CHAPTER - I

LITERATURE REVIEW

1.1 INTRODUCTION

Growth, development and physiological behaviour of multicellular organisms require the correct spatial and temporal expression of genes, coordinated by fine-tuned signaling systems along with intricate network of different metabolites with versatile bioactivity. Extensive research on animal system has revealed that this intercellular communication is largely mediated by steroids, peptides and other small bioactive compounds. Among these, peptides are the most common ligands probably because of their incredible number of sequence diversity and versatile range of post-translational modifications (Matsubayashi and Sakagami, 2006). Not surprisingly, peptides are the most commonly used bioactive molecules for intercellular communication in prokaryotes, fungi and animals. Do plants behave differently in this respect? Until very recently they appeared to be. Intercellular communication in plants has been thought to be exclusive playground of well-defined classical plant hormones for several decades (Raven *et al.*, 1992). So the concept of physiological regulation in plants was based upon our knowledge of so-called five classical plant hormones; namely auxin, gibberellins, cytokinins, ethylene and abscisic acid (Guern *et al.*, 1987). The paradox was how such a small number of chemically simple molecules could account for the observed diversity of cellular responses in plants (Lindsey *et al.*, 2002).

Research in recent years, however, has indicated that peptides may be widely used as 'chemical messengers' in plants as well. Endogenous bioactive plant derived peptides evoke specific cellular responses, and are involved in defense responses, callus growth, meristem organization, self-incompatibility, root-growth, leaf-shape maintenance, regulation of stomatal density and aperture, nodule development and organ abscission (Matsubayashi and Sakagami, 2006). Furthermore, there is some indication that plants contain peptides similar in structure and function to natriuretic peptides in animals for homeostatic regulation of water (Gehring, 1999).

In this review, I highlighted the current conceptual knowledge and brief historical viewpoint of peptide science; origin and importance of peptides in probiotic world; bioactivities of bacterial and animal peptides; structure, function, processing and signal-transduction properties of plant polypeptide hormones and the physiological role of

different isolated low molecular weight peptides related to germination, growth, development, senescence and oxidative stress mitigation. Special emphasis was also given on principles of isolation, purification and detection of plant peptides; mechanism of amylase induction during germination, cell cycle regulation, stomatal aperture control, heavy metal stress tolerance, and their association with isolated peptides along with peptide-phenolic interaction pattern during germination. Finally the review was concluded with a brief discussion about prospects of future studies of peptide ligands in plants.

1.2 PEPTIDE: DEFINITION & CONCEPT

Since plant synthesizes lots of low molecular weight peptides, proteins and they have certain physiological activities to control and maintain different metabolic, developmental and functional circuits; several works have been performed regarding their occurrence and effects. Both peptides and proteins bear versatile structural flexibilities and execute wide range of crucial functions in life processes. The true distinction between proteins and peptides are largely empirical because of lack of adequate theoretical understanding of their chemical behaviour and structural dynamism in biological system. In most cases peptides are only distinguished from proteins by means used for separating them. The term 'peptide' as distinct from 'protein' has come to be reserved for compounds having molecular weight less than, at most 10 000 or, if of higher molecular weight, having unusually simple amino acid composition. Natural compounds so far shown unequivocally to belong to this class don't exhibit denaturation in its usual sense or, particularly coagulation by heat (Synge, 1975). Peptides are the family of molecules formed from the linking, in a definite order, of various Amino Acids. The link between one Amino Acid residue and the next is an Amide Bond, sometimes referred to as peptide bond. Alternate definition of peptide relies on Amino Acid residues, i.e. peptides differ from proteins by virtue of their size. The dividing line is at approximately fifty Amino Acids in length, since naturally occurring proteins tend, at their smallest, to be hundreds of residues long. The monomers present as repeat units in peptides and proteins are α-amino acids (a carboxyl group and an amino group bounded to the same carbon atom). The α-Carbon is asymmetric, bonded to four different

substituent groups and acts as a chiral center. Almost all amino acids present in peptides have L-configuration. D-amino acids have been restricted only in small peptides of bacterial cell walls, peptide antibiotics and peptides in South American frog skin. Peptide bonds are created by the condensation of two amino acids, where one molecule of water is eliminated from α-carboxyl group of one amino acid and α-amino group of the other.

1.3 BRIEF HISTORICAL PERSPECTIVES

1.3.1 Concept of Peptide Bonds

The concept of peptide bond was first derived by Emil Fischer with E. Fourneau. Fischer first coined the name peptides (from 'pepsis' *i.e. digestion* or 'peptones' *i.e. digestion products of proteins*) in the 14th Meeting of German Scientists and Physician on Sept. 22, 1902 at Karlbarg. He first synthesized dipeptides, glycylglycine, from partial hydrolysis of diketopiperazine of glycine (Fischer, 1890). With Axhausen, he eventually succeeded in synthesizing a octapeptide, the sequence of which is L-Leu-$(Gly)_3$-L-Leu-$(Gly)_3$-L-Leu-$(Gly)_8$-Gly. Fischer first converted the dream into scientific reality that nature's most impressive and curious molecule can be recreated or reproduced in laboratory. In January 1906, at the Deutsche Chemische Gesellschaft at Barlin, Fischer illuminated the audience with his brilliant speculation on the synthetic availability of living matter. The onward journey in the golden history of peptide chemistry was further approached with the introduction of amino protecting benzyloxycarbonyl group in 1932 by Max Bergmann, a first rate protein chemist and Loenidas Zervas. But from 1920 to 1940, a serious question was raised, related to the existence of universality of peptide bonds in natural proteins. During mid 1930's, controversy was going in critical dimension due to lack of knowledge of adequate purification of natural peptides and reliable analytical data. Joseph Fruton and Max Bergmann first demonstrated that proteolytic enzymes were also able to cleave the synthetic peptide substrates, the result of which amino acids were generated as a product. In 1938, Bergmann and Zervas theorized that there was a periodicity of each amino acid in a protein. Later this generalization was proven wrong in the laboratory of William Stein. A British topologist, Dorothy Wrinch postulated with enthusiasm that amino acids were linked by =N-C(OH)= bond rather than peptide bond in globular

proteins but the theory was eventually discarded. Before the discovery of X-Ray crystallography, it was not possible for biochemists to elucidate the detailed structural analysis of proteins, peptides and peptide bonds. Some attempts were made by William Astbury in England in 1926 with keratin of hair, collagen silk etc. but satisfactory X-Ray diffraction pattern was established by the group of John Bernal in Cambridge in mid 1930s with pepsin and Hemoglobin. At that time, it was quite critical to predict the dimensions of peptide bond with fuzzy X-Ray data, but Linus Pauling, the first architect of protein chemistry, accurately established the C-C and C-N bond lengths (0.01 A°) from the examination of many organic compounds. In the late 1930s, Pauling and Corey embarked on a series of polypeptides and amino acids that laid the foundation of present understanding of protein structure. From X-ray diffraction studies of several crystals of amino acids and of simple dipeptides and tripeptides by Linus Pauling and Robert Corey, it was conclusively proved that the peptide C-N bond is somewhat shorter than the C-N bond in a simple amine (Edison, 2001). So a resonance is configured between the carbonyl oxygen and amide nitrogen and an electrical dipole is established between the partial negatively charged oxygen and partial positively charged nitrogen atom in peptide bond. On the basis of partial double bond character of peptide bond, Pauling concluded that peptide bond must be planer, which greatly restricts the structural dynamics of a protein. From many helical proteins, Pauling also formulated the length of N-H-O hydrogen bonds, present in many crystals all that were close to 2.9 A°. The backbone configuration of α-Helix which is the simplest arrangement of polypeptide chain with its rigid peptide bonds was derived by Pauling and Corey. In 1951, they published their helical structures where they claimed the repeat structure of 5.4 A° per turn (Wieland and Bodanszky, 1991).

1.3.2 Development of science of peptides

One of the important bioactive peptide hormones is insulin which revolutionizes the history of diabetic patients. Insulin was first isolated, purified and available in the form capable for therapeutic administration in 1921 (Bliss, 1984). In collaboration with biochemist James B Collip and physiologist J J R Macleod, Banting and Best carried out experiments that led to the discovery of insulin at the University of Toronto in Canada.

Charles Best, a young student, had volunteered to help Sir Frederick Grant Banting with the experiments. In the hot summer of 1921, Banting and Best decided to make one of the dog diabetic and to extract the pancreas of another dog whose ducts had been tied securely for some five to six weeks. It was the extraction of the material from this dog that proved to be the crucial turning point in their studies. Fortunately, the degenerated pancreas was kept chilled which made success possible. The low temperature prevented any remaining protein-digesting enzyme of the main gland from inactivating the extract they had isolated. The initial effects were not that dramatic, but the substance was lowering the sugar levels in the blood and urine. For the next few weeks they worked day and night on a succession of diabetic dogs, using the extract to produce life saving results. They also observed, that injecting too much of the extract resulted in excessively low blood sugar, or 'hypoglycemia'. By late July, they had succeeded in isolating the substance we all know as insulin (Wrenshall *et al.*, 1962). The extract was first referred to as "Isletin" after the secreting cells within the pancreas – the islands of Langerhans. Later, the name was changed to "Insulin" to aid spelling and pronunciation throughout the world. The word Insulin comes from the Greek "Insula" meaning 'island'. In 1928, insulin was declared as polypeptide and later its amino acid sequence was derived in 1955. For elucidating the entire structure of insulin, Fred Sanger of Cambridge University used 2,4-dinitrofluorobenzene as a marker for N-terminal amino acid of peptide. For his remarkable contribution in elucidating the entire structure of insulin from partial peptide fragments, he was awarded the Nobel Prize in Chemistry in 1958. At that time it was well-known that anterior pituitary hormones is under the control of hypothalamus, the reservoir of several peptide hormones of brain. By producing lesions in the median eminence of the hypothalamus, the importance of hypothalamic factor for the regulation of thyrotropin from pituitary was demonstrated (Greer, 1964). Thyrotropin releasing hormone (TRH) was first isolated from 1, 00,000 pig hypothalami and was confirmed by the pyro-GLU-HIS-PROamide with thyrotropin releasing properties. This is the molecule by which the hypothalamus through the pituitary regulates the functions of the thyroid gland.

Nobel Prize for Chemistry in 1955 was reserved for Vincent du Vigneaud for his remarkable contribution in peptide science. Oxytocin, the first hormone to be isolated,

structure determined and synthesized in the laboratory by Vigneaud, is now clinically used for the stimulation of uterine contraction in case of labor. It also plays pivotal role in milk ejection in lactating glands. In the course of isolation of oxytocin, the second hormone of the pituitary, vasopressin, a peptide with pressor and antidiuretic effects, was also isolated (Babu, 2011).

In 1898, Tigerstedt and Bergman published their observation that kidney extracts produce pressor effects. They characterized the substance and named it "renin" (Phillips and Schmidt-Ott, 1999). Forty years after the discovery of renin, two independent investigative groups in Buenos Aires and Indianapolis, headed by Drs Eduardo Braun-Menèndez and Irvine H. Page, respectively, identified the active polypeptide angiotensin that explained the pressor effect of renal hypertension (Page, 1939). The vasoactive peptide resulting from the action of rennin on an α-globulin was discovered by two group of scientists with the result that the peptide received two trivial names, angiotonin and hypertensin (Page and Helmer, 1939). After synthesis of the octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), it was named as angiotensin, which was derived from angiotensinogen (Braun-Menèndez and Page, 1958). Gastrin is another peptide hormone that stimulates the secretion of gastric acid (HCl) by the parietal cells of the stomach and aids in gastric motility. Its existence was first suggested in 1905 by the British physiologist John Sydney Edkins (Modlin *et al.*, 1997), and gastrins were isolated first in 1964 by Gregory and Tracy in Liverpool (Gregory and Tracy, 1964).

In later period, the first isolation of 800 mg of luteinizing hormone releasing hormone (LHRH or gonadotrophin, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) from ventral hypothalami of 1,65,000 pigs was achieved by 12 successive purification steps by Andrew V Schally and Roger Guillemin, who were awarded the Nobel prize for Medicine and Physiology in 1977. LHRH acts on the pituitary to promote rapid release of LH and follicle-stimulating hormone (FSH), which in turn regulates ovulation and spermatogenesis. LHRH and its analogs, find use as a nonsteroidal male and female contraceptive or as fertility agents (Gutte, 1995). Somatostatin, the tetra-decapeptide hormone released by the hypothalamus, plays an important physiological role as an inhibitor for the release of several hormones (glucagons, growth hormone, insulin and gastrin).

It has been known for eighty years that the adrenal is vital to the organism, but not until 1917 (Wheeler and Swale, 1917) was it shown that only the outer part, the cortex, is essential to life. No direct proof of a vital hormone of the cortex was published until 1927, when two groups of investigators, one in Cleveland (Rogoff and Stewart, 1927) and the other at Buffalo (Hartman *et al.,* 1927) demonstrated that an extract of the adrenal cortex could prolong the lives of completely adrenalectomized animals. After 1930, cortical controls of adrenal glands by peptide hormones were documented. Within six days after hypophysectomy the adrenals of the rat were reduced to one-half the weight of the controls; maximum atrophy was reached in thirty days. All zones showed marked reduction in the cytoplasm (Smith, 1930). In hypophysectomized rats removal of one adrenal did not lead to hypertrophy of the remaining gland as it did in normal animals (Reiss *et al.,* 1936). Pituitary preparations restored degenerating adrenal cortex or, in normal animals, could increase the size of the cortex as much as two-fold (Emery and Atwell, 1933). Adrenocorticotropic hormone (ACTH) or Corticotrophin was later isolated from anterior lobe of pituitary gland and characterized as 39 residue peptide. This doctrine was used in medicine for the treatment of hypophyseal insufficiencies and inflammatory processes. McCann and Brobeck in 1954 published the first experiments designed to evaluate ACTH-releasing substances in an animal in which ubiquitous ACTH release from stress was abolished (McCann, 1988). Saffran and Schally in 1955 developed an *in vitro* system of pituitary incubation and published that pressor posterior pituitary preparations caused a release of ACTH, but only from glands which were also incubated in the presence of norepinephrine. Independently of saffron and Schally, Guillemin and Rosenberg in 1955 used a hypothalamic-pituitary co-culture system to demonstrate increased ACTH release in the presence of hypothalami which could not be accounted for by the small amount of vasopressin released (McCann, 1992). The first important work on hypothalamic CRF was performed by Royce and Sayers (1958). They found that stalk median eminence extracts from beef would release ACTH in animals with acute median eminence lesions and went on to purify the active substance. They showed that it was a peptide and obtained preparations which were essentially free from vasopressin.

The incredible and ever increasing body of knowledge in this area clearly shows that the variety of peptides synthesized or isolated from natural sources are of tremendous significance. Due to the denaturation and enzymatic degradation of peptide substances in the gastrointestinal tract, at present, they find clinical use as parenteral, sublingual or intranasal administration. The predominant clinical use of peptide pharmaceuticals and their applications in diagnostics have shown significant potential use within its narrow limits.

1.4 ORIGIN OF LIFE AND ITS RELATION WITH PEPTIDES

1.4.1 Synthesis of Organic Molecules in Pre-biotic World

Historically, origin of life research has focused upon synthesizing organic monomers in reducing atmospheres and examining their assembly into structures ('proteinoids') and macromolecules (i.e. peptides and polynucleotides) whose roles symbolize life's most diagnostic properties, namely, its ability to replicate and evolve. Following naturally from the traditional theory that life arose within a reducing organic-rich aqueous broth, the earliest cells were assumed to have fermentative heterotrophs (Miller and Orgel, 1974). A long-standing paradox has been that the functions performed by the nucleic acid-protein translation apparatus were assumed to be essential for life to begin; yet this apparatus seems too complex to have arisen in the prebiotic milieu. The discovery of 'ribozymes' offers one potential solution to this paradox because both information storage and catalysis might have been achieved by a single class of compounds early in life's history (Cech, 1989).

The experiments performed by Harold Urey and Stanley Miller under simulated conditions resembling those thought at the time to have existed shortly after Earth first accreted from the primordial solar nebula. The experiment showed that many of the basic organic molecules that form the building blocks of modern life can be formed spontaneously from the reducing mixture of gases (methane, ammonia and hydrogen). Simple organic molecules are of course long way from fully functional self-replicating life forms; however, in an environment with no pre-existing life these molecules may have accumulated and provided a rich environment for chemical evolution ("soup theory"). On the other hand, spontaneous formation of complex polymers from

abiotically generated monomers under these conditions is not at all a straightforward process.

Current research explores in more detail the potential roles played by environmental conditions in prebiotic evolution and the origin of life. Some studies have recognized that prebiotic chemical processes required sustained energy sources that were actually available in the environment. A mildly reducing early atmosphere, sustained by thermal processes acting upon a more reduced crust and the upper mantle, might have sustained prebiotic organic synthesis (Kasting and Brown, 1999). Hydrothermal systems may have hosted prebiotic organic synthesis (Shock and Schulte, 1998). A cold early surface environment would have allowed prebiotically important species to survive and accumulate (Bada *et al.*, 1994). It therefore becomes important to define the nature of early habitable planetary environments on Earth and elsewhere. In suitable environment, amphiphilic molecules like 'lipids' assemble spontaneously into vesicles that resemble cellular membranes and that create chemical microenvironments favourable for the development of 'protometabolism' (Deamer and Oro, 1980). Similar amphiphilic compounds have been identified in meteorites. Other sources of complex molecules have been postulated, including sources of extra-terrestrial stellar or interstellar origin. For example, from spectral analyses, organic molecules are known to be present in comets and meteorites. In 2004, a team detected traces of polycyclic aromatic hydrocarbons (PAH's) in a nebula, the most complex molecule, to that date, found in space. Another unsolved issue in chemical evolution is the origin of homochirality. This is essential for both proteins and DNA, yet many prebiotic simulations produce a racemic, or equal mixture of left- and right-handed forms.

1.4.2 Formation of Amino Acids and Nitrogenous Derivatives

A small number of precursor molecules form the foundation for the synthesis of the complex array of organic compounds detected in the meteorites and in model prebiotic experiments (Miller and Orgel, 1974). High-energy molecules such as hydrogen cyanide, cyanoacetylene and formaldehyde are not likely to survive long in the environment, despite constant synthesis. However, awing to their reactivity, when

concentrated they undergo addition and polymerization reactions that generate bioorganic compounds rapidly in solution.

It has been suggested that life began with a self-replicating RNA molecules. The prebiotic synthesis of nucleic acid bases is a central issue in the RNA world hypothesis, one of the main proposals for the origin of life, based on the self-assembly of nucleic acid monomers (Saladino *et al.*, 2006). In the pioneering work, Ferris *et al.* obtained cytosine from urea and cyanoacetylene and from sodium cyanide solution and cyanoacetylene (Ferris *et al.* 1967). The isolation of the latter from spark discharges in methane/nitrogen mixtures suggests the relevance of this synthesis to the origin of life (Sanchez *et al.* 1966). Cyanoacetylene is present in the atmosphere of Titan, in comets, and in the interstellar medium and, thus, is indeed of prebiotic relevance (Clarke and Ferris, 1995). A quarter of the century later, Robertson and Miller reported the synthesis of cytosine in high yields (30-50%) upon heating various concentrations of urea and cyanoacetaldehyde in a sealed ampule (Robertson and Miller, 1995).

Many authors have opined about the synthesis of pyrimidines from urea and cyanoacetaldehyde and their relevance with prebiotic world (Shapiro, 1999 and 1998). The main concerns are the availability and instability of the reactants. In case of cyanoacetaldehyde, it could react with amino acids, undergo hydrolysis to generate formate and acetonitrile or form a dimer (Saladino *et al.*, 2005). Consequently, any cyanoacetaldehyde is unlikely to survive long enough to be available in sufficient quantity to produce the necessary concentration for cytosine synthesis. A way to overcome these problems, along with the problem of cytosine instability through deamination to form uracil, has been hypothesized in which ice-water solutions are used to generate the appropriate conditions for the reaction through the exclusion of solutes from concentrated interstitial brines in the ice matrix (Trinks *et al.*, 2005). This exclusion leads to a concentration enhancement in the microenvironments in the ice upon slow freezing of aqueous solutions with organic molecules (Shapiro, 2002). In addition, freezing extends the lifetime of labile molecules and gives time for them to be processed further, due not only to the protective effect of the ice but also to the low temperatures, which tend to retard reaction mechanisms that are prevalent in the liquid phase. Indeed, the unique environment generated in the interstitial liquid channels in ice, with high

pressures and strong gradients, could favour certain reactions, such as nucleotide polymerization (Abelson, 1966). When the icy solution under a reductive, methane-based atmosphere was subjected to spark discharge as an energy source for the first 72 hours, several organic products like triazines, cyanuric acid, ammeline; the pyrimidines cytosine, uracil and 2,4-diaminopyrimidine and the purine adenine were synthesized (Menor-Salvan *et al.*, 2009). The synthesis of pyrimidines from urea is possible under a methane/nitrogen atmosphere only at low temperature, in the solid phase. The ice matrix of prebiotic earth played the role of a protective medium for pyrimidines by diminishing the side chain reactions.

Assuming that the primitive atmosphere could have been reducing, Miller (Miller, 1953 and 1957) working at the University of Chicago in 1953, put the components of a reducing atmosphere (ammonia, methane, hydrogen and water) within an apparatus with a high energy source, like electrical discharge. After one week of sparking, the products were removed from the trap and analyzed by anion-cation-exchange chromatography. At present, 18 out of the 20 amino acids found in proteins have been synthesized by methods similar to Miller's classic experiment. Tryptophan and glutamine have not been identified among the reaction products. Interestingly enough, two amino acids, tyrosine and phenylalanine, have been produced only on heating mixtures of the presumed prebiotic gases to over 1000°C (Lemmon, 1970). These results are not consistent with the overall evolutionary hypothesis which says that the synthesis must have taken place at temperatures less than 150°C (Miller and Urey, 1959). Also, most amino acids are especially susceptible to decomposition by irreversible decarboxylation caused by heat.

Turning to the problems of the actual synthesis of amino acids, it is important to note the thermodynamic stability of the products formed in the reducing atmosphere that produced them (Bernstein, 2006). Simply stated, the reactions that create the amino acids also tend to destroy those (Bertrand *et al.* 2008). This is due in part to the strength of the energy source. One feature of Miller's apparatus and subsequent variations of his experiment is a trap suitable for the storage and/or the immediate removal of the products of the reaction. Thus, one must propose the existence of a primitive trap on earth during the early phases of the chemical evolutionary process. Without such, the destructive forces of electrical discharges or ultraviolet radiation would destroy the prebiotic

precursors of life that they had produced. A primitive-earth trap has been suggested by Bernal (Bernal, 1960); however, it seems precluded by Hull (Hull, 1960). Considering the thermodynamics of chemical evolution, especially the equilibrium concentrations of synthesized organic compounds, Hull demonstrated that the accumulation of amino acids on a primitive earth would result in a concentration hopelessly low and totally unsuitable as a starting material (Hull, 1960). Calculating not only the relative rates of formation of several amino acids, but also the rates of their decomposition, Hull found the resultant concentrations to be on the order of 10^{-12} moles/liter or less.

