response in terms of growth and seed production (Swarbreck et al. 2011).

WD is one of the major constraint depressing rice (*Oryza sativa* L.) production. The effect of WD varies with the variety, degree, and duration of stress and its coincidence with different growth stages (Gao et al. 2007). Poor seedling vigour, fertility and consequent reduction in yield have been the major problems provoked by the stress condition. NH_4^+ is the main form of nitrogen available to the young rice plants, which is assimilated by GS to glutamine. Glutamine serves as the main form of organic-nitrogen for transport through vascular tissues (Ishiyama et al. 2004b). Rice plants possess one gene (OsGS2) for chloroplastic GS2. The cytosolic GS1 gene family consists of three isoforms encoded by OsGS1;1, OsGS1;2 and OsGS1;3. OsGS1;1 and OsGS1;2 are expressed in all organs with higher expression in leaf blades and roots. In leaf, they are present as minor form in comparison to GS2. OsGS1;3 is expressed mainly in spikelets (Tabuchi et al. 2005). These isoforms have been shown to be regulated in a developmentally controlled manner as well as by light and nitrogen nutrition (Kamachi et al. 1991; Ishiyama et al. 2004a, b; Tabuchi et al. 2007; Kusano et al. 2011). However, the regulation of these isoforms during WD has

not been investigated in detail. The role of GS isoforms in controlling nitrogen metabolism during WD can be understood by studying their regulation in differentially drought tolerant rice varieties.

Resurrection plants have evolved a wide spectrum of adaptations to cope with the challenges of environmental stress (Oliver et al. 2011; Yobi et al. 2012). They have unusual capability to survive long dry period. They are able to recover complete physiological activity following repeated protoplasmic dehydration of fully differentiated tissues and hence, can serve as a system for studying metabolic events during dehydration and rehydration. *Selaginella bryopteris* belonging to the family Selaginellaceae, is a resurrection lycophyte growing in the hilly area of tropical regions. It is a creeping plant with simple scale-like leaves on branching stems from which roots also arises. It is capable of surviving almost complete dehydration for prolonged period (Deeba et al. 2009). A useful insight into the role of GS isoforms under WD conditions can be obtained by studying the enzyme in resurrection plants, *S. bryopteris*. With all these in view, the present investigation was undertaken with the aim of studying the effect of WD on GS isoforms in *Oryza sativa* and *Selaginella bryopteris*.

1.2. OBJECTIVES

1. To screen rice cultivars for drought tolerance.
2. To determine GS isoform pattern in leaf, stem and root of rice seedlings by Ion-exchange chromatography, RT-PCR and Immunoblot analysis.
3. To study the effect of WD on activities of GS isoforms in leaf, stem and root of drought sensitive and drought tolerant rice cultivars.
4. To investigate the transcriptional and translational regulations of GS isoforms in leaf, stem and root of drought sensitive and drought tolerant rice cultivars in response to WD.
5. To determine GS isoform pattern of *S. bryopteris* fronds.
6. To purify the GS isoforms from *S. bryopteris* fronds and determine their biochemical characteristics.
7. To study the regulation of GS isoforms during hydration cycle of *S. bryopteris* fronds.

1.3. LITERATURE REVIEW

Nitrogen is an essential nutrient for plant growth and productivity. Plant species have evolved specific strategies to acquire nitrogen from their environments and assimilate it into organic compounds. The nitrogen sources directly available to plants include inorganic nitrogen compounds, such as nitrate (NO_3^-) and NH_4^+, as well as organic compounds, such as amino acids (e.g. glycine, alanine, glutamic acid, aspartic acid) and small peptides (Schimel and Bennett 2004).

The main pathway of inorganic-nitrogen assimilation to organic form is the Glutamine synthetase - Glutamate synthase (GS-GOGAT) cycle (*Figure 1.1*).

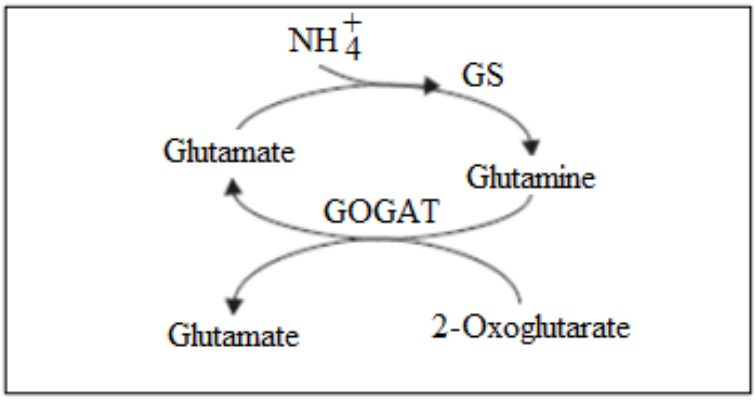

Figure 1.1. The glutamine synthetase-glutamate synthase (GS-GOGAT) cycle.

GS catalyzes the ATP-dependent condensation of NH_4^+ with glutamate to yield glutamine; GOGAT transfers the amide group of glutamine to α-ketoglutarate to subsequently produce glutamate (Bernard and Habash 2009). GS is a ubiquitous enzyme found in all higher plants, pteridophytes, bryophytes, algae, fungi and bacteria. The enzyme is involved in the assimilation of NH_4^+ produced via nitrate reduction, biological nitrogen fixation and catabolism of amino acids and, in the photosynthetic organism, in reassimilation of NH_4^+ evolved during photorespiration. In view of its key role in nitrogen metabolism, it has been studied quite extensively from varieties of prokaryotic and eukaryotic organisms. Information relevant to the present investigations is reviewed in the following subheadings.

1.3.1. GS isoforms in plants

GS exists in multiple isoforms in various plant organs such as leaves, roots, nodules, cotyledons and seeds. Leaves of several higher plants such as, barely (Mann et al. 1979), rice (Hirel and Gadal 1980), pumpkin (Kretovich et al. 1981), sorghum (Hirel and Gadal 1982), spinach (Hirel et al. 1982), potato (Teixeira et al. 2005) and wheat (Bernard et al. 2008) have been reported to contain two isoforms of GS that are either cytosolic (GS1) or plastidic (GS2). The cytosolic (GS1) isoform was the first to be eluted, followed by the plastidic (GS2) form (Becker et al. 1993; Tobin and Yamaya 2001), although the opposite elution pattern has been described in some species (Woodall et al. 1996; Orea et al. 2002). The cytosolic and chloroplastic localization of GS1 and GS2 has been further confirmed by immunological and molecular studies (Becker et al. 1993; Pereira et al. 1996; Bernard and Habash 2009). The relative abundance of GS isoforms varies within different organs of the same plant and within different plant species, depending on their photosynthetic type. McNally et al (1983) examined the relative abundance of GS1 and GS2 in leaves of a wide range of higher plants and on the basis of results obtained, classified plants into four different groups. Leaves of first group of plants contained only cytosolic form (GS1) of the enzyme, a pattern which is found in achlorophyllous higher plant parasites. The second group was characterized by having only the chloroplastic enzyme (GS2) as in tomato, spinach and mustard. The third group is represented by plants whose leaves contain GS1 as the minor component with chloroplastic isoform being the major form. Such a distribution profile of GS1 and GS2 is frequently encountered in C3 plants like barley (Mann et al. 1979), rice (Hirel and Gadal 1980), wheat (Tobin et al. 1985), sunflower (de la Haba et al. 1992) and Arabidopsis (Ishiyama et al. 2004a). The fourth group contains almost equal levels of GS1 and GS2 and such a situation is prevalent in most of the C4 species. Moreover, the relative proportion of GS1 and GS2 has been shown to be regulated in a developmentally controlled manner during vegetative and reproductive plant growth stages and particularly through senescence (Tobin et al. 1985; Kamachi et al. 1992; Kichey et al. 2005). They are also differentially regulated in response to environmental signals, such as, nitrogen status, light, biotic and abiotic stress (Oliveira and Coruzzi 1999; Bernard and Habash 2009). Majority of the studies on GS in roots indicate the presence of only cytosolic form of the enzyme (Mann et al. 1979; Ishiyama et al. 2004a; Ishiyama et al.

2004b; Bernard et al. 2008) though according to few reports substantial activity of GS was also reported to be associated with the plastid fraction of the root cells as in case of pea and alfalfa (Vezina et al. 1987). In fact, in roots of pea and alfalfa as high as about 50% of the total GS activity was recovered in the plastidic fraction when the plants were grown in NO_3^- containing media (Vezina et al. 1987). The isozymes of GS are not only found in leaves, stems, roots and seeds of plants but also recognized in root nodules of several leguminous plants such as, peanuts (Bonald et al. 1978; Tingey et al. 1987), alfalfa (Dunn et al. 1988), beans (Ortega et al. 1992), medicago (Stanford et al. 1993; Lima et al. 2006) and soybeans (Temple et al. 1996). In root nodules, the primary function of GS is the rapid assimilation of NH_4^+ released into the cytosol of the infected cells by N_2-fixing bacteroids (Atkins 1987). A marked increase in activity of GS during the development of nodule coinciding with the appearance of nitrogenases and legheamoglobin has been noted by several investigators (Cullimore and Benette 1988; Temple et al. 1996; Carvalho et al. 2003).

1.3.2. Structural and catalytic properties

1.3.2.1. Structural properties

GS has been purified and characterized from a variety of plant species and tissues such as leaves of pea rice (Hirel and Gadal 1980), spinach (Ericson 1985), jack pine (Vezina et al. 1988) and rapeseed (Ochs et al. 1995); roots of pea (Emes and Fowler 1979), rice (Hirel and Gadal 1980; Ishiyama et al. 2004b) douglas-fir (Bedell et al. 1995), *Arabidopsis* (Ishiyama 2004a); nodules of soybean (Mc Parland et al.1976), alfalfa (Groat and Schrader 1982), lupin (Mc Cormack et al. 1982) and bean (Cullimore et al. 1983). Molecular weight of the native GS protein from plant sources ranges from 350-400 kDa and it comprises of eight subunits of molecular mass of 38 to 43 kDa for the cytosolic GS and that of 44 to 45 kDa for the chloroplastic isoform. These subunits are arranged in a parallel, biplane, tetrahedral symmetry to form an octameric enzyme. The octameric as well as tetrameric states of both cytosolic and chloroplastic forms of enzymes from leaves of *Beta vulgaris* were found to be active whereas in roots only octameric form was enzymatically active (Mack and Tischner 1990). The atomic structure of maize cytosolic GS has recently been elucidated at 2.63-, 3.50- and 3.80-Å resolutions, indicating that plant

GS polypeptides (Type II) form decamers (Unno et al. 2006), which differ from the dodecameric structure of bacterial GS (Type I) (*Figure 1.2*).

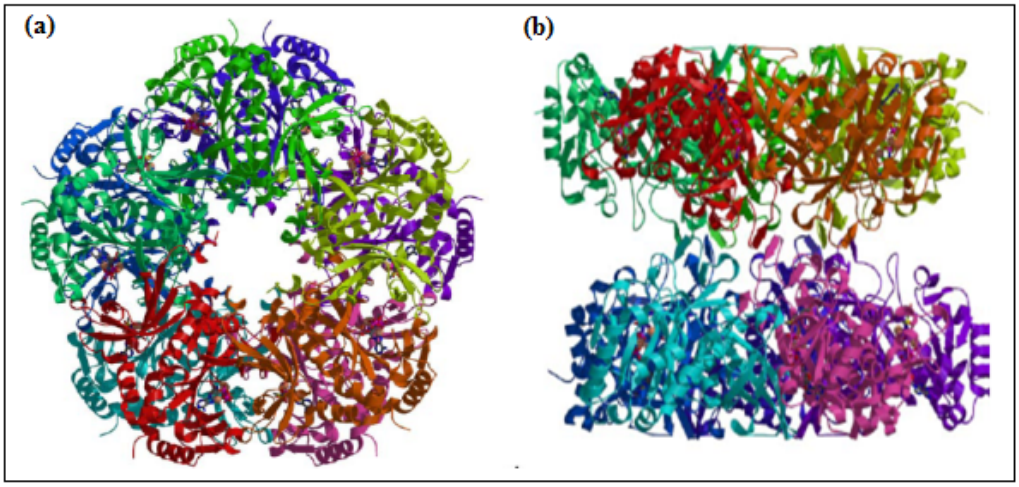

Figure 1.2. Structure of cytosolic GS from *Zea mays* (Maize) composed of two face-to-face pentameric rings of subunits contain 10 active sites, each between two neighbouring subunits within each ring. Source: Unno et al. 2006

1.3.2.2. Catalytic properties

In situ, the enzyme catalyses the following reaction which involves ATP-dependent amination of glutamate to glutamine:

$$\text{Glutamate} + \text{ATP} + NH^{+}_{4} \xrightarrow{Me^{2+}} \text{Glutamine} + \text{ADP} + \text{Pi} \qquad \text{(Reaction 1)}$$

Where, Me^{2+} can be magnesium or manganese. The reaction has been termed the 'biosynthetic' reaction and is considered the most physiologically relevant reaction that GS catalyzes.

The reaction is believed to proceed in two stages. In the first step γ-carboxylic group undergoes phosphorylation to yield γ-glutamylphosphate and in the next step phosphate group is replaced by amino group to form glutamine (*Figure1.3*). In addition of above physiological activity, isolated GS also catalyses the formation of γ-glutamylhydroxamate through either an ATP dependent semi-synthetase reaction (Reaction 2) or a transferase reaction (Reaction 3).

Glutamate + ATP + NH_2OH ⟹ γ-glutamylhydroxamate + ADP + Pi

(Reaction 2)

Glutamine + NH_2OH $\xrightarrow[Mn^{2+}, ADP]{Arsenate}$ γ-glutamylhydroxamate + NH_4^+

(Reaction 3)

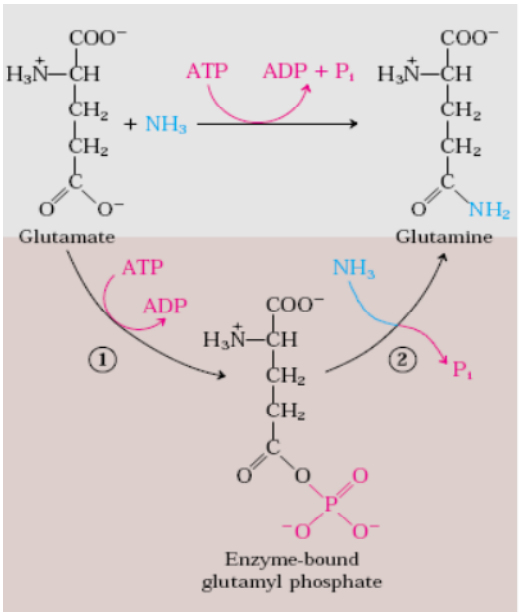

Figure 1.3. Reaction catalysed by glutamine synthetase.
Source: Nelson and Cox (2004)

The biosynthetic reaction of the enzyme has rarely been used for determination of GS activity in plants principally because, as compared to the semi-synthetase and transferase reactions, this activity is very low. Moreover, this assay is not suitable for use with unpurified extracts because of the presence of high activities of interfering enzyme such as glutaminase, ATPase, phosphatases and nucleotidases. On the other hand, semi-synthetase and transferase reactions have been generally used for determination of GS activity, since several folds higher activity of these reactions than biosynthetic activity offers a distinct advantage of increased sensitivity of the assay procedure (Lea et al. 1990).

1.3.3. Molecular characteristics

The primary translation product of GS2 has been shown to contain an N-terminal transit peptide which ostensibly facilitates its transport into chloroplasts and this transit

peptide is cleaved off after the enzyme is transported into the chloroplast. In contrast, such transit sequences have been shown to be absent in mRNA for GS1 isoform of the enzyme. Some key domains of the GS protein have been characterized, such as ATP-binding and glutamate-binding sites (Unno et al. 2006).

In angiosperms, GS2 is encoded by one gene and GS1 is encoded by multigene family consisting of three to five genes. Studies on GS1 gene families were first carried out in legume species, particularly *Phaseolus vulgaris* (Lara et al. 1983), *Pisum sativum* (Tingey et al. 1987) and *Medicago truncatula* (Stanford et al. 1993). GS gene families have also been described in non-legume plant species, such as *Zea mays* (Li et al. 1993), *Arabidopsis thaliana* (Ishiyama et al. 2004a), rice (Ishiyama et al. 2004b), potato (Teixeira et al. 2005), sugarcane (Nogueira et al. 2005) and wheat (Bernard et al. 2008). In cereal crops, the number of genes encoding cytosolic GS varies between three and five; wheat (GS1, GSr and GSe), rice (GS1;1, GS1;2. GS1;3), and maize (Gln1-1, Gln1-2, Gln1-3, Gln1-4 and Gln1-5). These genes are differentially expressed both in developmental and organ specific manner (Ishiyama et al. 2004b; Bernard et al. 2008).

GS gene and protein sequences are well conserved both within and across species. At the nucleotide level, cytosolic GS-encoding sequences are between 76 % and 82 % identical. At the amino acid level, the percentage similarity varies between 79 and 83 %, while the percentage identity varies between 88 and 93 %. Phylogenetic analyses of GS nucleotide or protein sequence have shown that chloroplastic and cytosolic GS emerged from gene duplication. The duplication occurred earlier than the monocot-dicot divergence (Bernard et al. 2008). Phylogenetic analyses of GS sequences retrieved from GenBank and plant transcript assembly databases gave further information on the evolution of the GS genes, in particular those encoding the cytosolic isoform. The topologies of the trees obtained with distance, parsimony, and likelihood phylogeny inference methods were overall similar (Pesole et al. 1991; Biesiadka and Legocki 1997; Swarbreck et al. 2011). The consensus tree obtained using maximum likelihood method indicated five monophyletic groups supported with high bootstrap values (*Figure 1.4*). One clade corresponded to the GS2 type while four additional clades included sequences encoding GS1.

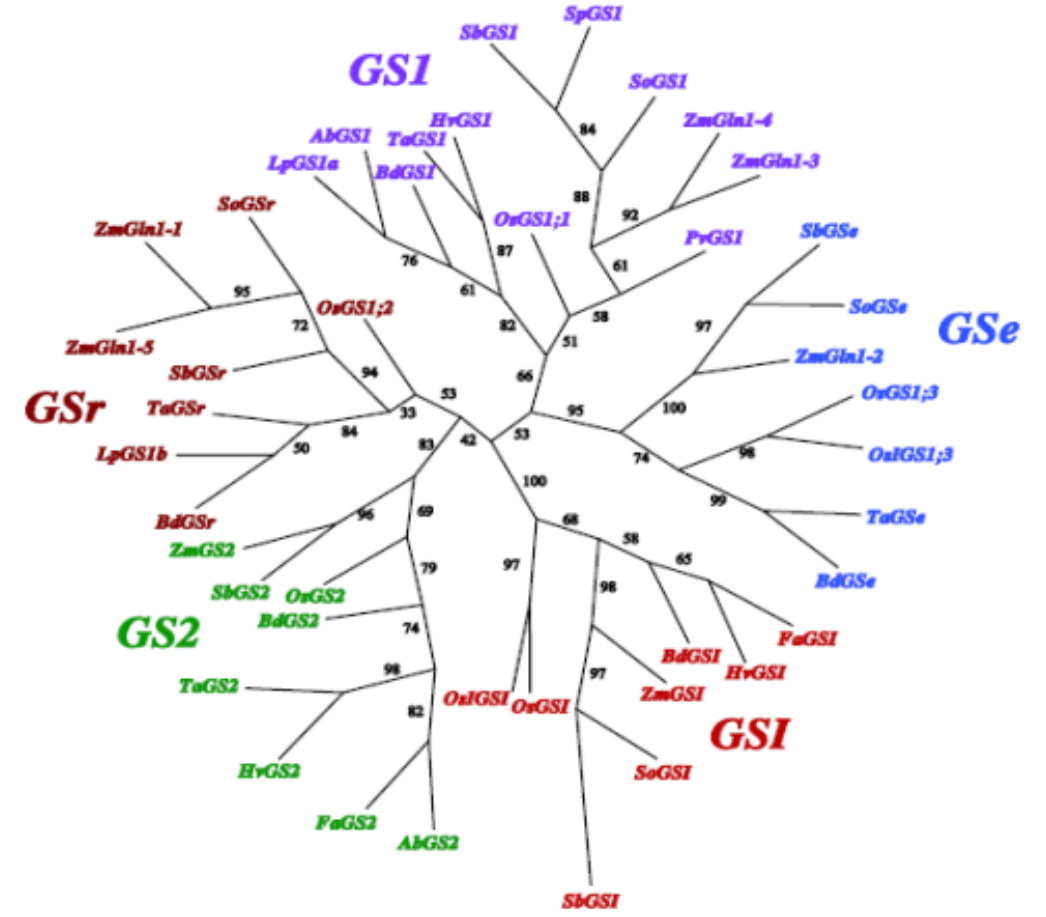

Figure 1.4. Unrooted phylogenetic tree of GS protein sequences from plant species of Poaceae family. The tree was calculated by using maximum likelyhood method. The reliabilities of each branch point were assessed by bootstrap analysis (100 replicates), and bootstrap values are displayed on the tree. GenBank accession number and transcript assembly number for each sequence used in the tree are listed below. *Brachypodium distachyon*: BdGS1, Bradi3g59970; BdGS2, Bradi5g24550; BdGSe, Bradi1g11450; BdGSI, Bradi3g27880; BdGSr, Bradi1g69530. *Festuca arundinacea*: FaGS2, TA701_4606; FaGSI, TA2341_4606. *Hordeum vulgare*: HvGS1, Q06378; HvGS2, P13564;HvGSI, AK252215. Lolium perenne: LpGS1a, ACR45959; LpGS1b, ACR45960. *Oryza sativa*: OsGS1;1, Os02g50240; OsGS1;2,Os03g12290; OsGS1;3, AAK18848; OsGS2, Os04g56400; OsGSI, Os10g31820. *Oryza sativa* Indica: OsIGln1;3, EEC76061; OsIGSI, EEC67088. *Panicum virgatum*: PvGS1, TA1886_38727. *Sorghum bicolor*: SbGS1, Sb04g028690; SbGS2, Sb06g031460; SbGSe,Sb01g010270; SbGSI, Sb01g020230; SbGSr, Sb01g042450. *Saccharum officinarum*: SoGS1, AAW21273; SoGSe, AAW21275; SoGSI, AAW21277; SoGSr, TA36550_4547. *Sorghum propinquum*: SpGS1, TA3613_132711. *Triticum aestivum*: TaGS1, AAZ30057; TaGS2, AAZ30060; TaGSe, AAR84349; TaGSI, TA75518_4565; TaGSr, AAR84347. *Triticum monococcum*: TmGS2, TA2112_4568. *Zea mays*: ZmGln1-1, P38559; ZmGln1-2, CAA46720; ZmGln1-5, P38563; ZmGln1-3, CAA46721; ZmGln1-4, CAA46722; ZmGS2, CAA46724; ZmGSI, GRMZM2G115646. *Avena barbata*, AbGS2, AbGSr, and AbGS1 were assembled from ESTs. Source: Swarbreck et al. 2011

The cytosolic GS genes are clustered into three phylogenetically distinct groups corresponding to GS1, GSr and GSe and an additional clade including GS1 and GSe groups, suggesting that these two groups are more related to each other than to the GSr group (Swarbreck et al. 2011).

1.3.4. Regulation of GS isoforms by light

Activity of GS in plant tissues is influenced by several environmental factors. Light is one of the important factors controlling the level of GS in photosynthetic tissues. An enhancement in GS activity during greening of leaves has been reported by several workers (Hirel et al. 1982; Canovas et al. 1986; Edward and Coruzzi 1989; Ghosh 2010). In all these investigations, light was found to exert a more profound influence on the appearance of GS2. In etiolated leaves of sorghum GS activity was detectable only in the cytoplasmic fraction and during greening increase in total activity was largely associated with the appearance of activity in the chloroplastic fraction (Hirel and Gadal 1982). On the other hand, etiolated sunflower cotyledons (de la Haba et al. 1992), and etiolated leaves of maize (Sakakibara et al. 1992) and *Pennisetum glaucum* (Ghosh 2010) were found to contain both GS isoforms and, on exposure to light the level of GS1 showed marginal increase and level of GS2 increased significantly. Functional expression analysis of the GS1 promoter in transgenic *Arabidopsis* also indicated the presence of regulatory sequences involved in the response to light (Avila et al. 2001).

In etiolated pea leaves, the steady-state levels of both the chloroplast-specific GS2 polypeptide and the GS2 mRNA increased during greening and are regulated by phytochrome (Tingey et al. 1987; 1988). Moreover, the fact is supported by the expression study of GS2 in mustard (Schmidt and Mohr 1989), wheat (Edwards and Coruzzi 1989), maize (Sakakibara et al. 1992), *Phaseolus* (Cock et al. 1991) and *Arabidopsis* (Peterman and Goodman 1991). In these studies GS2 was shown to be tightly regulated by light in a process at least in part mediated by phytochrome. However, Edward and Coruzzi (1989) noted and emphasized that white light elicited accumulation of GS2 mRNA several fold higher than that obtained with brief pulse of red light. Hence, they deduced that besides acting through phytochrome, light also exerted additional effect probably via its influence

in chloroplast metabolism. The level of GS1 gene expression was not significantly affected by light (Oliveira and Coruzzi 1999). According to some reports, light mediated enhancement in GS activity might involve activation of previously synthesized enzyme which was stored in inactive form in the plant tissue (Canovas et al. 1986; Ghosh 2010). The light dependent appearance of GS in *Pennisetum* seedling was inhibited by the photosynthetic inhibitor, dichlorophenyl dimethyl urea (DCMU) (Ghosh 2010). Recent studies on regulation of GS have also indicated the role of photosynthetic reactions in light dependent stimulation of GS. Exposure of sunflower plant to increasing CO_2 concentration in light caused concomitant increase in leaf starch and sugar contents and activities of both GS1 and GS2 (Larios et al. 2004).

1.3.5. Regulation of GS by nitrogen status

GS responds to nitrogen availability in the external medium as well as plant nitrogen status. Thus far, it has been difficult to establish a global model of GS response to variation in nitrogen availability in both roots and leaves. While NH_4^+ had no influence on appearance and activity of GS in sunflower cotyledons (de la Haba et al. 1988), it was reported to suppress the activity of GS in mustard cotyledons (Schmidt and Mohr 1989) and wheat cells (Fricke 1993).

Investigations have also been carried out on the changes in activities and expression of GS isoforms in response to nitrogen nutrition. In barley seedlings, the specific activity of GS was found to be much higher in leaves but declined in root in presence of NH_4^+ as compared to specific activities in respective organs of NO_3^- fed or N-free grown plants. With higher concentration of NH_4^+, the specific activity of GS1 rose in leaves but fell in roots. The activity of GS2 in leaves was also elevated with increasing concentration of NH_4^+ in the nutrient medium. The alterations in activities of GS1 and GS2 were correlated with changes in the subunit composition of the active holoenzyme (Mack 1995). NH_4^+ is supposed to substrate-induce the GS1 promotor of rice (Kozaki et al. 1991) and soybean (Hirel et al. 1987; Miao et al. 1991). Kozaki et al. (1992) showed that the GS2 promotor of rice is also activated by NH_4^+. Although some cytosolic GS gene members are up-regulated by the addition of NH_4^+, some are down-regulated or do not respond (Sakakibara et al. 1992; Ishiyama et al. 2004 a,b; Hirel et al. 2005; Kusano et al. 2011). In *Arabidopsis*,

GS1 mRNA and polypeptide accumulated in roots when plants were supplied with NH_4^+, however, the GS activity was maintained at a constant level. The discrepancy between the protein content and enzyme activity of GS1 was attributed to the kinetic properties and expression of four distinct isoforms encoded by *GLN1;1*, *GLN1;2, GLN1;3* and *GLN1;4* genes that function complementary to each other in *Arabidopsis*. GLN1;2 was significantly up-regulated by NH_4^+ and correlated with the rapid increase in total GS1 protein. However, GLN1;2 exhibited lower affinity to the substrates NH_4^+ and glutamate. In contrast, high affinity enzyme GLN1;1 was abundantly expressed in surface layer of root during nitrogen limitation and down regulated by NH_4^+ excess (Ishiyama et al. 2004a). Similarly in rice root the cytosolic OsGS1;1 and OsGS1;2 transcripts showed reciprocal response to NH_4^+ supply in the surface cell layers of roots. OsGS1;1 accumulated in the dermatogens, epidermis and endodermis under nitrogen limited conditions. By contrast, OsGS1;2 was abundantly expressed in the same cell layers under nitrogen sufficient conditions replenishing the loss of OsGS1;1 following NH_4^+ treatment (Ishiyama et al. 2004b). Recent study on quantitative comparative analyses between the metabolite profiles of a rice mutant lacking OsGS1;1 and its background wild type (WT). The mutant plants exhibited severe retardation of shoot growth in presence of NH_4^+ compared with the WT. Overaccumulation of free NH_4^+ in the leaf sheath and roots of the mutant indicated the importance of OsGS1;1 for NH_4^+ assimilation in both organs. The metabolite profiles of the mutant line revealed: (i) an imbalance in levels of sugars, amino acids and metabolites of the tricarboxylic acid (TCA) cycle, and (ii) overaccumulation of secondary metabolites, particularly in the roots under a continuous supply of NH_4^+. Metabolite-to-metabolite correlation analysis revealed the presence of mutant-specific networks between tryptamine and other primary metabolites in the roots. These results demonstrated a crucial function of OsGS1;1 in coordinating the global metabolic network in rice plants grown using NH_4^+ as the nitrogen source (Kusano et al. 2011).

NO_3^- had little effect on appearance of GS in mustard cotyledons (Schmidt and Mohr 1989) and scot pine seedlings, whereas in sunflower cotyledons (de la Haba et al. 1992) and maize leaves (Aguera et al. 1987) it strongly enhanced the level of the enzyme. NO_3^- enhanced the GS2 activity approximately by 4 fold in mesophyll cells and by 1.3 fold in bundle sheath cells of maize leaves but no enhancement was detected in GS1 level

(Sakakibara et al. 1992). Since the primary reaction of NO_3^- assimilation in C4 plants occur in mesophyll cells, the elevated activity was attributed to increased demand for assimilation of ammonia produced from nitrate reduction of these cells. An increase in the GS2 polypeptide content caused by NO_3^- nutrition was also found in pea roots (Vezina and Langlois 1989) and cultured rice cells (Hayakawa et al. 1990). Transcript stability is another means of GS1 regulation in response to nitrogen nutrition (Ortega et al. 2006). However, it is not clear whether plant nitrogen status or NO_3^- molecules interact with the cis-acting element at the 3' end of the GS1 transcript.

1.3.6. Regulation of GS isoforms by abiotic and biotic stress

Environmental stresses such as abiotic (drought, salinity and low or high temperatures) and biotic stresses are important factors which limit plant distribution and productivity (Hare et al.1999). Abiotic stress has toxic effects on plants and lead to metabolic changes, like loss of chloroplast activity, decreased photosynthetic rate and increased photorespiration rate (Winicov 1993). Teixeira and Fidalgo (2009) have suggested that nitrogen assimilation is more sensitive to water stress than CO_2-photosynthetic assimilation. The effect of abiotic stress on plant nitrogen metabolism has been studied with inhibition of protein synthesis, increased protein degradation and accumulation or depletion of protein and non-protein amino acids in a variety of monocots, dicots (Gilbert et al. 1998; Martinelli et al. 2007). Under drought conditions the expression of gene encoding ubiquitin related proteins and various proteases were found to be induced or enhanced, consistent with the requirement of protein degradation under stress conditions. All these processes ultimately lead to either acclimation to the stress conditions or to senescence and subsequent cell death. The response of GS to salt and drought stress hasn't been studied in much detail. During abiotic stress the abundance of GS2 polypeptide and its activity declined, whereas GS1 tended to increase or maintain the same level in the leaves (Bauer et al. 1997; Santos et al. 2004; Martinelli et al. 2007). The work of Hoshida et al (2000) also showed the importance of GS2 in salinity tolerance in *Oryza sativa*. Plant transformed with GS2 gene had increased photorespiratory capacity conferring resistance to salinity stress (Hoshida et al. 2000). The response of GS to abiotic stress in the roots is less clear, with studies on rice seedlings and potatoes showing decline in total GS activity in response to salt stress (Teixeira and Pereira 2007; Teixeira and Fidalgo 2009).

The accumulation of metabolite such as, proline, glycine betaine, polyols, polyamines and ions (i.e. potassium), is one of the mechanisms for stress tolerance (Kishor et al. 1995; Bajji et al. 2000). They act both by contributing to osmotic adjustment and by protecting proteins and cellular membranes. The studies of Brugiere et al (1999) have shown that silencing of a phloem specific GS (*Gln* 1-5) decreased proline production. GS thus plays a major role in regulating proline production consistent with the function of proline as a nitrogen source and a key metabolite synthesized in response to water stress. In potato leaf, in response to high salt levels GS2 regulation resides at the post-translational level while root GS1 is mainly regulated at a transcriptional level, with a differential expression of the GS1-encoding genes (Teixeira and Pereira 2007; Teixeira and Fidalgo 2009).

