2. LITERATURE REVIEW

2.1. Nomenclature and chemistry of *myo*-inositol and its derivatives

2.1.1. What is *myo*-inositol?

The inositols constitute an important group of naturally occurring polyhydric alcohols found in most plants and animals. There are nine isomers of inositols of which seven are *meso* forms and one DL pair. *Myo*-inositol is the most widely distributed inositol in plants and probably occurs in all living organisms. It occupies a central position in carbohydrate metabolism being the precursor of a number of metabolic products viz., inositol phosphates, phosphoinositides, cell wall polysaccharides, methylated derivatives and IAA conjugates. As a free cyclitol, *myo*-inositol has been found essential for normal growth and development of plant tissues. Lack of cellular level of inositol has been identified as the cause of "inositol-less-death" in *Saccharomyces cerevisiae.*

Myo-inositols serve as an essential nutrient for growth and development of all living organisms, like formation of sex units, an integral part of milk and precursors of principal storage phosphate in seeds. It also forms cell wall polysaccharides and an essential component of myelin in central nervous system. To account for the significance of diverse metabolic pathways centered around *myo*-inositol, studies on L-*myo*-inositol-1-phosphate synthase, the prime enzyme of *myo*-inositol biosynthesis is a prerequisite.

2.1.2. Chemical configuration of *myo*-inositol and its different forms

Inositols are hexahydroxy-cyclohexanes (cyclitols) containing three or more hydroxyl groups. The planar structures of the different isomers of this compound are presented in Fig-2.1. Out of nine forms of inositols, seven occur in nature, the exeptions being *epi*- and *allo*-inositol. *Myo*-, *chiro*- and *scyllo*-inositols constitute the major stereoisomers in plants (Valluru and Ende, 2011). Numbering of '*myo*-inositol' molecule is done clockwise and the carbons that bear hydroxyls projecting above the plane of the ring are assigned the lowest possible numbers (Loewus and Loewus, 1980). The strain free chair form (Fig-2.2) having an axial substitution at C-2 is the most stable form of *myo*-inositol (Anderson, 1972). The three dimensional molecular structure of *myo*-inositol-2-phosphate indicates that the phosphate group is attached to the axial oxygen atom of the molecule.