The yields of key amino acids such as aspartic and glutamic acids were very low in most experiments and not at all in proportion to the biological system concentrations (Tze-Fei-Wong and Bronskill, 1979). The total yield of these two compounds was less than 0.07%, while other important amino acids were not even present under the conditions producing these. One should keep these particular amino acids in mind, because they are very significant when one speculates on mechanisms for the polymerization of amino acids into polypeptides.

Amino acids produced under prebiotic conditions designated would more than likely contain equal parts of the D- and L-isomers (a racemic mixture) (Bernstein *et al.*, 2002). As noted, those amino acids found in living systems are of the L-α-configuration. Thus any hypothesis dealing with chemical evolution must ultimately account for the incorporation of the specific L-α-configuration over the other alternatives. All biological systems have the unique ability to differentiate between stereoisomers. This unique stereochemistry is required at the molecular level so that larger molecules will have the proper shape allowing them to carry out their varied and specific functions within the living cell. This shape is again important in determining the activity and the proper functioning of subcellular structures of the cell. There is a definite order and organization associated with living systems, and the stereochemistry of the basic building blocks is one of the key components of this beautiful structure (Cronin and Pizzarello, 1997).

1.4.3 Polymerization of Amino Acids and Development of Oligopeptides in Prebiotic World

The polymerization of amino acids can be achieved in biological system through the formation of peptide bonds. This process involves reversing the thermodynamic barrier, an energy barrier which does not allow monomers (amino acids) to spontaneously combine to form peptides unless they have been activated or energy is supplied. Several mechanisms for such polymerization have been proposed. After Hull (1960) stated the concentration of the prebiotic precursors in the oceans would never have reached an appreciable level for self-polymerization, researchers have sought other devices. Since water inhibits the formation of peptide bonds the first step to create a peptide often involves removing water. Fox (1960) created chains of amino acids by heating a purified concentration of amino acids to 150°C for about 14 hours. At this temperature, water and other volatile compounds vaporize. The heat drives off water molecule that is produced during peptide bond formation and forcing the reaction in forward direction. Fox obtained very long chains when he included high concentrations of the pure mixtures of glutamate, aspartic acid and lysine. When dissolved in hot water and allowed to cool, the polymer precipitates, forming spherical globules said by Fox to resemble coccoid bacteria, the so-called 'protenoids' (Fox, 1965). They are different from normal peptides in two important ways. The polymerization of amino acids by heating shows a marked degree of racemization of the optically active starting reagents, and stereoselective catalysts and surfaces would be nonexistent on a prebiotic earth (Kenyon and Steinman, 1969). Moreover, the side chains associated with lysine, glutamate and aspertate form over half of the peptide bonds. This second feature has led most origin of life researchers to drop protenoids as a viable candidate for the first living protein. Stanley Miller in particular has criticized thermal proteins as unlikely candidates because the conditions within a narrow range (150-180°C), and if the heating lasts too long (more than a day), then the thermal proteins are destroyed (Miller and Orgel, 1974). Thermal polymerization of amino acids was also achieved by Huber and Wachtershuser (1998) in their experimental modeling equivalent to volcanic or hydrothermal geoclimatic conditions. They converted amino acids into respective peptides by using coprecipitated (Ni,Fe)S and CO in conjunction with H_2S (or CH_3SH) as a catalyst and condensation agent at 100°C and pH 7 to 10 under anaerobic, aqueous conditions. These results also demonstrated that amino acids can be activated under geochemically relevant conditions. They supported a

thermophilic origin of life and an early appearance of peptides in the evolution of primordial metabolism.

The catalytic effects of the simple amino acid glycine on the formation of diproline and divaline in the prebiotically relevant salt-induced peptide formation (SIPF) reaction was investigated by Plankensteiner *et al.* (2005) in systems of different amino acid starting concentrations and using the two enantiomeric forms of the respective amino acid. They showed an improved applicability of the SIPF reaction to prebiotic conditions, especially at low amino acid concentrations, as presumably present in a primordial scenario. In this reaction chiral selectivity of the active copper complex were also observed. Brack (2007) in his review documented that the β-sheet-forming peptides can able to protect their amino acids from racemization. He also stated that the import of extraterrestrial amino acids represented the major supply, as evidenced by micrometeorite collections and simulation experiments in space and in the laboratory. According to his view, selective condensation of amino acids in water was achieved via N-carboxy anhydrides. Homochiral peptides with an alternating sequence of hydrophobic and hydrophilic amino acids is best for adopting stereoselective and thermostable β-pleated sheet structures. Some of the homochiral β-sheets strongly accelerate the hydrolysis of oligoribonucleotides. Even if peptides are not able to self-replicate, the accumulation of chemically active peptides on the primitive Earth appears plausible via thermostable and stereoselective β-sheets made of alternating sequences.

1.4.4 Importance of Peptides in the formation of Life

It is widely accepted that short peptides could be formed in the primordial soup and survive under its harsh conditions (Rode and Suwannachot, 1999). Furthermore, it was argued that peptides could actually self-replicate under these primitive conditions (Rode, 1998). Usually a catalyst like clay or some other mineral like pyrite is required for spontaneous generation of short peptide chains in water. The minerals interact with the C-terminus of another. Clay in particular can form pockets that may help exclude water. Now several mechanisms of formation of peptides could be envisioned from primordial soup in the microenvironment of clay pockets. These peptides were clearly short enough to be formed under prebiotic reactions like the SIPF reaction (Rode and Suwannachot,

1999). It should also be noted that self-replicating property of amyloid structure of prions are greatly favoured by SIPF mechanism (Rode *et al.*, 1999). In this connection, self-assembled amyloid structure of polypeptides are particularly considered important because this structure can be formed by diverge group of structurally unrelated proteins and all amyloid fibrils share similar biophysical and ultrastructural properties (Chiti *et al.*, 2001). Obviously it implies that the amyloid configuration actually represents an energy-favourable generic state for most proteins. Recent studies identified very short motifs of short peptide chains that are able to self-assemble into amyloid like fibril structure and/or into nanotubular arrangements (Reches *et al.*, 2002; Vauthey *et al.*, 2002). Some of these structures were demonstrated to be hollow and cylindrical or spherical. Thus, these structures allow the formation of an isolated aqueous environment and may form pockets, which can protect and catalyze the formation of RNA oligomers. Some of these self-assembled structures are durable in very harsh environments, including boiling, high pressure, autoclave treatment, and in various organic solvents (Reches and Gazit, 2003).

The formation of the self-assembled structures by the di- and tripeptides is especially intriguing in the context of the quest for the origin of life. These short peptides might be originated in extra-terrestrial atmosphere because dipeptides and larger amino acid polymers were found on carbonaceous chondritic meteorites (Meierhenrich *et al.*, 2004). Thus it seems reasonable to assume that such peptides were formed under prebiotic conditions and produced amyloid related self-assembled structures. Furthermore, a self-replicating amyloid forming peptide was recently constructed demonstrating a pathway to the enrichment of such fibrils of the prebiotic world (Takahashi and Mihara, 2004). This amyloid forming self-assembled peptides could contain small pores (Vauthey *et al.*, 2002). Such pores might enable small molecules to diffuse into the tube volume but inhibit larger molecule movement. Hence, NTP's monomers would be able to penetrate the structure whereas RNA oligomers would be blocked inside. This could give rise to a concentration mechanism of RNA inside the core of peptides. So local accumulation of self-assembling peptide fragments on clay particles produced an 'RNA oasis'. In this oasis, molecules of RNA could have survived and grow slowly inside a peptide structure functioning as an 'RNA factory'. This microenvironment would have given a 'Darwinian' advantage to RNA-Peptide

cooperative system. Such an evolutionary process could have given rise to primitive RNA polymerase machinery that functioned as the first enzyme of the prebiotic world (Carny and Gazit, 2005).

1.5 BACTERIAL PEPTIDES

1.5.1 Oligopeptide as prokaryotic communication molecule

Bacteria communicate with one another using chemical signal molecules. In general, gram-positive bacteria communicate using modified oligopeptides as signals and 'two component' type membrane-bound sensor Histidine kinases as receptors (Kim *et al.* 1992). Oligopeptide pheromones can be considered the first class of prokaryotic communication molecule to be detected as competence factors in *S. pneumoniae* and *B. subtilis* (Tomasz 1965) but their structures were only recently determined (Havarstein *et al.* 1995; Pestova *et al.* 1996; Solomon *et al.* 1996). The tremendous diversity of oligopeptides makes them especially suitable when a high degree of discrimination is required, as in the use of distinct oligopeptide mating pheromones and inhibitors involved in regulating the transfer of each of many conjugative plasmids in gram positive cocci (Clewell, 1993). Gram-positive oligopeptides are synthesized from larger precursor protein and transported outside the cell by ATP-binding-cassette (ABC) transporters. Bacteria direct the accumulation of minimal threshold stimulatory concentration of auto-inducing peptides and alter gene expression and therefore behaviour, in response. For example, *Bacillus subtilis* has two auto-inducing peptides functioning in the network arrangement that allows *B. subtilis* to commit to one of two mutually exclusive lifestyles: competence and sporulation (Waters and Bassler, 2005). Com X, a 10-amino acid peptide (Magnuson *et al.*, 1994, Solomon *et al.*, 1996) that is processed and secreted by Com Q (Bacon *et al.*, 2002), is detected by the membrane-bound Histidine sensor kinase Com P. In *Staphylococcus aureus* virulence factor production is largely under the control of the accessory gene regulator (agr) quorum sensing system. Each 'agr' gene produces cyclic autoinducing peptide (AIP) that activates its cognate receptor and executes virulence (George *et al.*, 2008). The swarming behaviour, i.e. rapid migrations over a surface by groups of elongated, hyperflagellated 'swarmer' cells were also controlled by cyclic peptides in *Serratia* sp. (Shapiro, 1998). Swarm cells of *Pseudomonas aeruginosa*,

Escherichia coli, Serratia marcescens, Burkholderia thailandensis and *Bacillus subtilis* show resistance to almost all standard antibiotics, except for the antimicrobial peptides (Lai *et al.* 2009).

1.6 ANTIMICROBIAL PEPTIDES

A plethora of novel gene-encoded antimicrobial peptides from animals, plants and bacteria has been described during the last two decades. Among the prokaryotes, productions of substances that can antagonize competitors are widespread, and antibiotic peptides are one of the major tools in such ecological context. One family of microbial defense peptide, bacteriocins are found in almost every bacterial species examined to date, and within a species tens or even hundreds of different kinds of bacteriocins are produced (James *et al.* 1991; Riley and Gordon, 1992). So bacteriocins constitute a structurally diverse group of peptides, and it was recently proposed that the bacteriocins can be classified into two broad categories: lanthionine containing (Class-I: lantibiotics) and non-lanthionine containing (Class-II) (Klaenhammer, 1988; Cotter *et al.* 2005). Lantibiotics are characterized by the inclusions of the unusual amino acids like α-lanthionine and β-lanthionine and the necessity for posttranslational processing to acquire their active forms. Lantibiotics were funther divided into two subgroups, A and B, based on structural features and their mode of killing (Jung and Sahl, 1991). Type A lantibiotics kill the target cell by depolarizing the cytoplasmic membrane (Schuller *et al.*, 1989). The most common food preservative Nisin, the archetypal and best-studied gram-positive bacteriocin, is type-A lantibiotic (Gross and Morell, 1971). Type-B lantibiotics function through enzyme inhibition. One example is merssacidin, which interferes with cell wall biosynthesis (Brotz *et al.* 1995). Class II bacteriocins are heat-stable ranging in size from 30 to 60 amino acids and act through the formation of pores in the cytoplasmic membrane (Riley and Wertz, 2002).

Antimicrobial peptides of eukaryotic origin can be divided into sub-groups on the basis of their amino acid composition and structure (Vizioli and Salzet, 2002; Boman 1995). One subgroup contains anionic antimicrobial peptides. Among these are small (721.6-823.8 Da) peptides present in surfactant extracts, bronchoalveolar lavage fluid and airway passage epithelial cells (Brogden *et al.*, 1996). They require zinc as a co-factor for

antimicrobial activity and are active against both gram-positive and gram-negative bacteria. A second subgroup contains cationic peptides, which are short (<40 amino acid residue), lack cysteine residues and sometimes have a hinge in the middle (Gennaro and Zanetti, 2000). In water, it exhibits a circular dichroism spectrum that is consistent with a distorted structure (Johansson *et al.*, 1998). A third subgroup contains cationic peptides enriched for specific amino acids like proline containing peptides from honeybees, tryptophan containing peptides from cattle or small histidine-rich salivary peptides. These peptides lack cysteine residues and are linear, although some can form extended coils (Otvos, 2002). A fourth subgroup of cationic and anionic peptides contains cysteine residues and form disulphide bonds and stable β-sheets. These are human or arthropod defensins which are crosslinked by disulphide bonds (Ganz, 2002). Finally there are anionic and cationic peptides that are fragments of larger proteins. Good examples of this class are lactoferricin from lactoferrin, casocidin I from human casein etc. These peptides kill micro-organisms through variable mode of action.

Cationic properties of many anti-microbial peptides result in an electrostatic attraction to the negative-charged microbial envelopes, such as lipopolysaccharide of gram-negative bacteria, through which microbial populations are categorically restricted but have lower toxicity for plant and animal cells (Zasloff, 2002). Regarding amino acid sequence, the increase in net positive charge is linked with enhanced antimicrobial activity. Likewise, a set of alanine substitution analogues showed the higher contribution to its antifungal activity results from its three cationic residues, thus reinforcing the importance of ionic attraction (Munoz *et al.*, 2007). Cell permeabilization is another important mechanism of peptides that leads to direct killing of microbes by disrupting membrane architecture and cell permeation. It is achieved through folding of peptides, which enhance their amphipathicity and thus their antimicrobial properties. However, some studies have argued that there is not always a complete correlation between cell permeation and antimicrobial activity (Epand and Vogel, 1999). Specific cationic peptides, called penetratins, are rich in arginine or aromatic residues and have the propensity to cross biological membranes in a nondestructive manner (Henriques *et al.*, 2006). Once internalized, antimicrobial peptides may combine with DNA, RNA and/or proteins, and interrupt DNA replication, RNA synthesis or enzyme activity, depending on

the nature of peptides (Marcos *et al.*, 1995). Relevant examples of peptides active against plant microbes are those from indolicidin or lactoferricin group (Ulvatne *et al.*, 2004). Due to basic properties of many antimicrobial peptides, their affinity with anionic DNA molecule is expected and in vitro binding has been demonstrated in a number of cases (Hsu *et al.*, 2005). Although such activity *in vivo* could alter cell homeostasis severely, it remains to be determined to what extend this property mediate peptide antimicrobial activity. Selected examples of natural antimicrobial peptides are enlisted Table 1:

Table 1.1 Bioactivities of some peptides isolated from different animals and their most probable sequences. Colours of different amino acid codes are given according to their hydropathy profile and chemical grouping

Sl. No.	Peptide	Source	Amino Acid Sequence	Pathogen	Proposed Mechanism	Reference
1.	**Megainin 2**	Frog	GIGKFLHSAKKFGK AFVALKAL	Bacteria, Fungi, *Fusarium oxysporum*	Permeabilizes bacterial membrane	Matsuzaki (1998)
2.	**Cecropin A**	Silk Moth	KWKFKKIEKMGRN IRDGIVKAGPAIEVI GSAKAI	Fungi, *Phytophthora infestans*	Membrane destabilizing	Gazit *et al.* (1996)
3.	**Cecropin B**	Insect	KWKVFKKIEKMGR NIRNGIVKAGPAIA VLGEAKAL	Bacteria & Fungi	Membrane lipid disruption	Alan and Earle, (2002)
4.	**Ib-AMP1**	Plant	QWGRRCCGWGPG RRYCVRWC	Fungi	Inhibits macromolecular synthesis	Van Loon *et al.* (2006)
5.	**Buforin II**	Toad	TRSSRAGLQFPVGR VHRLLRK	Bacteria	Binding to nucleic acid	Park *et al.*, (2000)
6.	**Apidaecin**	Honey bees	GNNRPVYIPQPRPP HPRI	Bacteria	Alter cell homeostasis	Casteels *et al.*, (1989)
7.	**Indolicidin**	Bovine	ILPWKWPWWPWR R	Fungi	Calcium-calmodulin interaction	Bhargava *et al.*, (2007)
8.	**Melittin**	Honey bee venome	GIGAVLKVLTTGLP ALISWIKRKRQQ	Bacteria, Virus	Cytolytic	Raghuraman and Chattopadhyay, (2007)
9.	**PR-39**	Porcine	RRRPRPPYLPRPRP P	Bacteria	Highly cationic and affinity towards nucleic acids	Boman *et al.*, (1995)
10.	**PAF26**	*Penicillium chrysogenum*	RKKWFW	Fungi, Gram(-) Bacteria	Electrostatic attraction with lipopolysaccharide	Munoz *et al.*, (2007)

1.7 GLIMPSES FROM VAST ARRAY OF BIOACTIVE ANIMAL PEPTIDES

In animals, most of the physiological interactions were achieved through peptide endocrine signal system. It was recently established that communication is possible because the nervous and immune systems share a common biochemical language involving shared ligands and receptors, including neurotransmittors, neuropeptides, peptide growth factor, neuroendocrine hormones and cytokines (Kelley *et al.*, 2007). A great deal of evidence has accumulated and confirmed that hormones secreted by the neuroendocrine system play an important role in communication and regulation of the cells of the immune system. Among peptide hormones, this has been clearly documented for prolactin, growth hormone and insulin-like-growth-factor-1 (IGF-1). It was recently proved that IGF-1 acts in cycling cells via insulin receptor substrate-1, phosphatidylinositol 3'-kinase and cyclin dependent kinase 2 (CDK2) and the proteins were inhibited by TNFα (Shen *et al.*, 2004). It is further believed that IGF-1 inactivation promotes cell survival, but at the same time seems to be associated with a reduction of life span. Lots of scientific data now point to the importance of critical homeostasis between peptide hormones and cytokines from the immune system. The role of proinflammatory cytokines in inducing resistance to IGF-1 (Kelley, 2004), growth hormone (Lang *et al.*, 2005), glucocorticoids (Pace *et al*, 2007), G-Protein coupled receptors such as catecholamines (Heijnen, 2007) and insulin (Hotamisligil, 2003) have been recently highlighted. These new findings underscore the importance of understanding the molecular details of communication systems between the immune and endocrine systems.

The family of natriuretic peptides (NP) constitutes another important peptide group that comprises at least eight structurally related amino acid peptides stored as three different prohormones: 126 amino acid atrial natriuretic peptide (ANP) prohormones, 108 amino acid brain natriuretic peptide (BNP) prohormones and 126 amino acid C-type natriuretic peptide (CNP) prohormones (Vesely, 1992). The prohormones ANP contains several peptides with blood pressure lowering properties, natriuretic properties, diuretic properties and/or kaliuretic properties (Vesely *et al.*, 1994). The name BNP was derived from its initial isolation from the procine brain (Hunt *et al.*, 1995). CNP has been detected in human coronary arteries and in the peripheral circulation in endothelial cells

of human veins and arteries of various sites (Komatsu *et al.*, 1992). CNP lacks significant natriuretic function, and serves as a regulator of vascular tone and growth in a paracrine or autocrine fashion (Cargil *et al.*, 1995).

One group of peptides released from wide range of nerves is considered as neuropeptides. Chemically distinct, they exhibit characteristic pattern of localization within the peripheral and central nervous system and possess the ability to stimulate a range of diverse biological activities. Substance P (SP) was the first neuropeptide to be discovered and was recognized as a sensory neurotransmitter by Lembeck (1953). Other neuropeptides have more recently secured their places in the archives through their potent and varied biological activities, distinct G-Protein coupled receptor (GPCR) activation and potential therapeutic targets (Hay *et al.*, 2004). A flare, which is visible in skin around the point of injury due to a spreading nerve-mediated axon reflex, involves stimulation of sensory nerves to release neuropeptides that include SP and GCRP. Another group, neuropeptide Y (NPY) was purified a decade after the sequencing of SP in 1982 by Tatemoto *et al.* (1982) using a novel identification process that selectively detected carboxy terminal amidated peptides. It is located in, and released from specific central neurons and peripheral neurons, sympathetic and sensory nerves. NPY was initially shown to exhibit a surprisingly weak direct vasoconstrictor response but a powerful potentiation of noradrenaline induced vasoconstriction in a variety of rabbit isolated blood vessels. Related nonmammalian neuropeptide PY exists in fish and neuropeptide F in *Drosophila melanogaster*. PY is found in the pancreas of several boney fishes but probably it was not the mammalian orthologue (Larhammar *et al.*, 2004). Some of the structural analogs of neuropeptides were also found in mammals like pancreatic polypeptide (PP) and Polypeptide Y (PYY), located in neuroendocrine cells of pancreas and terminal intestine, respectively. Together, NPY, PYY and PP exert wide ranging effects in central and peripheral target tissues. Neuropeptide Y (NPY) acts as an inhibitory neurotransmitter and anticonvulsant and antinociceptive actions, in addition to its hypertensive and appetite-stimulating effects (Brain and Cox, 2006). In the intestine, NPY and PYY act as potent anti-secretory agents in rodents and human tissues (Hyland and Cox, 2004).

Opioid peptides are another group that mimics the effect of opiates in the brain. Opioid peptides may be produced by the body itself, for example endorphins, or be absorbed from partially digested food (casomorphins, exorphins and rubiscolins). The pentapeptide methionine-enkephalin and leucine-enkephalin were first discovered in the brain and adrenal gland (Hughes *et al.*, 1975). Shortly after, enkephalin-containing peptides were identified throughout the central and peripheral nervous system, including the afferent neurons terminating in the heart (Tang *et al.*, 1982). The neural signaling of opioid peptides has been well characterized in multiple roles, including bradycardia, tachycardia, hypertension and hypotension (Jackson *et al.*, 2001). Three gene products, pro-opiomelanocortin, prodynorphin and proenkephalin are the precursors for endorphins, dynorphins and enkephalins, respectively (Barron, 1999). Over the past decade, accumulating evidence demonstrates that both proenkephalin and prodynorphin and their final products are expressed directly by cardiomyocytes from mammalian species, including rat, guinea pig, and dog besides their normal expression in central nervous system (Ventura *et al.*, 1998). Opioid peptides of myocardial origin have been shown to protect the heart against hypoxic and ischemic injury via activation of G_i-mediated signalling pathway (Pepe *et al.*, 2004).