Nitrogen nutrition has a significant impact on plant disease development. Reduced availability of nitrogen often increases the susceptibility of plant to diseases. Some evidences exist for the induction of GS gene in infected leaf cells in plants under pathogen attack (Olea et al. 2004; Pageau et al. 2006; Tavernier et al. 2007). GS1 was upregulated in leaves of tobacco (Pageau et al. 2006) and *Phaseolus vulgaris* (Tavernier et al. 2007) in response to viral and bacterial attack and during infection with pathogenic and non-pathogenic fungal strains. However, this induction did not depend on a hypersensitive response induction. Thus GS1 responded in the same manner as an early-response defence genes. The up-regulation of GS1 (*Gln-α*) in *P.vulgaris* was also accompanied by an increase in GS1 polypeptide abundance, but decline in overall activity, mainly resulting from decline in GS2 activity. Thus cytosolic GS regulated at transcript abundance level both by abiotic and biotic stress. However, it is unclear whether an increase in transcription occurs or mRNA transcript becomes more stable. Pathogen infection can also affect proline content and gene expression of proline metabolism. Fabro et al. (2004) found that inoculation of Arabidopsis with a virulent *P. syringae* produced a hypersensitive response led to increased proline synthesis around the inoculation site. The NH_4^+ imbalance thus created was compensated by plants assimilation of NH_4^+ into glutamine and glutamate.

1.3.7. Regulation of GS isoforms by senescence

Senescing leaves act as source of nitrogen for growing parts as their constituent proteins are hydrolysed and organic nitrogen is redistributed within the plant. The amides, glutamine and asparagines, are the major organic form for translocation of nitrogen in plants (Hayashi and Chino 1990). These amides are produced from glutamate and aspartate, respectively, using NH_4^+ released upon hydrolysis of Rubisco and other leaf proteins. Since GS is the main enzyme catalyzing the conversion glutamate and NH_4^+ to glutamine, a few studies have also been conducted on changes in GS activity in senescing leaves. GS activity is known to decrease rapidly during both natural and dark induced senescence (Kawakami and Watanabe 1988; Fischer and Feller 1994). The activities of GS1 and GS2 are differentially regulated by leaf age. The GS2 polypeptide level declined in parallel to Rubisco and other chloroplast enzymes during natural senescence in leaves of various plants. The decline in GS2 coincided with an increase in abundance of GS1 (Kamachi et al. 1992; Habash et al. 2001; Bernard et al. 2008). Similarly, in potato GS2 polypeptides and mRNAs were detected in leaves and their content decreased as leaves senesced, whereas GS1 was detected in non-photosynthetic tissues and in the later leaf senescing stages (Teixeira et al. 2005). Kichey et al (2005) studied the localization of GS1, GS2 and glutamate dehydrogenase (GDH) during natural senescence of the flag leaf and in the stem of wheat seedlings. In mature flag leaves, large amounts of GS1 were detected in the connections between the mestome sheath cells and the vascular cells, suggesting an active transfer of nitrogen organic molecules within the vascular system in the mature flag leaf. Parallel to leaf senescence, an increase of a GS1 polypeptide (GS1b) was detected in the mesophyll cytosol of senescing leaves, while the GS protein content represented by another polypetide (GS1a) in the phloem companion cells remained practically constant in both leaves and stems. Both GDH aminating activity and protein content were strongly induced in senescing flag leaves. The induction occurred both in the mitochondria and in the cytosol of phloem companion cells, suggesting that the shift in GDH cellular compartmentation is important during leaf nitrogen remobilization.

1.3.8. Physiological role of GS isoforms

Plants have evolved to capture and assimilate the available carbon and nitrogen from the environment and to store and relocate it efficiently. During the growth and

development of plants, nitrogen moves into and out of proteins in the different organs and transported between organs in a limited number of transport compounds that differ widely in their C/N ratio (Miflin and Habash 2002).

Plants have developed a variety of molecular strategies to use limiting nutrients with a maximum efficiency. Some of the organic nitrogen moves between compounds via the activity of transaminases and glutamine-amide transferases, but a significant portion is released as NH_4^+. For example, asparagine is a significant component of seed storage proteins in legumes and a major transport compound in cereals. It is metabolized to NH_4^+ and aspartate via the action of asparaginase. Similarly ureides, such as allantoin play an important role in nitrogen transport in legumes and their organic-nitrogen is released as NH_4^+ via the action of urease. Nitrogen containing into biomolecules also release NH_4^+ by plant metabolic activities in various physiological processes and such as photorespiration, the biosynthesis of phenylpropanoids. Thus over the life of a plant, nitrogen is released as NH_4^+ and refixed several times. Overall GS acts at the centre of nitrogen flow (*Figure 1.5*). As mentioned earlier, with a few exceptions leaves of most of higher plants contain cytosolic and chloroplastic GS though their relative proportion may vary markedly depending upon species and to some extent on the environmental factors.

1.3.8.1. Physiological role of cytosolic GS

In both C3 and C4 species, cytosolic GS is located in vascular bundle (Kichey et al. 2005; Martin et al. 2006). The promoter analysis of the GS3A gene of pea suggested that cytosolic GS is preferentially expressed in the vascular tissue of leaves (Edwards et al. 1990). Many subsequent studies have confirmed the importance of the location of GS1 in the phloem and related vascular tissues (Edwards et al. 1990). Furthermore, recent studies have shown that GS1 is located in intermediary cells, where nitrogen is exchanged between different cell types, such as the primary pit fields connecting the mestome sheath cells and the neighbouring parenchyma and vascular cells in wheat leaves (Kichey at al. 2005).

The localization of GS1 transcript and polypeptides in roots has led to suggestion that it is involved in assimilation of NH_4^+ from primary nitrogen uptake (Ishiyama et al.

2004a,b; Bernard et al. 2008). Furthermore, *in situ* hybridization study with rice has shown that the location of OsGS1;2 transcript is consistent with the site of NH_4^+ uptake (Sonoda et al. 2003; Ishiyama et al. 2004b; Tabuchi et al. 2007). A study of barley mutants deficient in GS2 showed a normal phenotype and no nitrogen deficiency when grown under nonphotorespiratory conditions (Wallsgrove et al. 1987; Leegood et al. 1995), suggesting that cytosolic GS is also involved in primary nitrogen assimilation. Recent study on quantitative comparative analyses between the metabolite profiles of a rice mutant lacking OsGS1;1 and its background wild type (WT). The mutant plants exhibited severe retardation of shoot growth in presence of NH_4^+ compared with the WT.

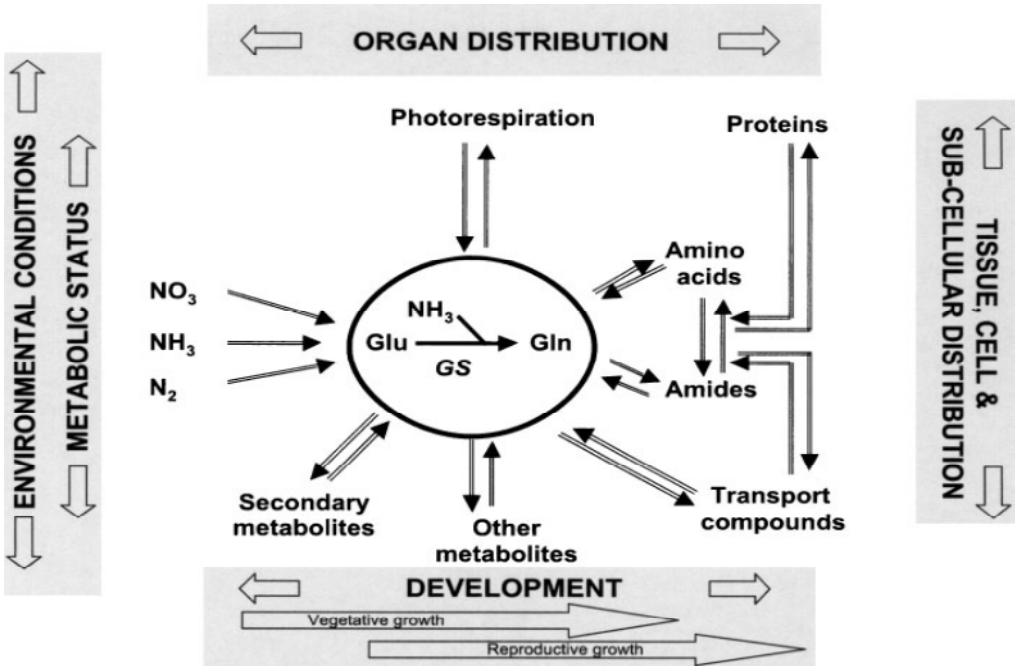

Figure 1.5. The central role of GS in the complex matrix of plant N metabolism. The central scheme encompasses the total role of GS. The boxes around the outside indicate the matrix of various locations and environments in which GS may be operating. Source: Mifilin and Habash 2002

Overaccumulation of free NH_4^+ in the leaf sheath and roots of the mutant indicated the importance of OsGS1;1 for NH_4^+ assimilation in both organs. The production of NH_4^+ that occurs either within the plants tissues or in nodules can significantly contribute to plant nitrogen nutrition. Cytosolic GS is a known to assimilate NH_4^+ from the three major types of nitrogen-fixing symbiotic association involving plant or either Rhizobium, actinomycetes or cyanobacteria (Rai et al. 2000). It also assimilates NH_4^+ produced by

endophytic diazotrophs colonizing the internal parts of some plants, such as sugarcane, without establishing symbiotic relationship (Nogueira et al. 2005). Cytosolic GS has also been implicated in remobilization of nitrogen for grain filling. Significant correlation were obtained between grain number/size and a locus of cytosolic GS protein (OsGS1;1) content in rice (Obara et al. 2004). In maize QTLs were found for GS activity, cytosolic GS locus (gln1) and grain yield (Hirel et al. 2001, 2007). These studies highlight the importance of cytosolic GS genes in determining several aspects of nitrogen use traits in the cereal crops with potential implications for breeding and agriculture (Hirel et al. 2007).

1.3.8.2. Physiological role of chloroplastic GS

The important function of GS2 is conceivably to assimilate NH_4^+ formed via NO_3^- reduction (Melo et al. 2003). Photorespiration, which is the side reaction of photosynthesis, has important implication in plant nitrogen metabolism since during the process substantial amounts of NH_4^+ is released in the reaction involving oxidative decarboxylation of glycine in mitochondria (Buchanan et al. 2002; Keys 2006). It has in fact estimated that in C3 plants the rate of ammonia evolution through photorespiratory cycle is about ten times the rate of NH_4^+ acquired through NO_3^- assimilation (Adriano et al. 2010). It has been unequivocally established that mitochondria do not contain GS. Since many higher plant species contain both chloroplastic and cytosolic GS isoforms in leaves, it was initially proposed that GS1 might be involved in reassimilation of photorespiratory NH_4^+. However, this contention was not supported by the subsequent studies. Barley mutant lacking chloroplastic GS accumulated ammonia in higher amounts only when grown under photorespiratory conditions (Wallsgrove et al. 1987; Blackwell et al. 1987), thereby indicating the importance of GS2 in reassimilation of photorespiratory NH_4^+. The role of GS2 was also supported by several other observations. In pea plants grown under conditions which favour higher photorespiratory activity (0.02 % CO_2), a four-fold increase in the level of GS2 mRNA was recorded whereas GS1 mRNA remained unaffected (Edward and Coruzzi 1989). Though the increase in GS2 mRNA was detectable after 14 days of the treatment, Woodall et al (1996) noted that activity of GS2 undergoes a much more rapid modulation even prior to the increase in level of transcript on altering the phorespiratory status of the plant. In their studies, during which the

photorespiratory rates were stimulated or depressed by lowering or increasing the growth temperature, caused much rapid response in activity of GS. The activity of GS2 in barley plant grown at 25^{o} C day temperature decreased by 75%, after three days at 15^{o} C with no effect on the cytosolic activity (Woodall et al. 1996).

1.3.9. The model plant, Rice (Oryza sativa L.)

Rice is one of the most important crops for mankind. It is the staple food of more than 60% of the world's population especially for most of the people of South-East Asia. It accounts for more than 50% of their daily calorie intake (Maclean et al. 2002). Among the rice growing countries in the world, India has the largest area under rice crop and ranks second in production next to China. Rice occupies about 23.3% of gross cropped area of India and plays vital role in the national food grain supply.

1.3.9.1. Taxonomy and Genome

The genus *Oryza* belongs to the tribe Oryzeae of the family Poaceae. There are 12 genera within the Oryzeae tribe (Vaughan 1994). The *Oryza* contains approximately 22 species, of which 20 are wild species and two; *O. sativa* and *O. glaberrima* are cultivated. *O. sativa* is most widely grown of the two cultivated species and is grown worldwide, including Asian, North and South American, European Union and African countries. However, *O. glaberrima* is grown solely in West African countries (Vaughan 1994). Among cereals, *O. sativa* has a relatively small (430 million base pairs) diploid genome (2n = 24) as compared to the significantly large genome sizes of sorghum, maize, barley, and wheat (about 750, 3000, 5000, and 16000 Mbp, respectively). Recent data place the number of genes in rice genome to about 50,000 (Goff et al 2002; Yu et al. 2002), reflecting even higher gene density. Moreover, rice contains relatively less repetitive DNA. Most other *Oryza* species are also diploid, however some are tetraploid (4n = 48) (Moore et al. 1995; Gale and Devos 1998).

1.3.9.2. Origin, Cultivation and Climate

O. sativa was cultivated in south-east Asia, India (*Figure 1.6*) and China between 8000 to 15000 years ago (Normile 2004). *O. glaberrima* was has been cultivated since approximately 1000 BC (Ahn et al. 1992; Murray 2005). Current cultivation of *O. sativa* is worldwide, over 110 countries.

Figure 1.6. Cultivation of *O. Sativa* in India.

Rice is grown from sea level to 3000 m and in both temperate and tropical climate. A varieties of water regimes are used, including unsubmerged upland rice (10% of total cultivation), moderately submerged lowland rice (irrigated, 45% or rainfed, 30%) and submerged rice (upto 6 m of water, 11% or floating 4%). Rice can grow in a wide range of soil types as well, including saline, alkaline and acid-sulfur soils (Ahn et al. 1992). The chemical properties of soils do not appear to be as important as physical ability of the soil to hold a flood (Scott et al. 2003). Proper growth and high production of rice crop is conditioned by temperature parameter at different growth stages. During flowering, it should range between 16º to 20 ºC, whereas during maturity 28º to 32 ºC temperature is optimum. The temperature beyond 35 ºC affects grain filling. During panicle initiation, 20º-22 ºC temperatures is ideal. The temperature below or above this range will adversely affect the growth and yield. Low temperature is one of the main limits on crop yield (McDonald 1994). Japonica cultivars are predominantly grown in temperate regions and can germinate and grow under low temperature (15º to 20 ºC) than the tropical and sub-tropical Indica cultivars. Temperature below 18 ºC at night during pollen formation results in sterile pollen in all cultivars (McDonald 1994).

Generally, rice plant requires about 14 hours of daylight every day during its growth period. When the rice plant is subjected to day length shorter than 12 hours, it will come to flowering. So, when rice is subjected to lower temperature and short duration of

sunshine, its vegetative phase will be reduced considerably. Too long or too short vegetative phase adversely affects the reproductive phase. In general, longer hours of sunshine with a temperature varying from 20º-35 ºC and abundant moisture supply are the ideal conditions for rice plant. Late maturing varieties are very sensitive to the day length and can be grown only during a specific season, while early maturing varieties can be grown at any time of the year but mostly during summer and kharif season (Vergara and Chang 1985).

1.3.9.3. O. sativa development

The life cycle of rice cultivars ranges from 110 to 150 days from germination to maturity, depending on the variety and the environment. *O. sativa* is an annual grass, bearing a fibrous root system as well as erect culms and developing long flat leaves (*Figure 1.7*). Rice plant forms multiple tillers, consisting of a culm and leaves, with or without panicle. The panicle emerges on the uppermost node of a culm, from within a flag-leaf sheath and bears the flowers in spikletes. Primary tillers emerge from nodes near the base of the main culm and secondary and tertiary tillers emerge sequentially from these.

Figure 1.7. Illustrating different parts of *O. sativa* plants

Single leaves develop alternatively on the culm, consisting of a sheath, which enclose the culm and a flat leaf blade. Rice cultivars can vary widely in the length, width, colour and pubescence of the leaves. Each spikelet has a single floret and two glumes. It is enclosed

by a rigid, keeled lemma which is sometimes extended to form an awn and partially envelops the smaller palea. The floret contains six stamens and a single plumose ovary with two branches. At anthesis, two lodules at the base of floret swell and force the lemma and palea apart as the stamens elongate and emerge. The stigma is sometime exposed as well. The fertilized ovary is caryopsis, meaning a small, single-seeded dry fruit with the pericarp and seed coat fused. It is commonly called a grain. The grain consists of an embryo, endosperm, pericarp and testa, surrounded by hull (the lemma and palea). Grain length varies with cultivar between 5 and 7 mm, and grains can be round, bold or slender (OECD 1999; McDonald 1979).

1.3.10. The model plant, Selaginella bryopteris L.

1.3.10.1. Origin, taxonomy and habitat

Selaginella, called spike moss, is an enigma in the plant kingdom. It belongs to the lycophyte lineage of vascular plants that arose over 400 million years ago during the Silurian and dominated the Earth's flora from the Devonian through the Carboniferous to the end of the Permian (Friedman 2011). The evolution of lycophytes was accompanied by the acquisition of roots with root caps, and typically simple and single-veined leaves called microphylls (Friedman 2011). Lycophyte includes families, the Lycopodiaceae (club mosses), the Isoeteaceae (quillworts), and the Selaginellaceae (spike mosses). *Selaginella* (family Selaginellaceae, order Selaginellales, class Lycopsida) constitutes a monophyletic group of plants with microphylls and adaxial, reniform sporangia (Banks 2009). It is represented by about 750 species in the world (Jermy 1990) and 65 species in India (Nisha et al. 2010). It grows in diverse array of arctic, temperate, tropical, and semiarid habitats (Banks 2009; Yobi et al. 2013). Although most spike moss species are susceptible to desiccation, a few species have evolved the ability to survive vegetative tissue drying, defined as the near complete loss (80-95%) of protoplasmic water (Tuba et al. 1998), and referred to as desiccation tolerant (DT) (Oliver et al. 2000). Such species include *S. lepidophylla* (Iturriaga et al. 2006), *S. bryopteris* (Deeba et al. 2009), and *S. tamariscina* (Wang et al. 2010). These DT species grow luxuriantly during rains exhibiting a lush green velvety landscape (*Figure 1.8*). During summer the plants undergo extreme desiccation. The fronds curl, become dry and virtually dead. The dry plants when left in water unfold their fronds, turn green and come back to active life (Sah et al. 2005).

1.3.10.2. Selaginella genome

The genome size of nine *Selaginella* species was determined by flow-cytometry analysis. The genome size estimates ranged from 84 - 110 Mb. Within the nine species the chromosome number varied between 2n = 16 to 2n = 27. Nuclear genome size appeared to be strongly correlated with chromosome number (Little et al. 2007).

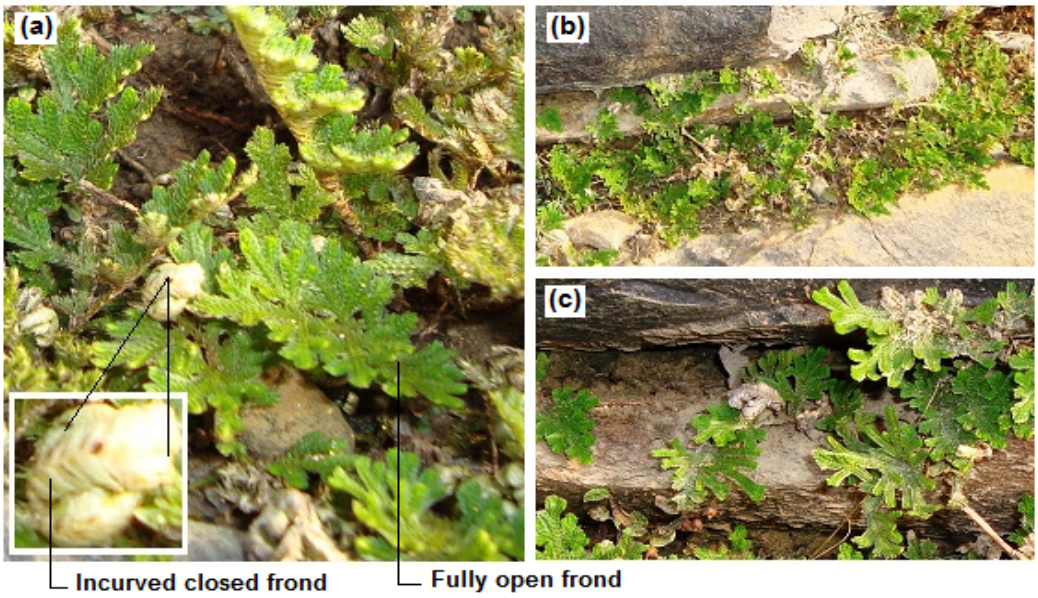

Figure 1.8. *Selaginella bryopteris* in natural habitat

The *Selaginella* genome was sequenced using whole-genome shotgun sequencing. The assembled genome size (212.60 Mbp) is twice that determined by flow cytometry (Wang et al. 2005; Little et al. 2007), indicating that the assembled genome includes two haplotypes of ~106 Mbp that are 98.50% identical at the nucleotide level. A deduced haplotype has 22,285 predicted protein-coding genes, of which 37% are supported by EST sequences (Axtell et al. 2007). Recently, Bank et al (2011) reported the genome sequence of *S. moellendprffti*. By comparing gene content in evolutionary diverse taxa, they found that the transition from a gametophyte to sporophyte - dominated life cycle required far fewer new genes than the transition from a non-seed vascular to a flowering plant. The secondary metabolic genes expanded extensively and in parallel in the lycophyte and angiosperm lineages. *Selaginella* differed in post-transcriptional gene regulation,

including small RNA regulation of repetitive elements, an absence of the tasiRNA pathway and extensive RNA editing of organellar genes.

1.3.10.3. Morphology and life cycle

Selaginella plants are 10-14 cm in size. Stem is erect, creeping and dichotomous branch with or without certain pattern, from which roots also arise (*Figure 1.8*). The dichotomous branches have two different sizes of leaves; where median is smaller than lateral ones (Jermy 1990). Microspore is much smaller than megaspore and usually has different colour (*Figure 1.9*).

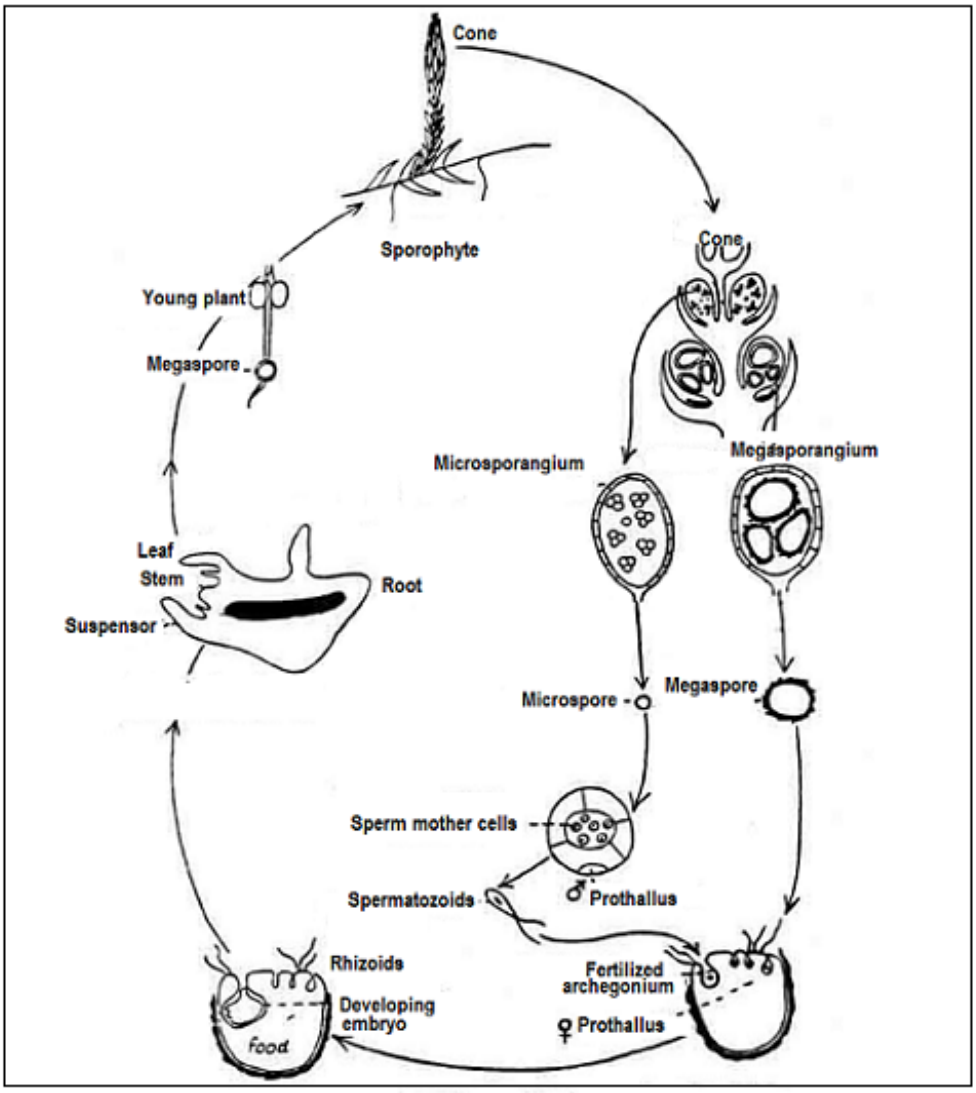

Figure 1.9. *Selaginella* life cycle.

The megaspores split open to reveal the female gametophyte. The Microspores develop many 100's of motile sperm, and when the spore bursts in contact with water these swim to the female gametophytes. This is a parallel to the situation with seed plants, in which the gametophyte is retained within the sporophyte.

1.3.10.4. Molecular properties

Biflavanoid is the major secondary metabolite of *Selaginella* species. *Selaginella* contains several secondary metabolites such as, alkaloids, phenolics (Flavanoid, tannin, saponin) and terpenoids (Chikmawati 2008). The different species of *Selaginella* varies in their secondary metabolite composition. The plant samples of the same species collected in different period, different environment or different locations, also shows variation in HPLC fingerprint. The therapeutic potential of *Selaginella* secondary metabolites has been well documented. *S. lepidophylla* has been used as a diuretic for uninary and kidney infections and in treatment of chronic gastritis and gastric cancer (Robles-Zepeda et al. 2011). Mishra et al (2011) have reported the anticancer properties of *S. bryopteris*. Total flavonoids from *S. tamariscina* have also been employed as an anti-diabetic treatment (Zheng et al. 2011). *Selaginella* species have also been studied for their therapeutic potential as anti-viral, anti-microbial, anti-inflammatory and anti-oxidant activities (Swamy et al. 2006; Aguilar et al. 2008; Tan et al. 2009; Liu et al. 2010; Wang et al. 2011).

1.3.10.5. Drought tolerance

Drought tolerance is extremely rare in vascular plants and only about 0.15% of species being known as resurrection plants (Oliver et al. 2000; Proctor and Tuba 2002). Although most spike moss species are susceptible to desiccation, a few species have evolved the ability to survive vegetative tissue drying, defined as the near complete loss (80-95%) of protoplasmic water (Tuba et al. 1998), and referred to as desiccation tolerant (DT) (Oliver et al. 2000). Such species include *S. lepidophylla* (Iturriaga et al. 2006), *S. bryopteris* (Deeba et al. 2009), and *S. tamariscina* (Wang et al. 2010).

The response of resurrection mosses to desiccation has been shown to rely on a combination of constitutive protection and repair mechanism (Oliver et al. 2005). In

contrast, DT angiosperm require a much longer time to dehydrate, presumably to allow sufficient time for developing adaptive responses and accumulation of key metabolite, such as sucrose, proline, to survive in the desiccation state (Oliver et al. 2000; Suzuki et al 2006). Since, lycophytes represents a plant lineage between mosses and angiosperms, they might be expected to exhibit both constitutive and inducible adaptive mechanism of drought tolerance (Banks et al. 2011). Several *Selaginella* species are able to survive on long drought and recover through rehydration (van Dijck et al. 2002). DT *Selaginella* species have high content of trehalose, a simple sugar responsible for endurance of heat and drought stress (Adams et al. 1990; Setyawan, 2011). The trehalose-6-phosphate synthase (TPS) gene of *S. lepidophylla* was homologous to that of yeast and bacteria (van Dijck et al. 2002). Several sugars (e.g., glucose, sucrose), sugar alcohols (e.g., inositol-1-phosphate, myoinositol, mannitol), and betaine, which act as osmoprotectants and/or hydroxyl radical scavengers were more abundant in *S. lepidophylla* at 100% and 50% relative water contents (Yobi et al. 2012).

A previous study by Harten and Eickmeier (1986) showed significant increase in activity of photosynthetic enzymes during hydration of DT *S. lepidophylla* fronds. However, a recent study has reported DT *Selaginella* species to retain their chlorophyll (and presumably their photosynthetic structures) during desiccation (Pandey et al. 2010). To understand the mechanisms of desiccation tolerance, Deeba et al (2009) carried out proteome based studies on detached *S. bryopteris* fronds to reveal proteins that were differentially expressed in response to dehydration and rehydration. It was observed that proteins involved in transport, targeting and degradation were expressed more in the desiccated fronds. The research work by Pandey et al (2010) indicated several desiccation induced changes in *S. bryopteris* fronds. The desiccated frond had the ability to recover complete physiological activities following rehydration, as all the measured traits returned to the control level. It is proposed that tolerance in *S. bryopteris* is likely to be a combination of (i) inward curling of the dehydrating fronds to minimize photo-oxidative damage (ii) complete recovery of photosynthetic activities (iii) high levels of antioxidative enzymes together with an osmoprotectant-proline. Thus the interplay of several mechanisms ensures optimal survival of *S. bryopteris*. Recent research on metabolic profiling of desiccation tolerant *S. lepidophylla* and desiccation sensitive *S. moellendorffi*

was compared at various hydration states using non-biased, global metabolomic profiling technology, based on GC/MS platform. *S. lepidophylla* retained significantly higher abundances of sucrose, mono- and polysaccharides and sugar alcohols than did *S. moellendorffi*. Aromatic amino acids and the well known osmoprotectant betain and flavanoids were also more abundant in *S. lepidophylla*. *Notably*, levels of γ-glutamyl amino acid, linked with glutathione metabolism in the detoxification of reactive oxygen species, and woth possible nitrogen remobilization following rehydration, were markedly higher in *S. lepidophylla*. Overall, *S. lepidophylla* appeared to tolerate desiccation in a constitutive manner using a wide range of metabolites with some inducible components (Yobi et al. 2012).

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CHAPTER 2

Regulation of glutamine synthetase isoforms in two differentially drought tolerant rice (*Oryza sativa* L.) cultivars under water deficit conditions

2.1. INTRODUCTION

Water deficit (WD) or dehydration is the most crucial environmental factor that limits crop productivity and the geographic distribution of several important crops such as rice, wheat and maize. The specific plant responses to WD are dependent on the rate and amount of water loss, the duration of the stress and the stage of plant development. Adaptation to WD at biochemical and molecular levels involves the activation/increased expression or induction of genes, transient increase in ABA level, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways. During prolonged period of WD, decrease in water availability for transport associated process also leads to limited uptake of nitrogen and reduced availability of CO_2 for photosynthesis as stomata are induced to close followed by disturbances in carbon and nitrogen metabolism (Foyer et al.1998; Xu and Zhou 2006). Acclimation to WD requires responses that allow essential reaction of primary metabolism to continue. Recent studies have shown nitrogen assimilation to be critical for plant acclimation to stress conditions. GS transcript and protein are shown to be regulated in response to both the plant status and environmental cues and hence, the enzyme constitutes a regulatory point at which environmental signals are integrated and translated into a plant response in terms of growth and seed production (Swarbreck et al. 2011).

WD is one of the major constraint depressing rice (*Oryza sativa* L.) production. The effect of WD varies with the variety, degree, growth stage and duration of stress. NH_4^+ is the main form of nitrogen available to the young rice plants, which is assimilated by GS to glutamine. Glutamine serves as the main form of organic nitrogen for transport through vascular tissues (Ishiyama et al. 2004b). Rice plants possess one gene (OsGS*2*) for GS2. The GS1 gene family consists of three isoforms encoded by OsGS1;1, OsGS1;2 and OsGS1;3. OsGS1:1 and OsGS1;2 are expressed in all organs with higher expression in leaf blades. They are present as minor form as compared to GS2. OsGS1:3 is expressed mainly in roots and spikelets, respectively (Tabuchi et al. 2005). These isoforms have been shown to be regulated by a developmentally controlled manner as well as by light and nitrogen nutrition (Kamachi et al. 1991; Kusano et al. 2011; Tabuchi et al. 2007). The role of GS isoforms in controlling N-metabolism during WD can be understood by studying their regulation in differentially drought tolerant rice varieties. Present study describes the regulation of GS isoforms in various organs of drought sensitive and tolerant cultivars of rice in response to WD.

2.2. MATERIALS AND METHODS

2.2.1. Chemicals and reagents

All the chemicals used in this investigation were purchased from Sigma-Aldrich, USA; E. Merck, Germany; Sisco Research Laboratory, India and HiMedia Laboratory, India. DEAE-Sephacel was purchased from Sigma-Aldrich, USA. Biogel P-2 and P-100 were from Bio-Rad, USA. Molecular biology kits were from Promega, USA; QIAGEN, Germany and Invitrogen, USA. GS-antibody was purchased from Agrisera, Sweden.