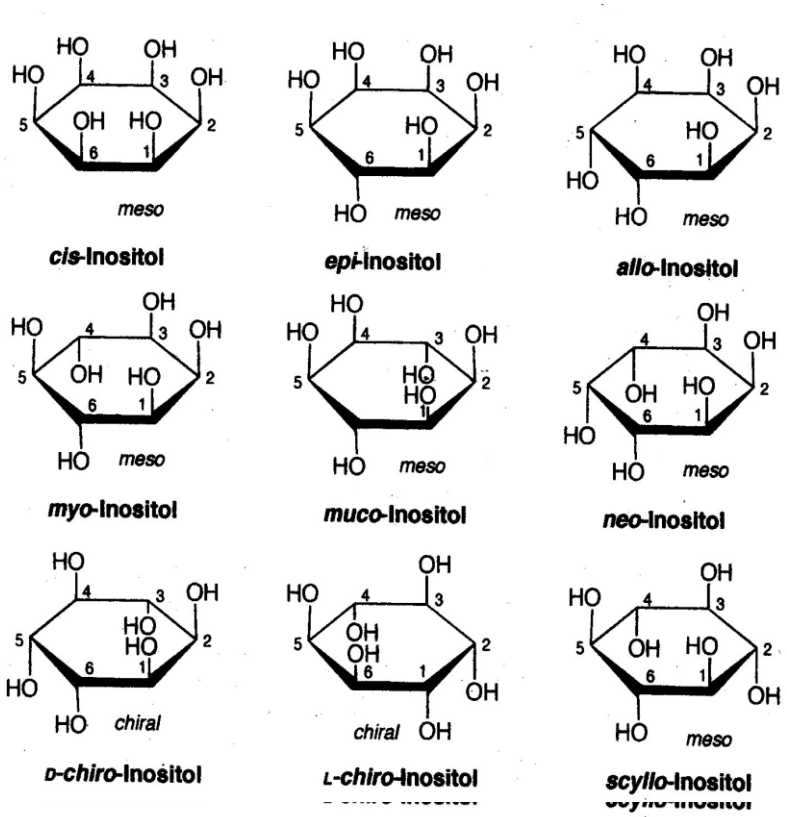


Fig-2.1 Structures of nine possible stereoisomers of inositol

Myo-inositol possesses hydroxyl groups which are secondary alcoholic groups and as such one molecule of *myo*-inositol can be esterified by a maximum of six phosphoric acid molecules by six phosphomonoester bonds generating inositol hexaphosphoric acid (phytic acid). Each *myo*-inositol molecule contains one axial hydroxyl group at C2 (Figure 2.1) and five equatorial hydroxyl groups. When reacted with enzymes or acid, the phytic acid leaves the last phosphate ester at C2, suggesting that the axial phosphate ester is most stable to hydrolysis (Tomlinson and Ballou, 1962).

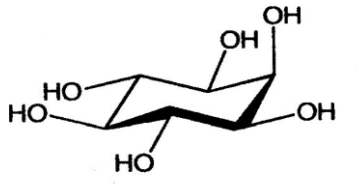
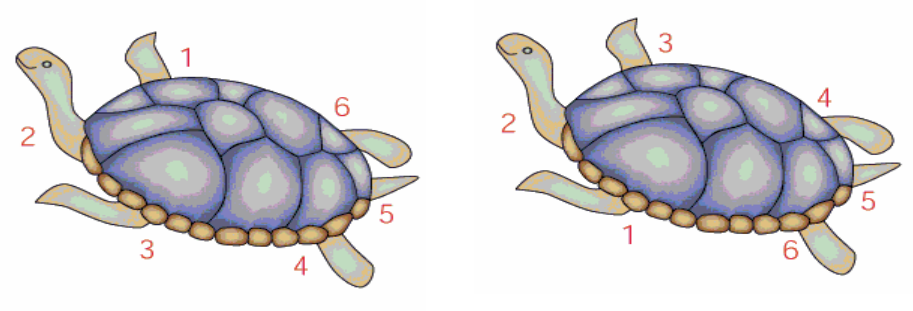


Fig-2.2 Chair form of *myo*-inositol

Numbering of *myo*-inositol has been simplified by visualizing an image of turtle (Fig.2.3) where the head of the turtle is defined as 2- position and is occupied by the axial hydroxyl group, while the five equatorial hydroxyl groups are symbolized by the four limbs and the tail of the turtle. Through this image, the D-ring numbering is assigned by using the right front limb of the turtle as the D-1 position, the head as D-2 position, the left front limb as D-3 position and so on (Agranoff, 1978), when the image is considered through anticlockwise direction. Similarly L-ring numbering is assigned by denoting the left front limb as the L1, head of the rurtle as L2 and so on, while proceeding clockwise.



D-numbering *L-numbering*

Fig-2. 3 Agranoff's turtle

As per Ogawa (1999) the sequential action of several phosphorylases synthesized phytic acid from *myo*-inositol -2-phosphate *in-vivo* from phytic acid or IP_6. Both plant and animal systems contain considerable quantity of phospholipid pool of the cell in the form of phosphoinositides or inositol phospholipid in which phosphatidylinositol or monophosphoinositide is the major *myo*-inositol lipid. Animal cells is recorded to contain about 2-12% of the total phospholipid (White, 1973) along with considerably lower amount of diphosphoinositide (phosphatidyl inositol-4-phosphate and triphosphoinositide (phosphatidyl inositol-4, 5- bisphosphate (Fig.2.4) [Dittmer and Douglas, 1969].