1.8 PLANT PEPTIDE HORMONES

Plant peptides can essentially be divided into two categories: bioactive peptides that are produced by selective action of peptidases on larger precursor proteins (Fricker *et al.*, 2006), and degraded peptides that result from the activity of proteolytic enzymes during protein turnover, may be utilized through nutritional recycling. Although both groups are the products of proteolysis, they differ in how they act within the cell. Recent findings indicate that first group has a key role in peptide signalling and are also involved in various aspects of plant growth regulation including defense responses, callus growth, meristem organization, self-incompatibility (SI), root growth, leaf shape regulation, nodule development and organ abscission (Matsubayashi and Sakagami, 2006). By definition, plant hormones or phytohormones are chemicals that regulate plant growth. Plant hormones are signal molecules produced within the plant, and occur in extremely low concentrations. Hormones regulate cellular processes in targeted cells through their

specific receptors (Kende and Zeevaart, 1997). So along with classical lipophilic growth regulators like cytokinins, gibberellins, abscisic acid, ethylene, brassinosteroids and jasmonates; peptides of the above class can be placed in 'hormone' category because they fundamentally maintain the basic properties of plant hormone. Among peptide hormones, one major group of secreted peptides can be categorically characterized by the presence of post-translational modification of prepropeptides mediated by specific transferases and by their small size (<20 amino acids) resulting from either proteolytic processing or programmed degradation (Matsubayashi, 2011). These peptide hormone groups include phytosulfokine [PSK] (Matsubayashi and Sakagami, 1996), hydroxyproline rich systemin [SYS] (Pearce *et al.*, 2001), tyrosine-sulfated peptides [PSY-1] (Amano *et al.*, 2007), tracheary element differentiation inhibitory factor [TDIF] (Ito *et al*, 2006), Clavata 3 [CLV3] (Fletcher *et al.*, 1999), C-terminally encoded growth factor (CEP 1) [Ohyama *et al*, 2008], root meristem growth factor [RGF] (Matsuzaki *et al.*, 2010) etc. Interestingly primary sequences of these peptides have common structural features and similar hydropathy profile. Primary precursor polypeptides for this class exhibit significant sequence diversity, with the exception of the conserved C-terminal domain that corresponds to the mature peptide sequences. It can be speculated that a number of amino acid substitution were accumulated within the sequences digested by proteolytic processing during molecular evolution (Matsubayashi, 2011). Hyper-variability of amino acid sequences outside of the mature peptide domain implies that the region removed by protease action are not under strong selection pressure, and thus, genes encoding paralogous secreted peptides with similar features may encode small post-translationally modified peptides (Matsubayashi, 2012). Recently some of the peptide hormones participated in the developmental programme was identified by *in silico* screening of the peptide families with these characteristics (Matsuzaki *et al.*, 2010).

1.8.1 Post-translational modification of peptide hormones

In plants, three types of post-translational modifications were identified, based on their primary sequences and biosynthetic pathways. But understanding the process of peptide secretion in soluble cargo to the apoplasm in plants is limited (Rojo and Denecke, 2008).

Some of the modifications of peptides are represented in flowchart (Figure 1.1) and in brief, they are also discussed below:

1.8.1a *Tyrosine sulfation:*

This modification of peptide is mediated by a specific enzyme *tyrosylprotein sulfotransferase* (TPST), which catalyzes the transfer of sulfate from 3′-phosphoadenosine 5′-phosphosulfate to phenolic group of tyrosine (Moore, 2003). Sulfated peptides play diverge role in plant growth, development and senescence programme (Zhou *et al.*, 2010).

1.8.1b *Proline hydroxylation:*

This modification is mediated by *prolyl-4-hydroxylase* (P4H), which catalyzes the oxidation of proline residues exclusively at fourth position carbon (Myllyharju, 2003). Hydroxyproline residues have been found in systemin, TDIF, CEP and CLV group of peptides. Among them CEP1 arrests root growth (Ohyama *et al.*, 2008) and TDIF suppresses xylem cell differentiation (Hirakawa *et al.*, 2010).

1.8.1c *Hydroxyproline arabinosylation:*

Hydroxyproline residues of some secreted peptide hormones like PSY1, CLV3 and CLE2 are further modified with arabinose chain (Ohyama, 2009). Arabinosylated peptides interact more strongly with their receptors along with enhancement of their bioactivity.

1.8.1d *Cysteine-rich peptides:*

Another major group of secreted peptide signals are 'cysteine-rich peptides', where even number of cysteine residues are present in their amino acid sequence, thus participating in the formation of intra-molecular disulfide bonds (Matsubayashi, 2011). This peptide group is represented by RALF (Wu *et al.*, 2007), stomagen (Hunt and Gray, 2009), pollen guidance factor (LURE peptides) (Okuda *et al.*, 2009), epidermal patterning factors (EPF1/EPF2) (Hara *et al.*, 2009) etc. RALF or rapid alkalinization factor is involved in various aspects of plant development, stomagen positively regulates stomatal density, LURE peptides are involved in pollen guidance and act as chemo-attractant for competent pollen tube, EPF1/EPF2 regulates epidermal cell patterning. So 'cysteine rich peptides' pursue different vital signals associated with growth development and reproduction.

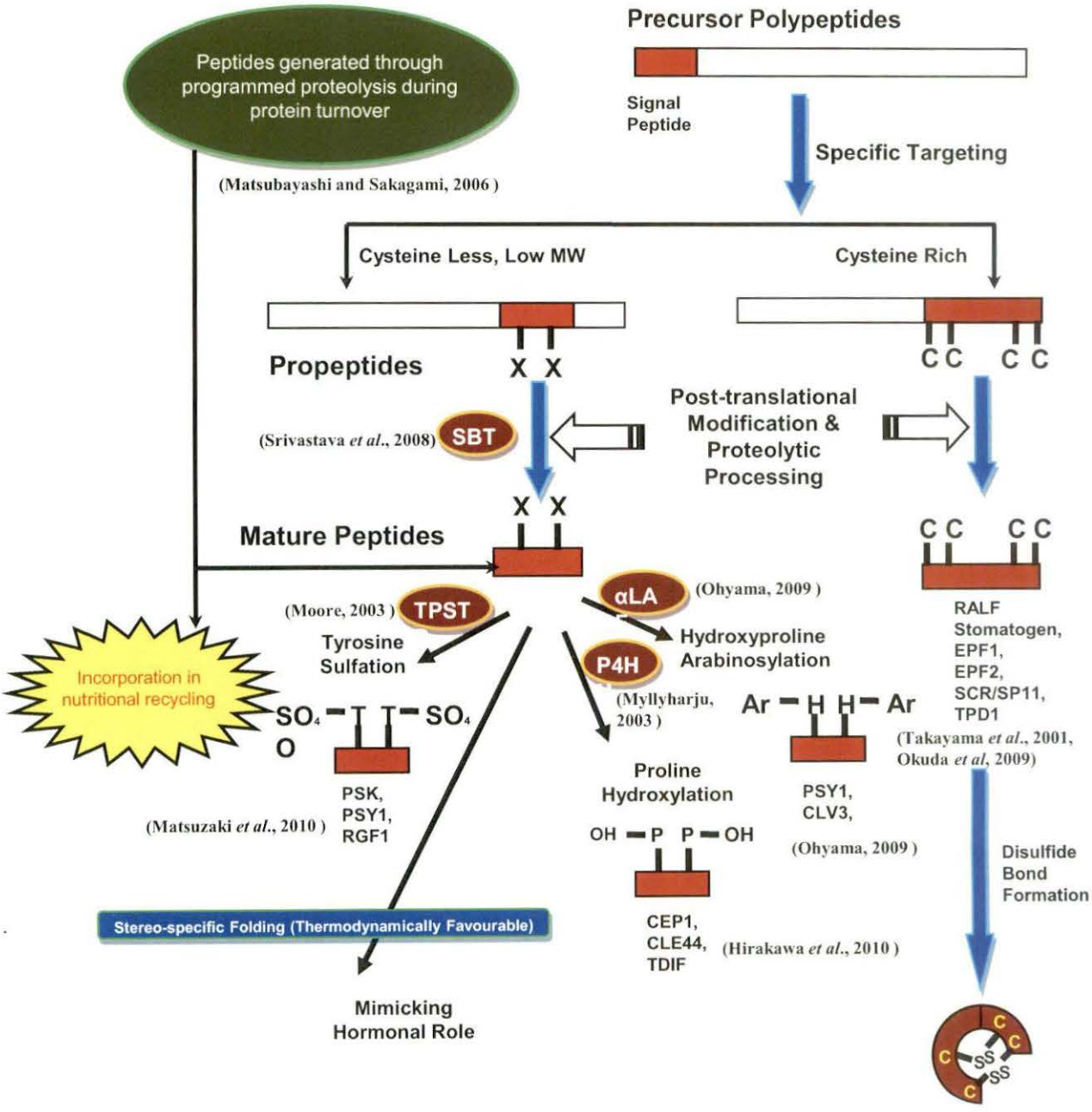

Figure 1.1 : Post-translational modification and proteolytic processing of peptides.

In general, low molecular weight peptides are produced after post-translational modification and proteolytic processing through secretary pathways. Secreted peptide hormone genes are initially translated as pre-propeptides, followed by selective removal of the N-terminal signal peptide by signal peptidases to afford propeptides. The sites of cleavage in the pre-propeptides can be predicted with a high degree of accuracy by Signal IP software (Bendtsen *et al.*, 2004). Many of these processed small peptides have been conserved during evolution, suggesting that these energy-expensive modified peptides have the physiological benefits worth their high cost in plants (Fukuda and Higashiyama, 2011). Here the current knowledge about the functional and signal transduction properties of some plant peptide hormones are represented in diagram (Figure 1.2) and summarized below:

1.8.2 Systemin: wound induced systemic response peptides

Higher plants respond to wounding by insects and pathogens through expression of a set of defense proteins in leaves and stems. In Solanaceous plants, wounding triggers the expression of serine protease inhibitors such as inhibitor I and II (Green and Ryan, 1972). These proteins accumulate not only in wounded leaves but also in undamaged leaves distal from the damage sites, indicating the presence of a mobile factor that induces a systemic defense response. Biochemical purification of this factor on the basis of its proteinase inhibitor-inducing activity led to the identification of a peptide named systemin (Pearce *et al.*, 1991). The pure substance was found to be an eighteen amino acid polypeptide with the sequence AVQSKPPSKRDPPKMQTD. Chemically synthesized systemin induces the expression of proteinase inhibitors in the leaves of young tomato plants when supplied at nanomolar levels through their cut petioles. Whole-leaf autoradiographic analyses (Narvaez-Vasquez *et al.*, 1995) showed that when [$_{14}$C]systemin was placed on fresh wounds, it was distributed throughout the wounded leaf within 30 min, then transported to the petiole, and finally into the upper leaves within 1 to 2 h of application. Tomato Systemin is produced by proteolytic processing of the C terminus of a 200-residue precursor called tomato prosystemin (McGurl *et al.*, 1992). Prosystemin orthologs have only been detected in Solanaceous species, suggesting that the systemin-mediated wound response is species specific. The signaling pathway for the

activation of defensive genes by systemin is mediated by lipid-derived intermediates (Farmer and Ryan, 1992). Systemin activates an intracellular cascade that results in the release of linolenic acid (LA) from membranes, with the LA subsequently converted to phytodienoic acid (PDA) and jasmonic acid (JA) (Vick and Zimmerman, 1984), both powerful inducers of defensive genes in plants (Doares *et al.*, 1995, Weiler, 1997). The significance of systemin in the defense response was revealed by experiments in which tomato plants were transformed with sense or antisense prosystemin cDNAs under the control of the constitutive 35S promoter (McGurl *et al.*, 1994). Overexpression of prosystemin resulted in constitutive expression of defense response genes, as if the plant were in a permanently wounded state. By contrast, transgenic plants expressing anti-sense systemin transcripts showed a severe depression of systemic proteinase inhibitor induction as well as decreased resistance towards herbivorous larvae (Orozco-Cardenas *et al.*, 1993). Recent reports suggest that systemin enhances the production of bioactive volatile compounds, increases plant attractivity towards parasitioid wasps, and activates genes involved in volatile production (Corrado *et al.*, 2007). Perception of systemin by the membrane-bound receptor SR 160 results in activation of MAP Kinase and expression of defense genes. Cosilencing of MAPKs i.e. MPK1 and MPK2 reduced kinase activity, Jasmonic Acid biosynthesis and expression of JA dependent defense genes. So MPK1 and MPK2 are essential components of the systemin signaling pathway and most likely function upstream of JA biosynthesis (Kandoth *et al.*, 2007).

A new class of peptides having similar signaling properties but little sequence homology to systemin have been found and termed Hydroxyproline-rich glycopeptides systemins (HypSys) (Narvaez-Vasquez *et al.*, 2007). Until recently these glycopeptides were thought to function only in protection from herbivore attack. However more recently, HypSys peptides isolated from petunia and sweet potato were found to induce defensin and sporamin B gene respectively and are involved in pathogenic defense. Sporamin B particularly codes for the major storage protein in tubers with trypsin inhibitory activity (Sun *et al.*, 2009). These recent discoveries expand the function and range of the Sys and HypSys family of glycopeptides and establish these unique inducible signaling molecules as potential components of defense pathways throughout the Eudicots (Pearce, 2001).

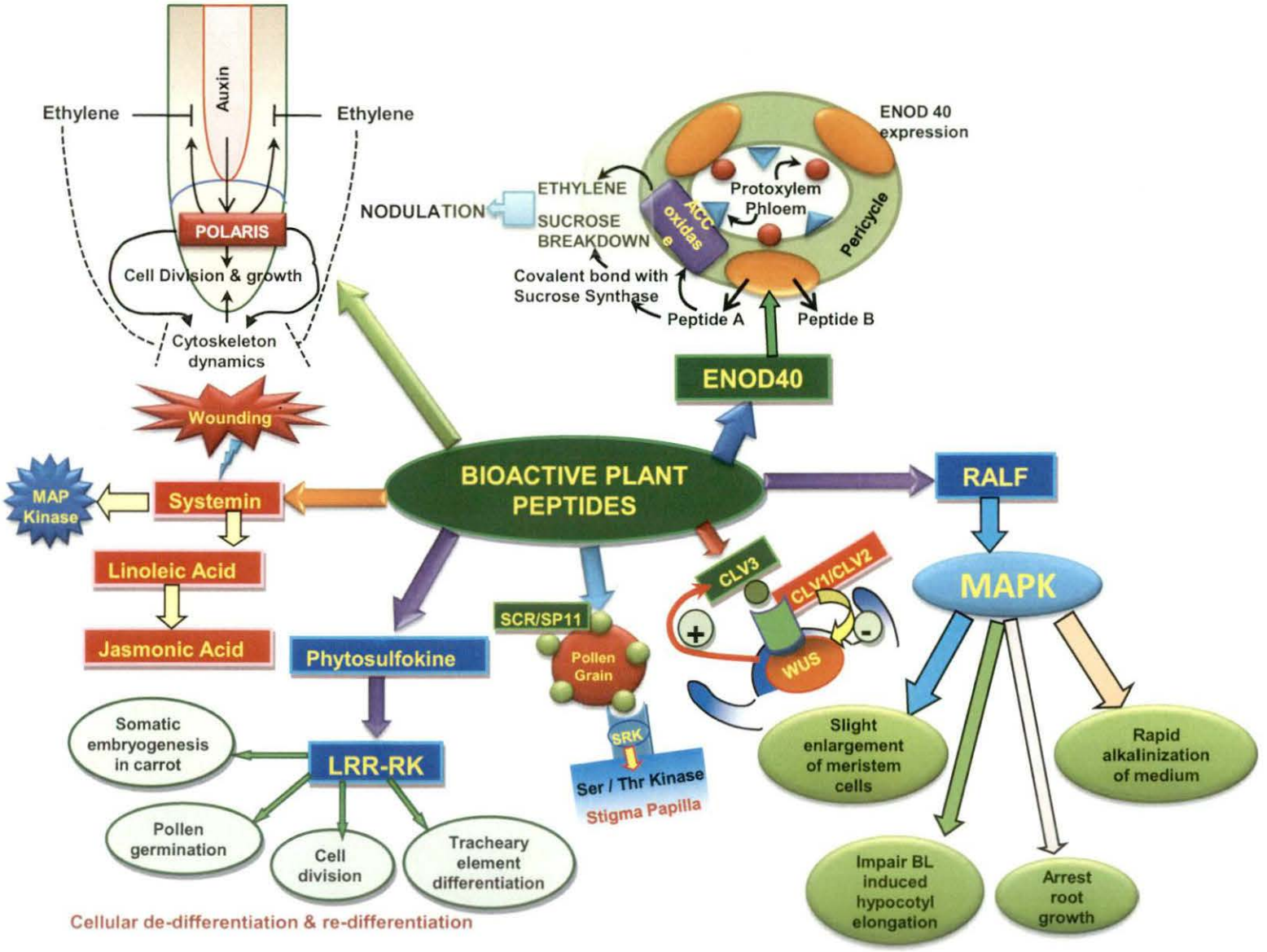

Figure 1.2 Functional aspects of some low molecular weight plant peptide hormones

1.8.3 Phytosulfokine: a peptide elicitor regulating growth and differentiation

The sulfated peptide phytosulfokine (PSK) is an intercellular signal peptide that plays a key role in growth and differentiation. Matsubayashi and Sakagami (1996) isolated and identified growth factors from conditioned medium that promoted the growth at low density of *Asparagus* mesophyll cells in tissue culture. They identified a sulfated pentapeptide [H-Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Gln-OH, abbreviated sYIsYTQ], named PSK-α, and a sulfated tetrapeptide [H-Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-OH], named PSK-β, that were active in the *Asparagus* cell system. Six genes encoding PSKs (AtPSK1–6) have been identified in *Arabidopsis*. Each encodes a preproprotein precursor of approximately 80 residues, with the YIYTQ peptide near their C-termini (Matsubayashi and Sakagami, 2006). Proteolytic processing of Arabidopsis phytosulfokine (*At*PSK$_4$) is dependent on subtilisin-like serine protease (Srivastava *et al.*, 2008). PSK apparently promotes cell division at nanomolar concentrations even at initial cell densities as low as 320 cells ml^{-1} (Matsubayashi and Sakagami, 1998). PSK also stimulates tracheary element differentiation of *Zinnia* mesophyll cells (Matsubayashi *et al.*, 1999) and somatic embryogenesis in carrot under defined conditions (Kobayashi *et al.*, 1999). These findings imply that PSK has important regulatory functions related to cell proliferation and differentiation in higher plants. It has also been demonstrated that PSK promotes various stages of root growth (Kutschmar *et al.*, 2008) including somatic embryogenesis (Hanai *et al.*, 2000; Igasaki *et al.*, 2003), adventitious bud formation, adventitious root formation (Yamakawa *et al.*, 1998), and pollen germination (Chen *et al.*, 2000). Besides controlling development, PSKs also have anti-pathogenic properties as observed against *Phoma narcissi* and *Botrytis tulipae* (Bahyryczl *et al.*, 2008). PSK precursor genes are redundantly distributed throughout the genome (Yang *et al.*, 2001) and are found in a variety of angiosperm and gymnosperm plant species (Lorbiecke and Sauter, 2002). Recently, four candidates for *PSK* genes were found in the *Arabidopsis* database by a BLAST homology search using the sequence of the PSK domains, and two of them were confirmed to be functional by site-directed mutagenesis, indicating that the *Arabidopsis* genome has a gene family encoding PSK precursors (Yang *et al.*, 2001). The preproPSK genes have been cloned from rice and *Arabidopsis*. The rice gene (*OsPSK*) encodes a peptide of 89 amino acids that has a 22-amino-acid signal peptide at the amino-terminal

end and a PSK sequence near the carboxy-terminal end (Yang *et al.*, 1999). The aspartic-acid residue just before the PSK sequence is important for tyrosine sulfation, indicating that the PSK precursor may be sulfated in a manner similar to that of animal peptides (Hanai *et al.*, 2000). The *Arabidopsis* genes (*AtPSK2* and *AtPSK3*) have similar structures to that of *OsPSK*. *AtPSK2* and *AtPSK3* have dibasic amino acids around the PSK sequence, which may be the processing sites, indicating an analogy to mammalian or yeast prohormones.

A PSK receptor was first identified in carrot (*Daucus carota*) as a leucine-rich repeat receptor kinase (LRR-RK) (Matsubayashi *et al.*, 2002). SDS-PAGE analysis of the labeled proteins indicates that a 120-kD protein and a minor 150-kD protein specifically interact with PSK. Based on the internal sequence of the PSK-binding protein, the 120-kD and 150-kD proteins were identified as LRR-RLKs derived from a single gene. The PSK-binding LRR-RLK is named PSKR1. Expression of PSKR1 has been detected throughout tissues of the leaves, apical meristem, hypocotyl, and root of carrot seedlings, although much higher expression has been detected in cultured carrot cells. The carrot PSK receptor, PSKR1, exhibits high percentage amino acid identity with several LRR-RLKs found in *Arabidopsis*. The *in vivo* function of PSK is currently being studied using knockout mutants of the genes for those LRR-RLKs.

1.8.4 SCR/SP11: Peptide mediated self perception

Self-incompatibility (SI) is a genetic system present in flowering plants that promotes outbreeding by rejecting self-pollen (Takayama and Isogai, 2005). In the case of the SI in *Brassica*, the recognition of self and nonself pollen at the stigmatic surface is genetically controlled by a multiallelic S-locus. The S-locus generally contains three highly polymorphic genes: S-locus protein 11 (SP11; or S-locus Cys-rich protein [SCR]), S-locus glycoprotein (SLG), and S-locus receptor kinase (SRK). SP11 encodes a secreted form of a small basic protein that localizes to the pollen coat (Takayama *et al.*, 2001; Iwano *et al.*, 2003). SRK encodes a membrane-spanning Ser/Thr receptor kinase that localizes to the papilla cell membrane and functions as the sole determinant of the SI phenotype of the stigma (Takasaki *et al.*, 2000). Biochemical studies have clearly shown that SP11 is a ligand for SRK, and the specificity of the SI response can now be

explained by the S-haplotype–specific interactions between the SP11 ligand and the membrane bound SRK receptor (Kachroo *et al.*, 2001; Takayama *et al.*, 2001). Bound SCR/SP11 induces *S*-haplotype-specific autophosphorylation of SRK. In contrast to the integral and membrane-anchored forms of SRK, which exhibited high affinity binding to SP11, the soluble form of SRK (eSRK) exhibited no high-affinity binding site. This finding suggests that the membrane anchorage is necessary for SRK to create a high-affinity binding site for SP11 (Shimosato *et al.* 2007). The membrane anchorage is necessary for SRK to maintain its configuration in the equilibrium state between the inactive monomeric or dimeric low-affinity form(s) and the dimeric active high-affinity form. Formation of the active dimer must be supported by the cell membrane, which restricts receptor diffusion in two dimensions (Tzahar *et al.*, 1997).