2.2.2. Plant materials and growth conditions

Rice (*Oryza sativa* L.) seeds (cv. Khitish, Pokkali, Triguna, Satabdi, IR-64, IR-8, PNR-519) were obtained from Rice Research Station, Chinsurah, West Bengal. Seeds were germinated in moist cotton bed at 30 $^{\circ}$C for 2-3 days under dark conditions. About 50 germinated seedlings were transferred to each pot containing a mixture of soil: soilrite (3:1 v/v). Seedlings were grown under 250 µmol m^{-2} s^{-1} photon flux density (16 h/8 h day/night regime) at 27±2 ^{o}C and 70-80 % relative humidity in a Plant Growth Chamber (Conviron, Canada). After 3 weeks of sowing, WD was imposed by withholding water. Seedlings

were harvested from individual pots at indicated days of stress treatment till day12, frozen in liquid nitrogen, and stored at -80 ºC for further analysis. Fresh leaf tissue immediately after harvest was used for determination of RWC, electrolyte leakage, proline and protein contents.

2.2.3. Determination of RWC

The relative water content (RWC) of leaves was measured according to Barrs and Weatherley (1962). 1 g of leaf tissue was weighed immediately after sampling to determine fresh weight (FW) and then rehydrated in water at 4 ^{o}C for 24 h and blotted dry and turgid weight (TW) was recorded. Finally the sample was dried in an oven at 80 ^{o}C for 48 h and dry weight (DW) was recorded. The leaf relative content was calculated using the following formula: RWC = [(FW – DW)/ (TW – DW)] x 100. The experiment was carried out in triplicates.

2.2.4. Electrolyte leakage assay

Electrolyte leakage was assayed by (Bhusan et al. 2007) estimating the ions leaching from the leaf into Milli-Q water. Leaf tissue was placed in 20 ml of Milli-Q water in two sets. The first set was kept at room temperature for 4 h, and its conductivity (C1) was recorded using a conductivity meter. The second set was autoclaved and its conductivity was also recorded (C2). Electrolyte leakage (1- C1/C2) x 100 was calculated. The experiment was carried out in triplicates.

2.2.5. Estimation of proline

Free proline content was estimated following the method of Bates et al (1973). The leaf tissue (100 mg) was powdered with the help of liquid nitrogen and extracted in 3% aqueous sulphosalicylic acid. The homogenate was centrifuged at 10,000 rpm for 10 min. and the supernatant was collected in a fresh vial. 2 ml of the supernatant was reacted with 2 ml of acid ninhydrin reagent (1.25 g ninhydrin dissolved in 30 ml of acetic acid at boiling temperature to which 20 ml of ortho-phosphoric acid was added) and 2 ml of glacial acetic acid and boiled at 100 ºC for 1 h. After termination of reaction on ice, the reaction mixture was extracted with 4 ml of toluene. The chromophore containing aqueous phase was aspirated, warmed to room temperature (RT) and the absorbance was recorded

at 520 nm. Proline concentration was calculated from a standard curve using 0-100 µg L-proline (*Figure 2.1*). The assay was done in triplicates. Amount of proline was expressed as µmole proline g^{-1} dry wt.

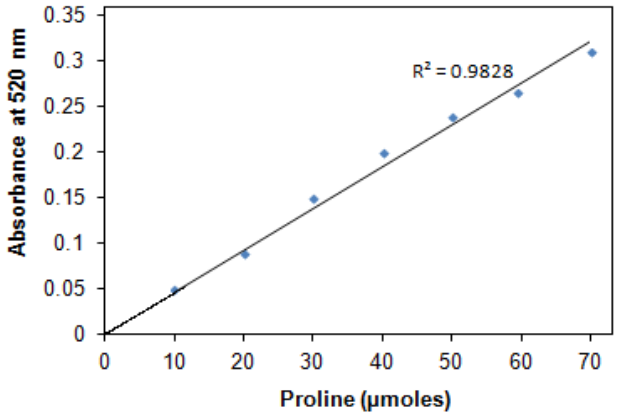

Figure 2.1. Standard curve for proline.

2.2.6. Protein estimation

One gram frozen tissue of rice (leaves, stems and roots) was homogenized in 5 ml of extraction buffer containing 50 mM Tris HCl (pH 8.0), 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15 % glycerol. The homogenate was filtered through four layers of muslin. The filtered homogenate was centrifuged at 10,000 rpm for 15 min at 4 ºC. Quantitative estimation of protein was carried out by following the method of Bradford (1976) using bovine serum albumin (BSA) as standard (*Figure 2.2*).

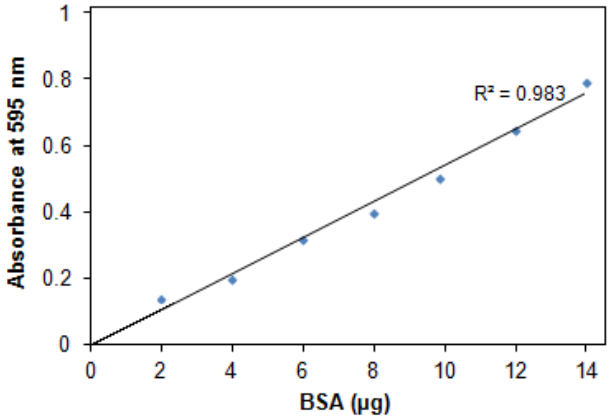

Figure 2.2. Standard curve for bovine serum albumin (BSA)

To 100 µl of protein sample, 3.0 ml of Bradford reagent [100 mg Coomassie Brilliant Blue G-250 was dissolved in 50 ml 95% ethanol; to the solution100 ml 85% (w/v) phosphoric acid was added and diluted to 1 litre] was added. The reaction mixture was incubated at RT for 10 min. followed by determination of absorbance at 595 nm. Amount of protein was expressed as mg protein g^{-1} dry wt.

2.2.7. GS extraction and assay

Frozen rice tissue (leaf, stem or root) was homogenized in liquid nitrogen and suspended in GS Extraction Buffer (5 ml g^{-1} fresh wt.) containing 50 mM Tris HCl (pH 8.0), 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15% glycerol. After filtering through four layers of muslin the extract was centrifuged at 10,000 rpm for 15 min at 4 ^{o}C. The supernatant was desalted on pre-equilibrated Biogel P-2 column. GS activity in the supernatant was determined by either transferase or semisynthetase reaction (Washitani and Sato 1977). For semisynthetase reaction, 1 ml reaction mixture contained 25 µmol Tris HCl (pH 7.5), 200 µmol glutamate, 10 µmol ATP, 5 mM hydroxylamine hydrochloride, 20 µmol $MgCl_2$, and 100 µl enzyme preparation. For transferase activity, the reaction mixture in a final volume of 1 ml contained 100 µmol Tris HCl (pH 7.5), 100 µmol glutamine, 60 µmol hydroxylamine hydrochloride, 20µmol sodium hydrogen arsenate, 1 µmol $MnCl_2$, and 10 µl of the enzyme preparation.

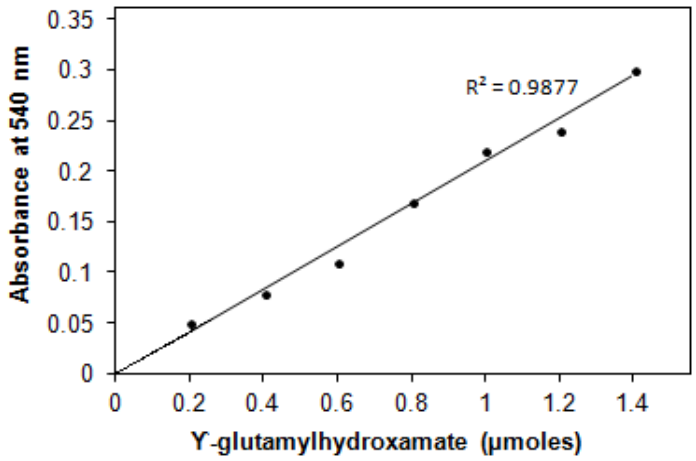

Figure 2.3. Standard curve for γ-glutamylhydroxamate.

The reaction was started by adding enzyme preparation and both the assays were carried out at 37 ^{o}C for 30 min. The reaction was terminated by adding 2 ml of $FeCl_3$ reagent (0.67 M $FeCl_3$, 0.37 M HCl and 20% (w/v) Tri-chloroacetic acid). After 20 min. the amount of γ-glutamylhydroxamate produced was determined spectrophotometrically by measuring the absorbance at 540 nm. γ-glutamylhydroxamate concentration was determined from a reference curve prepared with 0 - 2 µmole γ-glutamylhydroxamate (*Figure 2.3*). In blank, $FeCl_3$ reagent was added prior to the addition of enzyme preparation. One unit of GS activity represents 1.0 µmole of γ-glutamylhydroxamate produced 30 min^{-1}.

2.2.8. Separation of GS isoforms from leaf, stem and root of rice seedlings

GS isoforms in rice leaf, stem and root were separated by anion-exchange chromatography in a diethylaminoethyl (DEAE)-Sephacel column.

2.2.8.1. Preparation of DEAE-Sephacel

The pre-swollen DEAE-Sephacel slurry (Sigma-Aldrich, USA) was treated with 0.50 N HCl with slow stirring. The acid treated slurry was washed with distilled water till attaining pH 4. After discarding the supernatant, 0.50 N NaOH was added to the slurry followed by washing with distilled water till pH 6 to 7. Finally, the ion-exchange material was equilibrated with the GS Extraction Buffer, pH 8.0. About 10 ml of equilibrated slurry was packed into the column (10 x 2 cm) for separation of GS isoforms.

2.2.8.2. Separation of GS isoforms

All the steps of GS isoforms separation were performed at 4 ^{o}C. One gram of frozen rice tissue (leaf, stem or root) was homogenized in liquid nitrogen and suspended in 5 ml of GS extraction buffer. After filtering through four layers of muslin the extract was centrifuged at 10,000 rpm for 15 min at 4 ^{o}C. The homogenate was centrifuged at 10,000 rpm for 15 min. One ml of desalted supernatant was loaded onto a DEAE-Sephacel column (5x2 cm) pre-equilibrated with the GS extraction buffer. The column was washed with the same buffer until no protein was detectable in the eluate. Gradient elution of the adsorbed proteins was carried out by buffer containing, 0 - 0.50 M KCl. The flow rate was

maintained at 20 ml h^{-1}. Two ml fractions were collected and assayed for GS activity. The activities of chloroplastic and cytosolic isoforms were estimated from the area of the corresponding elution profile after fractionation. About 80-90 % of the total GS activity present in the crude extract was recovered after chromatographic separation.

2.2.9. RT- PCR analysis of GS genes

2.2.9.1. Isolation of total RNA from leaf, stem and root of rice seedlings

Total RNA was isolated from rice tissues by using TRIZOL reagent (Invitrogen, USA) following the manufacturer's instruction. 100 mg of plant tissue was homogenized in liquid nitrogen to fine powder and suspended in 1 ml of TRIZOL reagent. The homogenized sample was incubated at RT for 5 min to permit complete dissociation of the nucleoprotein complex. To the sample 0.20 ml of chloroform was added followed by vigorous shaking and incubation at RT for 2-3 min. The sample was centrifuged at 10,000 rpm for 15 min at 4 ºC. The aqueous phase was taken in a fresh tube and 0.50 ml of 100 % isopropanol was added. The tube was incubated at RT for 10 min followed by centrifugation at 10,000 rpm for 10 min at 4 ºC. The RNA pellet obtained after centrifugation was washed with 75% ethanol, air dried and dissolved in 25 µl of RNase free water.

2.2.9.2. Quantification of RNA

The purity and concentration of RNA were determined spectrophotometrically by measuring absorbance at 260 and 280 nm using a UV-spectrophotometer (Thermo, USA). The RNA concentration was calculated using the following formula:

$$\text{Concentration of RNA (µg /ml)} = A_{260} \text{ x dilution factor x } 40$$

The ratio of A_{260} and A_{280} determined the purity of the RNA preparation.

2.2.9.3. Agarose gel electrophoresis of RNA

1 % agarose in 1X TAE (Tris-acetate EDTA) buffer was melted in a microwave and then cooled to 50-60 ºC. It was then supplemented with 5µg ml^{-1} ethidium bromide. The melted agarose was then poured in a casting tray fitted with a teflon comb forming wells. RNA sample was mixed with RNA loading dye (1X) prior to loading in the wells. Electrophoresis was performed in a horizontal electrophoresis tank using 1X TAE buffer. RNA bands were visualized on a UV-transilluminator (Genei, India) (*Figure 2.4*).

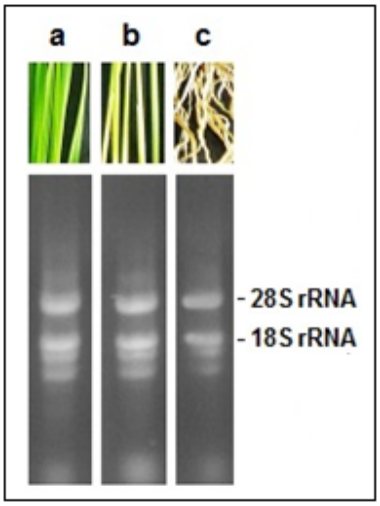

Figure 2.4. Agarose gel electrophoresis of total RNA isolated from (a) leaf, (b) stem and (c) root by using TRIZOL reagent

2.2.9.4. PCR Cloning of OsGS1;1, OsGS1;2 and OsGS2 ORF and sequence analysis

GS isoform genes were isolated by RT-PCR. The ImProm-II™ Reverse Transcription System (Promega, USA) was used for synthesis of first-strand cDNA in preparation for PCR amplification. The experimental RNA (1µg) and Oligo (dT) primer (0.50 µg) were combined in nuclease free water to a final volume of 5 µl. The tube was closed tightly and placed into a preheated 70 °C heat block for 5 min followed by immediate chilling in ice water for 5 min. To the tube other components of reverse transcriptase reaction were added. The reaction mixture in a final volume of 15 µl contained ImProm-II™ 5X Reaction Buffer, 4 µl; 25 mM $MgCl_2$, 1.20 µl; 10 mM dNTP mix, 1µl; Recombinant RNasin® Ribonuclease inhibitor, 20 units and 1µl ImProm-II™ reverse transcriptase. The reaction mix was incubated at 37 ^oC for 60 min. The reaction was terminated by incubation of reaction mix at 90 ^oC for 10 min. The cDNA was used as a template for PCR amplification in a 25 µl reaction mixture. Reaction contained selected couples of the following gene-specific primers: OsGS1;1-F (5′AGTATGGCT TCTCTCACCGATCTCGTC3’) and OsGS1;1-R (5′-GTACCTCGAGGGGCTTCCA GATGATGGTGGTC T-3′) for OsGS1;1; OsGS1;2-F (5′-GACTCATATGGCCAAC CTCACCGACCTCGTT-3′) and OsGS1;2-R (5′-TAGCGGCCGCGTTCTGCTTCCA CA GCAGCGTG-3′) for OsGS1;2 PCR was performed for 35 cycles. The PCR products were loaded and separated on 1% agarose TAE gel. The PCR products were then cloned

into pGEMT-Easy vector. For cloning the PCR product was extracted from the agarose gel by using Gel Extraction Kit (QIAGEN, Germany). The ligation reaction mixture in a total volume of 10 µl contained: 2X rapid ligation buffer, 5µl; pGEM-T-Easy vector, 50 ng (1µl); PCR product, 2µl; T4 DNA ligase (3weiss unit/µl), 1µl and 1µl H_2O. The reaction mixture incubated over night at 4 ºC was transformed into competent *E. coli* JM109 cells by heat shock at 42 ºC for 90 sec. The transformed cells were revived for 1.5 hours at 37 ºC in a shaking incubator. The transformants were selected in Ampicillin (50 µg ml^{-1}) agar plate supplemented with 20% IPTG and 2% (w/v) X-gal by incubating over night at 37 ºC. Plasmid was isolated from the recombinant colonies by Alkaline lysis method (Birnboirm and Dolly, 1979). The recombinant plasmids were analysed for the presence of DNA inserts by restriction digestion. The cloned PCR products were subjected to sequence analysis.

2.2.9.5. Quantative RT-PCR analysis of GS gene expression under WD

Semiquantitative RT-PCR was performed by using 5 µl of cDNA as template in 25 µl reaction mixture. Reaction contained selected couples of the following gene-specific primers: OsGS1;1-F (5′-AGTATGGCTTCTCTCACCGATCTCGTC-3') and OsGS1;1-R (5′-GTACCTCGAGGGGCTTCCAGATGATGGTGGTCT-3′) for OsGS1;1, OsGS1;2-F (5′-GACTCATATGGCCAACCTCACCGACCTCGTT-3′) and OsGS1;2-R (5′-TAGCGGCCGCGTTCTGCTTCCACAGCAGCGTG-3′) for OsGS1;2, OsGS2-F (5'-AGAACTTGGACGATGAATCGG-3') and OsGS2-R (5'-CATTTTATTTCGAG GGAAGG-3') for OsGS2 and OsActin-F (5'-GTCAGAATG GGATGATATGG-3') and OsActin-R (5'-TCTCCTTGCTCATCCTGTCAG-3') for actin. GS specific primers were designed according to the sequences of BAC clones represented in *Table 2.1*. PCR was performed for 27 to 29 cycles within a linear range of amplification of these genes. Expression of actin gene was used as a control to equalize cDNA quantity in different reactions. Seven microliters of the PCR products were loaded and separated on 1% agarose TAE gels. Gel was scanned using a gel documentation system (Spectronics, USA). The relative expression level of target genes under different experimental conditions were analysed using the software ImageAide version 3.06.04. The values were expressed relative to the standard. Results were repeated three times and representative one time gel pictures are shown.

Table 2.1. Detail of the BAC cloned genes of glutamine synthetase isoforms.

Gene Name	Product	BAC clone accession numbers	Chromosome location
OsGS1;1	GS1	AP004880 (P0487D09)	chr02
OsGS1;2	GS1	AC105364 (OJ1743A09)	chr03
OsGS2	GS2	AL662953(OSJNBa0011F23)	chr04

2.2.10. Immunoblot analysis of GS isoforms

2.2.10.1. Extraction of total soluble protein and SDS-PAGE

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out following the discontinuous method described by Laemmli (1970). Total soluble protein was resolved by 12.50 % SDS-PAGE (*Figure 2.5*). Resolving gel [30% acrylamide, 4 ml; 1.50 M Tris-Cl (pH8.8), 2.50 ml; 10 % SDS, 0.10 ml; 10% APS, 0.10 ml; TEMED 0.004 ml and 3.30 ml water] of 5 cm length was poured between two glass plates which were clamped together but held apart by plastic spacers and was allowed to set.

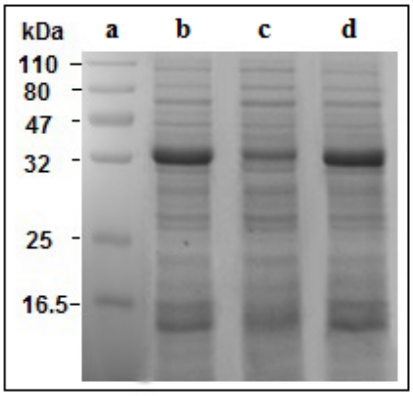

Figure 2.5. SDS-PAGE of total protein isolated from (a) protein marker, (b) leaf, (c) root, and (d) stem of rice seedlings.

The stacking gel (0.80 cm) [1M Tris HCl (pH 6.8), 0.38 ml; 10 % SDS, 0.03 ml; 10 % APS, 0.03 ml; TEMED, 0.003 ml; 2.10 ml water] was poured on the top of resolving gel and a plastic comb was placed on the stacking gel. After polymerization the comb was removed to provide loading wells. Glass plates with gel were placed in vertical electrophoresis system with running buffer tank containing running buffer [25 mM

TrisHCl (pH 8.0), 250 mM glycine, 0.10 % (w/v) SDS]. The protein sample was mixed with SDS gel loading buffer [50 mM TrisHCl (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.10 % bromophenol blue, 10 % glycerol] and heated in a boiling water bath for 10 min. Protein samples and prestained protein molecular weight marker were loaded in the wells and electric field was applied. When dye reached at the bottom of the tank, power was turned off. Gel was removed carefully from the glass plates and subjected to immunoblot analysis of GS isoforms.

2.2.10.2. Immunoblot analysis of GS isoforms

Proteins separated by 12.50 % SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane by semi-dry method using Electroblotting apparatus (Atto, Japan). The membrane was incubated with the anti-GS antibody (Agrisera, Sweden) raised against synthetic peptide from conserved region of GS1 and GS2. The reacted polypeptide was visualised with a secondary antibody, goat anti-rabbit IgG-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-p-toluidine (NBT/BCIP) detection kit (Invitrogen, USA). Broad range pre-stained standards were used as markers. Gel was scanned using a gel documentation system (Spectronics, USA). The relative expression level of target genes under different experimental conditions were analysed using the software ImageAide version 3.06.04. The values were expressed relative to the standard. Results were repeated three times and representative one time gel pictures are shown.

2.3. RESULTS

2.3.1. Screening of rice cultivars for tolerance to WD stress

Seven different varieties of rice (cv. IR-64, Khitish, Triguna, IR-8, Pokkali, PNR-519 and Satabadi) were screened for their drought tolerance characteristics. For this rice seedlings were grown for 3 weeks under controlled conditions and WD was imposed thereafter by withholding watering. Drought tolerance characteristic of rice cultivars was evaluated by monitoring changes in leaf relative water content (RWC), electrolyte leakage, proline and protein contents at indicated days of WD treatment.

2.3.1.1. Effect of WD on RWC in leaves of rice cultivars

The results of the effect of WD on RWC in leaves of different cultivars of rice are shown in *Figure 2.6*. The RWC measured at 3 h photoperiod was almost constant in well watered control plants.

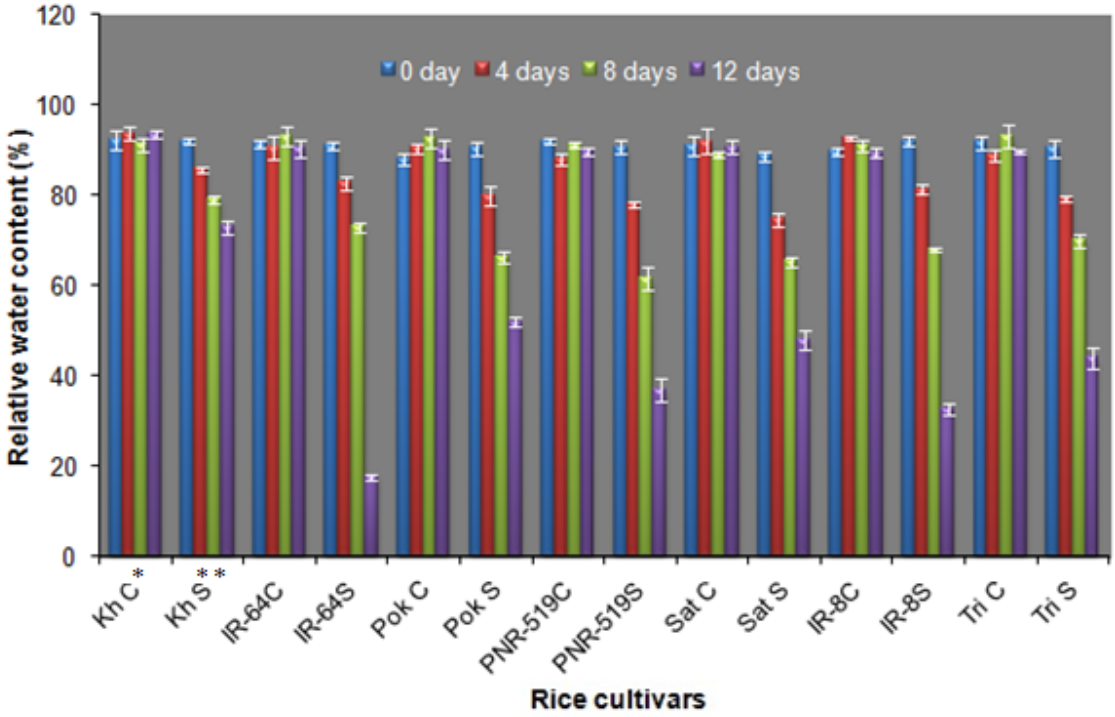

Figure 2.6. RWC in leaves of different rice (*O. sativa*) cultivars at 0, 4, 8 and 12 days of WD. All experiments were done in triplicates (n = 3), and average mean values of RWC were plotted against duration of WD. *C = Control, **S = WD treated

A differential effect of WD on rice cultivars was noted from day 8 of WD and became more significant on further treatment. Consequently, at 12 days of WD the decline was maximum in IR-64 (82 %); moderate in Pokkali (48 %), Satabadi (52 %), Triguna (56 %), PNR-519 (63 %), IR-8 (67 %); and least in Khitish (31 %).

2.3.1.2. Effect of WD on proline content in leaves of rice cultivars

The WD condition resulted in an increase in proline content in leaves of all the rice varieties. As can be seen from result in *Figure 2.7* that leaves of Khitish, Satabadi, Pokkali and Triguna cultivars had greater level of proline from the beginning of stress treatment. In these seedlings the accumulation of proline kept on increasing continuously throughout the duration of WD. However, in rice cultivars IR-8, PNR-519, and IR-64, proline content was maximum at day 12 of WD, followed by a severe decline on further dehydration.

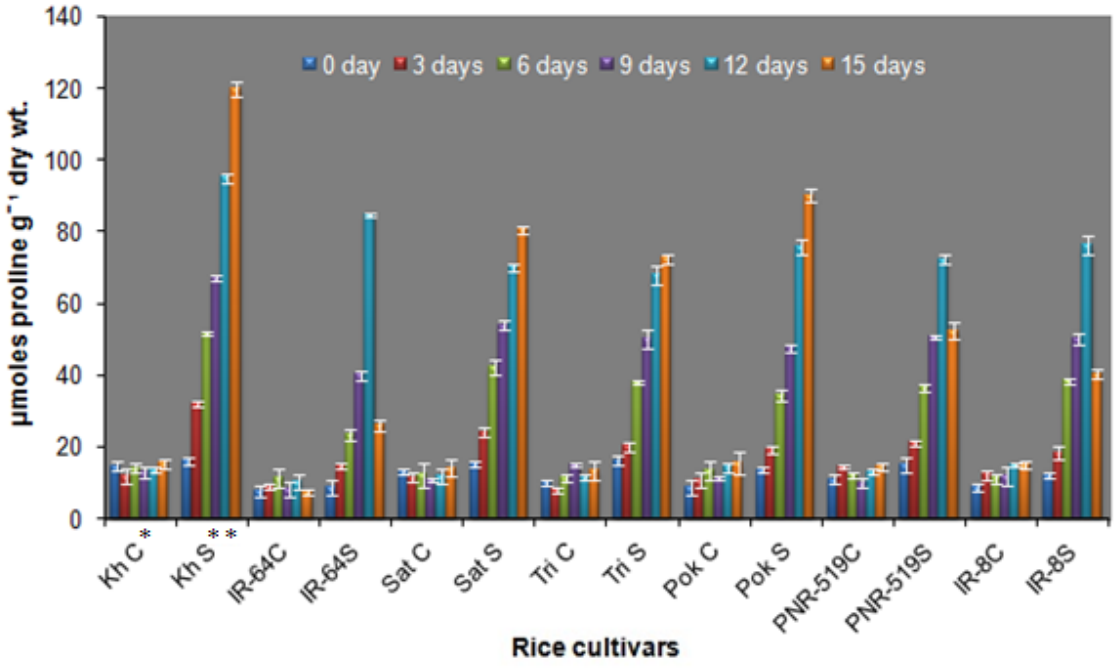

Figure 2.7. Proline content in leaves of different rice (*O. sativa*) cultivars at 0, 3, 6, 9, 12 and 15 days of WD. All experiments were done in triplicates (n = 3), and average mean values were plotted against duration of WD. *C = Control, **S = WD treated

At 15 days of WD, proline content increased to about 120, 90, 80 and 72 µmole g^{-1} dry wt. in Khitish, Pokkali, Satabadi and Triguna cultivars, respectively. Under similar condition

proline accumulation of about 52, 40 and 26 µmole g^{-1} dry wt. was noted in PNR-519, IR-8 and IR-64, respectively.

2.3.1.3. Effect of WD on protein content in leaves of rice cultivars

Influence of WD conditions on protein level in leaves of various rice cultivars is shown in *Figure 2.8.* As can be seen that total soluble protein increased throughout the experimental period in leaves of well watered control seedlings. WD treatment resulted in significant decline in protein level of all the varieties, however, the effect was variable. A comparison of protein content at day 12 of WD indicates that its decline was minimum in Khitish (15 %), moderate in Pokkali (33 %), Satabadi (35 %), Triguna (43 %), IR-8 (48 %), PNR-519 (48 %) and was highest in IR-64 (58 %).

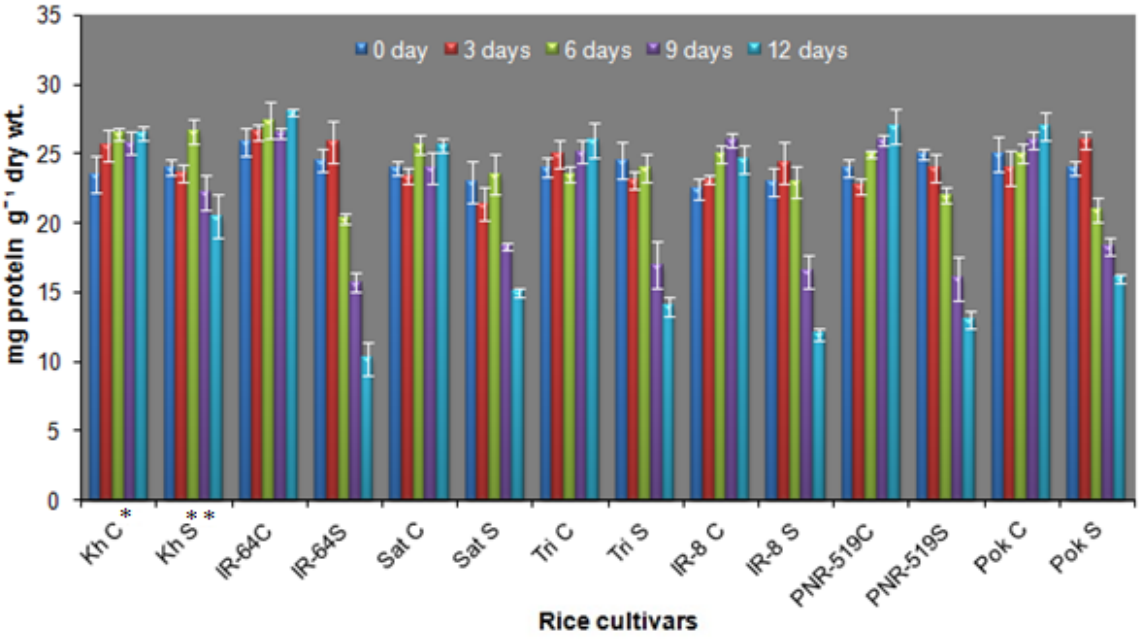

Figure 2.8. Protein content in leaves of different rice (*O. sativa*) cultivars at 0, 3, 6, 9, and 12 days of WD. All experiments were done in triplicates (n = 3), and average mean values were plotted against duration of WD. *C = Control, **S = WD treated

2.3.1.4. Effect of water-deficit on electrolyte leakage

The effect of WD on cell membrane integrity was evaluated by measuring electrolyte leakage from leaf. It is evident from the results in *Figure 2.9* that the electrolyte leakage was almost constant in well-watered control rice plants. Although, WD

treatment caused increase in electrolyte leakage from leaves, the response was variable in different rice cultivars. The effect of WD on leakage of electrolyte was more pronounced in IR-64, PNR-8 and IR-8 varieties. At 12 days of WD electrolyte leakage was enhanced by 3.6 and 2.6 folds in IR-64 and IR-8 cultivars, respectively. Electrolyte leakage was least in case of Khitish cultivar. Results of above studies indicated that *Oryza sativa* cv. IR-64 was the least tolerant, whereas *Oryza sativa* cv. Khitish was most tolerant to WD. Hence, IR-64 and Khitish cultivars were designated as drought-sensitive and drought-tolerant rice cultivar, respectively, and were selected for further studies.

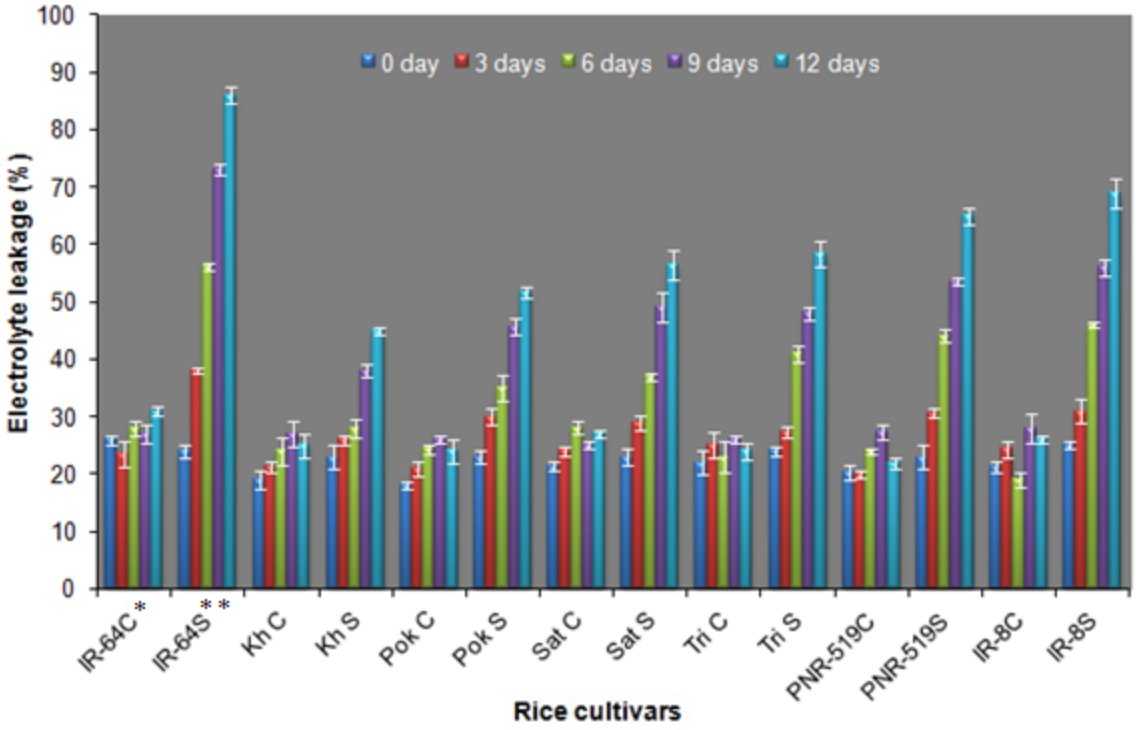

Figure 2.9. Electrolyte leakage from leaves of different rice (*O. sativa*) cultivars during 0, 3, 6, 9 and 12 days of WD. All experiments were done in triplicates (n = 3), and average mean values were plotted against duration of WD. *C = Control, **S = WD treated.