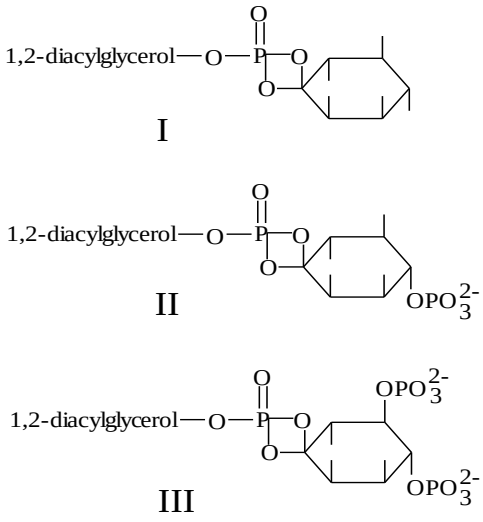


Fig-2.4 Structures of phosphatidylinositols: I. Monophosphoinositide; II. Diphosphoinositide and III. Triphosphoinositide

As compared to animal cells, most of the unsaturated fatty acid groups present in plant are phosphoinositides where phosphatidylinositol acts as a predominant phospholipid (Galliard, 1973). Similar compounds were reported from fungi and higher plants (Loewus and Loewus, 1980), while from tobacco leaves another six phosphosphingolipids were reported (Kaul and Lester, 1978).

2.1.3. Methyl esters of *myo*-inositol

Combined forms like the methyl esters of *myo*-, *scyllo*-, D-and L-*chiro*-and *muco*-inositols have been reported in plants which are considered to be the secondary metabolites and are important in the classification of plants (Plouvier, 1963, 1966; Dittrich *et al.*, 1972). Quite a few monomethyl esters viz., D/L bornesitol, D-ononitol and sequoyitol and three dimethyl esters among which are dimethyl esters are known to occur in plant tissues (Drobak, 1992).

Among the methyl esters of inositol, D-pinitol has been obtained from sugar pine and L-quebrachitol from rubber tree. According to Ogawa (1999) D-pinitol and L-quebrachitol are readily available in large quantities and useful as versatile raw materials for organic synthesis. Naturally occurring methyl derivatives of inositol have been presented in Fig-2.5.

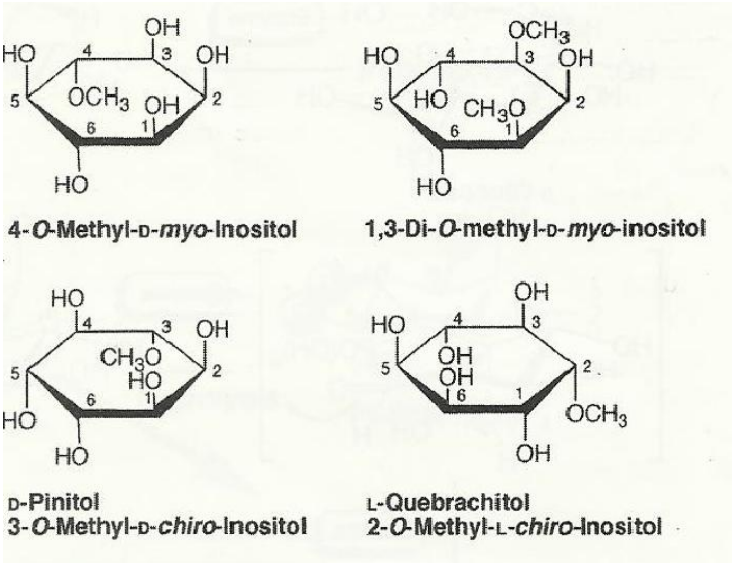


Fig-2.5 Naturally occurring inositol methyl esters

Pinitol (3-O-methyl D-*chiro*-inositol) is one example of an inositol methyl ester which is known as antihyperglycemic agent. This compound has been suggested to form part of the structure of a bovine liver-derived inositol phosphogylcans (IPGs) (Fonteles *et al*., 1996). Furthermore, pinitol has been found to be present in the leaves of *Bougainvillea*, a plant that has been used to treat diabetes mellitus in India (Narayanan *et al*., 1987).

2.1.4. Indole-3 acetyl esters of *myo*-inositol

Indole-3-acetyl esters of *myo*-inositol, 2-O-(IAA)-*myo*-inositol, 1D1-1-O-(IAA)-*myo* inositol viz., di-O-(IAA)-*myo*-inositol and tri-O-(IAA) *myo*-inositol are the indole-3-acetic acid (IAA) esters which are important IAA conjugates of *myo*-inositol (IAAMI) having physiological effects on the plants (Bandurski, 1978). 30% of the lower molecular weight derivatives of IAA the indole-3-acetyl-*myo*-inositol (IAAMI) are present in seeds of *Zea mays* and its transport from endosperm to shoot was 400 times in comparison to the rate of free IAA. This transport is thus adequate to serve as the