As opposed to the other known peptides, no further post-translational processing, except for the removal of signal peptide, is required to yield mature SCR/SP11 peptide. Instead, correct disulfide bond formation is a prerequisite for SCR/SP11 activity. Four disulfide bonds between eight conserved cysteine amino acids (C1–C8, C2–C5, C3–C6, and C4–C7) stabilize the structure and form a loop in the C3–C4 region of the protein (Mishima *et al.*, 2003). This C3–C4 region forms a hyper-variable domain depending on each *S*-haplotype and is considered one of the determinants affecting binding specificity to SRK. Another important domain was identified by site-directed mutagenesis, by which it was observed that the C3–C4 and C5–C6 regions contribute to this ligand-receptor interaction (Chookajorn *et al.*, 2004). The *Arabidopsis* genome also includes the large *SCR-related (SCRL)* gene family, which is homologous to *SCR/SP11* (Vanoosthuyse *et al.*, 2001). The *SCRL* family consists of 28 homologous genes encoding 4.4–9.5 kD basic and hydrophilic peptides that have the N-terminal signal peptide and the eight conserved cysteine residues. Some *SCRLs* are expressed in various tissues including flower buds, roots, stems, and leaves, but their functions remain to be characterized.

1.8.5 CLAVATA 3: Peptides for determining shoot apical meristem identity

The *Arabidopsis* genes *CLAVATA 1 (CLV 1), CLAVATA 2 (CLV2)* and *CLAVATA 3* (*CLV3*) appear to play important roles in the regulation of shoot meristem development. The *CLV* loci promote the transition towards differentiation of cells in the shoot and

floral meristems or restrict the proliferation of cells at the center of these meristems (Clark *et al.*, 1993; Clark *et al.*, 1995). Loss-of-function mutations in either *CLV1* or *CLV3* cause identical phenotypes, in which stem cells accumulate and there is a progressive enlargement of shoot and floral meristems. Double *clv1-clv3* mutants have the same phenotype, which suggests that *CLV1* and *CLV3* function in a common signaling pathway. *CLV3* encodes a 96-residue peptide containing an N-terminal secretion signal (Fletcher *et al.*, 1999). The protein does not contain a potential dibasic processing site that could be recognized by a processing enzyme, and anti-CLV3 antibodies detect an unprocessed polypeptide in *Arabidopsis* extracts, suggesting a lack of further processing in CLV3 biosynthesis. Both *CLV3* and *CLV1* are expressed in shoot apical meristems, and there is a strong possibility that CLV3 is a ligand for the CLV1 receptor-like kinase. *CLV3* is expressed in the surface L1 and L2 cell layers of the central zone, whereas *CLV1* is expressed in the inner L3 layer of the same zone (Clark *et al.*, 1997). In addition, CLV3 is transported through the secretory pathway and extracellular secretion is required for successfully activating the CLV1/CLV2 heterodimer receptor complex (Rojo *et al.*, 2002). *CLV3* acts as a negative regulator of *WUS* expression, a feedback regulatory loop exists in which *WUS* expression promotes the expression of *CLV3*, which in turn activates a signal transduction pathway that negatively regulates *WUS* expression. This feedback loop is essential for maintaining an optimal balance of stem cells in the SAM. Interestingly, over expression of *CLV3* results in a loss of meristem function in the root apical meristem (RAM) as well as the SAM, indicating that activation of a CLV like signaling pathway may also control cell fate in roots (Hobe *et al.*, 2003), and that the CLV3-like peptide might also be involved in regulating RAM growth.

1.8.6 Rapid Alkalinization Factor

During systemin purification, another peptide with 49 amino acid residues was identified, which causes a rapid alkalinization of the medium, and thus was named rapid alkalinization factor (RALF) (Pearce *et al.*, 2001). Like tobacco systemins (Pearce *et al.*, 2001), RALF induces a MAP kinase activity in the cultured cells. The MAP kinase activity of RALF is also induced much more rapidly than the activities induced by

systemins. Despite the similarities between RALF and the various systemin polypeptides in causing the alkalinization and MAP kinase responses, RALF is much larger than the systemin and does not induce the synthesis of tobacco trypsin inhibitors in leaves when supplied to young tobacco plants, suggesting a signaling role other than defense. RALF sequences possess a highly conserved 17-amino-acid COOH-terminal sequence motif containing two cysteine residues that have been found in many plant species, suggesting an essential role (Pearce *et al.*, 2001; Olsen *et al.*, 2002). Tomato RALF precursor cDNA encodes a 115-amino acid polypeptide containing a signal sequence at its N terminal and the RALF peptide at its C terminal (Pearce *et al.*, 2001). It is not known how mature RALF peptide is produced from its precursor, but a dibasic amino acid motif is located two residues upstream from the N-terminus of mature RALF. When supplied to *Arabidopsis* seedlings, synthetic tomato RALF peptide causes immediate arrest of root growth and slight enlargement of meristem cells. *RALF*-like genes have been identified in many plant species, and are expressed in various tissues including roots, suggesting that the peptide has basic physiological roles in plants other than arrest of root growth (Germain *et al.*, 2005, Pearce *et al.*, 2001). Although *Arabidopsis* has 34 genes encoding RALF-like peptides (Olsen *et al.*, 2002), their fundamental in vivo functions and possible target remain elusive (Matsubayashi and Sakagami, 2006). Because RALF precursor genes form a highly redundant family, it is difficult to perform genetic analysis of RALF signaling using the ligand.

1.8.7 ENOD 40: A Nodulation Factor

The early nodulin gene *ENOD40* is expressed in the nodule primordium when it develops in the root cortex of leguminous plants after infection by symbiotic rhizobia (Compaan *et al.*, 2001). A comparison of *Papilionoideae ENOD40* genes has revealed two conserved regions, I and II, located within the 5' end and the central part of the cDNA, respectively, and up to six domains of conserved RNA secondary structure (Gulyyaev and Roussis, 2007). The legume *ENOD40* genes do not contain conserved coding sequences except for two short ORFs, ORF A and ORF B, which encode small peptides. ORF A of 10–13 codons is located in the conserved region I and partially overlaps ORF B. Gene silencing of two *Medicago truncatula ENOD40* genes, *ENOD40-1* and *ENOD40-2*, demonstrated

that both genes are involved in nodule initiation and bacteroid development (Wan *et al.*, 2007). Orthologues of *ENOD40* have also been found in other nonlegume plants, suggesting roles outside of symbiosis (Kouchi *et al.*, 1999; Compaan *et al.*, 2003; Vleghels *et al.*, 2003; Gulyyaev and Roussis, 2007). Knockdown of *ENOD40* arrests functional nodule development, and its overexpression accelerates nodulation, indicating that this gene plays a central role in nodule development (Charon *et al.*, 1999). However, Overexpression of *ENOD40* causes no apparent aberration of plant growth, suggesting that this gene does not directly trigger cell division, but rather sensitizes cells to division-inducing signals. In rice, expression of *ENOD40* has been detected in stems, especially in parenchyma cells surrounding the protoxylem, suggesting that *ENOD40* plays a role in the development of vascular bundles. These findings indicate that *ENOD40* was originally involved in another plant developmental pathway, and was then recruited into the symbiotic nodulation pathway. The mechanism of *ENOD40* gene activity seems to be of a dual mode: one relying on the encoded short peptide(s) and the second depending on the transcript structure. The growing evidence of the role of plant short peptides supports the hypothesis that either the single *ENOD40* peptide A or both A and B peptides may have biological functions (Barciszewski and Legocki, 1997; Linsey, 2001; Wen *et al.*, 2004). The *G. max* ORF A and ORF B peptides of ENOD40 gene were found to interact with sucrose synthase (Rohrig *et al.*, 2002). ORF A and ORF B peptides antagonize *Zea mays* sucrose synthase phosphorylation, decreasing the efficiency of the enzyme's proteolysis (Hardin *et al.*, 2003). This competition between *ENOD40* peptides and plant-specific calcium-dependent kinase, an enzyme involved in regulation of diverse cellular processes (Klimecka and Muszynska, 2007), points to an *ENOD40* regulatory function. In addition to the above *in vitro* data, *ENOD40* peptide A activity was also confirmed in *Arabidopsis thaliana*, where it inhibited expansion of protoplasts similarly to overexpression of the full-length *ENOD40* gene (Guzzo *et al.*, 2005).

1.8.8 Atrial Natriuretic Peptides: For Regulating Water Homeostasis in Plants

In mammals, the natriuretic peptide (NP) was originally discovered in an extract of rat atria in 1981 (deBold *et al.*, 1981). The atrial natriuretic peptide is encoded by a 152 amino acid precursor protein, the preproANP. Mature ANP is obtained after removal of

signal peptide (proANP) and the cleavage of the propeptide at position 98 and deletion of the two carboxy-terminal amino acids, resulting in the C-terminal ANP peptide (99-126), which is then circularized through the formation of disulfide bond between amino acids 7 and 23 (Germain *et al.*, 2006). The biology of the natriuretic peptide (NP) system is complex, in animal system it regulates salt and water handling, promotes vasodilation and exerts favourable effects on the heart in the context of processes such as heart failure (Martinez-Rumayor *et al.*, 2008).

The first indications for NPs in plants came from radioimmunoassay on Florida beauty (*Dracena godseffiana*) (Versely and Gordano, 1991), where antibodies against the N-terminus (ANP, 1-98), the mid-portion (ANP, 31–67) and the C-terminus (ANP, 99–126) recognized the peptides in leaves and stems. Subsequently, it was demonstrated that synthetic rANP can induce stomatal opening in *Tradescantia* sp. in a concentration dependent manner (Gehring *et al.*, 1996). It was further noted that a synthetic peptide identical to the C-terminus (amino acids 99- 126) of ANP modulates the osmotically induced swelling of potato (*Solanum tubersosum*) mesophyll cell protoplast in a concentration and time dependent manner (Maryani *et al.*, 2001). Also the immunoreactant plant natriuretic peptide hormones (irPNP) have a role in radial water movements from the xylem of *Tradescantia multiflora* stems and hence in water and solute homoeostasis (Suwastika and Gehring, 1998). It is noteworthy that Na^+ is required in the medium for activity in animal systems, which is not the case in plants (Gehring *et al.*, 1996). This suggests that in plants NPs operate on processes other than Na^+ transport, such as K^+ transport or the synthesis of compatible solutes. Natriuretic peptides would represent the first known conserved hormonal system that is shared between animals and plants (Germain *et al.*, 2006). The sequence similarity and homology in the signaling mechanism strongly support that this mechanism, involved in homeostasis regulation, may have evolved before the last common ancestor of the two kingdoms, 1.6 billion years ago (Meyerowitz, 1999).

1.9 SOME RECENTLY DISCOVERED PEPTIDES PARTICIPATED IN PLANT DEVELOPMENTAL PROGRAMME

1.9.1 Polaris

POLARIS (*PLS*) was identified in a promoter trap transgenic line in which reporter gene expression is specially detected in the basal region of the embryo and the root tip (Topping *et al.*, 1994; Topping and Lindsey, 1997). It was later shown that *PLS* is also expressed in the leaf vasculature (Casson *et al.*, 2002). *PLS* encodes a short transcript of approximately 500 nucleotides, which contains a short ORF encoding a peptide with a predicted length of 36 amino acids and a predicted molecular mass of 4.6 kD (Casson *et al.*, 2002). Mutation of the initiation ATG codon of this ORF causes a complete loss of *PLS* gene function, indicating that *PLS* encodes a functional polypeptide rather than a biologically active RNA molecule. The predicted 36-amino acid peptide has no secretion signal, suggesting that it functions in the cytoplasm, although no direct evidence of intracellular localization has been reported (Matsubayashi and Sakagami, 2006). The *PLS* peptide has not yet been biochemically isolated, despite attempts using protein gel blot analysis combined with immunological methods. Physiological studies suggest *PLS* is required for correct cytokinin and ethylene signalling, which modulates root growth (Lindsey, 2001). Mutation in *PLS* results in an enhanced ethylene-response phenotype, defective auxin transport and homeostasis and altered microtubule sensitivity to inhibitors. New findings suggested that *PLS* is an essential component in the regulation of auxin homeostasis and root growth by restricting ethylene signaling (Chilley *et al.*, 2006). *PLS* transcription is activated at the root tip by the relatively high auxin concentration through which it accumulates and attenuates correct cell division at that position (Friml *et al.*, 2002; Blilou *et al.*, 2005). In root, *PLS* acts as negative regulator of ethylene signaling, which is inhibitory to cell division and expansion, and therefore root growth (Souter *et al.*, 2004). *PLS* is also required for correct lateral root initiation, presumably via ethylene-mediated control of auxin transport to the Pericycle (Ooi *et al.*, 2006). More experiments are required for determining the molecular events of auxin and ethylene interaction with *PLS* at root tip.

1.9.2 CLE Peptides

The CLAVATA 3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR) [CLE] peptides contain 12 or 13 amino acids, including hydroxylated proline residues that may or may not contain sugar modifications, and regulate various physiological and developmental processes in a non-cell-autonomous fashion. Recently CLE functional studies have pointed to their significance in governing meristematic activity in shoot or root apex and their signaling pathway are conserved in diverge land plants (Betsuyaku *et al.*, 2011). Overexpression studies and exogenous feeding assays have led to the conclusion that there are two major classes of CLEs (Katsir and Davies, 2011): A-type CLEs can induce termination of the root and/or shoot meristem activity through terminal differentiation of stem cells (Whitford *et al.*, 2008). In contrast, B-type CLEs don't induce termination, instead they can suppress xylem differentiation (Kinoshita *et al.*, 2007), and in *Zinnia*, the homologs of these peptides [TDIF, tracheary element differentiation inhibitory factor] suppress tracheary element differentiation from cultured mesophyll cells (Ito *et al.*, 2006). Some authors suggested that vascular patterning is a process controlled in time and space by different CLE peptides in conjugation with hormonal signaling (Whitford *et al.*, 2008).

1.9.3 EPF1/EPF2

The cysteine-rich EPF family is much smaller than the CLE family, and comprising eleven different sequences are obtained in *Arabidopsis* (Hara *et al.*, 2009). Genetic interaction studies in *Arabidopsis* suggest that LRR-containing receptors mediate EPF signaling (Katsir, 2011). Several EPF family members, including EPF1 and EPF2, function as the negative regulator of stomatal development. Actually stomatal development and density are negatively regulated by membrane proteins, including too many mouth (TMM) and ERECTA family of receptor-like kinases (ER_f) (Shpak, 2005). TMM and ER_f are thought to dimerize and activate in a tissue-specific manner (Yang and Sack, 1995). In this connection, at least two possible ligands of TMM-ER_f complex, epidermal patterning factor EPF1 and EPF2 are negative regulators of stomatal density, as established through extensive bioinformatic analysis (Ohki, 2011). Although EPF1 and EPF2 inhibit stomatal development through common receptors, their consequences on

epidermal patterning are also distinct (Hara *et al.*, 2009). EPF2 is expressed in protodermal cells that have not yet divided and regulates early decision that impact both stomatal and ground cell proliferation. Overexpression of EPF2 inhibits asymmetric division into stomatal lineage whereas loss of EPF2 increases asymmetric division, resulting in increased production of guard cells and neighbouring stomatal ground cell lineages (Hunt and Gray, 2009). In contrast, when EPF1 is over-expressed, protodermal cells divide asymmetrically but the resulting meristemoids don't differentiate further. On the other hand, epf1 mutants have characteristic defects related to incorrect orientation of asymmetric division, resulting in pairs of physically adjacent stomata (Hara *et al.*, 2007). In a nutshell, EPF peptides provide useful paradigms for peptide signaling strategies in epidermal and stomatal guard cell orientation.

1.9.4 Stomagen

Stomagen peptide ligand is a positive regulator of stomatal development and antagonizes the function of EPF1 and EPF2. Stomagen is expressed in the leaf mesophyll layers and over expression or application of chemically synthesized peptide increases both stomatal density and the presence of physically adjacent stomata (Sugano *et al.*, 2010). Like EPF1 and EPF2, STOMAGEN requires TMM to exert its cell fate and cell proliferation-promoting effects (Kondo *et al.*, 2010), suggesting a shared receptor. RNA interference knockdown of STOMAGEN, conversely, results in the appearance of fewer stomata and guard cells (Hunt *et al.*, 2010). TMM is required for stomagen to increase and for EPF1 and EPF2 to decrease stomatal densities, and stomagen didn't increase stomata in the absence of EPF1 and EPF2 (Hara *et al.*, 2009; Hunt and Gray, 2009). Thus all the positive and negative regulators of stomagen act on the putative signal receptor, TMM acts in a competitive fasthion but the over expression of stomagen could induce stomatal clustering abnormally as observed in plants with STOMAGEN under constitutive promoter (Kondo *et al.*, 2010). The external application of stomagen at concentrations as low as 10 nM also enhanced stomatal density fantastically as revealed from several other studies (Ohki *et al.*, 2011). In contrast, unfolded and misfolded stomagen, and stomagen in which Cys41 and Cys43 were replaced by serine, were ineffective, indicating that the conserved cysteine residues and their role in correct refolding are essential for stomagen

to exhibit activity (Pillitteri and Torii, 2012). So conserved cysteines are distinctive features of this family of peptides, and a long loop between these cysteines with an enormous variety in sequence and length could account for the functional diversity of these peptides (Kondo *et al.*, 2010)

1.10 ISOLATION & PURIFICATION OF PEPTIDES

1.10.1 Peptide isolation processes

Strategies and methodology for the isolation of peptides from plant biomass have recently received attention for three main reasons. First, plants containing unique pharmacologically active peptides have been found within natural products-based drug discovery programs (Gustafson *et al.*, 1994). Second, plants, like animals, are now known to make use of peptides as signal substances (Marx, 1996; Bergey *et al.*, 1996). Finally, genetically transformed plants ("transgenic plants") are now considered an attractive and cost-efficient alternative to bioreactor based systems for production of high value recombinant peptides (Whitelam, 2006). An extensive number of biologically important peptides like hormones, neurotransmitters and snake toxins, have been isolated from human and animal sources. Until now, however, only a limited number of polypeptides have been reported from plants. Consequently, numerous methods for the isolation of polypeptides from animal materials are described in the literature, whereas a discussion of procedures for the isolation of polypeptides from plant biomass is virtually lacking. The plant biomass constitutes a highly complex matrix containing many components, e.g., photosynthetic pigments, polysaccharides, tannins, and secondary metabolites that are not present in animal materials. Isolation procedures described for animal polypeptides therefore are generally not directly applicable to the isolation of polypeptides from plant materials.

A fractionation protocol for the isolation of a highly purified peptide fraction from aerial parts of *Viola arvensis* was described by Claeson *et al.*, 1998. The protocol involves pre-extraction with dichloromethane, extraction with ethanol (50%), removal of tannins with polyamide, removal of low-molecular-weight components with size-exclusion chromatography over Sephadex G-10, and final removal of salts and polysaccharides with solid-phase extraction using reversed-phase cartridges. Some of the

popular methods of plant peptide extraction precesses, assigned by different authors are represented in Figure 1.3. It is well known that peptides are not soluble in dichloromethane, but ubiquitous lipophilic substances such as chlorophyll, lipids, and other low-molecular-weight substances (e.g., terpenoids, phenylpropanoids, etc.) are extracted and thus removed. 50% aqueous ethanol has several advantages for isolation of peptides (Claeson *et al.*, 1998). This extract almost eliminates polysaccharide (Engelbrecht, 1969) or enzymes but all known peptides are solubilized. It needs no preservation from microbial growth. But alcoholic extraction always contains ubiquitous polyphenols which can be further removed through polyamine column (Cardellina *et al.*, 1993). In some protocol, urea and thiourea were also added to the peptide extraction medium to weaken the interactions between high molecular mass proteins and low molecular mass peptides and peptides were separated from medium through acetone precipitation (Fukutomi *et al.*, 2005).

It was recently described that antifungal peptides of 3184 Dalton were isolated from seeds of *Amaranthus hypochondriacus* through acidic extraction by 10% acetic acid for one hour (Rivillas-Acevedo and Soriano-García, 2007). Previous reports were also available where peptides were extracted with 5 (N) preheated acetic acid solutions as in the case of peptide isolation from germinating barley grains (Higgins, 1981). Preheated solutions particularly inhibit the function of proteolytic enzymes during extraction but simultaneously there is a chance of loss of bioactivity of thermolabile peptides. That is why the cold aqueous extraction in neutral pH is the most preferential choice for isolation of universal type of peptides.