Ready comparisons of the effect of WD on morphological and biochemical changes in seedlings of these two varieties are shown in *Figure 2.10*, *2.11* and *2.12*. IR-64 and Khitish seedlings were grown under similar conditions with same level of irrigation for three weeks and then subjected to WD for 12 days. WD treatment caused rapid decrease in the water content of IR-64 as compared to Khitish. The susceptibility of IR-64 to WD is

clearly visible in *Figure 10a*. At 12 days of WD, morphology of IR-64 seedlings altered significantly with drying of shoot and rolling of leaves, however, such features were not observable in Khitish seedlings throughout the experimental duration.

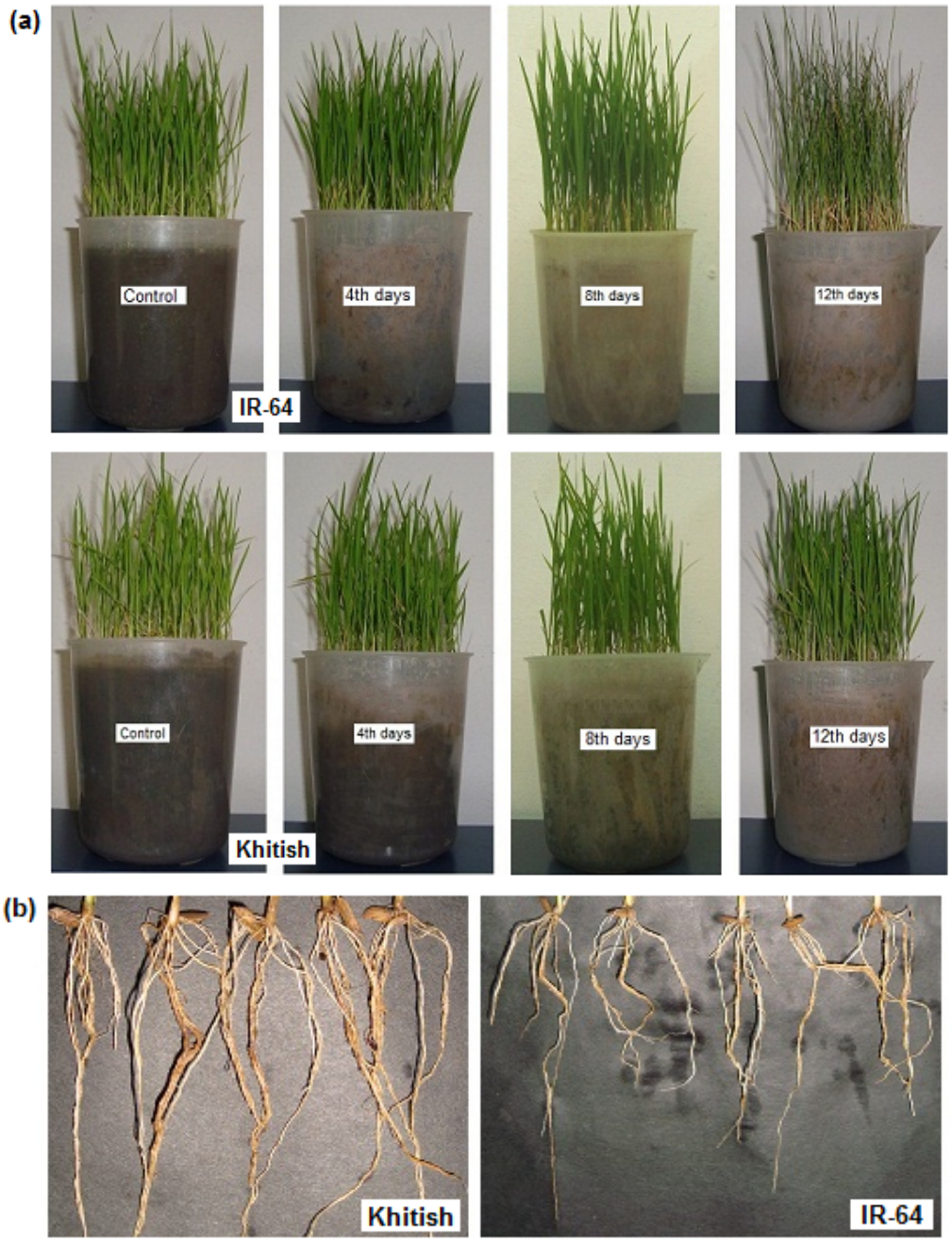

Figure 2.10. Effect of WD on IR-64 and Khitish cultivars of rice. Comparison of morphological characteristics of (a) shoot and (b) root of IR-64 and Khitish seedlings at 0, 4, 8 and 12 days of WD.

Drought tolerant and sensitive nature of Khitish and IR-64 are also evident from their root architectures. The root system of Khitish seedlings were more branched, longer and thicker as compared to that of IR-64 (*Figure 2.10 b*). The dry weight of leaves and roots of these seedlings were measured various days of WD treatment. The dry weights increased more significantly in IR-64 in comparison to Khitish. For example WD treatment for 12 days raised the dry wt. g^{-1} of leaves from 112 mg to 294 mg in IR-64 and from 115 mg to 151 mg in Khitish cultivar (*Figure 2.11a*). Under same condition, the dry wt. g^{-1} of root enhanced from 140 mg to 190 mg in IR-64, whereas it increased only marginally in Khitish cultivar (*Figure 2.11 b*). As a result RWC in leaf declined at a faster rate in IR-64 as compared to Khitish.

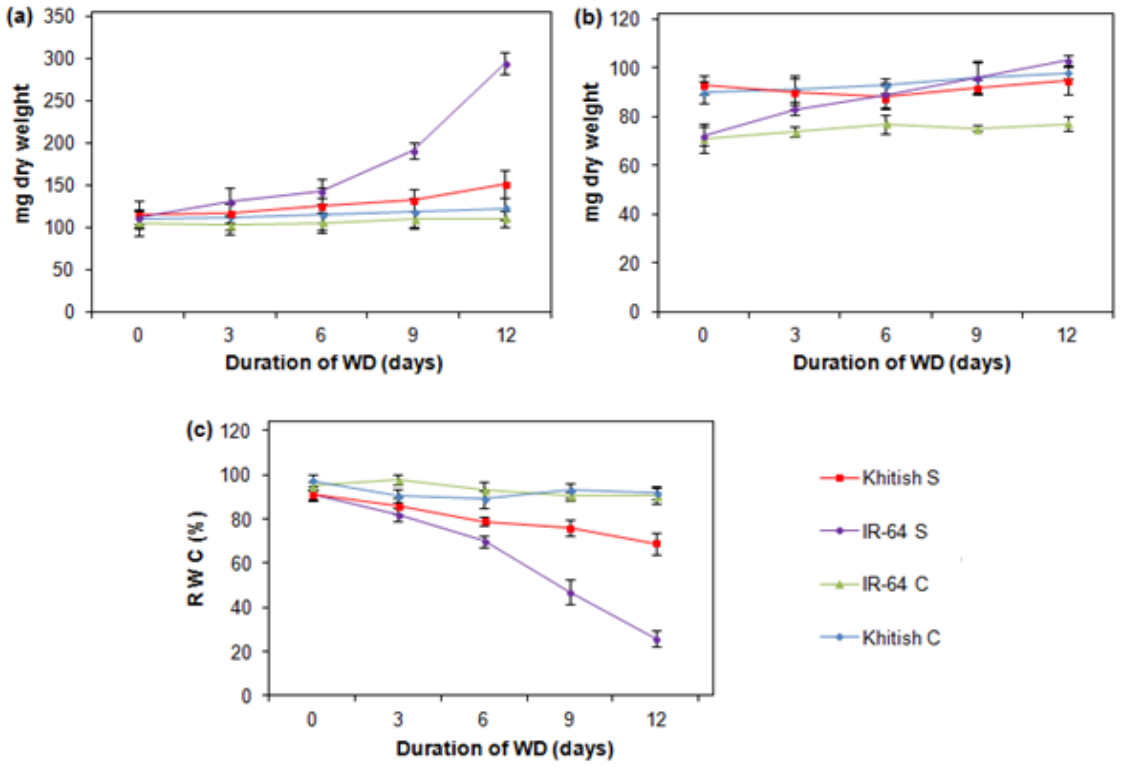

Figure 2.11. Effect of WD on dry weight and relative water content. Comparative analysis of dry weights of (a) leaf, and (b) root and (c) RWC of leaf between IR-64 and Khitish varieties of *O. sativa* in a time-dependent manner under WD conditions. Three week old seedlings were subjected to water-deficit and dry weight and RWC were determined at 0, 3, 6, 9 and 12 days of treatment. For determination of dry weight 0.50 g of fresh tissue was dried at 80 ºC for 48 h. All experiments were done in triplicates (n = 3), and average mean values were plotted against duration of WD.

WD treatment for 12 days reduced leaf RWC to 26 and 69 % in IR-64 and Khitish varieties, respectively (*Figure 2.11 c*). The protein contents in leaves and roots declined more rapidly in IR-64 seedlings. At the beginning of WD treatment protein content in leaves of both the cultivars was about 25 mg g^{-1} dry wt., which declined to 15 and 22 mg in IR-64 and Khitish, respectively, at day 9 of WD. At the end of WD treatment the soluble protein content in leaf and root was decreased by 58 % and 40 % in IR-64 and by 17 and 10 % in Khitish, respectively (*Figure 2.12 a, b*).

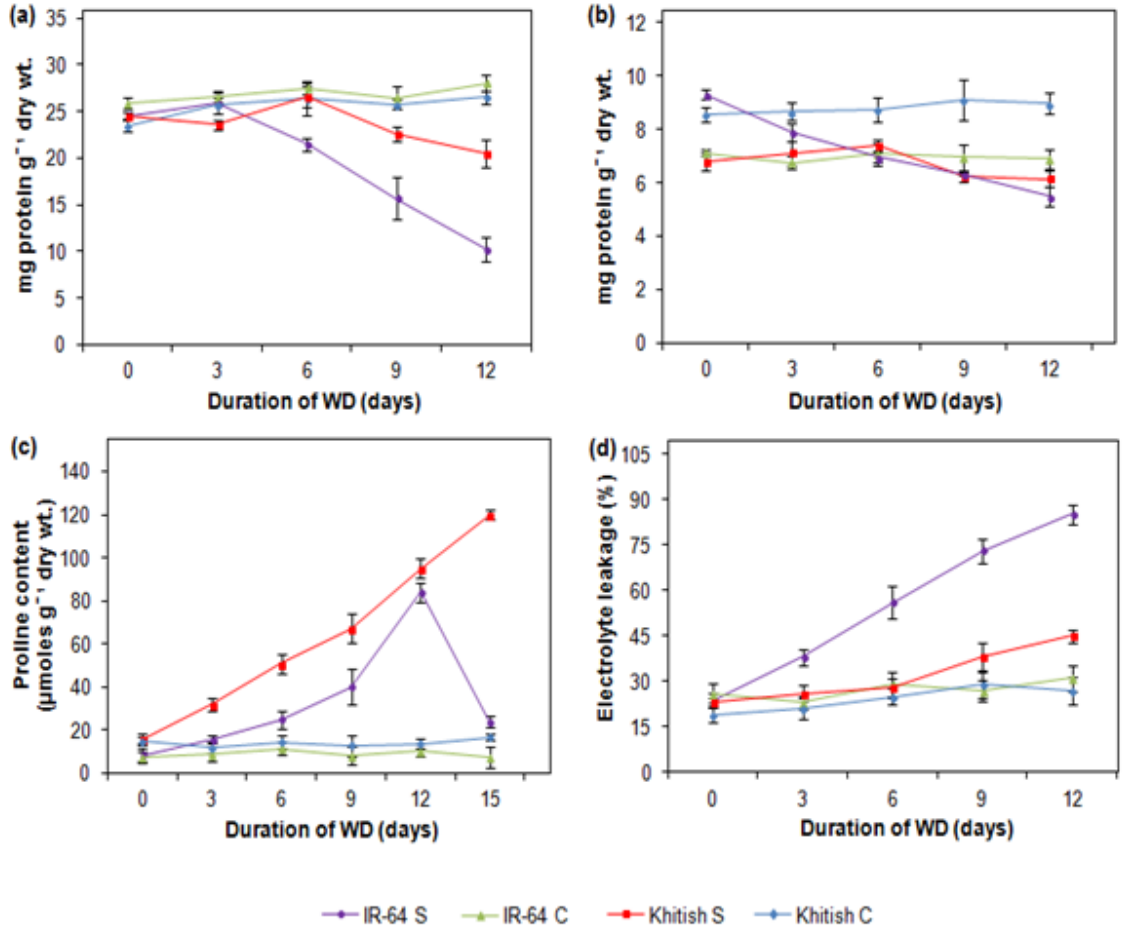

Figure 2.12. Effect of WD on protein content, proline content and electrolyte leakage. Comparative analysis of protein contents: (a) leaf, and (b) root; (c) proline content and (d) electrolyte leakage of leaf between IR-64 and Khitish varieties of *O. sativa* in a time-dependent manner under WD conditions. Three week old seedlings were subjected to water-deficit. All experiments were done in triplicates (*n*=3), and average mean values were plotted against duration of WD.

Although, the level of proline was increased during WD in leaves of both the varieties, its accumulation was greater in Khitish from the beginning of stress treatment (*Figure 2.12*

c). In IR-64 seedlings, proline content was maximum at day 12 of WD, followed by its severe decline on further dehydration. On the other hand, proline accumulation kept on increasing with WD in Khitish leaf. The electrolyte leakage was maintained at an almost constant level in Khitish until day 6 and increased marginally during later stages of WD. In contrast, a sharp rise in electrolyte leakage was noted in IR-64 cultivar (*Figure 2.12 d*).

2.3.2. Optimization of GS extraction and assay conditions

GS has been studied in many higher plants. However, the optimal conditions for its extraction vary with tissue as well as the plant species (Lea et al. 1990). Hence, the optimal conditions for extraction of the enzyme with respect to pH of the buffer and concentration of protective/stabilizing agents were established in order to ensure maximal extraction and recovery of the enzyme. Maximum recovery of the enzyme was obtained when 50 mM Tris HCl (pH 8.0) supplemented with 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15% glycerol was used as extraction buffer. The optimum pH of assay media for GS activity of was 7.5. The rate of semisynthetase activity rose proportionately upto 300 µl of enzyme extract. The amount of γ-glutamylhydroxamate produced increased proportionately with the reaction period upto 30 min when 300 µl of enzyme extract was used for assay. Hence in all the subsequent experiments GS activity was routinely determined over a period of 30 min with 300 µl of enzyme preparation.

2.3.3. GS isoforms in leaf, stem and root of rice varieties

GS was extracted from root, stem and leaf of three weeks old rice seedlings raised in 3:1mixture of soil and soilrite, under 250 µmol m^{-2} s^{-1} photon flux density (16h/8h day/night regime) at 27 ± 2 ^{o}C and 70-80 % relative humidity in a plant growth chamber.

The activity of GS in crude extract was determined by semisynthetase assay based on rate of formation of γ-glutamylhydroxamate. Anion-exchange chromatography was used to separate the isoforms of GS in leaf, stem and root extracts. As can be seen from result in *Figure 2.13a*, on subjecting the leaf extract to chromatography in DEAE-Sephacel column, the enzyme was resolved in two distinct peaks which were recovered with gradient elution buffer containing 0 - 0.50 M KCl. These two enzyme forms were

designated as GS1 and GS2, respectively. In case of the stem extract (*Figure 2.13 b*) also the enzyme activity was resolved into two peaks at the same salt concentration as GS1 and GS2 in the leaf extract. However, ion-exchange chromatography of root extract eluted almost the entire activity as a single peak at the same salt concentration as GS1 in leaf and stem extracts and no peak corresponding to GS2 was detectable (*Figure 2.13 c*). From the results in *Table-2.2* it is apparent that rice leaves contained 25 and 75 % of activity as GS1 and GS2, respectively. In stem most of the GS activity (70 %) was present as GS1 and rest 30 % activity represented as GS2.

2.3.4.Detection and quantization of GS isoforms mRNA and protein

2.3.4.1. Standardization of RT-PCR amplification of GS isoforms

Before performing the experiments on quantitative GS gene expression analysis, the reaction conditions for RT-PCR of full length GS isogenes were established. Total RNA was isolated from leaf, stem and root tissues of three week old rice seedlings. 1 µg of total RNA was used for synthesis of first strand cDNA by reverse transcriptase (RT) followed by PCR using gene specific forward and reverse primers. The number of cycles and annealing temperature were optimized for each gene specific primer pairs. To ascertain the PCR products as OsGS1;1, OsGS1;2 and OsGS2, the amplified PCR products (*Figure 2.14*) were cloned in pGEMT vector and sequenced. The sequence showed homology with OsGS sequences in the database.

2.3.4.2. Quantification of GS isoforms transcripts in rice seedlings

The transcript levels of GS isoform in various organs of rice plant were determined by RT-PCR. The PCR amplified full length OsGS1;1, OsGS1;2 and OsGS2 ORFs were resolved by agarose gel electrophoresis and quantified by ImageAide version 3.06.04, to calculate the level of gene expression. From the results in *Figure 2.15* it is apparent that among GS1 gene family, OsGS1;1 and OsGS1;2 transcripts were present in all the three organs, whereas OsGS2 was expressed in leaf and stem but not in root. In leaf tissue the OsGS2 mRNA level was considerably higher than that of cytosolic isoforms and its accumulation was found to be about 4.20 folds greater than OsGS1;1. OsGS2 was present as minor form in stem.

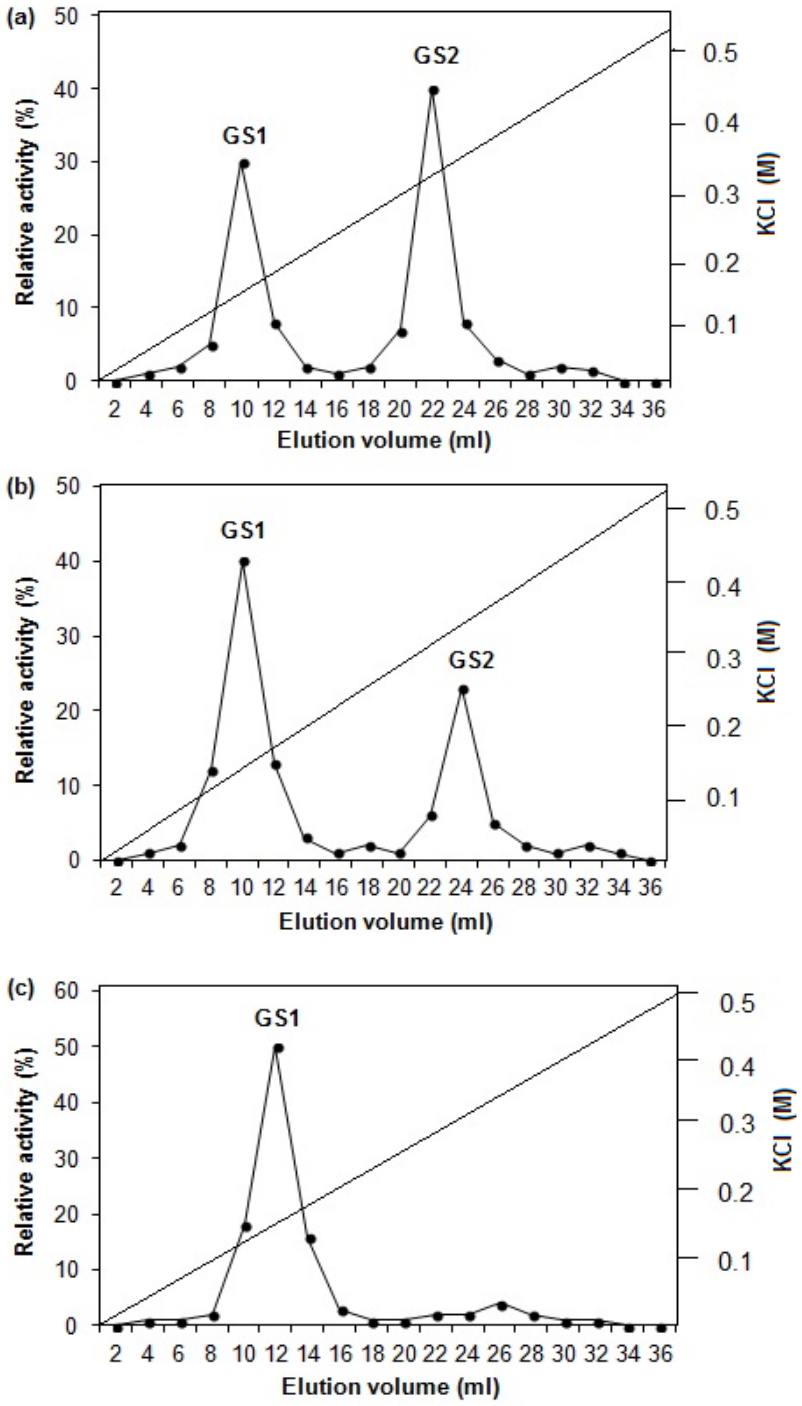

Figure 2.13. Elution profile during anion-exchange chromatography of GS isoforms (a) leaf, (b) stem and (c) root tissues of 3 weeks old rice seedlings. The enzyme activity in different fractions was assayed by the semisynthetase assay. One unit of GS activity represents 1.0 μmole of γ-glutamylhydroxamate produced 30 min^{-1}.

Table 2.2. Relative proportion of GS1 and GS2 in leaf, stem and root of rice seedlings.

Tissue	GS activity *				
	Total GS	GS1	GS2	Ratio GS2/GS1	Recovery
Leaf	31.25	6.10	22.70	3.72	92.16
Stem	13.20	8.21	3.80	0.46	90.90
Root	20.00	18.5	N.D.		92.50

N.D.: Not detectable

GS1 and GS2 were isolated by anion-exchange chromatography (Section 2.2.8.2) from leaf, stem and root tissues of 3 weeks old rice (*Oryza sativa* cv. IR-64) seedlings raised in plant growth chamber. The enzyme activity was assayed by the semisynthetase assay. *One unit of GS activity represents 1.0 µmole of γ-glutamylhydroxamate produced 30 min^{-1}.

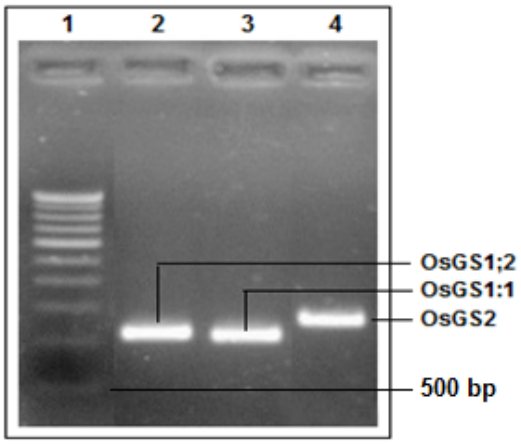

Figure 2.14. RT-PCR amplification of GS isoforms and agarose gel electrophoresis analysis of PCR products. Lane1- 500bp ladder, Lane2- OsGS1;2 (1171 bp), Lane3- OsGS1;1 (1039 bp) and Lane 4: OsGS2 (1255bp)

Among the GS1 isoforms OsGS1;1 was more abundant than OsGS1;2 in leaf. OsGS1;2 was the predominant form in stem and root of both IR-64 and Khitish cultivars. However, OsGS1;1 mRNA level was significantly greater in root and stem of IR-64 seedlings than that of Khitish. OsGS1;3 gene expression was not studied, as previous reports indicate its absence in vegetative stage of growth of rice seedlings (Ishiyama et al.2004b; Tabuchi et al. 2007).

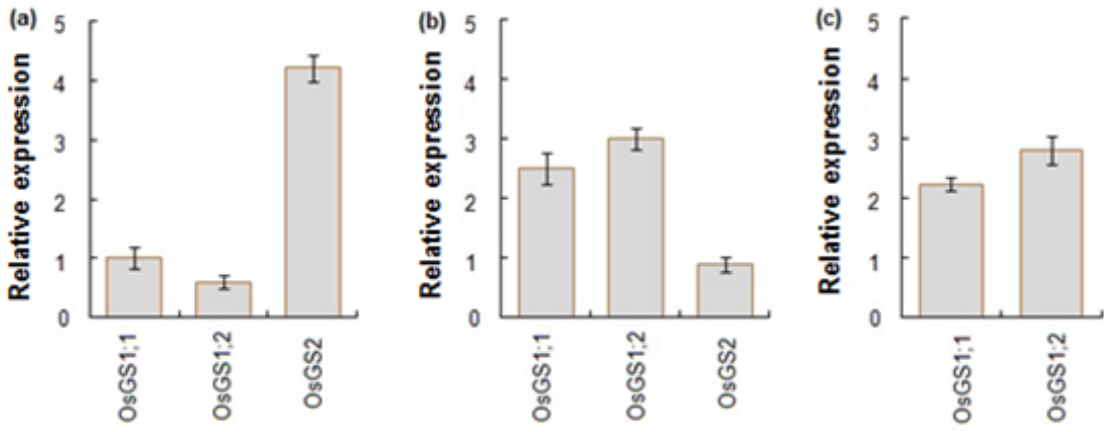

Figure 2.15. Quantitative analysis of OsGS1;1, OsGS1;2 and OsGS2 mRNA in (a) leaf, (b) stem and (c) root of rice (*Oryza sativa* cv. IR-64) seedlings.

2.3.4.3. Quantification of GS isoforms polypeptides in rice seedlings

The polypeptide levels of GS1 and GS2 isoforms in leaves, stems and roots of rice seedlings were determined by immunoblotting. Total soluble proteins (10 µg) of leaf, stem and root tissues were resolved by 12.50 % SDS-PAGE and transferred to PVDF membrane followed by probing the membrane with the anti-GS antibody raised against synthetic peptide from conserved region of GS1 and GS2. The reacted polypeptides were visualised with secondary antibody-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-p-toluidine (NBT/BCIP).

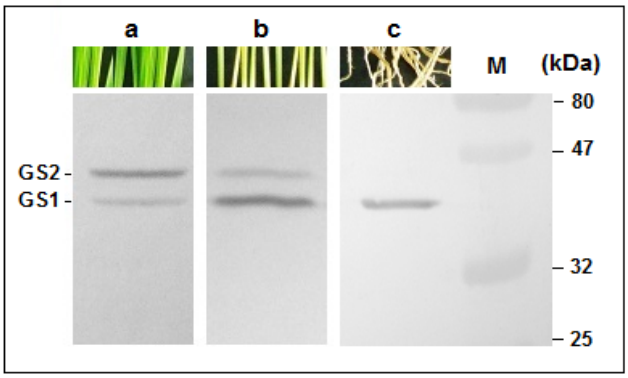

Figure 2.16. GS polypeptide in (a) leaf, (b) stem and (c) root of rice (*O. sativa* cv. IR-64) seedlings under normal conditions. GS polypeptides were detected by Immunoblotting using the anti-GS antibody against synthetic peptide from conserved region of GS1 and GS2. Immunoblot analysis was carried out with 10 µg of total soluble protein.

The result in *Figure 2.16* indicates the presence of two protein bands of approximately 39 and 43 kDa in leaf and stem that correspond to the molecular size of GS1 and GS2, respectively. The immunoblot analysis of root protein highlighted only one protein band corresponding to GS isoform. As the GS antibody was developed against conserved GS polypeptide, cytosolic isoforms could not be distinguished in immunoblot.

2.3.5. Effect of WD on activity and expression of GS isoforms in leaf, stem and root of IR-64 and Khitish seedlings

Rice seedlings of IR-64 and Khitish cultivars grown for 3 weeks under controlled conditions were subjected to WD by withholding watering and activity of GS1 and GS2 and the corresponding mRNA and polypeptide contents were simultaneously determined in leaf, stem and root tissue at 0, 4, 8 and 12 days of the treatment.

2.3.5.1. Effect of WD on total GS, GS1 and GS2 activities in leaf, stem and root of IR-64 and Khitish seedlings

Results of the influence of WD on total GS, GS1 and GS2 activities in leaves, stem and roots of IR-64 and Khitish cultivars are shown in Table 2.3. At the beginning of WD treatment the total GS activity was almost similar in both the cultivars. WD treatment caused rapid decline of GS activity in leaves and roots of IR-64 seedlings as compare to that of Khitish. In IR-64 leaf total GS activity decreased by 28 and 52 % at 8 and 12 days of WD, whereas in Khitish leaf a significant decrease of only 10 % was noted at day 12 of treatment. The rapid reduction in GS activity of IR-64 leaf was mainly due to disappearance of GS2 activity. For example, WD condition for 12 days reduced the GS2 activity from 23 to 11 in IR-64 and from 21 to 19 in Khitish cultivar. The differential rate of decline in GS2 activity in the two varieties decreased the ratio of GS2 to GS1 from 4.18 to 2.28 in IR-64 and from 4.00 to 3.75 in Khitish.

Total GS activity in stem of IR-64 and Khitish seedlings was almost unaffected by WD. However, alteration in both GS1 and GS2 activities was noted in stem of IR-64 seedling. In this seedling GS1 activity was reduced by 16, 36 and 41% on 4, 8 and 12 days of WD. On the other hand, the activity of GS2 was enhanced by 40 % at 12 days of stress

application. Thus the decline in GS1 activity was compensated by increase in GS2 activity, thus maintaining almost unchanged total GS activity. Alterations in GS1 and GS2 activities raised the ratio of GS2 to GS1 from 0.46 to 1.20 in IR-64 stem. Such change in ratio was not noticeable in Khitish stem as both GS1 and GS2 activities were almost constant throughout the WD treatment. The cytosolic GS1 was the only GS isoform in rice root. WD treatment had not much effect on GS activity in Khitish root. In contrast, the GS activity in roots of IR-64 cultivar was quite sensitive to WD. A rapid reduction in activity of about 50 and 60 % was noted at 8 and 12 days of WD treatment, respectively.

Table 2.3. Effect of WD on total GS, GS1 and GS2 activities in leaves, stem and roots of *O. sativa* cv. IR-64 and Khitish.

Rice Varieties	Tissues	Days of WD	GS activity*			Ratio
			Total GS	GS1	GS2	GS2:GS1
Khitish	Leaf	0	28.50 ± 1.51	5.28 ± 0.10	21.12 ± 2.42	4.00
		4	30.00 ± 2.12	5.45 ± 0.25	21.52 ± 1.84	3.95
		8	27.20 ± 2.40	5.25 ± 0.15	19.68 ± 2.80	3.75
		12	25.80 ± 3.21	5.11 ± 0.30	19.16 ± 3.41	3.75
	Stem	0	12.10 ± 1.10	7.89 ± 0.25	4.10 ± 0.02	0.52
		4	10.92 ± 1.25	6.77 ± 0.31	3.84 ± 0.03	0.56
		8	11.50 ± 2.50	7.10 ± 0.15	3.82 ± 0.10	0.53
		12	12.20 ± 1.80	7.00 ± 0.10	4.05 ± 0.02	0.57
	Root	0	17.00 ± 2.10	Total activity was present as GS1		
		4	16.70 ± 2.41			
		8	16.68 ± 2.15			
		12	15.69 ± 2.80			
IR-64	Leaf	0	32.06 ± 3.21	5.45 ± 0.02	22.78 ± 3.10	4.18
		4	34.00 ± 3.80	5.86 ± 0.14	22.45 ± 2.50	4.00
		8	23.00 ± 4.25	5.86 ± 0.14	15.88 ± 3.12	3.00
		12	17.22 ± 5.50	5.86 ± 0.14	11.69 ± 3.12	2.25
	Stem	0	12.40 ± 2.10	8.21 ± 2.11	3.80 ± 1.45	0.46
		4	11.95 ± 3.20	6.95 ± 1.61	4.32 ± 3.12	0.62
		8	11.38 ± 1.26	5.42 ± 2.23	5.35 ± 2.54	0.98
		12	11.60 ± 3.24	4.95 ± 3.41	6.00 ± 3.61	1.21
	Root	0	20.00 ± 3.22	Total activity was present as GS1		
		4	16.00 ± 2.65			
		8	10.00 ± 2.65			
		12	08.40 ± 1.82			

*Total GS activity was resolved into GS1 and GS2 by anion-exchange chromatography. Enzyme activity in different fractions was assayed by semisynthetase reaction. One unit of GS activity represents 1.0 μmole of γ-glutamylhydroxamate produced 30 min^{-1}.

2.3.5.2. Effect of WD on expression of GS1 and GS2 mRNA in leaf, stem and root of IR-64 and Khitish seedlings

Total RNA was isolated from various tissues of rice seedlings at indicated days of WD and mRNA levels of OsGS1;1, OsGS1;2 and OsGS2 were determined by semi quantitative RT-PCR .

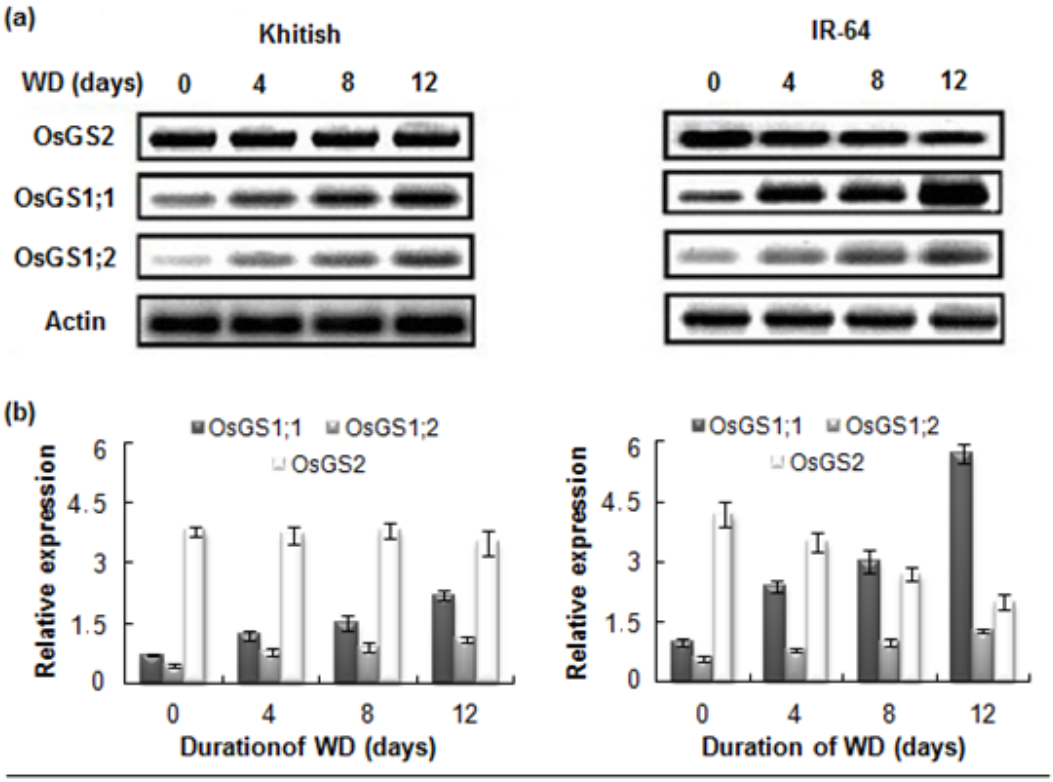

Figure 2.17. Effect of WD on expression of GS mRNA in leaves of *O. sativa* cv. IR-64 and Khitish. (a) Analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR. (b) Bar diagram of GS mRNA level

The results of WD mediated alteration in GS transcripts expression in leaf tissue are shown in *Figure 2.17*. Though, OsGS1;1 and OsGS1;2 mRNA levels increased in both IR-64 and Khitish cultivars in response to WD, expression level of OsGS1;1 mRNA was considerably higher in IR-64 leaf. OsGS1;1 mRNA level was increased by about 2.5 and 5 folds in IR-64 leaf and by 2 and 2.5 folds in Khitish leaf at 8 and 12 days of WD, respectively. On the other hand, OsGS1;2 mRNA content enhanced almost equally in both the varieties. It is also noticeable that OsGS2 mRNA level was significantly affected by WD in IR-64 cultivar. Its level declined by about 40 % at 8 days of WD. A further

reduction of upto 50 % was observed at 12 days of treatment. Under similar conditions no such change in OsGS2 expression was noticed in Khitish leaf.

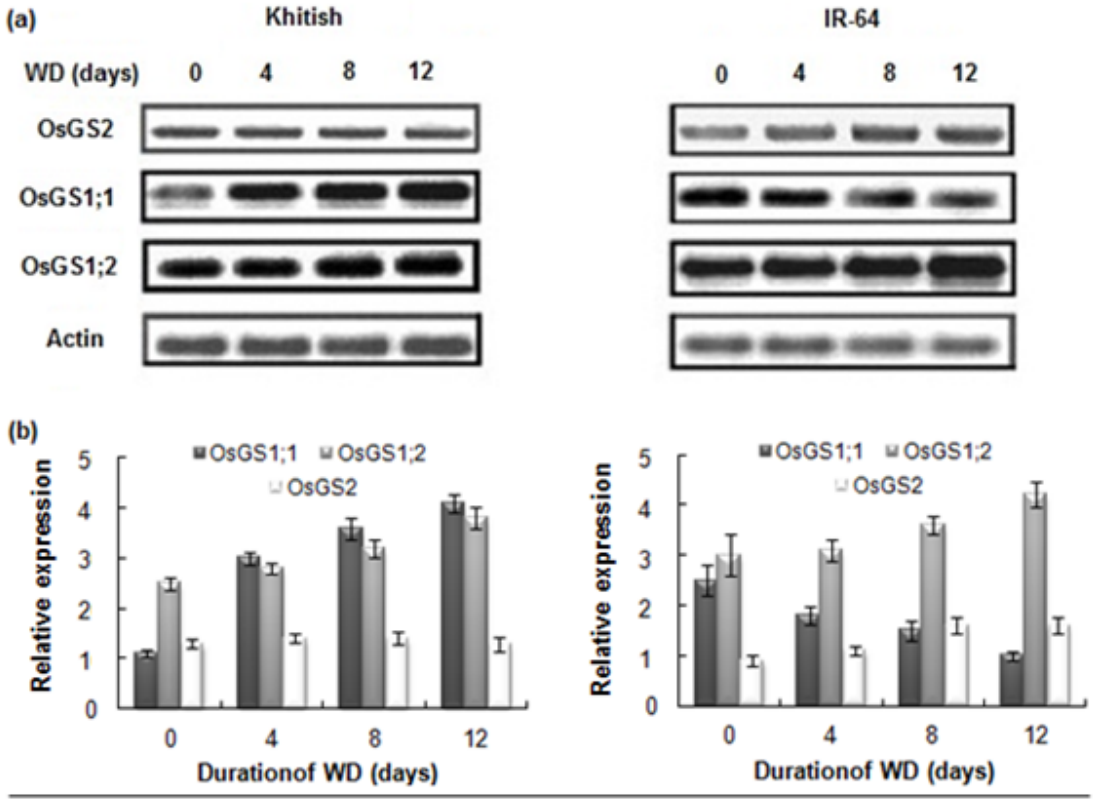

Figure 2.18. Effect of WD on expression of GS mRNA in stem of *O. sativa* cv. IR-64 and Khitish. (a) Analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR. (b) Bar diagram of GS mRNA level

The influence of WD on expression of GS mRNAs in stem of was significantly different from that of leaf. The results in *Figure 2.18* indicate a varietal variation in expression of OsGS1;1 in stem of IR-64 and Khitish seedling in response of WD. In stem, OsGS1;1 transcript content declined with the intensification of stress. Its level decreased to about 50 % at 8 days of WD followed by a further decline of upto 60% at 12 days of treatment. In contrast, OsGS1;1 mRNA was found to increase in Khitish stem with about 3 and 4 folds enhancement at 4 and 12 days of WD, respectively. The OsGS1;2 transcript responded almost equally in both the varieties, with almost 2 fold increase in its abundance at 12 days of stress treatment.

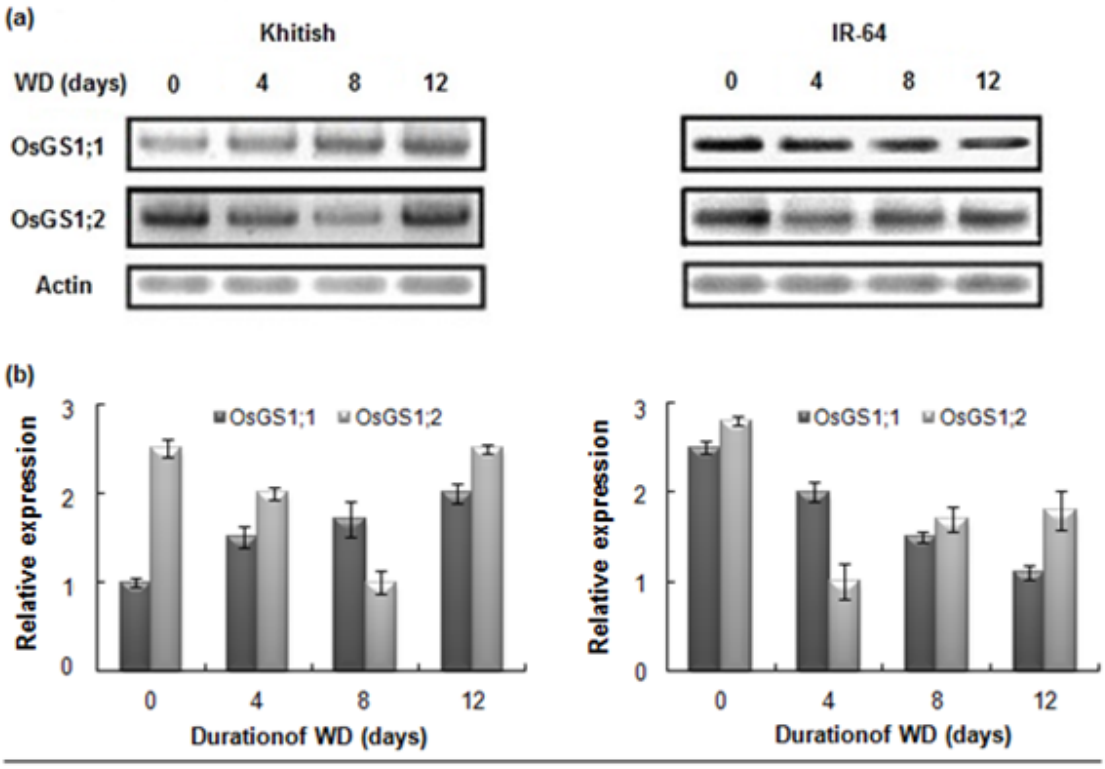

Figure 2.19. Effect of WD on expression of GS mRNA in root of *O. sativa* cv. IR-64 and Khitish (a) Analysis of OsGS1;1 and OsGS1;2 transcripts by RT-PCR. (b) Bar diagram of GS mRNA level

The chloroplastic GS2 mRNA was found to express in stem, but its mRNA level was significantly lower than that of cytosolic GS isoforms. A differential response of WD on GS2 mRNA expression was also noted. WD treatment for 12 days resulted in enhancement of OsGS2 transcript content by about 2 fold in IR-64 stem. However, such alteration in OsGS2 mRNA quantity was undetectable in Khitish stem, which maintained an almost constant level of the transcript throughout the treatment period.

Among GS transcripts only GS1 isoforms were expressed in root. As can be seen from the results in *Figure 2.19*, the expression of OsGS1;1 mRNA differed in root of the two cultivars. Its level was initially higher in IR-64 that declined with WD. At 8 days of WD the OsGS1;1 mRNA content was reduced to less than half in IR-64 root and increased to almost twice in Khitish root. Although, WD treatment resulted in initial decline of OsGS1;2 transcript content in roots of both the cultivar, the rate of decline was faster in IR-64, in comparison to Khitish. As a result, OsGS1;2 level fell to a minimum at day 4

and day 8 of WD in IR-64 and Khitish, respectively and then increased on further treatment.

2.3.5.3. Effect of WD on expression of GS1 and GS2 polypeptide in leaf, stem and root of IR-64 and Khitish seedlings

To ascertain whether the WD induced alterations in GS mRNA expression was reflected in corresponding polypeptides, immunoblot analysis of GS isoforms was carried out. The result of the effect of WD on expression of GS polypeptides in leaf tissue is shown in *Figure 2.20*. As can be seen that GS1 isoforms i.e. OsGS1;1 and OsGS1;2, couldn't be distinguished in immunoblot and is represented as single band. This is because of the development of GS antibody against conserved region of the GS polypeptide. As shown in *Figure 2.20a,* GS1 polypeptide accumulated in leaf of both IR-64 and Khitish seedlings with progress of WD.

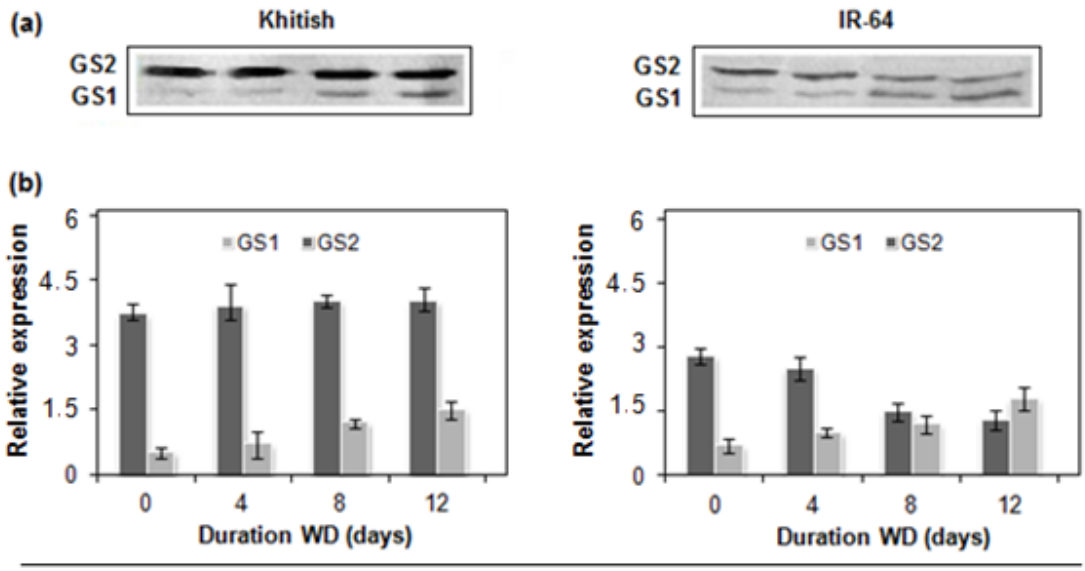

Figure 2.20. Effect of WD on expression of GS polypeptides in leaf of *O. sativa* cv. IR-64 and Khitish (a) Immunoblot analysis of GS1 and GS2 polypeptides (b) Bar diagram of GS polypeptide levels.

Its level increased by more than 2 fold by the end of the treatment. The GS2 polypeptide content of IR-64 leaf was reduced by about 50% at day 12 of WD, however, such change was not noticeable in Khitish leaf (*Figure 2.20b*).

The results in *Figure 2.21* show the effect of WD on expression of GS polypeptides in the stem of rice seedlings. WD significantly affected GS2 polypeptide expression in stem of IR-64 cultivar than that of Khitish cultivar. At 12 days of WD, GS2 polypeptide level rose by about 2 fold in IR-64 stem but remained almost unaltered in Khitish stem.

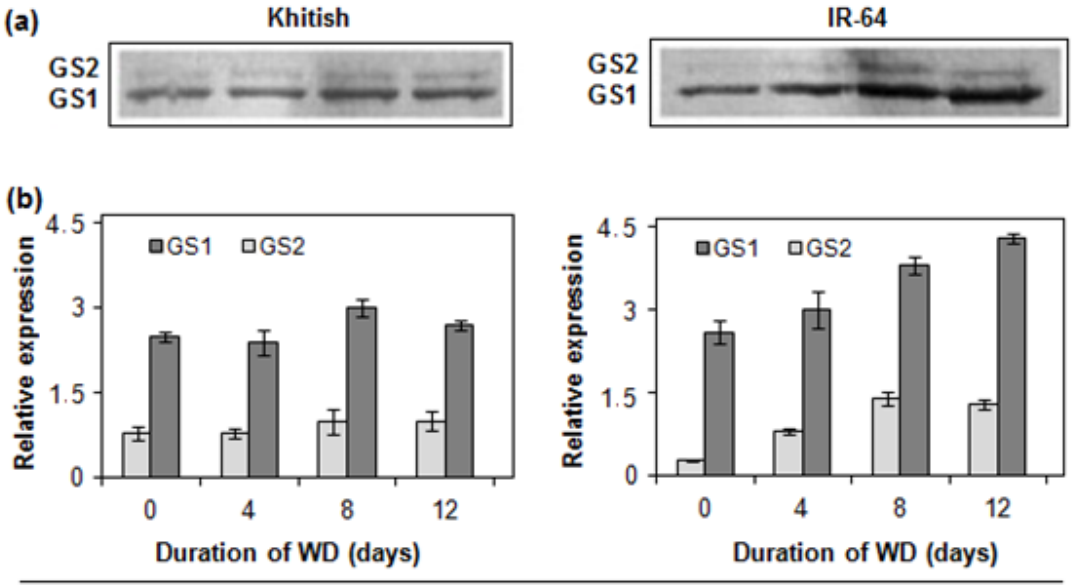

Figure 2.21. Effect of WD on expression of GS polypeptides in stem of *O. sativa* cv. IR-64 and Khitish; (a) Immunoblot analysis of GS1 and GS2 polypeptides (b) Bar diagram of GS polypeptide levels.

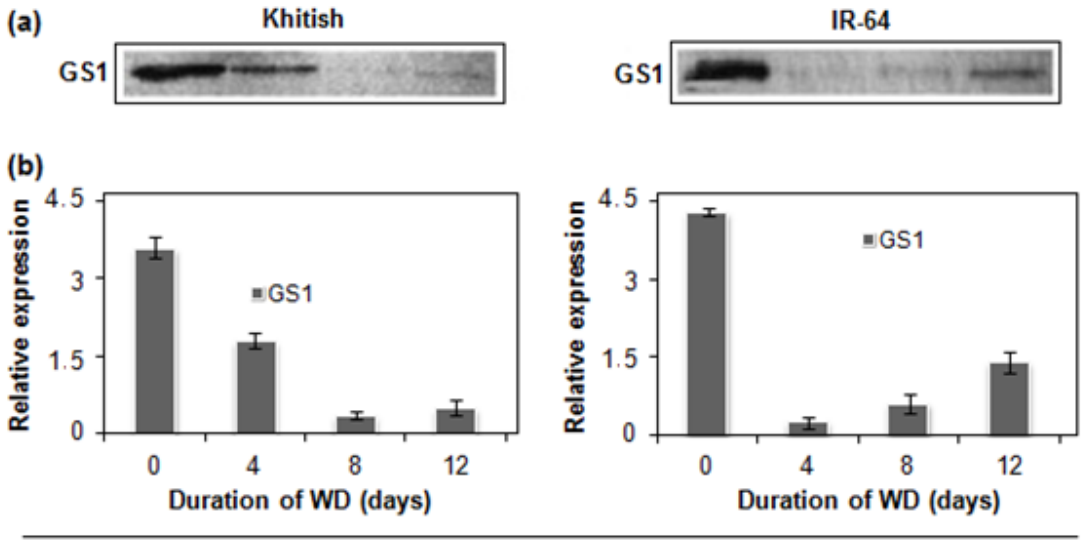

Figure 2.22. Effect of WD on expression of GS polypeptide in root of *O. sativa* cv. IR-64 and Khitish; (a) Immunoblot analysis of GS1 and GS2 polypeptide (b) Bar diagram of GS polypeptide levels.

Similarly the abundance of GS1 polypeptide was enhanced by 1.5 fold in IR-64 and didn't change significantly in Khitish. The immunoblot analysis of root proteins highlighted a single protein band of molecular size 39 kDa, which corresponds to the cytosolic GS1 isoform. In both IR-64 and Khitish cultivars, initial level of GS1 polypeptide was significantly higher. WD treatment resulted in drastic decline GS1 protein content. The rate of decline was faster in drought sensitive IR-64 as compared to Khitish. As a consequence its level was reached to a minimum on day 4 in IR-64 and on day 8 in Khitish. These declines began to reverse on further WD treatment in both the seedlings (*Figure 2.22*).

2.4. DISCUSSION

Environmental factors that impose water deficit (WD) stress, such as drought, salinity and temperature extremes, place major limits on plant productivity (Cushman and Bohnert 2000). The specific plant responses to WD are dependent on the amount and rate of water loss, duration of the stress and stages of plant development. Adaptation to WD at biochemical and molecular levels involves the activation or increased expression of several genes, transient increases in ABA levels, accumulation of compatible solutes and protective proteins, increased levels of antioxidants and suppression of energy-consuming pathways (Xiong et al. 2002; Waseem et al. 2011). During prolonged periods of WD, the decrease in water availability for transport-associated processes leads to changes in the concentrations of many metabolites, followed by disturbances in amino acid and carbohydrate metabolism. Acclimation to WD requires responses that allow essential reactions of primary metabolism to continue and enable the plant to tolerate WD (Foyer et al. 1998). Recent studies have indicated that nitrogen assimilation to be critical to plant acclimation to fluctuating environmental conditions (Swarbrek et al. 2011). However, the effect of WD on nitrogen metabolism remains relatively unexplored.

Rice is an important crop worldwide. It is also considered to be a model plant for monocots because of its relatively small genome size. WD is one of the major constraints depressing rice production (Jonaliza et al. 2004). Rice plants in paddy fields prefer to utilize ammonium as a major nitrogen source. GS serves for assimilation of ammonia to glutamine, which is the main form of organic nitrogen for transport through vascular tissues (Ishiyama et al. 2004b; Tabuchi et al. 2007). Present research work describes the effect of WD conditions on regulation of activity and expression of GS isoforms in leaf, stem and root of seedlings of two rice cultivars differently tolerant to WD conditions.

Dehydration tolerance in plants is attained by the maintenance of metabolic and physiological functions at low water status, which serve as the driving force for plant productivity. A few characteristics such as maintenance of RWC, osmotic adjustment and cell membrane stability are recognized as effective components of dehydration tolerance in many crops (Bhushan et al. 2007). RWC is considered to be the best integrated measure of plant water status, which represents variations in water potential (WP), turgor potential

(TP), and osmotic potential (OP). The choice of RWC as the best representation of plant water status in terms of genetic variation is also supported by genetic association between RWC and plant production under dehydration (Bhusan et al. 2007). Plants accumulate metabolites under stress conditions, which have been proposed as one of the mechanism of stress tolerance. Plant cells accumulate some kind of compatible solutes, such as proline, betaine, polyols, polyamines and ions (i.e. potassium) when subjected to abiotic and biotic stress. They act both by contributing to osmotic adjustment and by protecting proteins and cellular membranes. The beneficial roles of proline in conferring osmotolerance have been widely reported (Kishor et al. 1995; Bajji et al. 2000). It is shown to be involved in tolerance mechanisms against oxidative stress, which is the main strategy of plants to avoid detrimental effects of water stress (Vendruscolo et al. 2007). The WD stress on plant also results in inhibition of protein synthesis, increased protein degradation and accumulation or depletion of protein and non-protein amino acids in a variety of monocots and dicots (Gilbert et al. 1998). Moreover, the capacity to avoid or repair membrane damage during dehydration processes is also pivotal for the maintenance of membrane integrity, especially for those membranes in which functional proteins are embedded.

In the present study, rice varieties were initially screened for their dehydration tolerance characteristics by measuring RWC, accumulation of compatible solute like proline, and membrane permeability of ions and electrolytes. During WD, the dry wt. g^{-1} fresh wt. of root and leaf tissue of IR-64 seedling was increased significantly; however, no such change in dry weight was noted in Khitish. The RWC, protein and proline level declined markedly and the electrolyte leakage was increased sharply in IR-64 cultivar in response to WD treatment. Under similar condition, Khitish variety maintained relatively higher RWC, protein and proline level. Proline level kept on increasing continuously throughout the treatment period. Moreover, electrolyte leakage from Khitish leaf increased only marginally during WD. The results thus indicated more susceptibility of IR-64 to WD in comparison to Khitish and hence, were designated as drought- sensitive and tolerant- cultivar, respectively. These two cultivars were used for studying the effect of WD on regulation of GS isoforms.

GS isoforms in rice seedlings was determined by Anion exchange chromatography. GS activity in leaves and stem were resolved into two distinct peaks eluting at 0.15 M and 0.30 M and were designated as GS1 and GS2, respectively.GS activities in soluble fraction and isolated chloroplast were eluted from the column of DEAE-Sephacel at the salt concentrations corresponding to that of GS1 and GS2 thereby indicating their localization in cytosol and chloroplast, respectively. In rice root GS activity was localized only in cytosol. Anion exchange chromatography has commonly been used for separation of isoforms of GS from various plant tissues. This technique has been successfully employed for resolution of isoforms of GS from leaves of rice (Hirel and Gadal 1980), sorghum (Hirel and Gadal 1982), wheat (Tobin et al. 1985), maize (Becker et al. 1993), sunflower (Cabello et al. 1994, Larios et al. 2004), *Pennisetum glaucum* (Ghosh 2004), tobacco (Pageau et al. 2005) and potato (Teixeira et al. 2005); roots of rice, bean, maize (Suzuki et al.1981), pea and alfalfa (Vezina et al. 1987) and nodules of *Phaseolus vulgaris* (Robert and Wong 1986). As in the present study, leaves of several C_3 plants were found to contain most of the GS activity as GS2 (Mc Nally et al. 1983). Majority of studies conducted on roots indicate the presence only cytosolic isoform of GS in the tissue (Suzuki et al. 1981; Mack 1995; Ishiyama et al. 2004; Bernard et al. 2008). In few studies, substantial activity of GS has also been shown to be associated with plastid fraction of the root cells, such as in roots of pea and alfalfa (Vezina et al. 1987). Brugiere et al (1999) reported the occurrence of both GS1 and GS2 in phloem. They showed the major role of GS2 in stem in controlling proline production. In leaves of sorghum (Hirel and Gadal 1982) and soybean (Kang and Hymowitz 1988) GS activities in soluble fraction and isolated chloroplast were eluted from the column of DEAE-Sephacel at the salt concentrations corresponding to that of GS1 and GS2 thereby indicating their cytosolic and chloroplastic localization, respectively. The non-overlapping localization of GS1 and GS2 was further confirmed by subcellular and immunocytochemical studies. Immunolocalization studies in tobacco (Brugiere et al. 1999), pine (Canovas et al. 2007), potato (Pereira et al. 1995), rice (Tabuchi 2005) have shown predominant vascular location of GS1in different plant organs. A detailed immunolocalization study of mature flag leaf of wheat using anti-GS antibody showed the presence of GS2 label in the plastid of mesophyll parenchyma and in the plastid of parenchyma cells in the perifascicular sheath surrounding the vascular bundles (Bernard et al. 2008).

As in present study, a single chloroplastic GS2 is form has been reported in many higher plants. However, in soybean and alfalfa several GS2 isoforms have been identified (Zozaya-Garza and Sengupta Gopalan 1999). Two cytosolic GS isoforms, OsGS1;1 and OsGS1;2 were present in root, stem and leaf of IR-64 and Khitish seedling. Majority of studies indicated the presence of multiple homologous but distinct genes for cytosolic GS1 (Tingey and Corruzi 1987; Ireland and Lea 1999; Yamaya and Oak 2004; Canovas et al, 2007; Bernard et al. 2008). Earlier it was believed that GSr (OsGS1;2) is the only GS1 isoform expressed in rice root (Sakamoto et al. 1989). However, present investigation indicated the presence of both OsGS1;1 and OsGS1;2 in root of rice seedlings, as reported by Ishiyama et al (2004b). OsGS1;2 was the major cytosolic GS in root and stem, whereas OsGS1;1 was the major isoform of GS1 in leaf. Previous study (Ishiyama et al. 2004a,b) also showed approximately 2.5-fold greater abundance of OsGS1;2 as compared to OsGS1;1 in root. A varietal variation in expression of OsGS1;1 was noted in all the organs tested. At the beginning of WD treatment OsGS1;1 transcript level was significantly higher in IR-64 in comparison to Khitish. Genetic linkage studies using segregating mapping populations have implicated cytosolic GS genes with grain production in maize (Hirel et al. 2007; Fontaine et al. 2009), rice (Obara et al. 2004) and wheat (Habash et al. 2007). In rice crop significant correlations were obtained between grain number/ size and the locus for OsGS1;1 protein content (Obara et al. 2004). A higher level of OsGS1;1 in all the organs of IR-64 indicates that the cultivar can perform better under proper growth conditions.

To evaluate the effect of WD on GS isoform, activity of GS1 and GS2 and the corresponding mRNA and polypeptide contents were simultaneously monitored in different organs of the two cultivars, at various stages of WD. The WD mediated alteration in total GS activity in leaf and root was directly related to dehydration tolerance characteristics of rice varieties. The detailed view of regulation of GS isoforms in leaf of IR-64 and Khitish seedlings in response to WD has been depicted in *Figure 2.23*. Total GS activity declined significantly in IR-64 and didn't change markedly in Khitish cultivar. The decreased GS activity in IR-64 leaf was due to preferential reduction of GS2 activity and was correlated with decreased level of GS2 mRNA and protein. Under similar conditions, an almost constant GS2 mRNA and corresponding polypeptide maintained a

steady GS2 activity in Khitish leaf. The results suggest that WD mediated GS2 regulation resides mainly at the transcriptional and/or mRNA stability levels. As in the IR-64 seedlings, total GS activity declined to less than a quarter of its initial level during the natural senescence of rice leaves and this decline was mainly caused by a decrease in the GS2 level (Kamachi et al. 1991). Similarly, other studies have also shown the susceptibility of chloroplastic GS2 to other abiotic and biotic stresses as well as dark (Larios et al. 2004; Ghosh 2004; Santos et al. 2004; Pageau et al. 2005).

An important physiological function of GS2 is reassimilation of NH_4^+ produced during photorespiration (Wallsgrove et al. 1979). Photorespiration is a metabolic pathway in which CO_2 is released by light and is linked to Calvin-Benson cycle through the oxygenase activity of ribulose- bisphosphate carboxylase (Rubisco). Although photorespiration includes many metabolic steps which are performed across chloroplast, mitochondria and peroxisomes, several studies suggest that rate limiting step is the GS2 catalyzed reassimilation of ammonia (Hausler et al. 1994; Hosida et al. 2000). The phorespiration activity has been reported to be induced by abiotic stress and plays a protective role (Hoshida et al. 2000). The overexpression of GS2 in leaf of transgenic rice increased their photorespiration capacity and improved their salt tolerance. The transgenic rice line accumulating 1.5 fold more GS2 than the control plant, had an increased photorespiration capacity. They also retained more than 90% photosystem II activity when grown under osmotic stress treatment for two weeks indicating the physiological importance of GS2 in abiotic stress tolerance (Hoshida et al. 2000). Hence, in the present study a relatively unaltered GS2 expression in Khitish leaf could maintain the photorespiratory capacity of the plant at limited water availability that improves tolerance of the cultivar to WD.

WD treatment increased the expression of both OsGS1;1 and OsGS1;2 transcripts in leaf of IR-64 and Khitish cultivars. The time course of increase in GS1 transcripts corresponded with the accumulation of GS1 protein detected on Western blot. Although, the response of individual cytosolic GS genes to abiotic stress has not been studied earlier, the total GS1 transcript and polypeptide level have already been shown to accumulate in leaf during natural senescence and in response to biotic and abiotic stress (Kamachi et al.

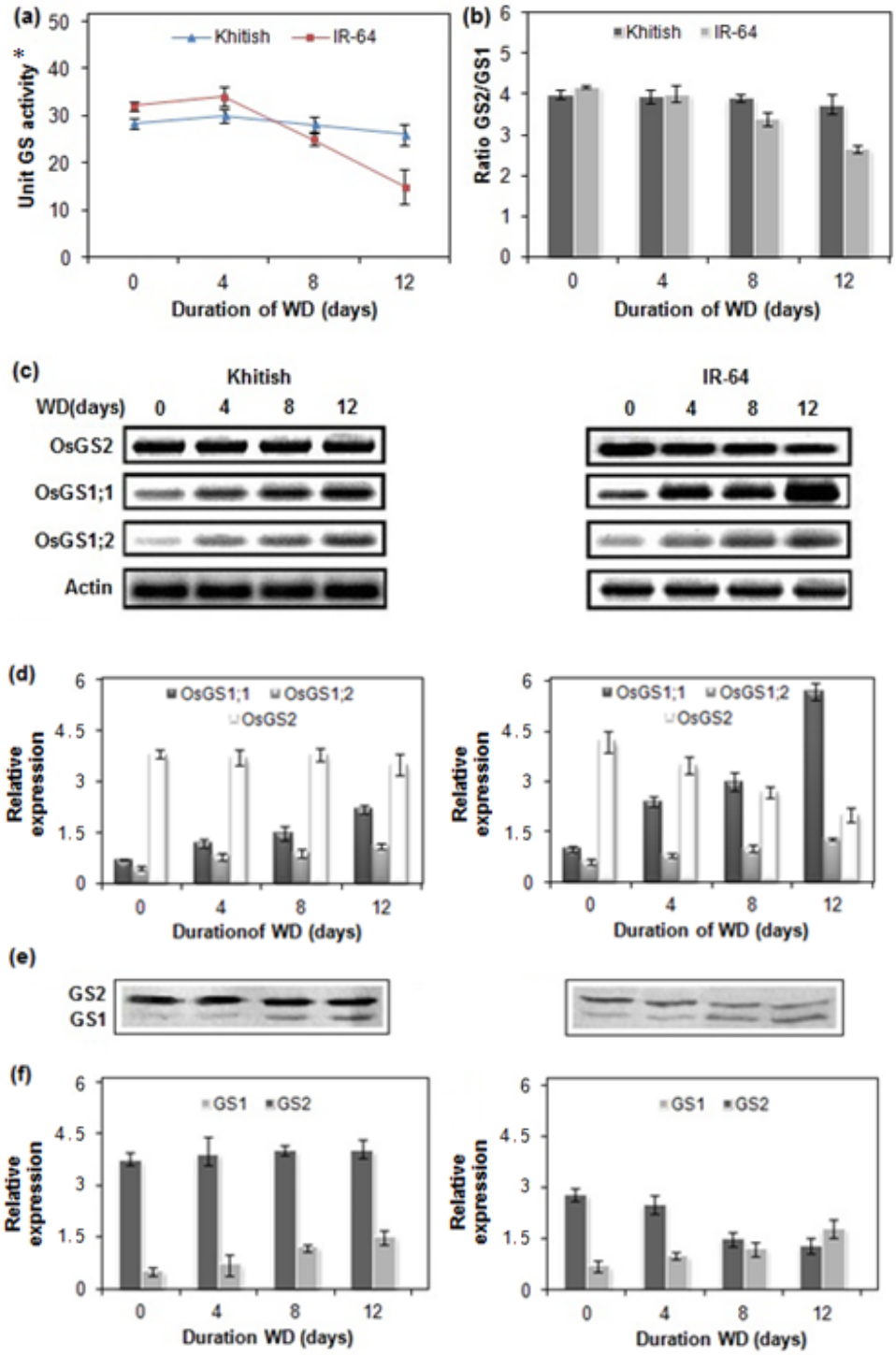

Figure 2.23. GS activity and expression in leaves of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment. (a) Relative change in total GS activity of IR-64 and Khitish, (b) Change in ratio of GS2/GS1 activity in IR-64 and Khitish seedlings , (c) Analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR,(d) Bar diagram for GS mRNA level (e)Western blot analysis of GS1 and GS2 polypeptide and (f) Bar diagram for GS polypeptide. Western blotting was carried out with 10µg of total soluble protein extracted from leaf. *One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1}.