1.10.2 Purification of Peptides

The least empirical procedures for separating peptides from proteins are dialysis and ultrafiltration with suitable membranes. Earlier authors purified soluble peptides from proteins through physical processes like dialysis, ultrafiltration and heat coagulation (Synge, 1955). In all separation procedures it should be noted that no control is exercised over the possible retention on the protein of smaller non-protein molecules by adsorption, ion-exchange etc. Thus peptides containing residues with basic, acidic, aromatic or higher paraffinic side chains would be expected to be selectively retained on protein, and this

may explain the rather simple amino acid composition of the peptide-like materials detected in ordinary tissues (Synge, 1955). Recently ion-exchange, size exclusion and reverse phase columns have been developed for purification of peptides from other components (Ooi *et al.*, 2006; Mant *et al.*, 2007). Different methods of recently developed column purification techniques suggested by different authors are summerized in Figure 1.4. High Performance Liquid Chromatography (HPLC) is the method of choice for quantification and purification in most cases (Mant *et al.*, 2007). Purified peptides can be obtained from hydrolysates by passing the pepsin and pancreatin digested products through G-50 gel filtration chromatography and C_{18}-based reverse phase HPLC (Megias *et al.*, 2004). Peptides below 10 KDa can also be separated from protein by Tricine-SDS-PAGE (Fukutomi *et al.*, 2005). RP-HPLC based purification and identification of kidney peptides were also achieved by the same authors (Fukutomi *et al.*, 2005). Recently purification of short antimicrobial peptides from earthworm by ultrafiltration, DE-52 ion exchange chromatography, Sephadex G-10 gel permeation and C-18 reversed phase HPLC techniques were documented (Liu *et al.*, 2004). In most of the studies low molecular weight peptides were separated from higher proteins through ultrafiltration with different molecular weight cut-off and the peptides were further purified through ion exchange and gel filtration chromatography in accordance with their charge and molecular size. Ultrafiltration membrane system were used for isolating peptides from the hydrolysates of rice bran (Hamada, 2000) and concentrating peptides in large scale after enzymatic hydrolysis of marine products (Vandanjon *et al.*, 2007). Purification of oligopeptides from lipochitooligosaccharide-induced tobacco cells was achieved by three-step procedure involving gel chromatography, ion exchange and reverse-phase HPLC (John *et al.*, 1997). 3500 Da MW cut-off ultrafiltration and DEAE cellulose ion exchange column DE52 was used for the purification of kiwi fruit peptide 'kissper' from the supernatant obtained upon precipitation of the soluble fraction with trifluoroacetic acid (Ciardiello *et al.*, 2008). LH-20 gel filtration chromatography is particularly useful for the purification of shot peptides below 3 KDa as documented in several journals. Hydrophobic interaction column (Sephadex LH-20) was utilized for the purification of antifungal peptide from the culture filtrate of endophytic bacterium *Paenibacillus* sp. (Senthil-kumar *et al.*, 2007) and *Streptomyces* sp. Strain M10 (Park *et al.*, 2008). Same

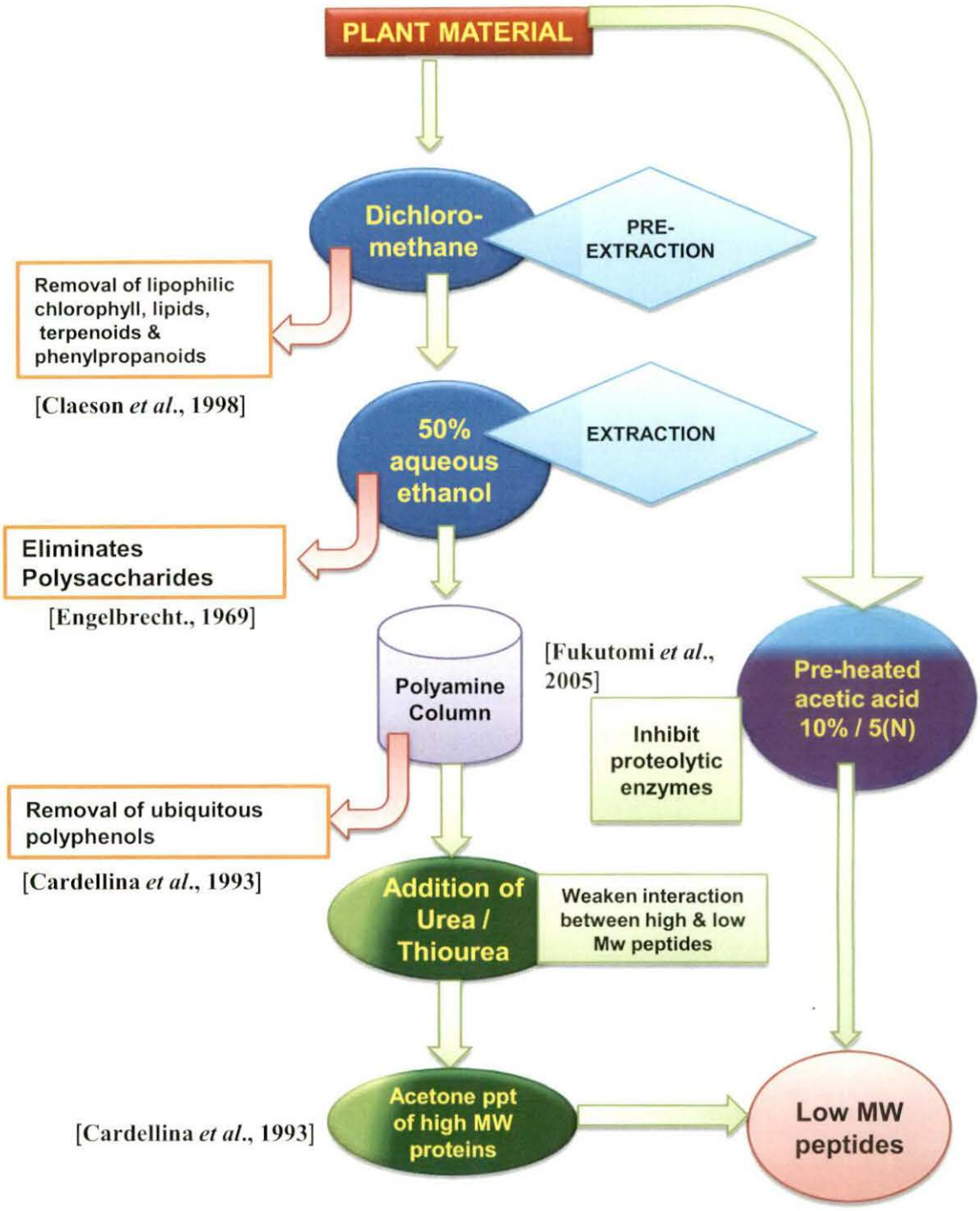

Figure 1.3 Solvent extraction processes of low molecular weight plant peptides

column was also used for isolation of amide linked peptide conjugate of Indole-3-Acetic Acid from *Phaseolus vulgaris* in last century (Blalek and Cohen, 1986).

1.10.3 Detection and Profiling of Peptides

The increasing demand for a functional analysis of gene products in order to understand the underlying physiology has made proteomics a highly valuable technology to produce functionally reliable data. This method is, however restricted to proteins that are bigger than 10 KDa. 2D-Gel Electrophoresis, which is the major tool used in proteomics, fails to analyze small peptides for two major reasons. First, peptides smaller than 10 KDa don't focus well under Isoelectric focusing. Second, the commonly used staining method like Commassie Brilliant Blue and Silver Staining are not efficient in staining small peptides; therefore the use of technologies other than traditional 2D-Gel Electrophoresis was necessary (Rill and Al-Sayah, 2004). Different modern techniques of detection of peptides are diagrammatized in Figure 1.5. But there are some conventional techniques for reliable detection and profiling of peptides, which are discussed below:

1.10.3a *Paper Chromatography and Ninhydrin based detection:*

Electrophoresis and chromatography have proved extremely useful techniques for separation and detection of small peptides prior to amino acid analysis and sequencing. Polarity determines the rate of migration of a peptide during chromatography, whereas both charge and size are the main determinant during electrophoresis (Mayes, 1984). The earliest attempt to combine electrophoresis with partition chromatography on paper was reported in 1948 by Haugaard and Kroner. In later period, the peptides from proteolytic digest were separated and identified by two-dimensional paper chromatography and electrophoresis, a technique of higher resolving power as described by Katz *et al.* (1959). The resulting pattern of peptide spots which were referred to as 'Fingerprints' or 'Peptide Maps' permitted the detection and characterization of peptides of a single amino acid difference (Katz *et al*, 1959). Ninhydrin is the best reliable reagent for identification of peptide spots in paper and thin layer chromatography. Since the first introduction of paper chromatography for the separation and identification of peptides, ninhydrin has been used most exclusively as a spraying reagent to reveal the position of the spots.

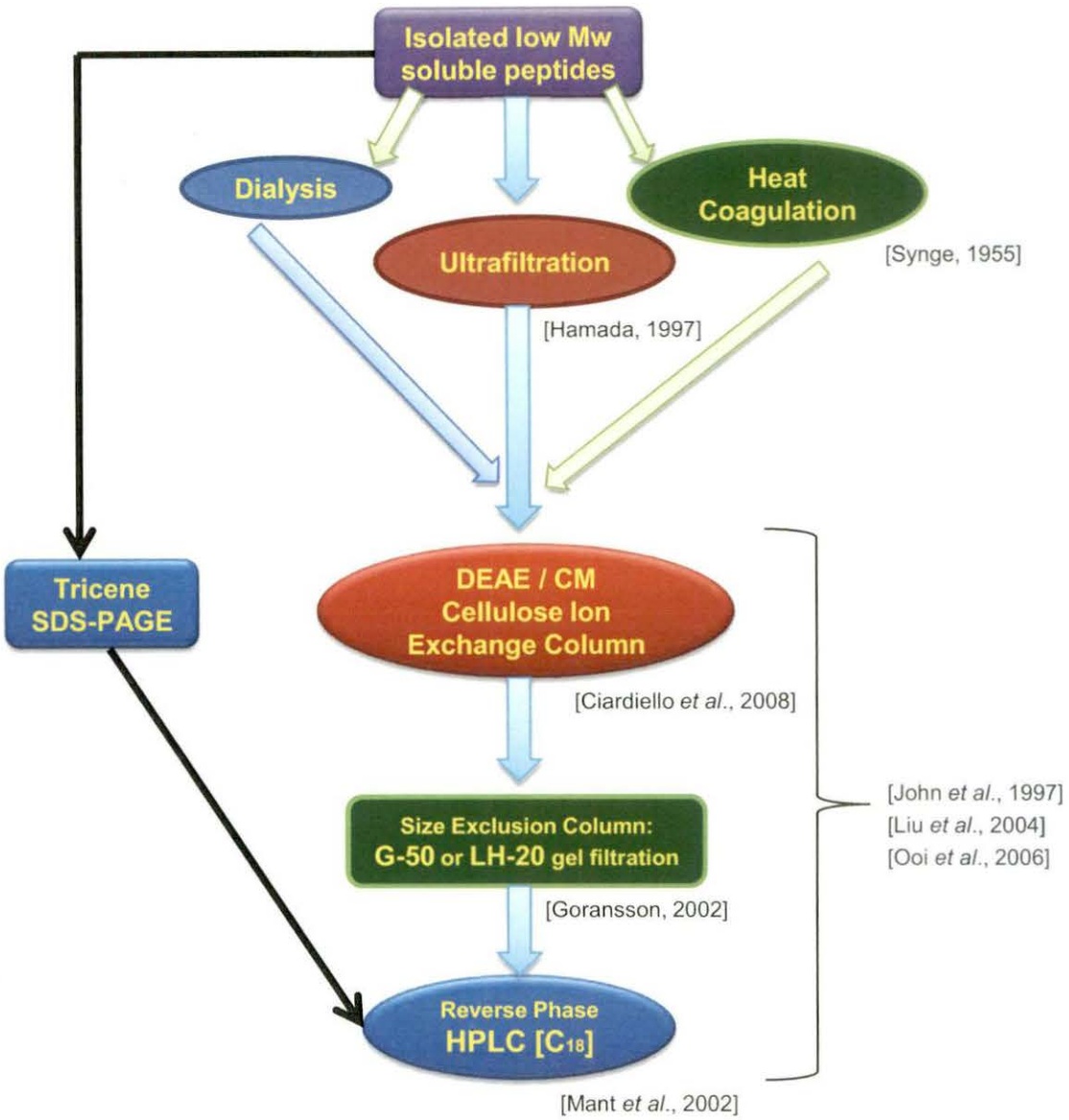

Figure 1.4 Suggested methods for purification of plant peptides

Ninhydrin, however, has two serious limitations; it doesn't reacts with cyclic peptides or with acylated amino acids or peptides, while with linear peptides, since only the terminal amino group enters into the reaction, the colour developed with a given amount of material decreases with increasing molecular weight (Rydon and Smith, 1952; Friedman, 2004). During the past several decades, considerable modifications including different heating times, temperatures, buffer systems, pH values of buffer solutions and solvents for ninhydrin reagents have complicated this method (Sun *et al*, 2005), but till today ninhydrin reagents are used frequently for detection of plant peptides (WenYan *et al.*, 2008). Paper chromatographic methods are also used for identification of peptide antibiotics (Awais *et al.*, 2008). The reliability and resolution of peptide spots on paper and thin layer chromatography are mainly dependent on solvent system and chamber saturation. Earlier authors developed most successful pattern of descending paper chromatography with the solvent mixture of n-Butanol-acetic acid-water (4:1:5) followed by electrophoresis with pyridine acetate buffer at pH 3.7 (Katz *et al.*, 1959). Peptides in the guttation liquid of germinating rice seedlings were also identified by ascending paper chromatography by using same solvent composition [Acetic acid: n-butanol: Water: 4:1:1 v/v] (Horiguchi, 1987). Very recently, Cu(II)-Ninhydrin positive α-peptides present in different spices were detected through circular paper chromatography with the mobile phase of Isopropanol:Water [4:1 v/v] (Nitya and Ramachandramurty, 2007). The technique of circular paper chromatography was also utilized during the last century for the isolation, conformation and estimation of peptides and amino acids in the high molecular weight proteins of marine algal species (Lewis and Gonzalves, 1962).

1.10.3b *Capillary Electrophoresis:*

Capillary electrophoresis is one of the most potential and excellent tool for peptide analysis and fingerprinting. Electrostatic interactions are believed to play a key role in the adsorption of peptides on the capillary wall. The analyte may be attracted to the capillary surface by Coulombic forces and hydrogen bonding with hydrophobic interaction joined in immobilizing the peptides at the surface between the capillary wall and the electrolyte solution (Surugau *et al.* 2008). Since peptides are amphoteric, they are ideally suited for electrophoretic analysis. In capillary zone electrophoresis, separation of neutral species is not possible; therefore it is important to maintain a charge on the peptides. So method

development in capillary zone electrophoresis for peptide identification is primarily focused on buffer composition: pH, ionic strength, the physical properties of buffering ion and addition of additives like detergents, ion pairing reagents, cyclodextrins and soluble polymers (Heiger *et al.*, 2008; Scriba and Psurek, 2008). Several authors recommended that capillary electrophoresis is the method of choice for monitoring of the separation and detection of diversified biologically active plant peptides. Basha (1997) suggested the separation process of peanut peptides extracted from seeds, leaves and cultured cells by capillary electrophoresis. Separation and identification of 18 amino acid polypeptide, Systemin were achieved by capillary electrophoresis and best capillary electrophoretic analysis were obtained in 25 mM phosphate buffer at acidic pH with a constant operating voltage of 30 kV (Mucha *et al.*, 1996). Ginseng polypeptide, isolated from ginseng roots and its modified peptides were determined by capillary zone electrophoresis under acidic and basic conditions by Kajiwara and Hemmings (1998). Peptide mapping were also efficiently performed by capillary zone electrophoresis as documented in case of casein hydrolysates (Macedo *et al.*, 2004).

1.10.3c *Amino acid analysis of peptides:*

Amino acid analysis is a classical analytical technique that characterizes proteins and peptides based on the composition of their constituent amino acids. Profiling and analysis of amino acid is widely applied in research, clinical facilities and industry. It is a fundamental technique in biological research, used to determine the concentration of peptide solutions, to confirm protein binding in antibody conjugates and for N-terminal analysis following enzymatic digestion (Halpine, 2005). But identification and quantification of amino acids in biological matrix and peptide is a challenging analytical task. Usually, the techniques are based on ion exchange separation coupled with post column derivatization (*e.g.* with ninhydrin, the 'classical' method) are considered more precise than those based on pre-column derivatization and reverse-phase high performance liquid chromatography (RP-HPLC), because the later techniques imply extensive sample manipulation before analysis and are affected by limited stability of the performed derivatives (Mengerink *et al.*, 2002). However, such RP-HPLC methods have the advantage of being accessible to most analytical laboratory, since they don't require expensive dedicated instruments. Another advantage of robotic sample derivatization is

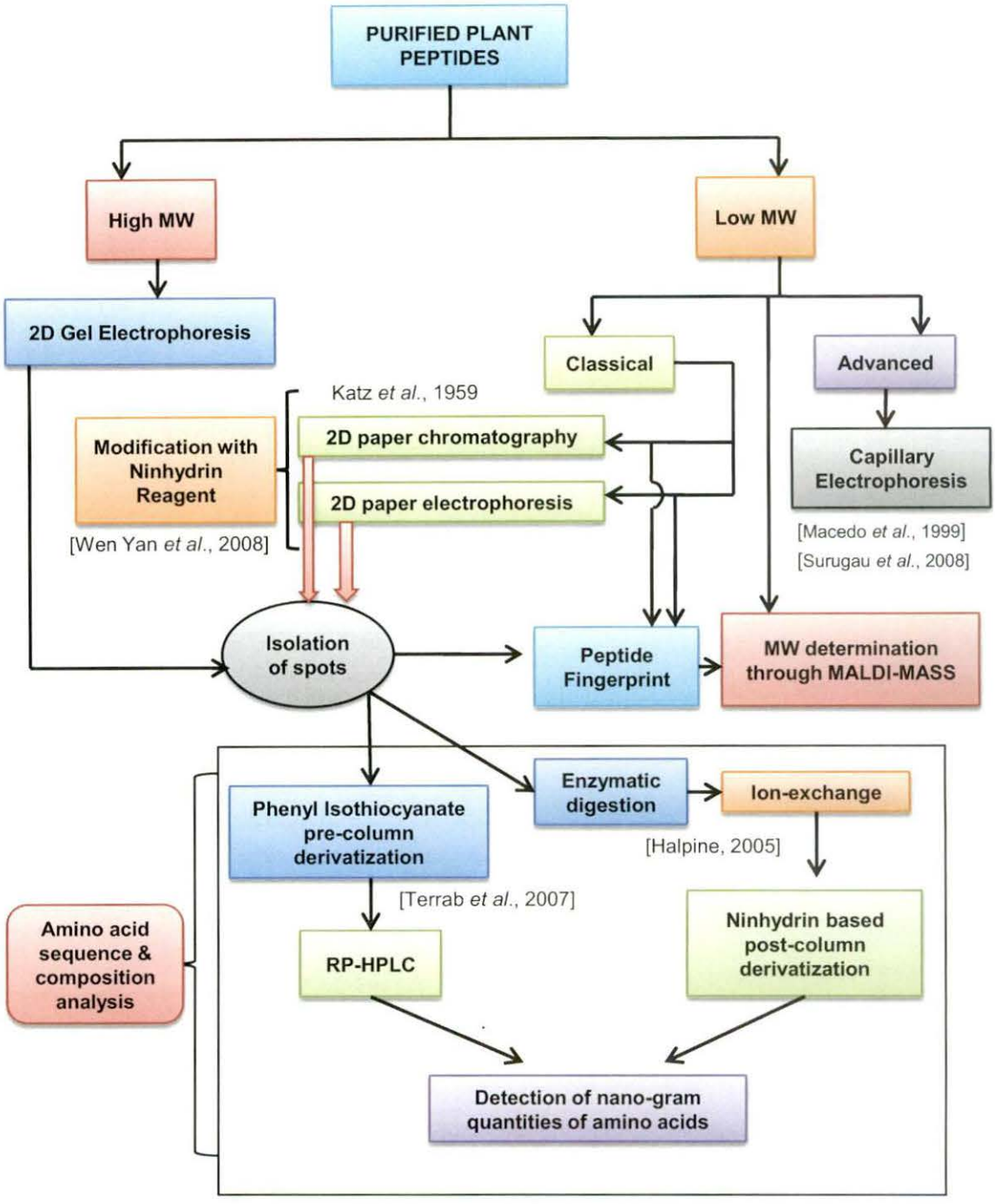

Figure 1.5 Detection of peptides by different instrumental techniques

that it is performed just before injection; therefore the time from reaction to injection is kept absolutely constant for all samples, thus avoiding differential degradation of labile derivatives (Bartolomeo and Maisano, 2006). During the past decades, the two most commonly used pre-column derivatization procedures; phenyl isothiocyanate (PITC) and *o*-Phthalaldehyde (OPA) were employed for the analysis of amino acids. Saunders *et al.* (1988) documented the differential distribution patterns of specific amino acids between the cytoplasm and vacuoles of whole leaf by RP-HPLC based derivatization methods mentioned above. Amino acid determination of raw seeds of *Canavalia ensiformis* were also performed by using HPLC coupled with fluorescence detector (Agbede, 2004). Hydrolysis of plant tissues by refluxing for 24 hours in a heating block at 110°C is the essential pre-requisite before such analysis. Almost same procedures were also followed in case of *Mucuna pruriens* seeds for OPA derivatization and amino acid analysis (Misra and Wagner, 2007). Free amino acids in leaf of cotton plants under water deficit were also evaluated by Marur *et al.* (1994) through RP-HPLC by utilizing o-Phthalaldehyde and 9-Fluorenylmethylchloroformate derivatives. Documents are also available where samples were analyzed for amino acids by RP-HPLC with pre-column PITC derivatization. The PITC derivatization method has the advantage of being highly sensitive, capable of detecting nanogram quantities of amino acids. Using this method eighteen amino acids were quantified in nectar samples collected from *Silene colorata* Poiret [Caryophyllaceae] (Terrab *et al.*, 2007). So the semi-automated method for amino acid derivatization and analysis has been validated by several authors and the validation parameters like specificity, linearity, accuracy, precision, limit of detection and quantification were screened several times (Bartolomeo and Maisano, 2006).

1.11 ROLE OF PEPTIDES IN GERMINATION AND AMYLASE INDUCTION

The amylolytic breakdown of starch is one of the central biochemical events in cereal seed germination. This degradation of starch is initiated by the induction of α-amylase, although the complete breakdown of starch also requires the concerted action of other glycolytic enzymes like β-amylase and de-branching enzymes (O'Neill *et al.*, 1990). Hydrolytic enzymes in seeds mainly act on reserve starch, whereas the degradation of transitory starch in leaves can be hydrolytic and/or phosphorolytic,

catalyzed by starch phosphorylase (Beck and Ziegler, 1989). In germinating cereal grains α-amylases are the most abundant starch degrading enzymes. The enzymes are secreted by aleurone cells into the starchy endosperm where they degrade the starch grains. Signal peptide dependent targeting of α-amylases of plastids and extracellular compartments were observed by expressing fused Green Fluorescence Protein (GFP) in transgenic tobacco or rice cells (Chen *et al.*, 2004). At the germinating stage of cereals, phytohormone gibberellin induces the synthesis and secretion of α-amylase in aleurone layer (Jones and Jacobsen, 1978). Gibberellic acid is *de novo* synthesized exclusively in embryonic organ, particularly in scuteller epithelium and then diffuses to the aleurone cells to induce the synthesis of hydrolytic enzymes at the transcriptional level (Kaneko *et al.*, 2002). In fact, α-amylase induction in cereal aleurone provides one of the best systems for studying hormonally and developmentally regulated gene expression in plants (Davies, 1995). In recent years, a number of components of GA signaling pathway leading to the *de novo* synthesis of α-amylase have been identified and how these factors control the response to GA is becoming clearer (Sasaki *et al.*, 2003; Washio, 2003). Also the compounds that have GA-like effects on cereal aleurone cells have frequently been discussed in literature (Kim *et al.*, 1994). Recently, the mastoparan analog Mas7 was shown to activate the induction of α-amylase in oat (*Avena fatua* L.) aleurone, and the involvement of heterotrimeric G-protein in GA signaling pathway was proven (Jones *et al.*, 1998). Mas7, a cationic amphiphilic tetradecapeptide that stimulate GDP/GTP exchange by heterotrimeric G-Proteins, specifically induced α-amylase gene expression and secretion in a very similar manner to GA_1. Though in rice, sulphuric acid can able to induce α-amylase production in aleurone tissue even in the absence of GA (Mitsunaga *et al.*, 2007), but no reports are available from plant peptides that can able to stimulate GTP/GDP exchange by G-Protein and induce amylases independently.

The biochemistry of germination of dicot plants is somewhat different. During the development of legume seeds on the parent plant, storage proteins are accumulated in protein storage vacuoles, especially in cotyledons. Following germination, the storage proteins are hydrolyzed to free amino acids, which serve as precursor for the synthesis of new proteins and other nitrogen containing compounds in the seedlings. Endopeptidases play key role in storage protein degradation producing oligopeptides. Cysteine

proteinases (CPs) [EC 3.4.22] are the major endopeptidases present in the cotyledons during early seedling growth and are assumed to be largely responsible for the mobilization of storage proteins (Shutov and Vaintraub, 1987; Muntz, 1996). Two types of cysteine proteinases, low-specificity enzymes for the papine family and Asn-specific from the legumain family are generally considered to be the major Endopeptidases responsible for degradation of seed storage proteins during early seedling growth (Zakharov *et al.*, 2004). But the oligopeptides that are produced by the action of endoproteinases generally provide nutrition to growing seedlings. Besides mastoparan like peptides and gibberellins, other molecules like sodium hypochlorite (NaOCl) has been reported to induce the production of α-amylase protein in mungbean cotyledon detached from embryonic axes (Kaneko et *al.*, 2002). It is known in dicots that detached cotyledons have reduced α-amylase activity as the axes are necessary to increase α-amylase activity following germination, however, NaOCl treatment can restore activity upto 70% of that seen in attached cotyledon. The detail mechanism has not been documented in all these cases and the entities of receptors of gibberellin like molecules on plasma membrane of dicot plant have not yet been confirmed.

In higher plants three families of proteins have been recognized to transport small peptides, the oligopeptides transporters (OPTS), the peptide transporter (PTR) and ATP binding cassettes [ABC Superfamily]. The first unambiguous demonstration of a pool of peptides in plant tissues described the presence of milimolar concentrations of small peptides in the endosperm of germinating cereal grains (Higgins and Payne, 1981). Here they serve as a supply of nutrients transported across the scutellum to support growth of the cereal embryo during the early stage of germination. Subsequently it is became apparent that small peptides and their transporter also play a significant role in control of plant cell differentiation and organogenesis in addition to the nutritional role (Yang *et al.*, 2000; Stacey *et al.*, 2002). The oligopeptide transporter (OPT) family can transport tetra- and pentapeptides whereas the peptide transporter (PTR) family can transport di- and tripeptide (Koh *et al.*, 2002). An important distinction between the two families is the much more selective nature of OPTs for peptides for only a certain amino acid composition and compared with the low selectivity of so far characterized di-/tripeptide transporters of the PTR family. Plant peptides like Phytosulfokines (PSKs), which are

sulfated tetra-/pentapeptide with mitogenic activity, are transported by specific peptide transporters. Although transduction of their mitogenic signal involves interaction of PSKs with an integral plasma membrane receptor (Yang *et al.*, 2000) during germination, but this role has yet to be verified. Given the metabolic complexity of plants there is great potential for (oligo)-peptides and their transporters to play a number of roles in plant physiology. During germination roles for plant PTRs and OPTs in host-pathogen interactions (Taylor *et al.*, 1972; Dietrich *et al.*, 2004), responses to mechanical stress of wounding (Karim *et al.*, 2005) and translocation of a number of molecules including plant hormone and metal ions around the plant in the form of peptide conjugates have also been suggested (Waterworth and Bray, 2006). Besides OPT and PTR families of transporters, plant ABC proteins are primary pumps, which use the energy of ATP hydrolysis to drive the transport of three peptides or peptide conjugates (Theodolou, 2000). *Arabidopsis* PTRs and OPTs appear to play a key role in acquisition of nitrogen, iron and phosphorus from the environment and subsequent transport around the growing seedlings (Steiner *et al.*, 1994). Recent exciting development in metabolomic analysis would yet reveal a more prominent role for peptides in nutrient redistribution and/or signaling in plants during germination and post-germination events.

1.12 PEPTIDES CONTROLLING CELL DIVISION

1.12.1 Cell Cycle and its regulation

Cell division plays a crucial role during all phases of plant development. The molecular analysis of cell division and its regulation in plants lags far behind such studies in yeast (*Saccharomyces cerevisiae*) and animals. Since the cell theory was proposed by Schleiden and Schwann in 1838, many approaches have been taken to elucidate how cells divide, but insight into the molecular basis of cell cycle control was initiated in yeast 25 years ago (Hartwell *et al.*, 1970). In plants as in all eukaryotes, the four basic phases of mitotic cell cycle are conserved. In addition to the coupled cycle where DNA replication (S phase) is followed by G_2 and M phase and hence give rise to daughter cells, alternative cycles also occur in certain developmental situations as in the case of endosperm development (Huntley and Murray, 1999). The cell cycles are regulated at multiple points, but major controls operate at the G_1-S and G_2-M phase boundary. These

transitions represent the onset of DNA replication and mitosis respectively. At the molecular level, cell cycle transitions in all period are controlled by specific type of serine-threonine protein kinases known as Cyclin Dependent Kinases (CDKs). In yeasts there is a single CDK involved in central cell cycle control known as Cdc28 in budding condition (Mendenhall and Hodge, 1998). Multiple cyclins are present in yeasts and the association of different cyclins with the CDK creates different kinase specificity. The timing of these activities during cell cycle is also controlled by cyclin association and cyclins are transcribed only during specific time windows and are also highly unstable proteins whose destruction is controlled in a cell-cycle dependent manner (Inze, 2000). Genome wide expression analyses of eukaryotic cells have enabled the identification of hundreds of the eukaryotic, cell cycle modulated genes, including those from plants (Spellman *et al.*, 1998; Cho *et al.*, 2001; Breyne *et al*, 2002). In addition, the completion of genomic sequence of *Arabidopsis* allowed searching for the core cell cycle regulatory genes based on their sequence homology (Vandepoele *et al.*, 2002). In the *Arabidopsis* genome, 61 core cell-cycle regulatory genes were identified based on their sequence similarities (Vandepoele *et al.*, 2002).

The plant cyclins represent the main classes of A, B and D type cyclins that are divided into three sub-groups of A_1, A_2, A_3, B_1, B_2 and D_1 to D_7 (Renaudin *et al.*, 1998; Vandepoele *et al.*, 2002). Although the genome sequence alone doesn't reveal the functions of the genes (Murray and Marks, 2001) current scientific knowledge indicates that cell cycle regulation is well conserved among eukaryotes (Mironov *et al.*, 1999). Similarity to mammalian cyclins, the A type cyclins are induced during late S phase while the B type cyclins are specific for G_2 to M phase. Like multicellular animal system, the plant D type cyclins are involved in cell cycle activation at the G1 to S transition phase (Himanen, 2003). In the D-type cyclins, so called PEST sequence were identified, which are involved in their rapid turn over at the end of G_1 phase (Soni *et al.*, 1995; Renaudin *et al.*, 1998). The G_1 to S specific D type cyclin of plants appear to be more divergent structure than their mammalian counterparts and it has been proposed that different members of D type cyclins may alternate in their binding to Cyclin Dependent Kinase A, reflecting plant specific regulation during G1 to S transition (Meijer and Murray, 2000). The sessile growth habit demands plants to respond continuously to the

flow of signal from the growth environment and D type cyclins may play a role in mediating these responses.

Although higher eukaryotes share the same theme of G_1-specific CDK activity and the ultimate role of transcription S-phase entry, the other proteins involved are not homologues used in yeast. The components present in both animals and plants CycDs, whose associated kinase activity is targeted to the Rb protein, resulting in the activation of E2F transcription factors (Oakenfull *et al.*, 2002). CycDs are the rate limiting components of cell cycle during the progression through G_1. Their expression in mammals is under the control of external signals such as serum growth factors, and they therefore are responsible for triggering the cell cycle in response to such mitogenic signals (Sherr, 1993). Similarly in plants, CycD levels and activity respond to signals such as hormones and carbohydrate levels are important in influencing decision by plant cells. In *Drosophila* and mammals CDK partner CycD is a variant CDK known as CDK4. However, there is no evidence for a CDK4 homologue in plants, and indeed there is now substantial evidence that the CDKA class is the partners of the plant CycDs in the control of G_1-S transition. A further significant difference between plant and animal CycDs is found in their substrate specificity. Human CycD kinases phosphorylate only the Rb protein, and histone H1 is a very poor substrate for CDK4-Cyclin D. Plant CDKA-CycD kinases phosphorylate histone H1 both *in vitro* and as immunoprecipitated from plant extract (Cockcroft *et al.*, 2000; Healy *et al.*, 2001).

Majority of the plant CycDs don't show strong cell cycle dependent mRNA regulation but may show tissue-specific expression. CycD1 is expressed at low or undetectable levels in liquid cultured cells, whereas CycD2 and D3 have been shown to have constant levels of mRNA from the day one of the growth cycle to early stationary phase on day seven (Riou-Khamlichi *et al.*, 2000). This indicates that the expression of the cyclins is dependent on active cell division or the cells being in a particular part of growth cycle. However, CycD3 mRNA levels were found to be strongly dependent on the continued presence of carbon source in the medium (Healy *et al.*, 2001; Riou-Khamlichi *et al.*, 2000).

Plant shape is elaborated by developmental signals regulating the time and orientation of cell division and cell enlargement. The mitogen activated protein kinases

(MAPKs) are utilized in eukaryotes to transient diverged stimuli such as mitogens, developmental cues or various stresses (Robinson and Cobb, 1997). They are integrated into a signalling molecule composed of three linked protein kinases: the MAPK, the MAPK-activating kinase (MEK) and the MEK-activating kinase (MEKK) (Madhani and Fink, 1998). MAPKs are required to reenter the cell cycle from both G1 and G2 phases. MAPKs stimulated by growth factors exert their effects on the cell cycle by influencing G1 specific cyclin expression (Lavoie *et al.*, 1996). A MAPK pathway is utilized at two points during mitotic cell division in *Xenopus*: to start oocyte maturation and meiosis-II from G_2-arrested oocytes and to arrest cell cycle progression in a metaphase-like state at the end of meiosis-II (Sagata, 1997). Bogre *et al.* (1999) reported that *Medicago sativa* MAP Kinase-3 (MMK3) protein could be found during all stages of cell cycle but its protein kinase activity was transient in mitosis and correlated with the timing of phragmoplast formation. Intact microtubules are required for MAPK activation because depolarization of microtubules abolished the activity. Plant MAPKs are responsible for the regulation of cytokinesis in cell cycle because MAPKs were found to be concentrated between the segregating chromosomes and localized at the mid-plane of cells in later stage of division (Bogre *et al.*, 1999).

1.12.2 Role of peptides in Cell Cycle Regulation

Although various hormones and growth factors in animal system are polypeptide, none of the previously known plant growth regulators is peptide in nature. However, the existence of peptidyl plant hormones has recently been indicated by the isolation of systemin that can initiate signal transduction to regulate the synthesis of different proteins from tomato (Ryan and Moura, 2002). Plant cells in low density suspension culture usually display strictly low mitotic activity which can be improved by supplementation with known plant hormones or defined nutrients. However, proliferation was activated by addition of conditioned medium from rapidly growing cells, suggesting that secreted mitogenic factors exist. Various efforts have been made to characterize such factors in different culture systems during the last few decades. To promote cellular growth at low cell population several researchers have successfully used specialized culture techniques such as nurse cultures, in which target cells are grown close to but physically separated from

high density nurse cells, suggesting that cell to cell communication mediated by a chemical factor is involved in cell growth (Raveh *et al.*, 1973). Matsubayashi and Sakagami (1996) observed that diluting mechanically dispersed *Asparagus* mesophyll cells in excess culture medium significantly reduces the rate of callus formation, even if sufficient amount of growth regulator and nutrients are supplied. The active factor was purified from the conditioned medium and identified as a sulfated peptide composed of only five amino acids. Due to the presence of sulfate esters, the peptide was named as 'Phytosulfokine' (PSK). Chemically synthesized PSK induces cellular dedifferentiation and proliferation of dispersed *Asparagus* mesophyll cells even at nanomolar concentrations. Identical structure of PSK was later identified and derived from cell lines of many plants like rice (Matsubayashi *et al.*, 1997), maize (Matsubayashi *et al.*, 1997), *Zinnia* (Matsubayashi *et al.*, 1999), carrot (Hanai *et al.*, 2000) and *Arabidopsis* (Yang *et al.*, 2001) indicating that it is widely distributed among the higher plants. Six genes encoding PSK (AtPSK 1-6) have been identified in *Arabidopsis* each encodes a preproprotein precursor of approximately 80 residues with N-terminal secretion signal (Yang *et al.*, 1999) and the YIYTQ peptide near C-termini (Matsubayashi and Sakagami, 2006).

The PSK binding protein was purified from microsomal fractions of carrot cells by different solubilization and ligand-based affinity chromatography using PSK Sepharose column (Matsubayashi *et al.*, 2002). Based on the internal sequence of PSK binding protein, the 120-150 KDa protein was identified as LRR2-RLK derived from a single gene (Matsubayashi and Sakagami,2006). Sequence information from the carrot protein (DcPSKR1) was used to identify an ortholog in *Arabidopsis*, AtPSKR1. AtPSKR1 over-expressing plants showed delayed senescence, and, as a result, leaves continued to expand, resulting in larger leaves than the wild-type.

Besides phytosulfokines, other low molecular weight peptides are also involved in cell cycle regulation, cell proliferation and differentiation. Among them, role of CLV3, CLE and RALF peptides are considered very important. Recent studies demonstrated that a family of plant-specific genes, CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR) (CLE), which has at least 31 members in *Arabidopsis* genome, are able to generate extracellular peptides to regulate cell division and differentiation (Fiers *et al.*,

2007). Plants maintain pool of totipotent stem cells throughout their entire life. These stem cells are embedded within specialized tissues called meristem which forms the growing point of the organisms. It was suggested that non-autonomous signalling between the stem cell pool and the organizing centre is responsible for homeostasis of stem cell number (Brand *et al.*, 2000; Schoof *et al.*, 2000; Reddy and Meyerowitz, 2005). Fundamental to this mechanism is the negative feedback regulation between the homeodomain transcription factor WUSCHEL (WUS) and the short secreted peptide CLV3. WUS is expressed in organizing center and is essential for the maintenance of stem cell fate and expression of CLV3 (Schoof *et al.*, 2000). CLV3 in turn is secreted by stem cells and acts as a non-cell autonomous signal to repress WUS expression in the organizing centre via a complex signaling pathway (Rojo *et al.*, 2002).

Like CLV3, CLE family of dodecapeptides of *Arabidopsis thaliana* suppresses xylem cell development at a concentration of 10 picoM and promotes cell division (Ito *et al.*, 2006). A-type of CLE peptides promote cell differentiation in root and shoot apical meristem, whereas B-type peptides inhibit the differentiation of tracheary elements as observed in *Zinnia elegans*. The B-type CLE41 peptide promoted proliferation of vascular cells, although differentiation was delayed in phloem and xylem cell lineages. However, in combination of both A- and B-type CLE, massive proliferation of vascular cells was observed. This proliferation relied on auxin signaling because it was enhanced by exogenous application of synthetic auxin, decreased by an auxin polar transport inhibitor, and abolished by a mutation in the 'MONOPTEROS' auxin response factor (Whitford *et al.*, 2008). CLE peptides probably interact with membrane-bound, leucine rich repeat receptor-like kinases (LRR-RLKs) to execute the decision between cell proliferation and differentiation (Fiers *et al.*, 2007).

1.13 STOMATAL GUARD CELL REGULATION

Stomata are pores formed by a pair of specialized guard cells, which exist in the surface of aerial parts of higher plants. The most conspicuous role of stomata is the regulation of transpiration and photosynthesis. It also increases the uptake of carbon dioxide for photosynthesis (Willmer 1983). However, if water supplies are limited the plant's priority changes from maximizing assimilation to restricting transpiration, while

maintaining as much assimilation as possible (Mansfield *et al.* 1990). In their simplest design stomata are small, permanently open pores; in more advanced designs they are hydraulically operated valves whose openings are adjustable depending on specific demands (Ziegler 1987).

Stomatal guard cells respond to a range of stimuli, causing changes in cell turgor and stomatal pore, due to fluxes of cations and anions across membranes (Ward *et al.*, 1995; Willmer and Fricker, 1996). For the majority of signals, the molecular identity of the sensors of guard cells is not known, with the notable exception of blue light: the phototropins PHOT1 and PHOT2 were shown to be the blue light receptors in *Arabidopsis* guard cells (Kinoshita *et al.*, 2001). Much evidence supports a role for calcium both in the promotion of stomatal closing and in the inhibition of stomatal opening (Finkelstein, 2006). Calcium ion influx across the plasma membrane of guard cells was increased in response to plant hormone abscisic acid (Staxen *et al.,* 1996) and auxin (Irving *et al.*, 1992), other stimuli such as elevated CO_2 (Webb *et al.*, 1996) and oxidative stress (McAinsh *et al.*, 1996), as detected through calcium imaging and patch clamp analysis. In stomatal guard cells, *myo*-inositol-triphosphate (IP_3) is another important component in stimulus response coupling pathways (Leckie *et al.*, 1998). IP_3 can able to activate inward K^+ channels, whilst at the same time activating an inward current that depolarizes the plasma membrane (Blatt *et al.*, 1990). The inhibition of inward K^+ channels by IP_3 is indirect and is probably mediated by Ca^{2+} (Blatt *et al.*, 1990) in a process that may involve protein phosphatase activity (MacRobbie, 1997).

Stomatal opening is driven by plasma membrane proton extruding H^+-ATPases. H^+-ATPases can drive K^+ uptake via K^+_{in} channels (Kwak *et al.*, 2001). A 14-3-3 protein has been found to bind to the phosphorylated C-terminus of the guard cell H^+-ATPases. The 14-3-3 protein seems to dissociate from H^+-ATPase upon dephosphorylation of the C-terminal domain. Recent studies have shown that guard cell phototropins are phosphorylated by blue light and that they bind the 14-3-3 protein upon phosphorylation (Kinoshita *et al.*, 2003).

1.13.1 Role of Peptides in Stomatal Opening and Closing

In recent years, researchers have concentrated on the mechanism of rhythmic stomatal movement induced by environmental stresses (Allen *et al.*, 2000; Pei *et al.*, 2000; Yang *et al.*, 2003). Stomatal opening is associated with net influx of K^+ and the plasmalemma and either influx of chloride or synthesis of malate in the cytoplasm, whereas stomatal closing follows net efflux of K^+ and anions from guard cells (Thiel *et al.*, 1992; Roelfsema *et al.*, 2001). Evidences shows that cytosolic calcium may also play a central and primary role in stomatal movement and changes in endogenous calcium regulate the stomatal guard cell behaviour (Allen *et al.*, 1999 and 2000; Pei *et al.*, 2000; Yang *et al.*, 2003). Through molecular genetic analysis it was demonstrated that ABA-induced H_2O_2 production and H_2O_2-activated Ca^{2+} channels were important mechanism for ABA-induced stomatal oscillation (Pei et al., 2000; Staxen *et al.*, 1999). Very recently Zhang *et al.* (2009) reported that there is a positive correlation between ABA-sensing and stomatal closure in *Arabidopsis thaliana.* Among peptide hormones, plant natriuretic peptide immunoanalogues (irPNP) have been shown to affect a number of biological processes including stomatal guard cell movement, ion fluxes and osmoticum-dependent water transport. Evidence has been obtained which suggest that these proteins have a function in modulating plant water and solutes homeostasis (Gehring, 1999). Exogenous application of plant natriuretic peptide (PNP) stimulate stomatal opening (Billington *et al.*, 1997; Maryani *et al.*, 2001) and activates H^+-ATPase pumping system (Maryani *et al.*, 2001). Moreover, irPNP rapidly and specifically induces the transient elevation of cGMP levels in maize root in stele tissues (Pharmawati *et al.*, 1998) and stomatal guard cell protoplast (Pharmawati *et al.*, 2001). Stomata opened by irPNP are induced to close in the presence of guanylate cyclase inhibitor, LY 83583 (Wang *et al.*, 2007). These findings suggest the presence of PNP receptors that contain guanylate cyclase domains. Such domains have been identified in the receptors NPR-A and NPR-B for ANP in vertebrates (Chinkers *et al.*, 1989). Maryani *et al.*, (2001) also observed the enhancement of osmoticum dependent volume changes in leaf mesophyll protoplasts through the application of natriuretic peptides. The effect of cGMP on stomatal opening appears to be linked with Ca^{2+} levels. ANP, irPNP and 8-Br-cGMP all induce stomatal opening and this is inhibited by compounds that lower intracellular Ca^{2+} levels such as ethylene glycol

bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), ruthenium red and procaine (Pharmawati *et al.*, 2001). The immuno-reactants (irPNP) directly or indirectly modulate net ion fluxes across plant membranes leading to rapid net influx of H^+ and delayed net influx of Na^+ and K^+ in maize stellar tissues (Pharmawati *et al.*, 1999). Antibody mediated tissue printing revealed that immunoreactive peptides were concentrated in vascular tissues of leaves, petioles and stems. Phloem associated cells, xylem cells and parenchymatic xylem cells showed the strongest immunoreaction (Maryani *et al.*, 2001). *In silico* analysis has established the evolutionary and functional relationship of irPNP-like molecules within the superfamily of expansins, pollen allergens and distantly related molecules such as endoglucanases (Ludidi *et al.*, 2002; Gehring and Irving, 2003).

Besides natriuretic peptides another secretory peptide gene of *Arabidopsis thaliana*, EPIDERMAL PATTERNING FACTOR 1 (EPF1) enforces the asymmetric cell division during the development of stomatal precursors and controls stomatal patterning. EPF1 activity was dependent on the TOO MANY MOUTHS receptor like proteins and ERECTA family receptor kinase, suggested that EPF1 may provide positional cell interpreted by these receptor (Hara *et al.*, 2007).

1.13.2 Stomatal Behaviour during Senescence

Senescence is a normal, energy-dependent developmental process that is controlled by the plants' own genetic programme (Taiz and Zeiger, 2006). The senescence syndrome in plants has been studied from various view point and by using several types of experimental material: seeds, leaves, flowers, fruits and whole plants. In leaves, the disappearance of chlorophyll and the hydrolysis of leaf proteins are the two most frequently observed criteria of senescence (Thimann and Satler, 1979). It is known that stomatal closing induces senescence (Thimann and Salter, 1979) while treatments allowing stomata to stay open delay this process which was proved for *Hibiscus* cutting where open stomata enabled escape of internal ethylene from leaf tissues (Kirk *et al.*, 1986). There is, however, little or no evidence for the implication of ethylene in the senescence of leaves. Stomatal apertures regulation is associated with senescence programme as stomatal conductance is the main mechanism by which plant controls gaseous exchange and leaf temperature (Jones, 1998). Phytohormones like ABA and

cytokinins which affect stomatal closure and opening respectively can also able to control senescence. ABA induced stomatal closure were examined in *Arabidopsis* wild-type plants and in an ethylene over-producing mutant (*eto*-1-1). Using isolated epidermal peels, stomata of wild-type plants were found to close within a few minutes in response to ABA, whereas stomata of the *eto*-1-1 mutant showed a similar but less sensitive ABA response. In addition, ABA-induced stomatal closure could be inhibited by the application of ethylene or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid [ACC] (Tanaka *et al.*, 2005). It was observed that ethylene delays stomatal closure by inhibiting the ABA signaling pathway. The same inhibitory effects of ethylene on stomatal closure were observed in ABA-irrigated plants and the plants in drought condition (Tanaka *et al.*, 2005). When the detached leaves of *Brassica,* Capsicum and *Richinus* were exposed to stress, continuous increase in ethylene content was found (Aharoni, 1978) but decrease in ethylene production was observed in *Gerbera jamesonii* (Olivella *et al.*, 1998). Also it was documented that stomatal closure was not always regulated by ethylene as in the case of drought-exposed *Rosmarinus officinalis* leaves (Munne-Bosch *et al.*, 2002).