1991; Pageau et al. 2005; Teixeira et al. 2005; Bernard and Habash 2009). In natural senescing potato plant GS1 transcript accumulation was coupled to increased synthesis of corresponding polypeptide (Teixeira et al. 2005). However, during natural senescence of rice leaves translatable GS1 mRNA level increased by four folds without affecting the corresponding polypeptide content (Kamachi et al. 1991). Similarly, the GS1 protein quantity has also been shown to increase in *Nicotiana tabaccum* leaf during *Potyvirus* infection (Pageau et al. 2005). In the present study, WD mediated increase in GS1 transcript and protein expression in rice leaf didn't correspond with GS1 activity. A similar type of response to GS1 mRNA overexpression was observed in *Arabidopsis* root. In this tissue the nitrogen nutrition mediated increase in GS1 transcript and polypeptide was related to the maintenance of GS1 activity rather than increase (Ishiyama et al. 2004a). A lack of correlation between constitutive overexpression of GS1 mRNA and abundance of corresponding polypeptide and activity has been reported earlier in leaf of Alfalfa (Ortega et al. 2001). WD treatment caused significantly greater OsGS1;1 accumulation in leaf of IR-64 as compared to that of Khitish. The increased OsGS1;1 in IR-64 leaf could be due to its higher rate of protein degradation (*Figure 2.12*), conforming to role of the isoform in reassimilation of nitrogen released from protein breakdown. The contention is supported by previous studies showing the localization of GS1 protein in companion cells and vascular parenchyma cells in senescing leaf blade of rice (Kamachi et al.1992) and wheat (Kichey et al. 2005) plants. The research work by Tabuchi et al (2005) with OsGS1;1 knockout mutant also showed the importance of OsGS1;1 in remobilization and reutilization of nitrogen in rice plant. Knockout mutants created by the insertion of Tos17 into the exon of OsGS1;1 were screened and characterized. Homozygously inserted mutants exhibited a severe retardation in growth rate and grain filling when grown under normal nitrogen fertilizer concentrations. Reintroduction of OsGS1;1 cDNA under the control of its own promoter into the mutant successfully complemented the slow growth phenotype.

In contrast to leaf, WD treatment reduced GS1 activity and OsGS1;1 transcript level in stem and root of IR-64 seedlings (*Figure 2.24 and 2.25*). Nitrogen remobilization from protein breakdown constitutes the major source of nitrogen in vascular tissue and glutamine is the most abundant free amino acid for transport in rice plant (Tabuchi et al.

2007). The repression in OsGS1;1 might result from remobilization and transport of high concentration of glutamine to stem and root from increased protein degradation in IR-64 leaf. The transcriptional down-regulation of OsGS1;1 has already been documented in presence of NH_4^+ in roots of *Arabidopsis* and rice seedlings (Ishiyama et al.2004a; Ishiyama et al. 2004b; Kusano et al. 2011). Several other rice genes associated with N-uptake and metabolism, such as, OsGS1;2, OsNADH-GOGAT1, OsAMT1;1 and OsAMT1;2, are also regulated by exogenous NH_4^+ ions (Ishiyama et al. 2004; Sonoda et al. 2003; Tabuchi et al.2007). However, pharmacological studies have suggested glutamine rather than NH_4^+ ions, being the real signaling molecule in regulation of expression of these genes (Oliveira and Coruzzi 1999; Tabuchi et al.2007). Oliveira and Coruzzi (1999) reported the association of metabolic regulation of GS1with relative abundance of carbon skeleton verses amino acids accumulated in the root tissue. Their results suggested a negative feedback regulation of GS1 by glutamine or the downstream nitrogen metabolites.

A comparison of kinetic properties of OsGS1;1 and OsGS1;2 encoded GS isoforms in rice root was carried out by Ishiyama et al. (2004b). The Vmax values were approximately 2-fold higher with OsGS1;1 than with OsGS1;2. In addition, OsGS1;1 exhibited extremely high substrate affinity for ammonium, as indicated from its Km value: the Km for ammonium was 2.7-fold lower in OsGS1;1 than in OsGS1;2. The result supported the importance of OsGS1;1 in promoting the rapid conversion of ammonium to glutamine even under low ammonium conditions (Ishiyama et al. 2004b). Similarly, in *Arabidopsis* root, GLN1;1 exhibited an extremely high affinity for ammonium (Km < 10 µM) as compared to GLN1;2 (Km = 2450 µM) (Ishiyama et al. 2004a). The implication of OsGS1;1 in NH_4^+ assimilation has been further indicated by the over accumulation of free ammonium in the leaf sheath and roots of the rice mutant lacking OsGS1;1(Kusano et al. 2011). In the present investigations WD mediated decrease in GS1 activity in stem and root of IR-64 seedling might correlate with reduction in OsGS1;1 mRNA level. Hence, OsGS1;1 seems to play significant role in performance of plant under stress condition.

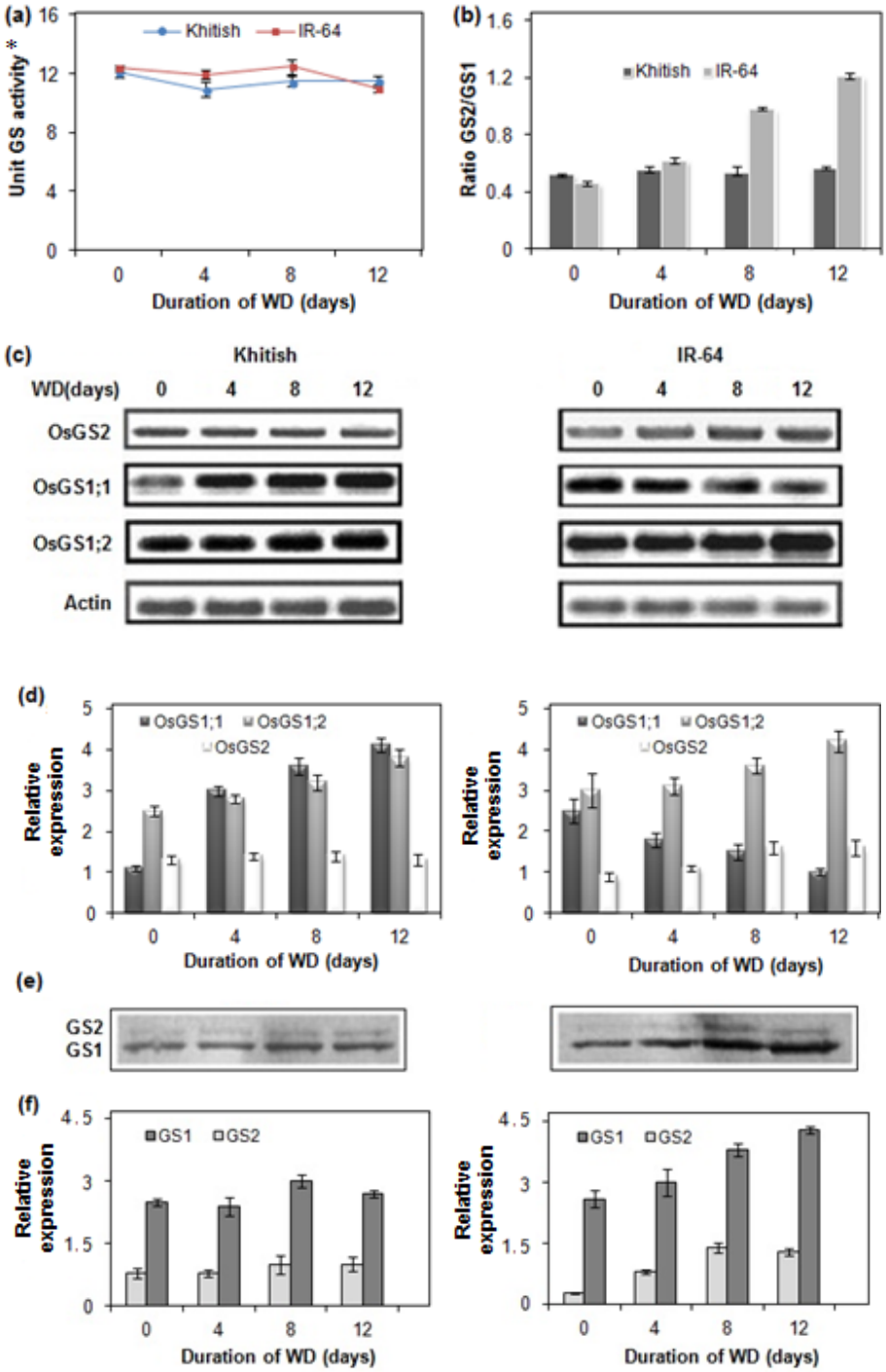

Figure 2.24. GS activity and expression in stem of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment. (a) Relative change in total GS activity of IR-64 and Khitish, (b) Change in ratio of GS2/GS1 activity in IR-64 and Khitish seedlings , (c) Analysis of OsGS1;1, OsGS1;2 and OsGS2 transcripts by RT-PCR,(d) Bar diagram for GS mRNA level (e)Western blot analysis of GS1 and GS2 polypeptide and (f) Bar diagram for GS polypeptide. Western blotting was carried out with 10µg of total soluble protein extracted from leaf. *One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1}.

The decline in GS1 activity in IR-64 stem was compensated by increment in GS2 protein and activity, maintaining almost unchanged total GS activity. The increased GS2 protein can be due to observed increase in total protein content of stem during WD. As in the present study, a tissue specific response of WD stress was noted in *Lupinus albus* with strikingly increase in concentration of N and S in stem with intensification of water stress. At 13 days of WD, the ratio of stem protein concentration of WD to control plant increased from 0.8 to 1.3 (Pinheiro et al. 2001). Translation of some proteins has also been found to increase in stem in other type of stress conditions as well. Recently, Moller et al (2011) in their proteomic based study indicated an increase in synthesis of some proteins of chloroplastic transcription and translation machinery under N-starvation condition. The condition is quite similar to that encountered by rice plant under WD leading to limited uptake of nutrient from soil. Thus, a different metabolic status may contribute to the maintenance of GS protein and activity in stem during WD.

WD treatment had no differential effect on expression of OsGS1;2. The transcript level of OsGS1;2 was found to enhance progressively and almost equally in leaf and stem of IR-64 and Khitish seedlings. However, in roots of both the cultivars the highly expressed OsGS1;2 was found to decline initially followed by an increased accumulation on further dehydration. The rate of decline was faster in IR-64 root in comparison to Khitish root. The time course of OsGS1;2 mRNA was reflected in GS1 polypeptide abundance in all the three tissues. The result is supported by study of Ishiyama and co-worker (2004a) on effect of ammonium nutrition in *Arabidopsis* root GS isoforms. Arabidopsis root contained four different isoforms of GS1, among them amount of GS1;1, GS1;3, and GS1;4 mRNA decreased and GS1;2 mRNA was increased by ammonium nutrition. The time course increase in GS1;2 mRNA corresponded with the accumulation of GS1 protein detected on the western blot. However, the increase in GS1;2 mRNA and protein was not correlated with total GS1 activity. The lack of correlation was due to lower affinity of GS1;2 for ammonium as compared to other isoform. Furthermore, GS1;2 has been reported to overexpress in leaves and roots by ammonium supply. GS1;2 knockout mutants of *Arabidopsis* displayed lower GS activity, higher ammonium concentration, and reduced rosette biomass compared with the wild type (WT) under ample nitrogen supply only.

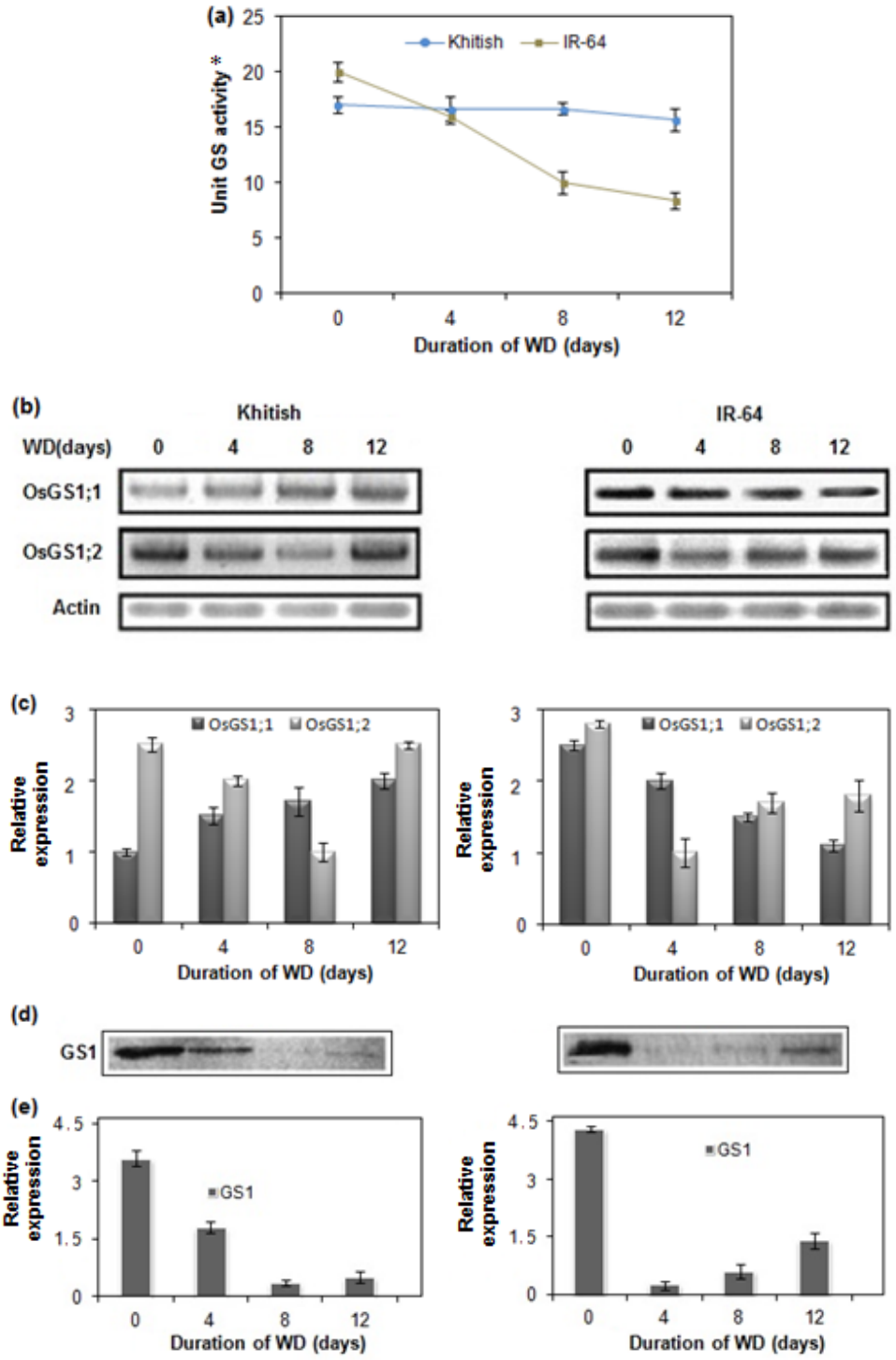

Figure 2.25. GS activity and expression in root of *O. sativa* cultivars at 0, 4, 8, and 12 days of WD treatment; (a) Relative change in total GS activity of IR-64 and Khitish, (b) Analysis of OsGS1;1 and OsGS1;2 OsGS2 transcripts by RT-PCR, (c) Bar diagram for GS mRNA level, (d) Western blot analysis of GS1 and GS2 polypeptide and (e) Bar diagram for GS polypeptide. Western blotting was carried out with 10µg of total soluble protein extracted from leaf. *One unit of GS activity represents 1.0 µmol of γ-glutamylhydroxamate produced 30 min^{-1} .

However, it showed no significant difference from the wild type under nitrogen limiting conditions (Lothier et al. 2010). A similar nitrogen limiting condition prevailed during WD. IR-64 seedlings being more susceptible to WD showed faster decline in OsGS1;2 mRNA and protein in root with minimum expression levels at day 4 of treatment due to limited uptake of nitrogen from soil. The enhancement in OsGS1;2 expression on further intensification of WD might be related to increased accumulation of ammonium ion due to observed increased protein degradation.

In conclusion, the regulation of GS isoforms by WD was organ specific. Two GS isoforms i.e. GS1;1 and GS2 were differentially regulated in drought sensitive IR-64 and drought tolerant Khitish cultivars of rice. GS2 was the major GS isoform in leaf. GS2 expression in leaves decreased in IR-64 seedlings and remained almost unaltered in Khitish seedlings in response to WD. The maintenance of GS2 expression in leaf might be associated with the maintenance of photosynthetic and phorespiratory capacity of the Khitish and hence, with WD tolerance characteristics of the cultivar. The GS1;1 isoform has been shown to be involved in remobilization and reutilization of nitrogen during senescence and other stress conditions characterized by high rate of protein degradation. A higher substrate affinity of the enzyme for ammonium signifies its promoting the rapid conversion of ammonium to glutamine even under low ammonium conditions. Hence, from the results it can be inferred that a relatively maintained OsGS2 and the over-expression of OsGS1;1 in might contribute to improved drought tolerance characteristics of *Oryza sativa* cv. Khitish.

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CHAPTER 3

Studies on GS isoform in *Selaginella bryopteris* L. and its regulation during water deficit stress

3.1. INTRODUCTION

Plant growth is greatly affected by drought stress. It is a composite stress condition that includes soil WD, increased daytime temperature, and reduced nutrient availability. However, the most important factor limiting growth and impairing plant productivity is the drop in water availability to the plant. Plants survive the extreme water deficit (WD) conditions either by avoiding stressful events or by activating adaptive stress mechanisms. Most crop species are very sensitive to soil water potential and only rarely survive soil water deficit that drive leaf water potential to a lower value (Proctor and Pence 2002). However, resurrection plants can survive with less than 5% of their total water in the vegetative tissues and are able to regain normal metabolism and growth within several hours of rewatering (Ramanjulu and Bartels 2002; Jiang et al. 2007; Deeba et al. 2009).

In plants, WD induces complex change in nitrogen metabolism due to decreased water availability for transport leading to limited uptake of nitrogen nutrients. The effect of WD on nitrogen metabolism of resurrection plants has been studied with inhibition of protein synthesis, increased protein degradation and accumulation or depletion of protein and non-protein amino acids (Martinelli et al. 2007; Whittaker et al. 2007; Yobi et al. 2013). Nitrogen-rich amino acids such as glutamine, glutamate, arginine, aspartate, citrulline, asparagine, 3-(3-hydroxyphenyl) propionate, N-6-trimethyllysine, trans-4-hydroxyproline, and ophthalmate have been shown to accumulate in dehydrated state (Martinelli et al. 2007; Oliver et al. 2011; Yobi et al. 2013). Resurrection plants are also able to synthesize new proteins even at very low RWC (Bartels and Sunkar 2005). In

resurrection angiosperm *Craterostigma plantagineum*, protective proteins, such as hydrophilins, detoxifying enzymes**,** degradation and transport proteins, were overexpressed under dehydration (Bartels and Sunkar 2005; Deeba et al. 2009).

GS is the key enzyme of nitrogen assimilation and remobilization. GS together with GOGAT forms the GS-GOGAT cycle. Glutamate produced by the cycle is the preferential amino-donor for the different aminotransferase reactions for subsequent amino acid inter-conversions (Ford and Lea 2007). In most of the higher plants, GS and GOGAT exist as multiple isoforms. The cytosolic GS1 is considered to be operational along with the NADH-dependent isoform of GOGAT (NADH-GOGAT, EC 1.4.1.14) in the cytosol. On the other hand, chloroplastic GS2 together with the ferredoxin-dependent isoform of glutamate synthase (Fd-GOGAT, EC 1.4.7.1), is reported to be responsible for the chloroplastic GS/GOGAT cycle (Lea and Ireland 1999; Skopelitis et al. 2006). Finally, glutamate dehydrogenase (GDH) is also directly involved in glutamate metabolism and it's *in vivo* role is still under debate. In drought sensitive higher plants, GS is highly susceptible to WD conditions. Both the cytosolic and chloroplastic GS activities decline during WD (present study, Rice paper). Present study was undertaken with the aim to search dehydration tolerant isoforms of GS. Since resurrection plants have the ability to survive long dry period, they could be an excellent system for searching dehydration tolerant GS.

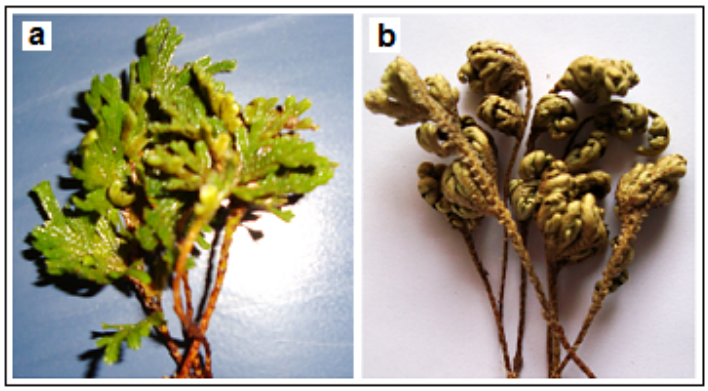

Figure3.1. *S. bryopteris* fronds; (a) hydrated, and (b) rehydrated.

Selaginella bryopteris belonging to the family Selaginellaceae, is a resurrection lycophyte growing in the hilly area of tropical regions (Deeba et al. 2009). It is a creeping or

ascendant plant with simple, scale-like leaves on branching stems from which roots also arise. It is capable of surviving almost complete dehydration for prolonged period. Under dry conditions, fronds of *Selaginella* roll into brown balls and become green on rehydration. The plant has the unique feature with detached fronds possessing a similar level of desiccation tolerance as that of whole plant. Present investigation describes isolation and characterization of GS from S. *bryopteris*. Further studies were carried out to determine the regulation of GS expression during dehydration and rehydration of *Selaginella* fronds and its relationship with other enzymes of ammonium metabolism.

3.2. MATERIALS AND METHODS

3.2.1. Chemicals and reagents

The required chemicals and reagents used in this investigation were purchased from different companies as described in section 2.2.1.

3.2.2. Plant material

Selaginella bryopteris plants were collected from natural habitat (Darjeeling District, West Bengal, India, *latitude* 26°31' and 27°13' N and *longitude* 87°59' and 88°53'E). There, the plants grow on rocky slope, on a thin layer of organo-mineral rich, dark mountain soil. Plants were harvested and kept in water until the beginning of experiment. For dehydration, freshly detached fronds from well hydrated plant were kept in a growth chamber (Conviron, Canada), at 25 ^{o}C and ambient photoperiod. Rehydration is achieved by keeping the dehydrated fronds in moist filter paper. The plants were sampled at various stages of dehydration and rehydration. Sampling times were determined by visual appraisal of the fronds using decolouration and folding as benchmarks, at which RWC of fronds was determined.

3.2.3. Determination of RWC

The relative water content (RWC) of *Selaginella* fronds was measured according to Barrs and Weatherley (1962) as described in section 2.2.3.

3.2.4. Pigments estimation

For pigments determination, fronds were extracted in 80% chilled acetone followed by centrifugation in 10,000 rpm for 10 minutes at 4 ºC. The supernatant was taken for determination of photosynthetic pigments. Pigment contents were calculated according to the formulas of Lichtentaler and Wellburnn (1985). Chlorophyll *a* (mg/g) = ((12.7 x A_{663} - 2.69 x A_{645}) v/w), chlorophyll *b* (mg/g) = ((22.9 x A_{645} - 4.68 x A_{663}) v/w), and carotenoid (mg/g) = ((1000 x A_{470}) - (3.27 x chlorophyll *a* + 1.04 x chlorophyll *b*)) /227 v/w). The experiments were done in triplicates.

3.2.5. Proline estimation

Free proline was quantified according to Bates et al (1973) as described in section 2.2.5.

3.2.6. Protein estimation

Quantitative estimation of protein was carried out by the method of Bradford (1976) as described in section 2.2.6.

3.2.7. Ammonium estimation

Ammonium was estimated by the phenol hypochlorite colorimetric method (Russell, 1944) using ammonium sulphate as standard (*Figure 3.2*). One gram of *Selaginella* fronds was homogenized in 5 ml of Selaginella extraction buffer (SE-Buffer) containing 50 mM phosphate buffer (pH 6.5), 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 15 % glycerol. The homogenate was filtered through four layers of muslin and was centrifuged at 10,000 rpm for 15 min at 4 ºC. After centrifugation the supernatant was collected in fresh tube. For quantification of ammonia-nitrogen, the procedure involved consecutive addition of 2 ml of phenol solution (10 g of reagent grade phenol dissolved in 100 ml of 95% v/v ethyl alcohol), 2 ml of 0.5% sodium nitroprusside and 5 ml of oxidizing reagent (prepared by mixing of alkaline-sodium citrate and sodium hypochlorite) to 1 ml of the plant extract. The reaction contents were mixed thoroughly after each addition. The colour was allowed to develop at room temperature (22-25 ºC) for 1 h and the absorbance was recorded at 640 nm in a spectrophotometer. The assay was done in triplicates. Amount of ammonia was expressed as µg NH_4^+ g^{-1} dry wt.

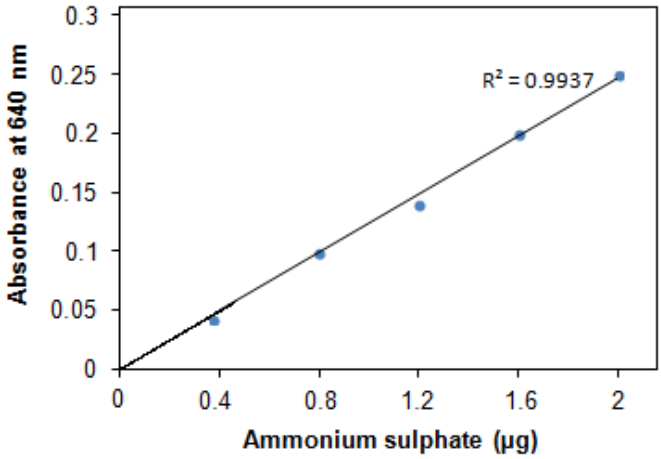

Figure 3.2. Standard curve for ammonium sulphate.

3.2.8. Separation of GS isoforms from S. bryopteris fronds

All the steps of isoenzyme separation were performed at 4 ^{o}C. One gram of *Selaginella* fronds was homogenized in 5 ml of SE-Buffer. The homogenate was centrifuged at 10,000 rpm for 15 min. One ml of desalted supernatant was loaded onto a DEAE-Sephacel column (5x1 cm) pre-equilibrated with the SE-Buffer. The column was washed with the same buffer until no protein was detectable in the eluate. The adsorbed proteins were eluted by gradient elution using 0 - 0.5 M KCl in SE-Buffer. The flow rate was maintained at 20 ml h^{-1}. 2 ml fractions were collected and assayed for GS activity. About 80-90 % of the total GS activity present in the crude extract was recovered after chromatographic separation.

3.2.9. Separation of cytosolic and chloroplastic fraction of S. bryopteris fronds

Chloroplasts were isolated from *Selaginella* fronds following the method of Rathnam and Edward (1976). Fronds were homogenised in a pre-chilled blender with approximately five volumes of chloroplast isolation buffer (300 mM sucrose, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1mM each of $MgCl_2$ and $MnCl_2$). The homogenate was sieved through four layers of muslin cloth and centrifuged at 1500 rpm for 10 min. The supernatant was centrifuged at 15,000 rpm (15K) for 20 min to obtain the crude chloroplast pellet. The 15K supernatant was fractionated by 40-70 % ammonium sulphate precipitation. The precipitated pellet was suspended in 2.5 ml of SE-Buffer, dialysed and used to measure cytosolic GS activity. The isolated chloroplast fraction was homogenized

in SE-Buffer and centrifuged at 15000 rpm for 40 min. The dialysed supernatant was used as a source of chloroplastic GS.

3.2.10. Enzyme assays

One gram *Selaginella* fronds were homogenized in 5ml of SE-Buffer. The homogenate was centrifuged at 10,000 rpm for 15 min at 4^{o}C. The supernatant was desalted on pre-equilibrated Biogel P-2 column and used for determination of GS, NADH-GOGAT and GDH activity.

3.2.10.1. Glutamine synthetase

GS activity was determined by semisynthetase reaction (Washitani and Sato 1977). A 1 ml reaction mixture contained 50 µmol acetate buffer (pH 5.5), 200 µmol glutamate, 10 µmol ATP, 5 µmol hydroxylamine hydrochloride, 20 µmol $MgCl_2$, and 100 µl enzyme preparation. The reaction mixture was incubated at 37 ^{o}C for 30 min. and terminated by adding 2 ml of $FeCl_3$ reagent (0.67 M $FeCl_3$, 0.37 M HCl and 20% (w/v) Tri-chloroacetic acid). After 20 min, the amount of γ-glutamylhydroxamate produced was determined spectrophotometrically by measuring the absorbance at 540 nm. One unit of enzyme activity is defined as the amount catalyzing the formation of 1.0 µmol of γ-glutamylhydroxamate 30 min^{-1} at 37^{o}C.

3.2.10.2. Glutamate oxoglutarate amino transferase (GOGAT)

GOGAT (EC 2.6.1.53) was assayed by determining the rate of glutamine-dependent NADH oxidation (Turano et al. 1996). The reaction mixture contained 5 µmol L-glutamine, 5 µmol 2-oxoglutarate, 0.08 µmol NADH, 5 µmol EDTA, 50 µmol Tris buffer (final pH 7.5) and 100 µl of enzyme preparation in final volume of 1 ml. One unit of enzyme activity is defined as the amount catalyzing the formation of 1.0 µmol of glutamate min^{-1} at 37^{o}C.

3.2.10.3. Glutamate dehydrogenase (GDH Aminating)

GDH (EC 1.4.1.2) was assayed by determining the rate of 2-oxoglutarate-dependent NADH oxidation (Suzuki et al. 1994). The reaction mixture in final volume 1 ml, contained 150 µmol NH_4Cl, 1 µmol $CaCl_2$, 0.3 µmol NADH, 20 µmol 2-oxoglutarate,

and 100 µmol Tris buffer (final pH 7.5) and 100 µl enzyme preparation. One unit of GDH represents the reduction of 1 µmol NADH min^{-1} at 30°C.

3.2.11. Partial purification of GS from S. bryopteris fronds

All steps of purification were carried out at 4 ºC. Five gram of *Selaginella* fronds were homogenized in 25 ml of SE-Buffer, pH 6.5. The homogenate was filtered through four layers of muslin and was centrifuged at 12,000 rpm for 20 min. The supernatant so obtained, was designated as crude extract. Solid ammonium sulphate was added to it gradually with continuous stirring to bring the final concentration to 30% saturation. After 30 min, the precipitated proteins were removed by centrifugation at 10,000 rpm for 20 min. The supernatant was decanted and further addition of ammonium sulphate was added to it to achieve 70% saturation. After allowing it to stand for 30 min, it was centrifuged at 10,000 rpm for 20 min. The pellet was dissolved in 1 ml of SE-Buffer and loaded onto gel-filtration column (Biogel P-100, Bio-Rad, 1 × 25 cm) pre-equilibrated with SE-buffer and eluted with the same buffer at a flow rate of 10 ml h^{-1}. The fractions thus obtained were checked for GS activity. The active fractions were pooled and subjected to ion-exchange chromatography onto a DEAE-sephacel column (2 × 15 cm) pre-equilibrated with SE-buffer. The column was initially washed with the 100mM SE-Buffer, pH 6.5 to remove unbound proteins. Thereafter, elution of the bound protein was carried out by gradient elution using 0-0.50 M KCl in the buffer. The flow rate was maintained at 20 ml h^{-1}. Fractions of 2 ml were collected and analyzed for protein and GS activity. The active fractions eluted as a single peak were pooled and stored at 4°C.

3.2.12. Characterization of GS

The kinetic properties of the partially purified enzyme were determined with respect to pH optima, temperature optima, *Km* for glutamate and thermostability. The pH optimum was determined by measuring the enzyme activity at pH 2.5-8.5 in the following buffers: 100 mM glycine (pH 2.5), 100 mM sodium acetate (pH 4.5, 5.5), 100 mM phosphate buffer (pH 6.5), and 100 mM Tris-HCl (pH 7.5 and 8.5). For determination of Km for glutamate, substrate concentrations were varied in the range of 12.5-300 µM followed by measuring the activity under standard conditions. Km value for glutamate was determined by Lineweaver-Burk plot. The optimum temperature for activity was

determined at optimum pH and at temperatures ranging from 15 to 80 ºC. For determination of thermal stability, the enzyme was pre-incubated at 15-80 ºC for 30 min, followed by measurement of activity at standard temperature and pH.

3.2.13. SDS-PAGE analysis

Total soluble proteins were resolved by SDS-PAGE (Laemmli 1970) as described in section 2.2.10.1.

3.2.14. Immunoblot analysis of GS and GDH protein

Total soluble proteins (10 µg) were resolved by 12 % SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Polypeptide detection was performed using polyclonal antibody raised against synthetic peptide from conserved region of GS and tobacco GDH (Agrisera, Sweden). The reacted polypeptide was visualised with a goat anti-rabbit IgG-alkaline phosphatase conjugate using colorimetric based nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate-p-toluidine (NBT/BCIP) detection kit (Invitrogen, USA). Broad range pre-stained standards were used as markers.

3.2.15. Bioinformatic analysis

Amino acid sequences of plant GS isoforms were retrieved from GenBank databases (*Table 3.2*). Alignment of the coding regions was performed by the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) program (Robert 2004; http://www.ebi.ac.uk) and multiple align show (http:/bioinformatics.org/sms/).

3.3. RESULTS

3.3.1. Determination of dehydration-rehydration kinetics of S. bryopteris fronds

Preliminary experiment was carried out to determine the dehydration-rehydration kinetics of *S. bryopteris* fronds by measuring relative water content (RWC). As shown in *Figure 3.3a*, RWC decreased from 95 % to 70 % at 3h of dehydration and further decreased to 5% at 24 h of treatment. However, fronds were able to recover completely from dehydration by massive uptake of water within a much shorter duration of rewatering.

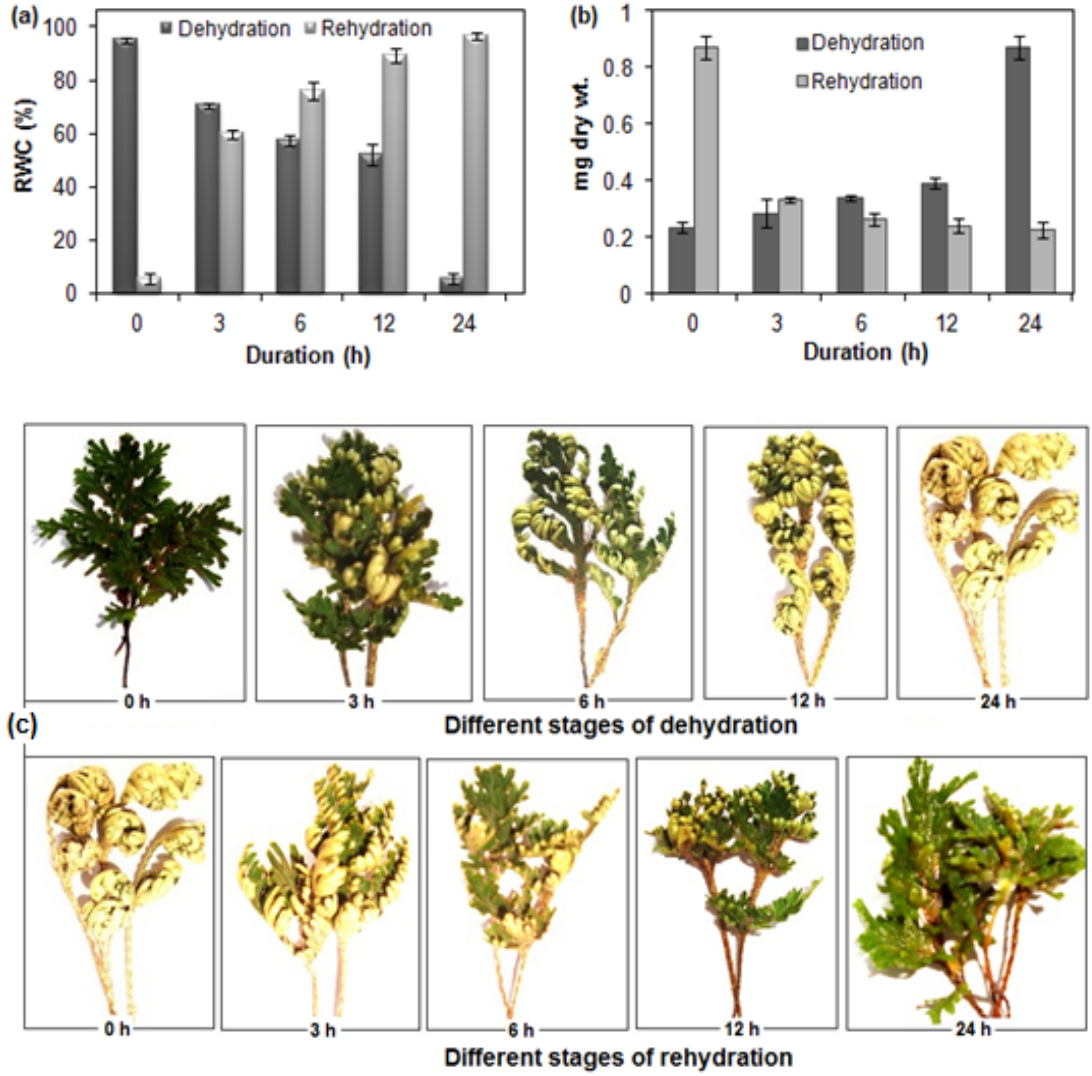

Figure 3.3. Effect of dehydration-rehydration on (a) RWC, (b) dry weight, and (c) morphology of *S. byopteris* fronds. Each point is the mean of three samples ±SE.

Consequently, RWC increased to 60 % at 3h, 76 % at 6 h and then gradually to 96 % at 24 h of rehydration. The dry weight (mg dry wt. g^{-1} tissue) of fronds altered considerably during various stages of dehydration-rehydration. Its value increased steadily till 12 h followed by a sharper increase at 24h of treatment. Similarly, rehydration of dried fronds for 3h reduced dry weight of fronds by about 60% and followed by a gradual decrease till the end of the treatment (*Figure 3.3b*). The phenotype of the dehydrated fronds was different from hydrated one. The dehydrated fronds showed inward curling and brownish appearance. It regained original morphology with opening of adaxial surface and greening on rehydration. Greening of fronds was clearly visualized at 3 h of hydration with complete green appearance at 24 h of hydration (*Figure 3.3c*). To ascertain whether the observed colour transition was correlated with changes in plant pigment levels, chlorophyll a (chl a), chlorophyll b (chl b) and carotenoid contents were monitored during various stages of dehydration-rehydration.

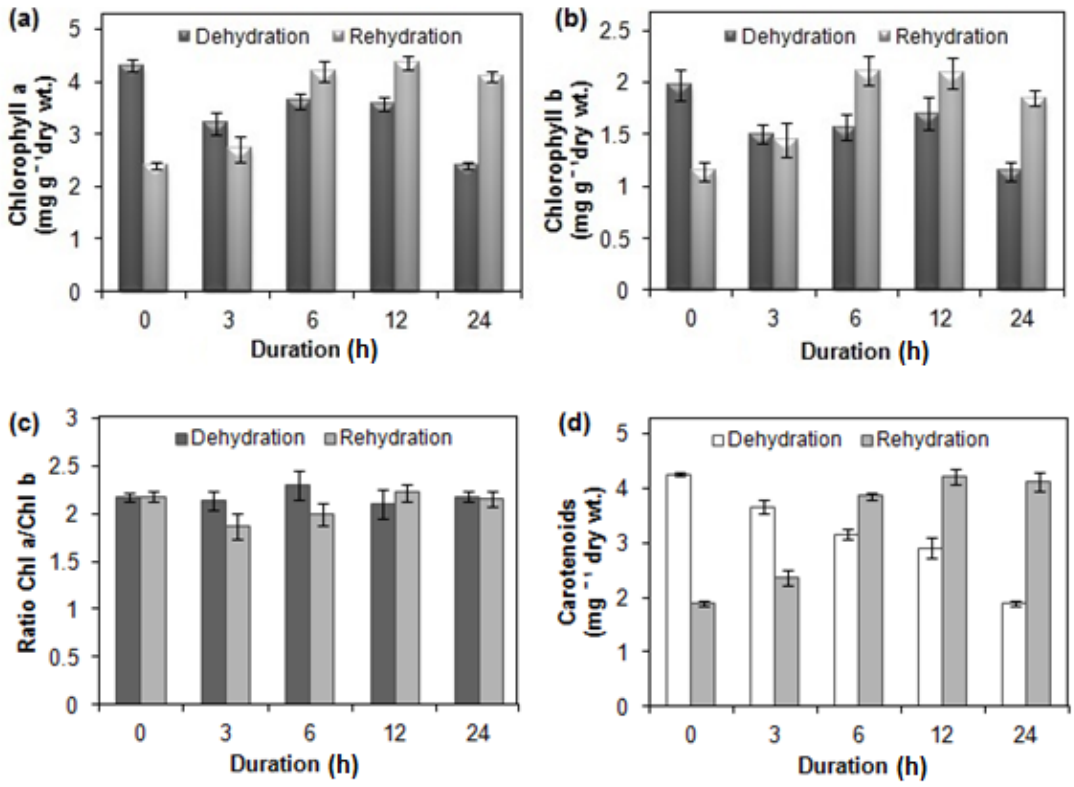

Figure 3.4. Effect of dehydration-rehydration on (a) Chlorophyll a, (b) Chlorophyll b, (c) Ratio of Chlorophyll a and Chlorophyll b, (d) Carotenoid contents of *S. bryopteris* fronds. Each point is the mean of three samples ±SE.

Although, chl a and chl b contents didn't alter significantly during 12 h of dehydration, however, their levels declined by about 40 % at 24 h of treatment. Rehydration of fronds restored chl a and chl b contents to a value at the beginning of dehydration treatment (*Figure 3.4 a, b*). However, the ratio of Chl a to chl b remained unchanged throughout the hydration cycle (*Figure 3.4 c*). In contrast to chlorophyll, a noticeable alteration in carotenoid level was detected. The carotenoid content gradually reduced on dehydration with about 50% decrease at 24 h of treatment. The carotenoid content was regained on rehydration of frond (*Figure 3.4 d*).

3.3.2. Effect of dehydration-rehydration of S. bryopteris fronds on protein, proline and ammonium contents

The change in total protein, free ammonium and proline contents were monitored at various stages of dehydration-rehydration treatment. The total protein content decreased by about 30 % and 50 % at 3 h and 24 h of dehydration, respectively and the decrease was reversed on subsequent rehydration of the fronds (*Figure 3.5 a*).

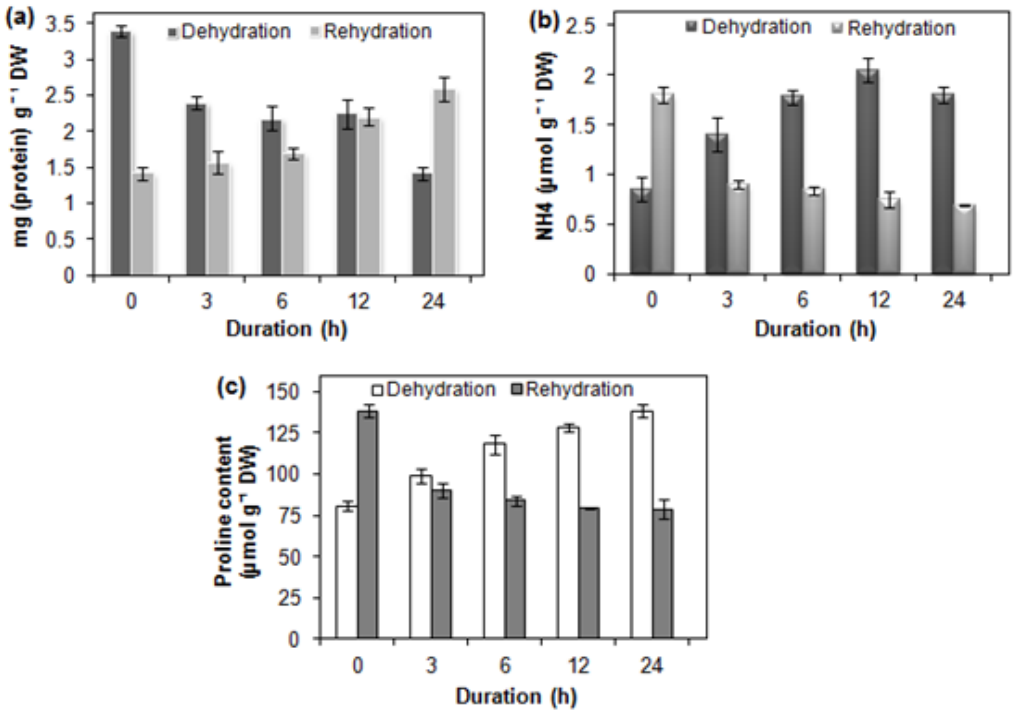

Figure 3.5. Effect of dehydration-rehydration on (a) total soluble protein, (b) ammonium, and (c) proline contents of *S. byopteris* fronds. Each point is the mean of three samples ±SE.

Present study showed a close relationship between the alteration in protein content and accumulation of ammonium. Dehydration mediated decline in protein level paralleled a significant increase in free ammonium content. Ammonium level rose by almost 2.5 folds at 12 h of dehydration, but didn't change markedly on further intensification of stress. Subsequent rehydration of the fronds for 3 h lowered the ammonium content sharply by about 50 %, followed by a steady decrease on further rehydration (*Figure 3.5 b*). The change in proline content during dehydration-rehydration of *Selaginella* fronds was also examined. Proline content increased progressively from the beginning with almost two folds accumulation at 24 h of dehydration treatment. Its level fell sharply to about 40 % on subsequent rehydration for 3 h and didn't alter significantly on further rehydration (*Figure 3.5c*).

3.3.3. Optimization of GS extraction and assay conditions

The optimal conditions for extraction of the enzyme with respect to molarity and pH of the extraction buffer and concentration of protective/ stabilizing agent was established in order to ensure maximal extraction and recovery of the enzyme from *Selaginella* fronds. Maximum recovery of the enzyme obtained when 50 mM Phosphate buffer (pH 6.5) buffer containing 1 mM $MgCl_2$, 2 mM cysteine hydrochloride and 10% glycerol was used as extraction buffer. The reaction conditions for GS activity assay were standardized by varying the pH of assay buffer as well as concentrations of other reaction components such as glutamate, ATP and $MgCl_2$. Maximum activity of the enzyme was obtained in the reaction mix containing 50 µmol acetate buffer (pH 5.5), 200 µmol glutamate, 10 µmol ATP, 5 µmol hydroxylamine hydrochloride, 20 µmol $MgCl_2$, and 100 µl enzyme preparation.

3.3.4. GS isoforms in S. bryopteris fronds

Selaginella fronds were extracted in SE-Buffer and subjected to anion-exchange chromatography in DEAE-Sephacel column. The elution of adsorbed protein by 0 - 0.5M KCl gradient revealed only one peak of GS activity (*Figure 3.6 a*). To ascertain the subcellular localization of the enzyme, GS activity was determined in chloroplastic and cytosolic fractions of the frond. GS activity was only present in the cytosolic fraction and was completely undetectable in the chloroplastic fraction. Anion-exchange

chromatography of the cytosolic fraction showed peak of GS activity eluting from the column at the same salt concentration as in the total *Selaginella* frond extract. The chloroplastic fraction did't reveals any GS activity peak. Immunoblot analysis of total soluble protein of the frond using GS antibody also indicated the presence of single immunoreactive band of molecular weight about 39 kDa (*Figure 3.6 b*).

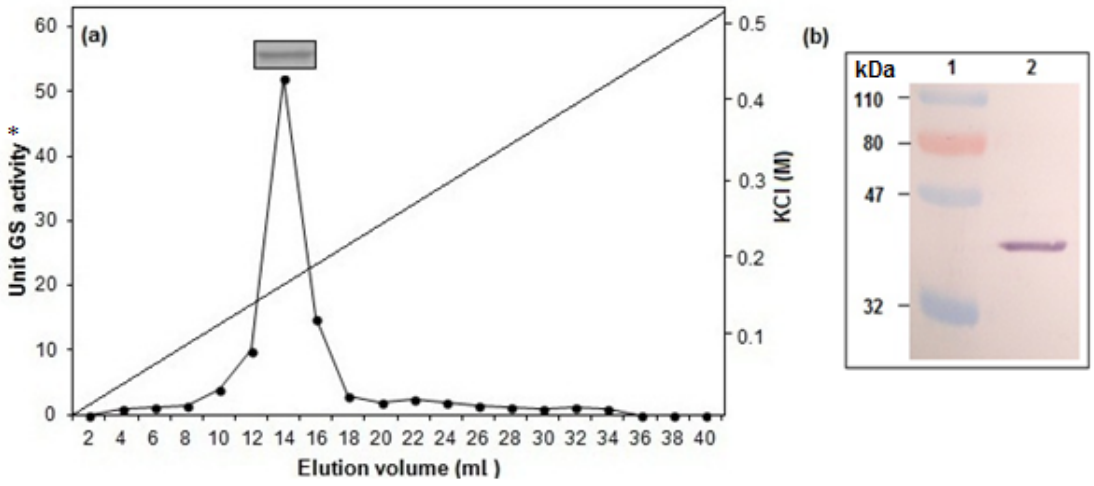

Figure3.6. GS in *S. bryopteris* fronds (a) Anion-exchange chromatography of *Selaginella* frond extract for GS activity in DEAE-sephacel column. Immunoblot analysis of peak fraction showing the one immunoreactive GS band (inset). (b) Approx. 20 µg of protein resolved on a 10 % SDS-PAGE gel and immunoblotted on a PVDF membrane and probed with GS antibody. *One unit of GS activity represents 1 µmole of γ-glutamylhydroxamate produced 30 min^{-1}.

3.3.5. Partial purification of GS from S. bryopteris fronds

GS was partially purified from *S. bryopteris* fronds by $(NH_4)_2SO_4$ precipitation, gel-filtration and anion-exchange chromatography as described in method section and the results are summarized in *Table-3.1*. The crude extract was subjected to ammonium sulphate fractionation and about 82 % of enzyme was recovered in 30-70 % saturated ammonium sulphate fraction. The 30-70 % pellet dissolved in 1ml of GS extraction buffer and subjected to gel-filtration chromatography in Biogel P-100 column. It resulted in purification of enzyme by 15.67 fold with specific activity of 359.40 U mg^{-1} protein and recovery of enzyme was 33.60 %**.** The active fractions were pooled, loaded onto DEAE-sephacel column and proteins bound to the column matrix were eluted by using elution buffer containing 0 to 0.50 M KCl. Anion-exchange chromatography resulted in

enhancement of specific activity to 662.50. The yield and purification fold of the enzyme purified were found to be 15.40 % and 28.89, respectively.

Table 3.1. Purification of GS from *S. bryopteris* fronds.

Purification steps	Total Activity (U*)	Total Protein (mg)	Specific Activity U/mg protein	Purification fold	Yield (%)
Crude	344.00	15.0	22.93	1.00	100
$(NH_4)_2SO_4$ (30-70%)	280.80	6.20	45.16	1.96	82
Biogel P-100	115.60	0.32	359.37	15.67	33.60
DEAE-Sephacel	53.00	0.08	662.50	28.89	15.40

*1U=1.0 µmol of γ-glutamylhydroxamate min^{-1} at 37°C.

3.3.6. Kinetic properties of S. bryopteris GS

The optimum pH for semisynthetase activity of GS was determined over a range of pH 2.5 to 8.5. The enzyme had pH optimum of 5.5. The enzyme exhibited 88.60 % and 66 % activity at pH 4.5 and 6.5, respectively, as compared to that at its optimum pH 5.5 (*Figure 3.7 a*). Effect of temperature on GS activity was also studied. For determining the effect of temperature, the GS reaction was carried out at different temperature ranging from 15 to 80 °C. The rate of reaction increased with increasing temperature and the calculated Q_{10} value (between 50 and 60 °C) was 1.13. The result in *Figure 3.7b* indicate that the optimum temperature for *Selaginella* GS was 60°C. At 70°C, GS gave 35% lower activity than that at its optimum temperature. Thermal stability of GS was determined by pre-incubating the partially purified enzyme preparation for 30 min. at the specified temperatures. The result in *Figure 3.7 c* reveals that activity of GS remains unaffected on pre-incubation of enzyme till 60 °C. Further increase in pre-incubating temperature to 70 °C resulted in significant loss in GS activity by about 80%. The Km value for glutamate as determined by Lineweaver-Burk plot was 2.4 mM (*Figure 3.7d*).

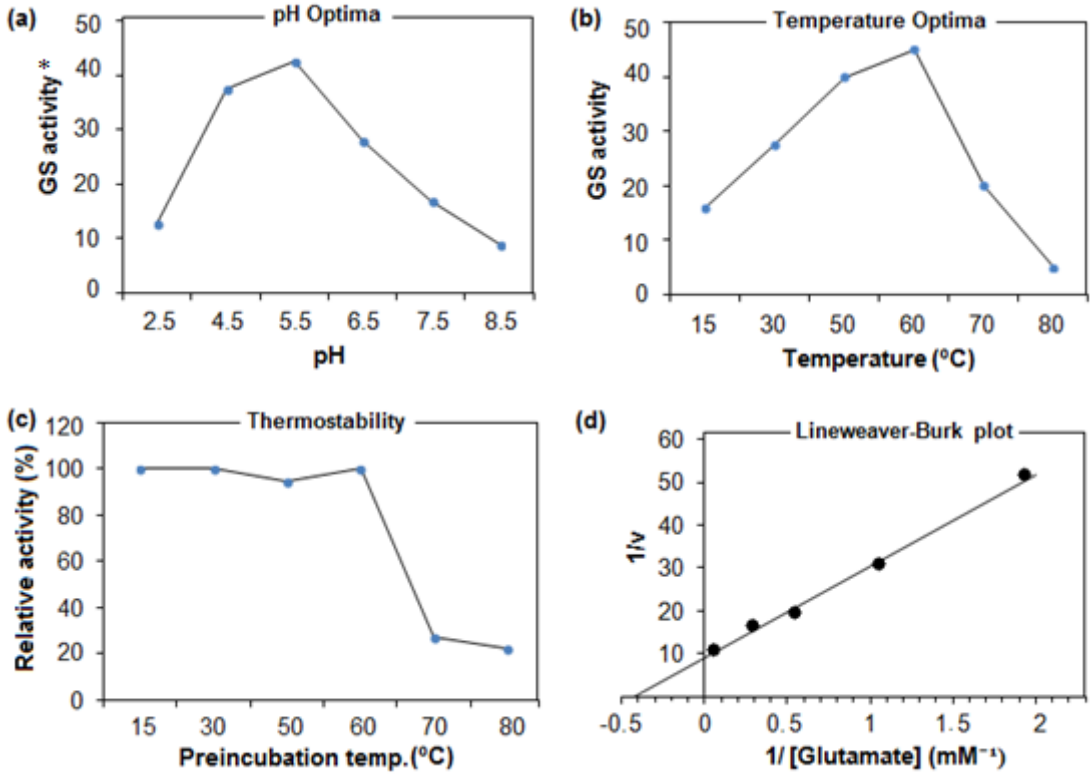

Figure 3.7. Kinetic properties of GS from *S. bryopteris* (a) Effect of pH on GS activity, (b) Effect of temperature on GS activity, (c) Effect of temperature on stability of GS, and (d) Km value for glutamate (Lineweaver-Burk plot). * One unit of GS activity represents 1.0 µmole of γ-glutamylhydroxamate produced 30 min^{-1}.

3.3.7. Sequence homology between GS of Selaginella and other angiosperms

Genome sequence analysis of *S. moellendorffii* has identified only ORF encoding GS (SmGS) (Grigoriev et al. 2011). Amino acid sequence of the ORF was aligned with the coding region of cytosolic and chloroplastic GS sequence in the GenBank database (*Table 3.2*) by using MUSCLE program (Robert 2004). From the results in *Figure 3.8* it is evident that SmGS sequence alignment highlighted amino acid residues His-249, Asp-56, Glu-297, Cys-92, Cys-303 and Cys-368, which are known to be highly conserved across all GS sequences. The polypeptide length and molecular weight were similar to that of cytosolic GS. In addition, SmGS lacked transit peptide sequence, the highly conserved motif for cleavage of transit peptide and lacked the highly conserved cysteine residues of chloroplastic GS2. Hence, SmGS encodes cytosolic GS isoform.

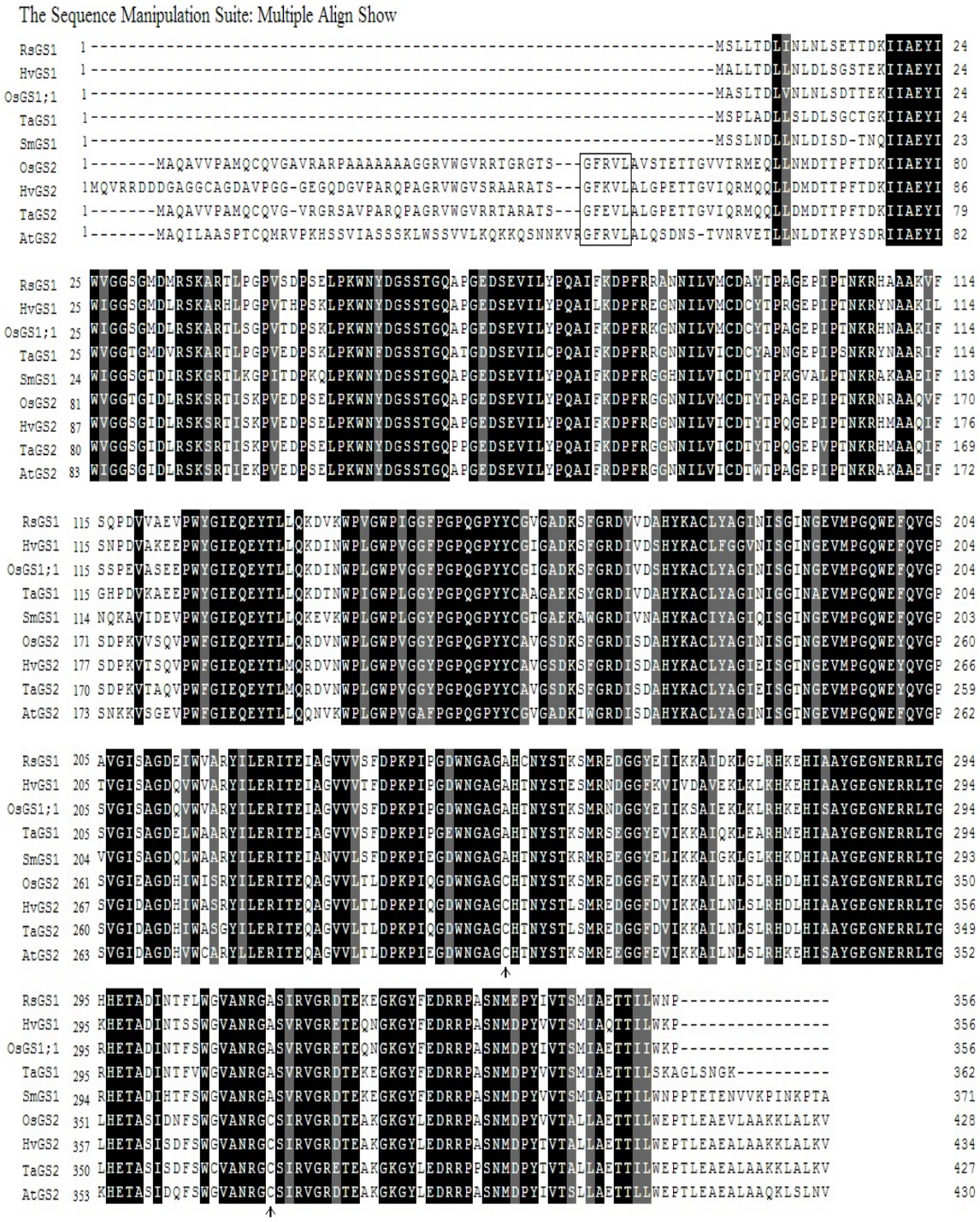

Figure 3.8. Alignment of SmGS1 of *Selaginella moellendorffii* with GS1 and GS2 proteins from different plants. The conserved motif indicating the position of cleavage site for the transit peptide are encircled by a box on GS sequences and the two cysteine residue conserved only in GS2 (Choi et al. 1999), is indicated by the two small arrows.

Table 3.2. Details of plants and accession numbers of GS amino acid sequences.

Species	Accession No.	Localization	References
Oryza sativa	P1465	Shoot	Sakomato et al. 1989
Selaginella moellendorffii	EFJ27072	Shoot	Grigoriev et al. 2011
Hordeum vulgare	Q06378	Shoot	Marigo et al. 1993
Triticum aestivum	AAR84349	Shoot	Bernard et al. 2008
Raphanus sativus	BAA04996	Shoot	Watanabe et al. 1994
Oryza sativa	P14655	Chloroplast	Sakomato et al. 1989
Hordeum vulgare	P13564	Chloroplast	Stroman et al. 1990
Triticum aestivum	AAZ30060	Chloroplast	Bernard et al. 2008
Arabidopsis thaliana	Q43127	Chloroplast	Peterman et al. 1991

3.3.8. Effect of dehydration-rehydration on GS/GOGAT activity and expression of GS

To examine the effect of hydration cycle on GS, *S. bryopteris* fronds were subjected to dehydration for 24 h (5% RWC) followed by rehydration for the same duration (95 % RWC) and GS activity was monitored at various stages of treatment. As can be seen from the result in *Figure 3.9 a*. the activity of GS didn't change markedly except at 24 h when it decreased to about 25 %. Its activity remained unchanged throughout the rehydration period. As the cytosolic GS has been shown to be operational along with NADH-dependent isoform of GOGAT and GS activity in *Selaginella* was localized in the cytosol, the activity profile of only NADH-GOGAT was examined. The NADH-GOGAT activity didn't alter significantly during dehydration-rehydration cycle (*Figure 3.9 b*). Immunoblot analysis of GS protein at various stage of dehydration-rehydration reveals that GS protein level increased marginally during dehydration with maximum 2 fold accumulation at 24 h treatment. Rehydration of frond for 3 h lowered of GS polypeptide content by 2 fold and the level remain maintained till the end of the treatment (*Figure 3.9 c, d*).

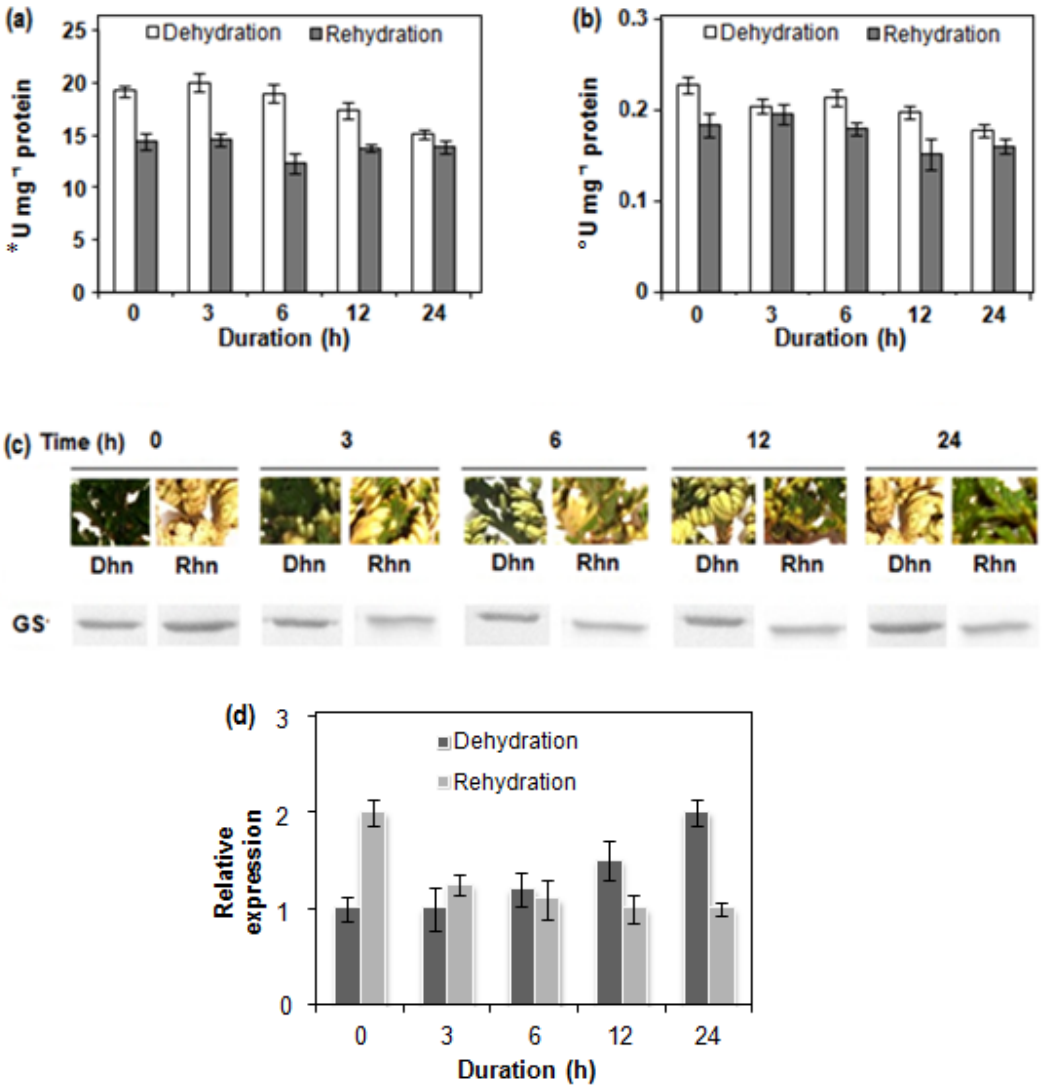

Figure 3.9. GS/GOGAT activities and GS expression during dehydration-rehydration of *S. bryopteris* fronds; (a) Total GS activity, (b) NADH-GOGAT activity, (c) Immunoblot analysis of GS, and (d) Bar diagram for GS polypeptide content. Each point is the mean of three samples ±SE. *One unit of GS activity represents 1.0 µmole of γ-glutamylhydroxamate produced 30 min^{-1} and °one unit of NADH-GOGAT activity represents 1.0 µmol of glutamate produced min^{-1} at 37 °C.