Cytokinins are often considered as ABA antagonists in many processes including stomatal opening, but the effects are species specific and depend on cytokinin-type, concentration and method of application (Pospisilova, 2003). On the other hand, the standard preservative solution (8-hydroxyquinoline citrate and 2% sucrose) caused closing of stomata in both species and that is why both are considered as 'anti-transpirants' (De Stigter, 1981). They are often included in the commercial preservative for cut-flower to diminish transpirational losses and maintain flower turgidity (Skutnik and Lukaszewska, 2001). Paradoxically, their effects on leaves were mostly reported as negative (Skutnik *et al.*, 2001) and was also confirmed in plant species of *Hosta* and *Zantedeschia* (Wachowicz *et al.*, 2006). The preservatives hasten leaf senescence not only by accelerating chlorophyll degradation and proteolysis (Skutnik *et al.*, 2004) but also by decreasing stomatal aperture (Wachowicz *et al.*, 2006).

1.14 ROLE OF PEPTIDES IN CALLOGENESIS, MORPHOGENESIS AND DIFFERENTIATION

1.14.1 Peptides regulating Callogenesis

The commercial use of plant tissue culture involves the production of large number of plantlets from embryogenic calli with minimum input expenses. The main factors which determine growth, development and micropropagation of plants *in vitro* are: the genetic configuration of plant species, the physical environment and the chemical media for *in vitro* culture. One of the earliest reports was published by Overbeek *et al.* (1941), who succeeded in growing immature *Datura* embryos in culture by including the liquid endosperm of *Cocos nucifera* (coconut milk) in their culture medium. Coconut milk was shown to stimulate cell division in other cultivated tissues and its use as a supplement was adopted in many laboratories (Archibald, 1954; Wiggans, 1954). Other complex plant juices and liquid endosperms have been shown to possess stimulatory property more or less similar to those of coconut milk. These include liquid endosperm from immature corn (Netien *et al.*, 1951), tomato juice (Straus and La Rue, 1954), immature fruits and seeds (Steward and Shantz, 1959), organic juice, malt extract, casein hydrolysates, leaf extracts, sap from a number of plants and tumour extracts (Butenko, 1968).

Great interest has always been attached to the identification of the active constituents of natural fluid used in tissue culture medium. Straus (1960) has shown that tomato juice, yeast extract or casein hydrolysates function by supplying a form of organic nitrogen to *in vitro* cultured explants. Besides nutritional supplements these natural fluids may also contain growth promoting and callogenic substances, most of which were analyzed in recent decades.

In contrast to animal cells a high proportion of plant cells, even when fully differentiated, can dedifferentiate and proliferate *in vitro* as totipotent stem cells called calli, following treatment with plant hormones such as auxin and cytokinin. Callus cells differentiate into various organs, which eventually form a new plant, indicating that plant cells from a given tissue can differentiate into cells of all tissue type (Skoog and Miller, 1957). The relative rate of dedifferentiation and callus growth *in vitro*, however, strictly depends on the initial cell density, even if sufficient amount of auxin and cytokinins are

supplied. Cellular dedifferentiation and callus formation significantly suppressed at low initial cell density. Interestingly, this suppression is elevated by addition of conditioned medium in which cells have previously been grown at high density (Jorgensen *et al.*, 1992; Folling *et al.*, 1995).

Phytosulfokine (PSK), a five amino acid sulfated peptide that has been detected in conditioned medium of plant cell cultures is the primary signal molecule responsible for cell-to-cell communication (Matsubayashi and Sakagami, 1996). Addition of chemically synthesized PSK to culture medium even at nanomolar concentration significantly increases the rate of callus growth even when the initial cell population is below the critical density. Sulfated tyrosine residues are often found in secreted peptides in animals (Huttner, 1982) but, to date, PSK is the only example of post-translational sulfation of tyrosine residues in plants. PSK with an identical structure is present in conditioned medium derived from many plant cell lines, including dicotyledon and monocotyledon, which indicates that the peptide is widely distributed in higher plants. PSK also stimulates tracheary element differentiation of *Zinnia* mesophyll cells without intervening cell division (Matsubayashi *et al.*, 1999) and stimulates somatic embryogenesis in carrot (Kobayashi *et al.*, 1999). Such cellular dedifferentiation and redifferentiation, however, cannot be induced by PSK alone, but require in addition to certain ratios and concentrations of auxin and cytokinins (Matsubayashi, 2003).

In *Arabidopsis thaliana*, phytosulfokines (AtPSK) are derived from larger prepropeptide precursors by a specific subtilisin serine protease (Srivastava *et al.*, 2008). Each of the AtPSK precursors has specific subtilase recognition site signature (dibasic amino acid) 8-10 residues upstream from the mature peptide (Barr, 1991). High levels of Subtilase expression may not have anything to do directly with shoot recognition. However, higher levels of subtilase expression might promote the proliferation of callus from which shoots are derived. Similar reasoning was used by Hanai *et al.*, (2000) to explain the stimulatory effects of PSKs on somatic embryo formation in carrot. They concluded that PSKs might promote the proliferation of cells giving rise to somatic embryos, rather than influencing the formation of somatic embryos.

1.14.2 Role of peptides in morphogenesis and meristem differentiation

Since the identification of systemin, secretory and non-secretory peptides have been shown to regulate various aspects of plant growth and development (Matsubayashi and Sakagami, 2006). Several small secretory peptides encoded by short open reading frame are involved in the morphogenesis of multicellular organisms (Hashimoto *et al.*, 2008). Recent studies demonstrated that a family of plant-specific genes, CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR) (CLE), which has at least 31 members in *Arabidopsis* genome, can able to generate 26 extracellular CLE peptides which regulate cell division and differentiation (Sawa *et al.*, 2006). A hydroxyl 12-amino acid peptide derived from the conserved CLE motif of CLV3 promotes cell differentiation, whereas another CLE-derived peptide suppresses the differentiation. CLV3 type of processed peptides of CLE ligand family is particularly required for meristem maintenance and performs through CLV1/CLV2 receptor mediated signalling system (Trotochaud *et al.*, 1999; Jun *et al.*, 2008).The peptide ligand family INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) is responsible for floral abscission and the IDA mediated regulation of floral abscission through HAESA (HAE) AND HAESA-LIKE (HSL) receptors was established recently (Stenvik *et al.*, 2008). Intriguingly, both IDA and CLV3 depend on closely related receptor-like kinases (RLKs) for their functional identity (Stenvik *et al.*, 2008). Another 23 amino acid peptide ligand AtPep1 induces the response of defence genes in the innate immune response and, when exogenously applied, induces cell alkalinization (Huffaker *et al.*, 2006). Mature AtPep1 is found at the C-terminal conserved part of PROPEP1, one of the six members of the PROPEP family in *Arabidopsis*. AtPep1 activates the expression of defense genes PDF1.2 (encoding defensin) and its own precursor gene PROPEP through the Jasmonate/Ethylene signaling pathway. The elicitation of this defensin was blocked in mutant plants deficient in Jasmonate/Ethylene and Salicylate pathways, and in wild-type by treatment with diphenylene iodonium chloride, an inhibitor of hydrogen peroxide production. Probably the defence peptide like systemin and defensin modulate signaling through Jasmonate and Salicylate pathways with the production of H_2O_2 as toxic intermediate (Huffaker and Ryan, 2006).

Leaf shape of a plant is determined by polar cell expansion and proliferation along the leaf axes. In *Arabidopsis thaliana*, ROTUNDIFOLIA (ROT4) gene controls polar cell proliferation in lateral organs and regulates proximal-distal development of leaf. The ROT4 open-reading frame encodes a novel small peptide that was not recognized previously in the *Arabidopsis* genome annotation. Plants having dominant mutant of these genes possess short leaves and floral organs. Phylogenetic analysis indicates that ROT4 defines a novel seed plant-specific family of small peptide in *Arabidopsis* named ROT4 LIKE (RTFL) (Narita, 2004). Loss-of-function mutations in several RTFL genes were aphenotypic, suggesting that there may be some functional redundancy between family members.

Root growth and development angiosperm is also controlled by 15-amino acid peptide ligand C-TERMINALLY ENCODED PEPTIDE 1 (CEP1). CEP1 is mainly expressed in lateral root primordia and when overexpressed or externally applied, significantly arrests root growth (Ohyama *et al.*, 2008). So this peptide was capable of repressing cell division potential in the root apical meristem. Again the rate and plane of cell division and anisotropic cell growth are critical for root development. In *Arabidopsis thaliana*, 36-amino acid peptide POLARIS (PLS) is required for correct root growth and vascular development. According to Chilley *et al.* (2006), PLS expression is repressed by ethylene and induced by auxin. Mutation in PLS results in an enhanced ethylene-response phenotype, defective auxin transport and homeostasis and cultured microtubule sensitivity to inhibitors (Chilley *et al.*, 2006).

There are several methods that can be used to dissect components of a signaling pathway, including (i) bioassays, where synthetic peptide ligands are used to screen for mutated insensitive receptors, (ii) genetic suppressor and activation screens and (iii) ligand fishing. But unfortunately, the sequences of only a few classes of peptide signals in plants are known and only a few plant peptide ligand-receptor pairs have been documented. Even less is known about the downstream effectors of peptide-based signalling, although there are indications of the involvement of MAPK cascades similar to those found in animals (Cho *et al*, 2008). How different signalling inputs are integrated by a cell and how they elicit responses in different situation remains a central problem for understanding the responses of plants in developmental cues.

1.15 PEPTIDES AND REGULATION OF OXIDATIVE STRESS

1.15.1 Glutathione mediated control of Oxidative Stress

The ultra-low molecular weight peptide glutathione (GSH: γ-glitamylcysteinyl glycine) plays a crucial role in plant metabolism and stress response. It is an abundant and ubiquitous thiol and plays significant roles in the storage and transport of reduced sulphur, the synthesis of proteins and nucleic acids and as a modulator of enzyme activity (May *et al.*, 1998). The reduced form of glutathione (GSH) is considered to protect the cells from oxidative damage, based on its redox buffering action and abundance in the cell (Ogawa, 2005). The level of glutathione has also been shown to correlate with the adaptation of plants to extremes of temperature, in the tolerance of plants to xenobiotics and to biotic and abiotic environmental stresses. In addition, the size of reduced glutathione pool shows marked alterations in response to environmental conditions (May *et al.*, 1998). Actually the balance between the reduced (GSH) and oxidized (GSSG) forms of these tripeptides play a fundamental role in basic physiological and metabolic processes in plants. Recently a remarkable amount of evidence has explained the glutathione associated events in plants, in particular, growth and development including cell differentiation, cell death and senescence, pathogen resistance, and enzymatic regulation. One of the major themes that have emerged from *in vitro* studies is that GSH promotes cell proliferations while GSSG promotes organized development. Thus *in vitro* plant regeneration can be manipulated by the application of this redox compound in culture medium (Yeung *et al.*, 2005). The rate-limiting steps in glutathione biosynthesis is catalyzed by γ-glutaminyl cysteine synthatase, the activity of which is tightly regulated at different stages. The enzyme encodes the regulatory mechanism based on redox-sensitive disulfide bridges. *In vitro* analysis suggests a link between high cellular glutathione levels and associated down regulation of its biosynthesis by this enzyme in plant stress defence mechanism.

In plant cells protection against peroxidation is achieved by several antioxidants like vitamin-E and C or glutathione (Finckh and Kunert, 1985). The lipophilic vitamin E, however observed to be the most effective radical chain-breaking substances. It has to be reductively regenerated by water-soluble GSH either directly or via a system consisting

of GSH and water-soluble vitamin-C (McCay, 1985). To maintain a high level of active GSH GSSG has to be rapidly reduced. This reaction is catalyzed by the enzyme glutathione reductase in the presence of NADPH. Increased production of antioxidant and elevated activity of antioxidative enzymes like glutathione reductase seems to be a general strategy to improve tolerance against toxic oxidations. Enhanced level of GSH prevented lipid peroxidation in higher plants (Schmidt and Kunert, 1986). Cells also possess a range of peroxidases which includes glutathione and ascorbate peroxidases to counter oxidative stress and photo-damage generated during photosynthesis (Moon *et al.*, 2002). In mammals and also in Solanaceous family of angiosperm, phospholipids hydroperoxide glutathione peroxidases were found to be associated with both soluble and membrane fractions, that reduces lipid hydroperoxide (Jung *et al.*, 2002). Phospholipid hydroperoxides are key intermediate in the lipid peroxidation chain reaction, one of the major types of oxidative damage in cells associated with membrane perturbation, inactivation of membrane proteins and cell lysis. Thus, this peroxidase is an important cellular enzyme capable of halting membrane lipid peroxidation and oxidative damage (Chen *et al.*, 2004).

1.15.2 Peptides as molecular alphabets of abiotic stress tolerance

During growth and development, a plant has to cope with a range of different internal and external stresses. The ability to adapt to metabolic and environmental changes is essential for survival of growing seedlings. The production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O^{\cdot}{}_2^-$) and its more toxic derivatives, hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$), is increased when plants are exposed to various biotic and abiotic stresses (Asada, 1994; Dat *et al.*, 2000). Abiotic stress negatively influences survival, biomass production and accumulation, and grain yield of most crops (Khanna *et al.*, 1998; Grover *et al.*, 1998). Different crop ecosystems are affected by different abiotic stress factors, and to a differential extent (Khan *et al.*, 2007).

Plants have a number of different defence mechanisms by which they respond to oxidative stress. These include the production of both non-enzymatic antioxidants such as ascorbate and glutathione and enzymatic antioxidants such as catalase, superoxide dismutase and ascorbate peroxidase. Plant peptides have a definite role in abiotic stress

tolerance. In winter wheat, it was observed that small defensin peptides (γ-thionin, γ-purothionin etc.) and non-specific lipid transfer proteins (LTPs) were induced during low temperature hardening. Regulation of defensin transcripts was clearly different from that of LTPs under controlled environments. The γ-thionin and γ-purothionin transcripts were not expressed in unhardened plants grown at 20° C and strongly upregulated after 1-3 days hardened at 2° C; whereas the LTP transcripts were constitutively expressed in plants growing at 20° C and gradually increased to maximum levels following 14-28 days after hardening (Gaudet *et al.*, 2003). Generally defensin related peptides confer resistance to snow moulds and other fungi but it was observed that γ-thionin expression was also associated with freezing resistance among different genotypes of winter wheat (Gaudet *et al.*, 2003). LTPs are a family of peptides capable of moving various kinds of lipid molecules *in vitro* and *in vivo* (Kadar, 1996). But LTPs have now revealed to be involved in many other biological functions. Non-specific LTPs are sub-divided into two multigene subfamilies that differ in molecular mass: ns-LTP1 (9 kDa) and ns-LPT2 (7 kDa) (Stanislava, 2007). Because an LTP has a signal peptide indicative of a secretory protein, and is observed mainly in the cell walls and cuticle, the primary role of plant LTP could be the assembly of cutin and was in the surface layers (Cameron *et al.*, 2006). LTP may also be responsive to environmental stresses, including salt, drought, abscisic acid (ABA) and cold treatment (Hong *et al.*, 2001; Yubero-Serrano *et al.*, 2003; Wu *et al.*, 2007). The expression of LTPs genes is developmentally and spatially regulated (Kader, 1996). Organ and tissue specificity show a high level of diversity in different species. In some species, the expression is differentially regulated by pathogens, defense-related signal molecules, abiotic and environmental stresses (Wu *et al.*, 2007). LTP1 is found primarily in aerial organs, whereas LTP2 is expressed in roots. Both peptides are located in the aleurone layer of cereal grain endosperm (Capocchi *et al.*, 2005). LTP1 is an abundant grain protein, while LTP2 represents a minor fraction. They are secreted protein with a hydrophobic N-terminal sequence and localization is restricted to peripheral cells, such as the epidermis of leaves or the aleurone layers (Douliez *et al.*, 2000). Recent data suggests that they also may have intracellular localization in protein storage vacuoles and lipid containing vesicles (Dani *et al.*, 2005).

1.16 ROLE OF PEPTIDES IN HEAVY METAL STRESS TOLERANCE

1.16.1 Detoxification of heavy metals with peptides

Heavy metals whose densities exceed 5g / cm^3 (Elmsley, 2001), come in two varieties- those that are and those that are not essential to organisms. Essential heavy metals, such as copper and zinc, are required as cofactors in redox reactions and ligand interactions, as well as for charge stabilization, charge shielding, and water ionization during biocatalysis (Elmsley, 2001; Voet and Voet, 2004). Nonessential heavy metals, such as arsenic, cadmium, lead and mercury, are not required as cofactors, but instead interfere with those that are and/or stimulate the effects of supraoptimal levels of essential heavy metals (Rea *et al.*, 2004). Plants respond to heavy metal toxicity in a variety of different ways. Such responses include immobilization, exclusion, chelation and compartmentalization of the metal ions, and the expression of more general stress response mechanisms such as ethylene and stress proteins (Cobbett, 2000). One recurrent general mechanism for heavy metal detoxification in plant and other organisms is the chelation of the metal a ligand and, in some cases, a subsequent compartmentalization of the ligand-metal complex. Plants make two types of peptide metal-binding ligands; metallothionins (MTs) and Phytochelatins (PCs). MTs were identified as cadmium binding proteins in mammalian tissues (Kagi, 1991). After the structures of PCs had been elucidated, it was proposed that PCs were the functional equivalent of MTs (Grill *et al.*, 1987). Actually PCs have now been classified as class-III MTs which play relatively independent functions in respect to other PCs during metal detoxification and/or metabolism (Cobbett, 2000). PCs are mostly active for detoxification of cadmium like heavy metals. They are structurally related to glutathione (GSH: γ-Glu-Cys-Gly) and were presumed to be the product of same biosynthetic path. A number of structural variants of PCs for example, $(\gamma\text{-Glu-Cys})_n$-β-Ala, $(\gamma\text{-Glu-Cys})_n$-Ser, and $(\gamma\text{-Glu-Cys})_n$-Gln were identified in different plant species (Rauser, 1995 and 1999). PC-Cd complexes are sequestered to the vacuoles by an efficient catalytic enzyme named Phytochelatin Synthase. PCs can also form complexes with Pb, Ag and Hg *in vitro* (Mehra *et al.*, 1996; Rauser, 1999). PCs were induced to varying levels by a wide range of metal ions. The most effective metals appeared to be Ag, Arsenate, Cd, Cu, Hg and Pb ions. However the only PCs complexes identified *in vitro* were with Cd, Ag and Cu ions. The clearest evidence for the role of PCs in heavy

metal detoxification comes from characterization of PC-synthase deficient mutants of *Arabidopsis* and fission yeast. A comparison of a relative sensitivity of *Arabidopsis* and fission yeast mutants to different heavy metals revealed a similar but not identical pattern (Ha *et al.*, 1999). In both organisms PCs appear to play an important role in Cd and arsenate detoxification and no apparent role in the detoxification of Zn, Ni and selenite ions (Cobbett, 2000). Two types of PC-heavy metal complexes were particularly involved in vacuolar sequestration: [1] High M_r (HMW) and [2] Low M_r (LMW). The transport and pumping of heavy metals through phytochelatin are ATP dependent and mostly linked with proton antiporter system (Oritz *et al.*, 1995). In *Brassica napus*, during Cd- exposure, high ratios of [PCs]/[Cd] and [Glutathione]/[Cd] in the phloem sap were observed whereas, only traces of PCs were detected in the xylem sap (Mendoza-Cozatl, 2008). These results suggest that phloem is the major vascular system for long distance source to sink transport of Cd as PC-Cd and Glutathione-Cd complexes. In case of water hyacinth (*Eichhornia crassipes*) phytochelatin PC_3 and PC_4 are primarily found in HMW-Cd complex. So thiol peptides are also served as an efficient and reliable heavy metal chelator in aquatic hyper-accumulators (Jian et al., 2008). But the mercury tolerance strategies of the plant like salt march (*Halinione portulacoides*) are somewhat different. In this plant metal resistance is achieved through immobilization of metals in root cell wall rather than metal chelation in the cytosolic fraction. Nevertheless phytochelatins were demonstrated to chelate mercury under environmental exposure (Valega *et al.*, 2009).

1.16.2 Peptide transporter and their role in heavy metal storage and transport

Plants, being rooted in soil, are unable to escape toxicity resulting from the presence of heavy metals in the soil. But there are a small number of terrestrial plant species that not only can tolerate high levels of toxic heavy metals in the soil but also can accumulate those metals to unusually high levels in their shoot biomass. These fascinating plant species, first coined hyperaccumulators by Brooks *et al.* (1977), are loosely categorized as plants that can accumulate metals in the shoot from 100- to 1000- fold higher than normal, nonaccumulator plants (McGrath *et al.*, 2002). Hyperaccumulating plant species have been identified for a number of heavy metals, including nickel (Ni), zinc (Zn) and

cadmium (Cd), as well as for the metalloids selenium and arsenic. One of the distinctive hallmarks of metal hyperaccumulators are their ability to translocated efficiently most of the absorbed metals from the root to the shoot. A second hallmark of this hyperaccumulator is the extreme metal tolerance, which is exhibited both in roots and shoots. Mechanism of metal tolerance can involve both ion transporters that transport the metal out of the cytoplasm (either into an internal compartment or out of the cell), and the synthesis of metal-binding ligands that can detoxify the metals in the cytoplasm (Clemens, 2001).

Plants have developed homeostatic mechanisms to ensure appropriate concentrations of transition metals and to minimize the damage from exposure to toxic heavy metals. This homeostasis requires a regulated network of transport, mobilization and sequestration processes to maintain uptake, allocation and detoxification of metal ions. Two common mechanisms are known that plants utilize to eliminate excess metal ions from the cytoplasm; efflux into the apoplastic space and compartmentalization (Kim *et al.*, 2006). Vacuole of the plant cells are the main storage compartments for toxic metals. The known proteins that mediate transport and detoxification of heavy metals in plants belong to the following families: the cation diffusion facilitator (*CDF*) family (Blaudez *et al.*, 2003), the heavy metal transporting P_{1B}-ATPases (*HMA*) family (Hammond *et al.*, 2006), the natural resistance associated macrophage protein (*NRAMP*) family (Weber *et al.*, 2004) and the *ABC* family (Lee *et al.*, 2005). *CDF* family of transporters regulates cytoplasmic efflux of transitional metals like zinc, cobalt, cadmium and nickel. They are also called metal tolerance proteins (*MTP*) (Talke *et al.*, 2006). *CDF* family of proteins is highly expressed in hyperaccumulator plants like *Arabidopsis halleri* and *Thlaspi caerulescens*. Higher transcript levels of MTP genes during heavy metal exposure suggest a role in adjustment of metal homeostasis in the hyperaccumulator plants (van de Mortel *et al.*, 2006). The *HMA* family members are involved in pumping cations across the membranes and out of the cytoplasm using hydrolyzed ATP as an energy source. In *Arabidopsis thaliana*, *HMA2* and *HMA4* regulate the mobilization of zinc from root-to-shoot *via* xylem loading and are thought to be plasma-membrane localized. It may be possible that *HMA4* causes cadmium hyper-accumulation and contributes to cadmium tolerance by mediating cytoplasmic cadmium efflux in root and

leaf cells (Verret *et al.*, 2004). The *NRAMP* gene family also plays a role in metal homeostasis. Identification and characterization of transporters in metal homeostasis has shed light on possible entry pathways into the plant and the mechanism of metal tolerance for toxic metals such as cadmium and lead. Heavy metal treatment of plants induces the transcription of many genes (Weber *et al.*, 2004). However, the mechanism by which the heavy metal is sensed and the downstream signaling pathways leading to gene induction have yet to be elucidated.

Phytochelatin (PCs) mediated detoxification of heavy metals is well known. A putative phytochelatin transporter (CeHMT-1) is recently discovered in *C. elegans* (Vatamaniuk *et al.*, 2005). To date, the identification of an *Arabidopsis* gene(s) encoding the vacuolar transporter for PCs has not been reported. But phytochelatins are small peptides, and completion of the *Arabidopsis* genome sequence has revealed a number of potential peptide transporter families for phytochelatins. Peptide transporters have been placed into two groups based on their energy source: (1) the oligopeptide transporter (OPT) and peptide transporters (PTR) families which use proton-motive force, and (2) ATP-binding cassette (ABC-type) transporters which use ATP hydrolysis as an energy source. A MgATP-energized transport pathway for PCs and PC- Cd^{2+} complexes, analogous to ABC-type transporter, has been characterized in vacuolar membrane vesicles isolated from oat roots (Salt and Rauser, 1995). Recently the *Arabidopsis* ABC-type transporter AtPDR12 was shown to contribute to lead resistance by serving to exclude lead and/or lead containing compounds (Lee *et al.*, 2005). Not only in *Arabidopsis*, but also the peptide transporters play vital roles in tolerance and sequestration of heavy metals in almost all angiosperm families.

1.16.3 Seed priming and mechanism of oxidative stress tolerance

Rapid and uniform field emergence is the essential prerequisite to improve yield, quality and ultimately profits in annual crops. Uneven or poor germination and subsequently non-homogeneous seedling growth can lead to great financial losses, by e.g., reduced possibilities for mechanization, or lower prices of non-homogeneous plant batches (Ghiyasi *et al.*, 2008). One pragmatic approach to increase crop production is seed invigoration (Farooq *et al.*, 2006). Seed invigoration strategies include hydropriming,

osmopriming, osmohardening, elicitor based hardening, hormonal-priming, matripriming and others (Kao *et al.*, 2005; Windauer *et al.*, 2007). The invigoration persists under oxidative stress conditions like salinity (Abdul Jaleel *et al.*, 2007), heavy metal toxicity, temperature extremes (Wahid and Sabbir, 2005), hypoxia (Ruan *et al.*, 2002) and drought (Du and Tuong, 2002). Pre-sowing seed treatments (seed priming) can also enhance germination index and establishment in many crops like maize, wheat and rice (Ghiyasi *et al.*, 2008b).

The three early phases of germination are: (i) imbibition, (ii) lag phase, and (iii) protrusion of the radicle through the testa (Simon, 1984). Priming is a procedure that particularly hydrates seed, so that germination processes begin, but radicle emergence doesn't occur. There are reports that hydration of seeds up to, but not exceeding, the lag phase with priming permits early DNA replication (Bray *et al.*, 1989), increased RNA and protein synthesis (Fu *et al.*, 1988), greater ATP availability (Mazor *et al.*, 1984), faster embryo growth (Dahal *et al.*, 1990), repair of deteriorated seed parts (Karssen *et al.*, 1989; Saha *et al.*, 1990), and reduced leakage of metabolites (Styer and Cantliffe, 1983) compared with checks. Priming of wheat seed in osmoticum or water may improve germination and emergence (Ashraf and Abu-Shakra, 1978) and promote vigorous root growth (Carceller and Soriano, 1972) under low soil water potential. Osmotica that have shown good potential to enhance germination, emergence, growth, and/or grain yield of crop plants include solutions of potassium hydrogen phosphate (KH_2PO_4) monobasic (Das and Choudhury, 1996), polyethylene glycol (PEG) (Dell'Aquila and Taranto, 1986) and potassium chloride (KCl) (Misra and Dwibedi, 1980). Water has also been used successfully as a seed priming agent for wheat improvement (Harris *et al.*, 2001). Seed pretreatment with hydrogen peroxide (H_2O_2) can also improve stress tolerance due to its biological activity against oxidative stress. When maize seeds were pretreated with hydrogen peroxide solution, they exhibited enhanced heat tolerance, α-amylase activity and solute sugar contents and decreased H_2O_2 production, solute-leakage and malondialdehyde (MDA) (Wahid *et al.*, 2008). Pretreatment also improved net photosynthesis and vegetative growth and decreased membrane permeability under oxidative stress in some dicot plants. Advancement of radicle meristem cells into the S and G_2 phases of cell cycle and replicative DNA synthesis has been reported to occur

during priming (Gurushinghe *et al.*, 1999). It was observed that the changes in metabolite levels are important events during seed priming (Wahid *et al.*, 2008). As revealed from microarray studies, seed protein synthesis is a global phenomenon that initiates the up- or down-regulation of a number of germination related genes (Gallardo *et al.*, 2001; Soeda *et al.*, 2005). Natural or artificial seed priming induces the mobilization and solubilization of globulins and the synthesis of late embryogenesis abundant proteins (Capron *et al.*, 2000; Gamboa-deBuen *et al.*, 2006). Antioxidant enzymes-including superoxide dismutase, catalase, and glutathione reductase -were also expressed during seed priming, through which oxidative stress can be manipulated by plants during seedling stage (Bailly *et al.*, 2000). Among other pre-germination metabolic changes, seed priming decreased the level of malondialdehyde (Bailly *et al.*, 1998, 2000), changed saturated and unsaturated fatty acids (Walters *et al.*, 2005), and induced α-amylase to increase the soluble sugar pool, thus improving seedling emergence and other related attributes (Mwale *et al.*, 2003; Farooq *et al.*, 2006).

1.16.4 Peptide Mediated Seed Priming

Seed priming (osmoconditioning) increases vigour as well as accelerate germination, improve stress resistance and enhance plant growth and productivity (Pattan *et al.*, 2001; Burguieres *et al.*, 2006), but priming agents may differ greatly in their effectiveness. It was observed that priming-induced improvements in germination and seedling growth were associated with *de novo* protein synthesis, low molecular weight peptide accumulation, membrane repair mechanisms and a greater availability of germination substrates which resulted in a rapid and energetic start (Mwale *et al.*, 2003). From the metabolic changes during priming it is plausible that the priming treatments reprogrammed the gene expression for antioxidant synthesis (including peptide antioxidants) and mobilized germination substrates in greater amounts (Wahid *et al.*, 2007). It was already stated thiol peptides are mainly responsible for oxidative stress tolerance. Antioxidant thiol peptide is regarded as one of the major determinants of cellular redox homeostasis. Glutathione and Metallothionins are involved in the ascorbate / glutathione cycle and in the regulation of protein thiol – disulphide redox status of plants in response to abiotic and biotic stress (Mullineaux and Rausch, 2005). But very

few efforts are made for judging the efficiency of peptides as an inducer of stress tolerance. In recent times, it was established that seed priming with reduced glutathione mitigated the oxidative salt stress experienced by different cultivars of canola (*Brassica napus* L.) (Kattab, 2007). Seed priming with reduced glutathione improve seedling resistance probably by increasing the activities of antioxidant enzymes like superoxide dismutase, polyphenol peroxidase and oxidase as well as ascorbate peroxidase and oxidase (Khattab, 2007), but more scientific investigations are required to elucidate the mechanism of stress tolerance on the gene level.

Efforts were also made for stimulating the enzymes of phenyl propanoid pathway (PPP) in mung bean sprouts through pentose phosphate and shikimate pathways by natural peptide elicitors derived from fish protein hydrolysates (FPH) (Randhir *et al.*, 2004). Fish hydrolysate derived peptides also enhanced the antioxidant activity in the elicited fenugreek sprouts as measured through the scavenging of free-radical like 1,1-diphenyl-2-picrylhydrazyl (DPPH) and inhibition of β-carotene bleaching (Randhir *et al.*, 2004). During elicitation, it was detected that the increased activity of antioxidants were also correlated with high guaiacol peroxidase (GPX) activity indicating that polymerizing phenolics required during lignification with growth have antioxidant function (Randhir *et al.*, 2004). Both in the dark and light germinated sprouts of corn, higher glucose-6-phosphate dehydrogenase (G6PDH) activity was observed only in fish protein hydrolysate primed treatments during early germination possibly due to the carbohydrate mobilization from the cotyledons directed towards the high nutrient requirements of the growing sprout (Randhir and Shetty, 2005). Very recently, Randhir *et al.*, (2009) also improved the pharmacological properties of *Mucuna pruriens* sprouts by priming the seeds with peptide elicitors of fish protein hydrolysates. After elicitation with peptides, Parkinson's disease relevant L-DOPA concentration was enhanced in *Mucuna* seedlings; whereas anti-diabetes relevant α-amylase and α-glucosidase inhibition percent were high in the cotyledons and decreased following elicitation and sprouting (Randhir *et al.*, 2009). Peptides are indispensable elements during germination, growth and development of sprouts as revealed from the expression of different peptide transporters both in monocots and dicots during different stages of their growth and development (Waterworth *et al*, 2005; Komarova *et al.*, 2008). Peptide transporters also play a critical role during seed

development (Song *et al.*, 1997). All these facts particularly explain the biological significance of peptide priming through which the metabolic circuits can be manipulated for developing stress tolerance.

1.16.5 Peptide expression under heavy metal stress

Many organisms respond to cytotoxic effects of heavy metals by synthesizing metal-chelating proteins or peptides. The predominant class of such molecules in plant, algae, and some fungi are the small, cysteine-rich peptides referred to as phytochelatins, Cd^{2+} binding peptides, cadystins or γ-glutamyl peptides (Howe and Merchant, 1992). These molecules have the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (Steffens *et al.*, 1986), where n can range from 2 to 11 depending on the species from which the peptides are isolated and the conditions of their induction (Grill *et al.*, 1987). A wealth of biochemical evidence from experiments conducted *in vivo* supports a model in which phytochelatins are synthesized via a post-translationally activated, metal-dependent, enzymatic pathways from precursor GSH (Scheller *et al.*, 1987). The synthesis of phytochelatin from glutathione (Grill *et al.* 1989), homo-glutathione, hydroxymethyl-glutathione (Klapheck *et al.*, 1995) or γ-glutamylcysteine (Hayashi *et al.*, 1991) is catalyzed by a transpeptidase, named phytochelatin synthase, which is a constitutive enzyme requiring post-translational activation by heavy metals (De Knecht *et al.*, 1995; Chen *et al.*, 1995). Phytochelatin synthase (PCS) has been shown to be activated by a broad range of metals and metalloids, in particular Cd, Ag, Pb, Cu, Hg, Zn, Sn, Au and As both *in vivo* and *in vitro* (Maitani *et al.*, 1996). But phytochelatin-mediated detoxification might not be the most effective strategy to cope with toxic exposure to heavy metals in hyper-tolerant plants. Phytochelatin synthase genes seems to have been lost in a number of animals and fungal lineages, possibly as a consequence of the evolution of more effective and more specific matallothionein-based metal sequestration systems (Schat *et al.*, 2002). Phytochelatins are enzymatically synthesized peptides whereas metallothioneins are gene-encoded polypeptides (Cobbett and Goldsbrough, 2002). The completion of the *Arabidopsis* genome sequence has allowed the identification of entire suite of metallothionein genes in higher plants. Expression of type-IV metallothionein is restricted to developing seeds. Type-IV metallothionein genes contain promoter sequences with homology to ABA-

response elements and their expression is regulated by ABA (White and Rivin, 1995). Type-III metallothioneins are expressed at high levels in the fruits like banana, apple and kiwi, as they ripen (Reid and Ross, 1997). Metallothioneins are mainly associated with copper homeostasis as revealed from reporter genes of *Arabidopsis* metallothionein promoters (Himelblau *et al.*, 1998).

But the ubiquitous occurrence of phytochelatin throughout the plant kingdom is still enigmatic. Somehow the phytochelatins are involved in metal micronutrient homeostasis under non-toxic physiological conditions, particularly because plants exposed to normal nutritional micronutrients appear to contain phytochelatin at low, but detectable concentrations (Schat *et al*, 2002). The manipulation of phytochelatin and metallothionein expression is one of the potential mechanisms for understanding the capacity of plants for phytoremediation (Maity *et al.*, 2005). Tissue-specific expression of genes of phytochelatin biosynthetic pathway on metal tolerance and accumulation will lead to indication on their usefulness in this endeavor.

1.17 INTERACTION OF PEPTIDES WITH PHENOLIC COMPOUNDS

1.17.1 Phenolic compounds and seed germination

Phenolic compounds are wide-spread in seeds, fruits, and other tissues, occurring either in a free state or conjugated with sugars as glycosides or esters (Harborne, 1998). Numerous studies have shown that many phenolics are inhibitory (allelopathic) to germinating seeds or growing plants (Williams and Hoagland, 1982). It is also clear that phenolics may have indirect effects on physiological processes, through more non-specific effects on intermediary metabolism. For example, many phenolics are capable of inhibiting ATP synthesis in mitochondria, of uncoupling respiration and of inhibiting ion absorption in roots (Stenlid, 1970). Phenolics may react with plant hormones by synergism or inhibition and both situations were recorded in case of plant growth stimulation by gibberellic acid. There is evidence that dihydroconiferyl alcohol in lettuce has a synergistic effect on the GA_3 stimulated elongation of hypocotyls; by contrast substitution of dihydroconiferyl alcohol by any of several common hydroxycinnamic acids reverses this effect (Kamisaka and Shibata, 1977). It was also observed that tannins have generally been shown in most plant systems to have an antagonistic effect on GA_3

activity (Corcoranm *et al.*, 1972). Many phenolic compounds present in seeds are behaved as germination inhibitors in several plant species. For example, ρ-hydroxybenzoic acid acts as the major phenolic germination inhibitor of papaya seeds (Chow and Lin, 1991). In normal rye caryopsis, five phenolic acids were detected: ferulic, sinapic, vanillic, caffeic and ρ-coumaric, three of which were found in the free phenolic fractions. Cells of unripe rye grains reacted to water stress by lowering the level of total phenolic compounds. The enforced dehydration stimulated the processes of precocious germination of unripe caryopsis. Here also the phenolic acids were considered as inhibitors of germination because faster germination was induced only after the lowering of the level of phenolic acids after the dehydration treatment (Weidner *et al.*, 2000). The presence of endogenous phenolics like salicylic, syringic, and chlorogenic acids and catechol in small seeds of *Artiplex tringularis* could account for germination inhibition in these seeds (Khan and Ungar, 1986). In *Artiplex,* inhibition of germination by exogenous application of all highly active phenols was alleviated by the application of gibberellic acid and kinetin.

Accumulations of free phenolics in the seed coats are highly influenced by the physical environment of storage conditions. High temperature storage induced Navy beans (*Phaseolus vulgaris*) contained higher levels of hydroxycinnamic acids (especially ferulic acids) than control in their seed coats and cotyledons (Srisuma *et al.,* 1989). Large increases of free hydroxycinnamic acid content are generally associated with increased hardening in beans. In faba beans (*Vicia faba* L.), a loss in total phenolics, total tannins and proanthocyanidins was found with increased darkness of testa and cotyledons during storage (Nasar-Abbas *et al.*, 2008). Age-related changes of phenolic profile during storage were observed in seeds of beech (*Fagus sylvatica* L.). In *Fagus* germination capacity was strongly and positively correlated with the total phenolic compounds, UV-absorbing phenolics and antioxidant accumulation but a strong, negative correlation was found between germination capacity and reactive oxygen species (ROS). As the phenolics are generally participated in scavenging free-radicals, these compounds could play a significant role in maintaining seed viability of beech (Pukacka and Ratajczak, 2007). Changes in free phenolic acids during stratification were also established. In sugar maple, inhibitory phenolics were declined between 36 and 68% of the original

concentration after stratification (Enu-Kwesi and Dumbroff, 1980). In almost all cases, *p*-Coumaric acid was the principal phenolic component which possesses marked inhibitory properties during seed germination. The localization and intensity of cytoplasmic and apoplastic deposits of phenolic compounds during embryogenesis and storage are one of the important decisive factor of germination of common angiosperm seeds.

1.17.2 Phenolic Compounds and peptide interaction during germinating seeds

The localization and intensity of cytoplasmic and apoplastic deposits of phenolic compounds and the expression of peptides change between embryogenesis and at least upto one week after seed germination. During seed maturation phenolic compounds were localized in small vesicles or more precisely in vesicular-shaped endoplasmic reticulum (ER). In the dormant seeds, the deposits of phenolic compounds were mostly concentrated between the plasmalemma and the cell wall (Zobel *et al.*, 1989). The occurrence of phenolic deposits and peptide signaling paralleled with embryo activity at different stages of development like mitoses, synthesis of DNA, RNA and protein and mobilization of storage materials. Phenolic compounds and related enzymes such as phenol biosynthesizing enzymes (phenyl alanine ammonia lyase) and phenol catabolizing enzymes (polyphenol oxidase and peroxidase) are determinants for crop utilization as human food because they influence product properties and also regulate germination and embryo development related peptide expression and signaling (Dicko *et al.*, 2006). During analysis of enzymic hydrolysis of cereal storage proteins, ferulic acid and its derivative/complex were detected in the glutenin fraction of wheat and its peptide products, but neither in albumin-globulin and gliadin fractions nor in their hydrolysates (Karamac *et al.*, 2007). This indicates positive interaction between ferulic acid and the hydrolysates of glutenin fraction. In cereal grains like wheat, triticle and rye ferulic acids can exist as an extractable form as free, esterified and glycosylated phenolic constituents (Weidner *et al.*, 1999) as well as an insoluble-bound form occurring in the outer layers of grains (Kim *et al.*, 2006). Ferulic acid and its derivatives are also responsible for the antioxidant properties of cereal grains (Yang *et al.*, 2001; Kim *et al.*, 2006). These compounds are proven to play a significant role in the dormancy of cereal caryopsis (Weidner *et al.*, 1999). On the other hand, transport and nutritional properties of

hydrolysates depend on the size of peptides obtained during proteolysis (Karamac *et al.*, 2007). So interaction of phenolic acids and the hydrolyzed peptides generated during germination may regulate precise time of radicle emergence and oxidative stress tolerance.

It is well-known that tannins particularly interfere with the digestion of proteins due to tannin-protein reactions. Non-tannin polyphenols can also undergo oxidation of semiquinone and quinones. These can undergo further oxidative polymerization as well as coupling to proteins, by a wide variety of chemical reactions. Some of these reactions may serve to protect the plant against infections, parasites or predators (Synge, 1975). So polyphenols in plants bind to the proteins/peptides leading to the formation of soluble or insoluble peptide-polyphenol complexes which could significantly influence their biological properties (Synge, 1975). Recent NMR and molecular modeling studies have revealed that the strength of the interactions could be positively correlated with polyphenol hydrophobicity and the flexibility of peptide chains (Richard *et al.*, 2006). It has become increasingly clear that peptide-polyphenol reactions regulate different phases of growth and morphogenesis of plants. Indeed, it is possible that this function has determined the evolutionary history of some of these polyphenols as abiotic and biotic stress modulators of living system.

1.17.3 Future perspective

Beyond doubt, now it can be concluded that secreted plant peptides are important regulators of plant growth, development, and physiology. But till now, identification of novel peptide is critical and challenging task. In the classical research approach, peptides have been identified by repeated purifications based on their specific biological activities. The feasibility of using this approach, however, largely depends on the quality and sensibility of the bioassay system. In fact, the major limitation of this approach is the lack of an established bioassay system to detect peptide hormones with an unexpected class of function. Obviously the alternative strategy is required for defining novel class of peptides to overcome the shortage of bioassay-based approaches. There are other problems too. When the low molecular weight peptides are encoded by genes, they are often missed during gene annotation and in the era of genomics, if a gene is not

annotated, it will not be investigated. Again identification of additional peptide signals by sequence homology searches has had limited success due to high sequence heterogeneity among processed peptides. Bioinformatic approaches are now taken for identification of unannotated secreted peptides through empirical analysis of transcriptional activity by genome-wide tiling hybridization (Yamada *et al.*, 2003), single-linkage clustering of the preproproteins (with signal peptides) with published genome data and reverse transcription PCR output (Lease and Walker, 2006).

It is now known that small post-translationally modified peptides are produced through a secretary pathway after post-translational proteolytic processing and modifications. This post-translational modification requires considerably higher energy compared with normal peptides (Matsubayashi, 2010). Nevertheless, a number of post-translationally modified peptides have been evolutionary conserved, suggesting that these 'expensive' peptides afford physiological merits in favour of better survival of plants. It can be speculated that the structure of unmodified small peptides are thermodynamically more unstable in cellular atmosphere than modified peptides, with substituted functional groups because of more specific interactions with their receptors and intra-chain covalent/non-covalent bonds among substituted groups. So, the proteolytically processed modified peptides can be an indicative of biologically active peptides. The peptidomics approach targeting these specifically modified peptides has now being identified successfully as a novel peptide hormone and recently the specific bioactivity of some putative peptide ligands have uncovered through *in silico* gene screening approaches (Ohyama *et al.*, 2008).

Definitely future research will be directed at identifying new peptide genes and their products through more precise genetic, biochemical and bioinformatic tools. However, it will equally be aimed at the receptors that interact with these molecules, because many orphan receptor kinases are still uncharacterized with no known ligands. The availability of yeast two-hybrid and proteomic technologies along with virtual molecular dynamic simulation techniques will be expected to solve the path of entire peptide signals which will redefine the cellular communication of plants in near future.

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