3.3.9. Effect of dehydration-rehydration of S. bryopteris fronds *on activity and expression of GDH*

The influence of dehydration-rehydration of fronds on aminating and deaminating activities of GDH was determined The aminating GDH activity was found to increase on dehydration and at 12 h became almost twice with no considerable alteration thereafter. Dehydration mediated enhancement in GDH activity was reversed on rehydration.

Rehydration of dehydrated fronds for 3h lowered the GDH activity by 50 % and the activity further reduced to 33 % at 24h of treatment (*Figure 3.10a*).

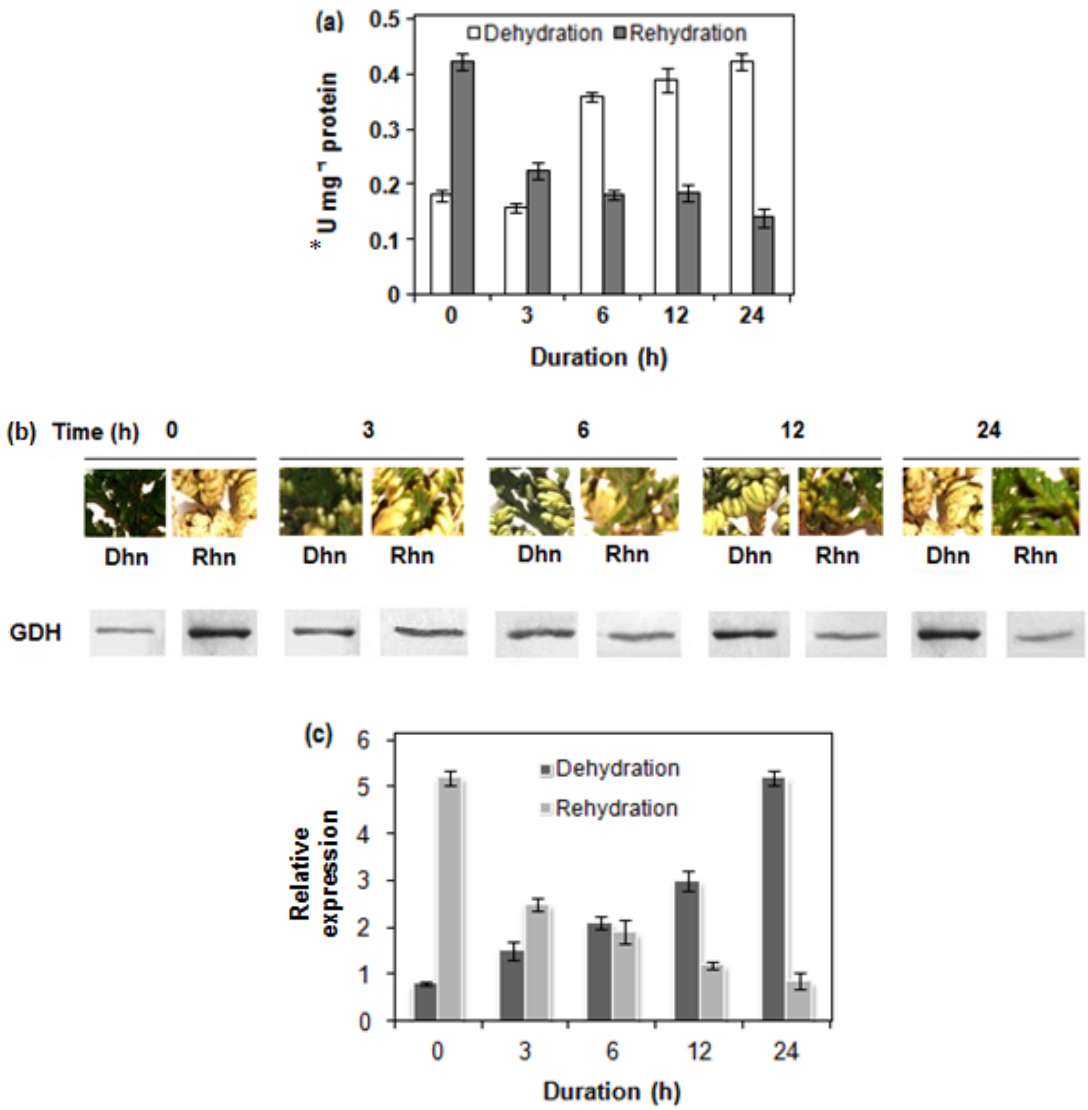

Figure 3.10. GDH activity and GDH expression during dehydration-rehydration of *S. bryopteris* fronds; (a) aminating GDH, (b) Immunoblot analysis of GDH, and (c) Bar diagram for GS polypeptide content. Each point is the mean of three samples ±SE. *One unit of GDH represents the reduction of 1 µmol NADH min^{-1} at 30^{o}C.

A comparison of GDH activity with corresponding protein content indicates that the time course of GDH protein correlated with GDH activity. Dehydration treatment for 12 h induced the GDH polypeptide expression by almost 2.5 folds, which again declined on rehydration (*Figure 3.10b*). The aminating GDH activity remained unaltered during entire dehydration-rehydration cycle.

3.4. DISCUSSION

Resurrection plants have evolved a wide spectrum of adaptations to cope with the challenges of environmental stress. They have unusual capability to survive long dry period and hence, can serve as a system for studying metabolic events during dehydration and rehydration. Several species of the *Selaginella* genus, including *S. lepidophylla, S. bryopteris and S. tamariscina,* have evolved the desiccation tolerance or ability to survive dry conditions (Yobi et al. 2012). They are able to recover complete physiological activity following repeated protoplasmic dehydration of fully differentiated tissues. Present study describes partial purification and properties of glutamine synthetase isolated from *S. bryopteris* fronds. Further studies were carried out to determine the regulation of GS expression during dehydration and rehydration of *Selaginella* fronds and its relationship with other enzymes of ammonia metabolism.

The dehydration-rehydration kinetics of *Selaginella* frond was determined by monitoring changes in RWC. The RWC decreased from 95 to 5 % within 24 h of dehydration treatment. However, fronds were able to recover completely from dehydration status by massive uptake of water within a much shorter duration of rewatering driven by the extreme osmotic gradient between the dried fronds and the environment. The results dehydration- rehydration kinetics was significantly different from that reported by Deeba et al (2009). They showed a relatively rapid rate of dehydration of the fronds in comparison to the present study. The deviation in the rate of RWC decline could be due to the differences in plant habitat or humidity level during sampling. Several resurrection plants, such as *Craterostigma plantagineum* (Norwood et al. 2000), *Xerophyta viscose* (Peters et al. 2007), *Sporobolus stapfianus* (Oliver et al. 2011) have the ability to survive almost complete (< 5%) tissue dehydration. However, most of the angiosperms cannot survive dehydration of their vegetative tissue to 20-30 % RWC, which translates to between -5 and -10 MPa (Proctor and Pence, 2002). Dehydration treatment caused significant changes in morphology of fronds, like, inward curling, disappearance of green colour and brownish appearance. The original morphology was regained on rehydration, with opening of adaxial surface and greening of the desiccated fronds. The folding of fronds during drying of plants has been proposed to prevent light-chlorophyll interaction and light-inducd damage (Farrant and Sherwin 1998). Hydration cycle mediated

phenotypic changes in detached fronds were not completely correlated to chl a and chl b contents. Although, the fronds were almost brown at 24 h of dehydration, they contained significantly high level of chl a and chl b indicating constitutive presence of the pigment molecules. As in the present investigations, constitutive presence of chl a and chl b and maintenance of their ratio during dehydration were also noted by Pandey et al (2010). Chlorophyll content has been shown to decline rapidly in plants under stress and during leaf senescence (Sims and Gamon 2010). The insensitivity of chlorophyll contents to dehydration might serve as one of the factor for dehydration tolerant characteristics of *S. bryopteris* plant. In contrast to chlorophyll pigments, dehydration resulted in severe decline in the level of carotenoid, which can be associated with browning of the fronds. The protein content of fronds reduced considerably during dehydration treatment. The breakdown of soluble and insoluble proteins and accumulation of amino acids during dehydration are features of many plants (Bernacchia 1996; Martinelli et al. 2007). However in resurrection plant *Sporobolus stapfianus*, the total soluble protein content declined at the beginning of desiccation and the decrease was reversed in the remaining step of dehydration (Martinelli et al. 2007). Present study showed a close relationship between the alteration in protein content and accumulation of ammonium. The dehydration mediated decline in protein level paralleled a significant increase in free ammonium content. Subsequent rehydration of the fronds lowered the ammonium content. Previous research work by Martinelli et al (2007) also showed dehydration mediated accumulation of ammonium in leaves of *Sporobolus stapfianus*, however, the fate of accumulated ammonium ions on rehydration of resurrection plants has not been studied so far. It has been well proposed that metabolite accumulation is one of the mechanisms for stress tolerance. Several solutes, such as, proline, glycine betaine, polyols, polyamines and ions (i.e. potassium), can accumulate during WD conditions. They act both by contributing to osmotic adjustment and by protecting proteins and cellular membranes. The beneficial roles of proline in conferring osmotolerance have been widely reported (Kishor et al. 1995; Bajji et al. 2000). *Selaginella* fronds were found to accumulate proline in markedly high quantity on dehydration. Recent study on metabolic profiling of *S. lepidophylla* during dehydration-rehydration cycle indicated the accumulation of several nitrogen rich amino acids including proline in the dehydrated fronds (Yobi et al. 2012). The increased accumulation of nitrogen-rich amino acids might reflect an adaptation to the

nitrogen-limiting conditions typically encountered by *Selaginella* and other dehydration tolerant species in their natural habitat of rocky outcrops with nitrogen-poor soils (Burke, 2002). Alternatively, these amino acids might provide a nitrogen reservoir useful during the early stages of rehydration before the recovery of photosynthetic activity (Martinelli et al. 2007).

The regulation of nitrogen metabolism enzymes was studied during dehydration and rehydration of *S. bryopteris* fronds. Initial screening of *S. bryopteris* frond extract by anion exchange chromatography indicated the presence of only one GS isoform. The cytosolic localization of the isoform was confirmed by the presence of GS activity only in the soluble fraction but not in the chloroplastic fraction of the fronds. Furthermore, immonoblot analysis of the total soluble protein as well as the GS activity fraction showed the presence of single band of molecular size about 39 kDa. GS is present as cytosolic (GS1) and chloroplastic (GS2) isoforms in most of the higher plant. GS1 is encoded by two to five genes, whereas there is a single nuclear gene encoding GS2 (Bernard et al. 2008; Bernard and Habash 2009). The multiplicity of the cytosolic isoforms couldn't be established in the present study. The information on isoforms of GS in resurrection lycophytes is limited. The complete genome sequence of *S. moellendorffi* has shown the presence of an ORF encoding GS (SmGS). Amino acid sequence corresponding to the ORF was aligned with other higher plant cytosolic and chloroplastic GS. The size of ORF was similar to cytosolic GS of higher plants. Moreover, the lack of chloroplastic GS2 specific transit peptide sequence, conserved cleavage site for transit peptide and other conserved amino acids, suggested cytosolic origin of SmGS.

The GS enzyme was partially purified from *S. bryopteris* fronds by ammonium sulphate precipitation, gel-filtration and ion-exchange chromatography. The kinetic properties of GS from *S. bryopteris* were considerably different from that of higher plants. The enzyme showed pH optima of around 5.5, which is slightly more acidic in comparison to cytosolic and chloroplastic GS from higher plants. The enzyme was remarkably thermostable as it didn't lose activity on pre-incubation at temperature upto 60 ^{o}C. At this temperature GS from most of the higher plants underwent almost complete denaturation (Lea et al. 1990). For example, rice leaves GS2 was highly heat labile and its activity

diminished by 80% of the initial activity after 15 min of treatment at 40 ^{o}C and it lost activity completely when kept for 60 min at this temperature (Hirel and Gadal 1980). GS from *S. bryopteris* showed higher considerably temperature optima of about 60 ^{o}C. The semi-synthetase activity of the enzyme showed hyperbolic response with increasing concentration of glutamate. The Km value for glutamate was lower than that reported for enzymes from leaves of higher plants (Hirel and Gadal 1980; Hirel and Gadal 1982; Cullimore et al. 1983; Ericson et al. 1985; Bedell et al. 1995) indicating higher affinity of the *Selaginella* GS for glutamate. Hence, GS from *S. bryopteris* was superior in kinetic properties as compared to that of higher plants. Purification and kinetic properties of GS from resurrection plants have not been reported earlier.

The effect of dehydration-rehydration cycle on activity and expression of GS was examined. The activity of GS did not alter markedly indicating its lower sensitivity to dehydration stress. The information on response of resurrection plant GS to dehydration is limited. In resurrection angiosperm *Sporobolus stafianus*, desiccation tolerant young leaves had higher total GS activity at the end of dehydration stress and were shown to maintain high chloroplastic and cytosolic GS protein content during the entire stress period (Martinelli et al. 2007). In contrast, GS from some higher plants has shown susceptibility to abiotic stress (Teixeira and Pereira 2007; present study). Total GS activity decreased during drought and salt stress and most of the decline was in GS2 activity. The reduction in GS2 activity correlated with corresponding polypeptide (Lutts et al. 1999; Santos et al. 2004). Immonoblot analysis of *Selaginella* GS at various stages of dehydration-rehydration indicated its increased accumulation during dehydration and reduction in level on subsequent rehydration. However, GS polypeptide content reduced on subsequent rehydration. As in the present study, increased expression of cytosolic GS transcript and polypeptide has been reported in higher plants during natural senescence and in response to biotic and abiotic stress (Bernard and Habash 2009). However, the enhanced expression didn't correlate with GS activity (Kamachi et al. 1991; Ishiyama *et al.* 2004a). As the cytosolic GS has been shown to be operational along with NADH-dependent isoform of GOGAT and in *Selaginella* fronds GS activity was present only in the cytosol, the activity profile of only NADH-GOGAT was examined. The NADH-GOGAT activity didn't alter significantly during dehydration-rehydration cycle. In

contrast, GOGAT activity was found to decline during dehydration in both desiccation tolerant young leaves and desiccation sensitive old leaves of *Sporobolus stapfianus* (Martinelli et al. 2007).

Since ammonium level increased during dehydration and both GS and GOGAT activity remained unaltered, it was likely that senescence-like process was induced during dehydration. However, the conditions seem to be reversed by significant increase in immunoreactive α-GDH polypeptide and aminating GDH activity. The aminating GDH activity increased progressively from the beginning of dehydration and became about 2 fold by the end of dehydration. The dehydration mediated increase in GDH activity was reversed on rehydration. A comparison of GDH activity with corresponding protein content indicates that the time course of GDH protein correlated with GDH activity. The desiccation tolerant young leaves of *Sporobolous stapfianus* also showed higher activity of both aminating and deaminating GDH in comparison to desiccation sensitive older leaves (Martinelli et al. 2007). The dehydration mediated enhancement in aminating GDH activity in the present investigations could be related to increased protein degradation producing high concentration of ammonium and α-ketoglutarate from deamination of aminoacids. The contention is supported by research work of Yobi et al (2012) reporting increased accumulation of α-ketoglutarate during dehydration of desiccation tolerant *Selaginella lepidophylla*. There are reports on increased aminating activity and expression of GDH under intracellular hyperammonia conditions due to either exogenous ammonium, senescence induced high proteolytic activity or abiotic stress (Kumar et al. 2000; Hoai et al. 2003; Skopeltis et al. 2006). The aminating GDH activity increased with increasing salt stress in salt-tolerant rice (*Oryza sativa*) cultivar, whereas it decreased in the salt-sensitive one (Kumar et al. 2000). Furthermore, high NaCl has been shown to induce the formation of reactive oxygen species, which in turn induces the synthesis of GDH in tobacco and grapevine. The inhibition of GS in these plants resulted in the incorporation of ammonia via GDH into [^{15}N] glutamate and [^{15}N] proline in the presence of high salt (Skopelitis et al. 2006). Hence, the enhanced level of proline during dehydration of *S. bryopteris* fronds could also be correlated with enhanced GDH activity. Present study concludes that *S. bryopteris* frond contained only one isoform of GS localised in cytosol. The enzyme was superior in kinetic properties in comparison to that of higher plants. The enzyme showed

tolerance to dehydration. The activity GS /GOGAT remained largely unaltered in dehydration treated *Selaginella* fronds. Moreover, GDH aminating activity and expression were increased during dehydration. GDH thus seems to be involved in amelioration of toxicity of excess ammonia generated during dehydration. The relatively maintained GS/GOGAT activities and increased activity and expression of GDH also correlate with dehydration mediated increase in glutamate, the substrate for proline biosynthesis. Hence, GDH along with GS/GOGAT plays important role in regulation of ammonia assimilation during WD in *S. bryopteris*.

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General Discussion and Conclusion

Nitrogen is an essential nutrient for plant growth and productivity. In higher plants inorganic nitrogen is assimilated predominantly via Glutamine synthetase (GS; EC 6.3.1.2)/ Glutamate synthase (Glutamate oxo-glutarate ammonium transferase, GOGAT; EC 1.4.7.1) pathway. In higher plants, GS exists in multiple isoforms that are either cytosolic (GS1) or plastidic (GS2). GS isoforms are also differentially regulated in response to environmental signals, such as, nitrogen status, light, biotic and abiotic stress.

Drought is important environmental stress factors limiting plant productivity in cultivated areas worldwide. The stress condition is due to soil water deficit, increased daytime temperature and reduced nutrient availability. Among these water deficit (WD) is the most important factor for plant growth (Oliver et al. 2011). The prolonged period of WD leads to limited uptake of nitrogen from soil and reduced availability of CO_2 for photosynthesis as stomata are induced to close followed by disturbances in carbon and nitrogen metabolism (Foyer et al.1998; Xu and Zhou 2006). Acclimation to WD requires responses that allow the primary metabolism to continue. Recent studies have shown nitrogen assimilation to be critical for plant acclimation to stress conditions. The enzyme GS constitutes a regulatory point at which environmental signals are integrated and translated into a plant response in terms of growth and productivity (Swarbreck et al. 2011).

Rice (*Oryza sativa* L.) plant growth and productivity are adversely affected by WD. The effect of WD varies with the variety, degree, and duration of stress and its coincidence with different growth stages (Gao et al. 2007). NH_4^+ is the main form of nitrogen available to the young rice plants, which is assimilated by GS to glutamine. Glutamine serves as the main form of organic-nitrogen for transport through vascular tissues (Ishiyama et al. 2004b). Rice plants possess one gene (OsGS2) for chloroplastic GS2. The cytosolic GS1 gene family consists of three isoforms encoded by OsGS1;1, OsGS1;2 and OsGS1;3. These isoforms have been shown to be regulated in a developmentally controlled manner as well as by light and nitrogen nutrition (Kamachi et

al. 1991; Ishiyama et al. 2004a, b; Kusano et al. 2011). However, the regulation of these isoforms during WD has not been investigated in detail.

Resurrection plants have evolved a wide spectrum of adaptations to cope with the challenges of environmental stress (Oliver et al. 2011; Yobi et al. 2012). *Selaginella bryopteris* belonging to the family Selaginellaceae, is a resurrection lycophyte growing in the hilly area of tropical regions (Deeba et al. 2009). A useful insight into the role of GS isoforms under WD conditions can be obtained by studying the enzyme in *S. bryopteris*. Present investigations were carried out primarily to gain some insight into the regulation of glutamine synthetase isoforms during WD in two differentially drought tolerant rice (*Oryza sativa* L.) cultivars and in *S. bryopteris* (L.).

Rice cultivars were screened for their drought tolerance characteristics by monitoring changes in leaf relative water content (RWC), electrolyte leakage, proline and protein contents. During water deficit, RWC, protein and proline level declined markedly and the electrolyte leakage increased sharply in *O. sativa* cv. IR-64. Under similar condition, *O. sativa* cv. Khitish variety maintained relatively higher RWC, protein and proline level and showed least leakage of electrolyte. The morphological changes associated with WD were more prominent in IR-64 seedling in comparison to Khitish seedlings. Hence, IR-64 and Khitish cultivars were designated as drought-sensitive and tolerant-cultivar, respectively. These two cultivars were used further to study the effect of WD on regulation of GS isoforms.

GS was extracted from root, stem and leaf of three weeks old rice seedlings. The isoforms of GS in leaf, stem and root extracts were separated by anion-exchange chromatography on DEAE-Sephacel column using gradient elution technique. In leaf and stem extract, the enzyme was resolved into two peaks corresponding to cytosolic GS1 and chloroplastic GS2. In root extract entire activity was eluted as single peak at the same salt concentration as GS1. Rice leaves contained 25 and 75 % of activity as GS1 and GS2, respectively. In stem most of the GS activity (70 %) was present as GS1 and rest 30 % activity represented as GS2.

The transcript levels of GS isoform in various organs of rice plant were determined by RT-PCR. Among GS1 gene family, OsGS1;1 and OsGS1;2 transcripts were present in all the three organs, whereas OsGS2 was expressed in leaf and stem but not in root. In leaf tissue the OsGS2 mRNA level was considerably higher than that of cytosolic isoforms,

but it was present as minor form in stem. Among the GS1 isoforms OsGS1;1 was more abundant than OsGS1;2 in leaf. OsGS1;2 was the predominant form in stem and root. However, OsGS1;1 mRNA level was significantly greater in root and stem of IR-64 seedlings than that of Khitish.

The polypeptide levels of GS1 and GS2 isoforms in leaves, stems and roots of rice seedlings were determined by immunoblotting. Two GS protein bands of approximately 39 and 43 kDa that correspond to the molecular size of GS1 and GS2, respectively, were present in leaf and stem. The immunoblot analysis of root protein highlighted only one protein band corresponding to GS1 isoform. As the GS antibody was developed against conserved GS polypeptide, cytosolic isoforms could not be distinguished in immunoblot.

Effect of WD on total GS, GS1 and GS2 activities in leaf, stem and root of IR-64 and Khitish seedlings were determined. WD had a profound effect of total GS activity in leaves and roots of IR-64 seedlings as compare to that of Khitish. The rapid reduction in GS activity of IR-64 leaf was mainly due to disappearance of GS2 activity. The GS2 activity decrease in IR-64 leaf was correlated with decreased level of GS2 mRNA and protein. As for activity, the GS2 mRNA and protein remained almost unaltered in Khitish leaf. The results suggested WD mediated GS2 regulation mainly at the transcriptional and /or mRNA stability levels. GS2 is known to catalyse the rate limiting step of photorespiration i.e. reassimilation of ammonia. The photorespiration activity has been reported to be induced by abiotic stress and plays a protective role. Hence, a relatively unaltered activity and expression of GS2 in Khitish leaf could maintain the photorespiratory capacity of the plant at limited water availability that improves tolerance of the cultivar to WD.

Although, WD treatment increased the expression of both OsGS1;1 and OsGS1;2 transcripts in leaf of IR-64 and Khitish cultivars, expression level of OsGS1;1 mRNA was considerably higher in IR-64 leaf. The increased OsGS1;1 in IR-64 leaf could be due to its higher rate of protein degradation (*Figure 2.12*), conforming to role of the isoform in reassimilation of nitrogen released from protein breakdown. The time course of increase in GS1 transcripts corresponded with the accumulation of GS1 protein detected on Western blot. WD mediated increase in GS1 transcript and polypeptide was related to the maintenance of GS1 activity rather than increase. A similar type of response to GS1 mRNA over expression was observed in *Arabidopsis* root. In this tissue the nitrogen

nutrition mediated increase in GS1 transcript and polypeptide was related to the maintenance of GS1 activity rather than increase (Ishiyama et al. 2004a).

Total GS activity in stem of IR-64 and Khitish seedlings was almost unaffected by WD. However, alteration in both GS1 and GS2 activities was noted in stem of IR-64 seedling. GS1 activity was reduced and GS2 activity enhanced on stress application. The decline in GS1 activity was compensated by increase in GS2 activity, thus maintaining almost unchanged total GS activity. A varietal variation was noted in expression of OsGS1;1 and OsGS2 in response of WD. OsGS1;1 transcript content declined in IR-64 and enhanced in Khitish with the intensification of stress. The repression in OsGS1;1 in IR-64 stem might result from remobilization and transport of high concentration of glutamine to stem and root from increased protein degradation in IR-64 leaf. The reduction in OsGS1;1 mRNA in IR-64 stem was not reflected in GS1 polypepeptide abundance. The results thus indicated a different metabolic status of stem during WD maintaining a constant GS activity. A tissue specific response of WD has been noted earlier with role of stem in survival of plant under stress (Pinheiro et al. 2001).

The cytosolic GS1 was the only GS isoform in rice root. The GS activity in roots of IR-64 cultivar was quite sensitive to WD and reduced to more than half at the end of treatment. As in stem, the mRNA level of OsGS1;1 differed in root of the two cultivars. Its level was initially higher in IR-64 that declined with WD. WD mediated decrease in GS1 activity in stem and root of IR-64 seedling might correlate with reduction in OsGS1;1 mRNA level. The GS1;1 gene has been previously reported to be involved in remobilization and reutilization of nitrogen in rice plant during senescence (Tabuchi et al. 2007). Moreover, a comparison of kinetic properties of OsGS1;1 and OsGS1;2 encoded GS isoforms in rice root has shown that OsGS1;1 exhibit higher Vmax and lower Km value for substrate (Ishiyama et al. 2004b). Hence, OsGS1;1 seems to play significant role in performance of plant under stress condition.

In the present study, the two rice varieties did not differ much in expression pattern of OsGS1;2 during WD, despite difference in their drought tolerance properties. Its transcript level was found to enhance in leaf and stem. However, in root the highly expressed OsGS1;2 was found to decline initially followed by an increased accumulation on further dehydration. Although, the time course of OsGS1;2 mRNA was reflected in GS1 polypeptide abundance, it was not correlated with total GS activity in all the three

tissues. The result is supported by study of Ishiyama et al (2004b) on effect of ammonium nutrition in Arabidopsis root GS isoforms. The time course increase in GS1;2 mRNA corresponded with the accumulation of GS1 protein detected on the western blot. However, the increase in GS1;2 mRNA and protein was not correlated with total GS1 activity. The lack of correlation could be due to lower affinity of GS1;2 for ammonium as compared to other isoform.

The effect of WD in regulation of GS of *S. bryopteris* was also studied. Preliminary experiment was carried out to determine the dehydration-rehydration kinetics of *S. bryopteris* fronds by measuring relative water content (RWC). The RWC decreased from 95 to 5 % within 24 h of dehydration treatment. However, fronds were able to recover completely from dehydration status by massive uptake of water within a much shorter duration of rewatering driven by the extreme osmotic gradient between the dried fronds and the environment. Dehydration treatment caused significant changes in morphology of fronds, like, inward curling, disappearance of green colour and brownish appearance. The original morphology was regained on rehydration, with opening of adaxial surface and greening of the desiccated fronds. Dehydration mediated browning of fronds was not completely correlated to the change in chl a and chl b contents. As in the present investigations, constitutive presence of chl a and chl b and maintenance of their ratio during dehydration were also noted by Pandey et al (2010). The protein content of fronds reduced considerably during dehydration and this decline paralleled significant increase in free ammonium content. *Selaginella* fronds were found to accumulate proline in markedly high quantity on dehydration. Recent study on metabolic profiling of *S. lepidophylla* during dehydration-rehydration cycle indicated the accumulation of several nitrogen rich amino acids including proline in the dehydrated fronds (Yobi et al. 2012). The increased accumulation of nitrogen-rich amino acids might reflect an adaptation to the nitrogen-limiting conditions typically encountered by *Selaginella* and other dehydration tolerant species in their natural habitat of rocky outcrops with nitrogen-poor soils (Burke, 2002).

The regulation of GS was studied during dehydration and rehydration of *S. bryopteris* fronds. Anion-exchange chromatography followed by immunoblot analysis of GS indicated the cytosolic localization of GS. Alignment of GS amino acid sequence of *Selaginella* in database with that of higher plant cytosolic and chloroplastic GS suggested cytosolic origin of SmGS. GS was partially purified from *S. bryopteris* fronds by ammonium sulphate precipitation, gel-filtration and ion-exchange chromatography. The enzyme showed pH optima of around 5.5. The enzyme was remarkably thermostable as it

didn't lose activity on pre-incubation at temperature upto 60^{o}C. GS from *S. bryopteris* showed considerably higher temperature optima of about 60 ^{o}C. The Km value (2.4 mM) for glutamate was lower than that reported for enzymes from leaves of higher plants (Cullimore et al. 1983; Ericson et al. 1985; Bedell et al. 1995). The effect of dehydration-rehydration cycle on activity and expression of GS was examined. The activity of GS didn't alter markedly indicating its lower sensitivity to dehydration stress. Immonoblot analysis of *Selaginella* GS at various stages of dehydration-rehydration indicated its increased accumulation during dehydration and reduction in level on subsequent rehydration. A lack of correlation between GS expression and activity has been reported earlier (Kamachi et al. 1991; Ishiyama et al. 2004a; Bernard and Habash 2009). As in case of GS, NADH-GOGAT activity didn't alter significantly during dehydration-rehydration cycle.

Since GS and GOGAT activity remained unaltered and ammonia concentration was increased, it was likely that senescence-like process was induced during dehydration. In plants, glutamate dehydrogenase (GDH) constitutes an alternative route for ammonia assimilation. Both GDH aminating activity and polypeptides were found to increase during dehydration and thus seem to reverse the condition of ammonia toxicity. The dehydration mediated increase in GDH activity was reversed on rehydration. The dehydration mediated enhancement in aminating GDH activity in the present investigations could be related to increased protein degradation producing high concentration of ammonium and α-ketoglutarate from deamination of amino acids. The contention is supported by research work of Yobi et al (2012) reporting increased accumulation of α-ketoglutarate during dehydration of desiccation tolerant *Selaginella lepidophylla*. In another study, the inhibition of GS in grapevine plant resulted in the incorporation of ammonia via GDH into [^{15}N] glutamate and [^{15}N] proline in the presence of high salt (Skopelitis et al. 2006). Hence, the enhanced level of proline during dehydration of *S. bryopteris* fronds could also be correlated with enhanced GDH activity.

From the present investigations, it can be concluded that in rice plant the regulation of GS isoforms by WD was organ specific. Two GS isoforms i.e. GS1;1 and GS2 were differentially regulated in drought-sensitive and -tolerant rice cultivars. GS2 is the major GS isoform in leaf and its over-expression in leaf has been found to be associated with the maintenance of photosynthetic and photorespiratory capacity of the plant. The GS1;1 isoform has been shown to be involved in remobilization and reutilization of nitrogen. A higher substrate affinity of the enzyme for ammonium signifies its promotion of the rapid

conversion of ammonium to glutamine even under low ammonium conditions. Hence, it can be inferred that a relatively maintained OsGS2 and the over-expression of OsGS1;1 may contribute to improved drought tolerance characteristics of *Oryza sativa* cv. Khitish.

GS from *S. bryopteris* showed tolerance to dehydration. The activity GS /GOGAT remained largely unaltered in dehydration treated *Selaginella* fronds. Moreover, GDH aminating activity and expression were increased during dehydration. GDH thus seems to be involved in amelioration of toxicity of excess ammonia generated during dehydration. The relatively maintained GS/GOGAT activities and increased activity and expression of GDH also correlate with dehydration mediated increase in glutamate, the substrate for proline biosynthesis. Hence, GDH along with GS/GOGAT plays important role in regulation of ammonia assimilation during WD in *S. bryopteris.* A comparison of GS of *S. bryopteris* and *O. sativa* indicated that in *S. bryopteris* GS was only present in cytosol. The properties of cytosolic GS from the two sources also varied. GS from *S. bryopteris* was remarkably thermostable as it didn't lose activity on pre-incubation at temperature upto 60^{o}C. At this temperature GS from rice plants undergo almost complete denaturation (Lea et al. 1990). The temperature optima of *Selaginella* GS was also higher than that of rice. *Selaginella* enzyme showed more affinity for the substrate glutamate. The susceptibility of GS to WD in *O. sativa* was correlated to their drought tolerance properties. In contrast, the enzyme from *S. bryopteris* showed insensitivity to WD.

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