seed auxin precursor for the free IAA diffusing downward from the shoot-tip. The first identified seed auxin precursor was indole-3-acetyl-*myo*-inositol. Interconversion between esterified IAA and free IAA occurs in the growing shoot. Free IAA may be limiting for plant growth, but the fact that this free hormone remains in equilibrium with its conjugates suggests a new paradigm that influences plant growth (Basak, 2013). It is proposed that the non-hormone moiety is involved in targeting of IAA within the plant or itself act as messenger (Michalczuk and Bandurski, 1982).

2.1.5. Inositol derived antibiotics

Streptomycin and Kanamycin are some antibiotics which contains diamino and diaminodeoxy derivatives of scyllo-inositol. Production of such aminoglycoside antibiotics is the reason for the development of an era of synthetic carbohydrates (Umezawa, 1974). Other antibiotic like Hygromycin is an aminodeoxy derivative of neo-inositol, while Kasugamycin has (+) chiro-inositol as its constituent. The derivatives of *myo*-inositol added for the chemical modification of these antibiotics in order to enhance the potency of these drugs (Ogawa, 1999).

Aminoglycosides containing a 2-deoxystreptamine unit are a large class of clinically important antibiotics. It has been found that deoxytriptamine is derived from D-glucose and that 2-deoxy-*scyllo*-inosose alongwith 2-deoxy-*scyllo*-inosamine is the two key intermediates in the biosynthetic pathway for 2-deoxystreptamine containing aminoglycosides synthesis (Goda and Akhtar, 1987). An efficient route for the synthesis of these two key intermediates of deosystreptamine has been established (Yu and Spencer, 2001).

Hygromycin-A produced by *Streptomyces hygroscopicus* provides distinct carbon skeletons for the development of antibacterial agents. The Hygromycin- A biosynthetic gene cluster has been identified, cloned and sequenced. The gene cluster has 29 ORFs of which one is assigned to the biosynthesis of one of the three key moieties of hygromycin A, 2L-2amino-2-deoxy-4,5-*O*-methylene-neo-inositol (Palaniappan *et al*., 2006). The importance of Hygromycin-A lies in the fact that it offeres a disticnt carbon skeleton and binding mode for other antibiotics that targets the ribosome of pathogen bacteria. As such, it has the potential to generate new antibiotics against drug resistant pathogens. Thus, semisynthetic programs based on hygromycin A have become attractive (Hayashi *et al*., 1997; Jaynes *et al*., 1992). It may be safely concluded that *myo*-inositol and its derivatives have great potential as starting materials for design of new bioactive compounds (Ogawa, 1999).

2.2. *Myo*-inositol biosynthesis

Biosynthesis of *myo*-inositol takes place by irreversible isomerization of D-glucose-6-phosphate to L-*myo*-inositol-1-phosphate by a NAD-dependent oxido-reductase, L-*myo*-inositol-1-phosphate synthase (D-Glucose-6-phosphate-1 L-*myo*-inositol-phosphate syntase; EC 5.5.1.4), hereinafter called 'MIPS.' Free *myo*-inositol is generated as the end product of this enzymic reaction by cleaving the intermediate product, L-*myo*-inositol-1-phosphate by a phsopahatse, L-*myo*-inositol-1-phosphate phosphatase (L-*myo*-ionositol-1-phosphate phosphohydrolase; EC 3.1.3.25). Lateron, *myo*-inositol may also be converted to chiro-inositol by epimerization of its C3 hydroxyl group (Larner *et al.,* 2010) [Fig-2.6].

It is proposed that the *myo*-inositol-1-phosphate synthase reaction is consisted of three partial reactions, with the formation of 5-keto-glucose-6-phosphate and *myo*-inosoe-2, 1-phosphate, the two postulated enzyme-bound intermediates. The existence of *myo*-inososе-2, 1-phosphate, is evidenced from the study of partially purified testis enzyme which exhibited the presence of myo-inososе 2, 1-phosphate (Chen and Eisenberg, 1975) that proves the formation of 5-keto-glucose-6-phosphate (Kiely and Fletcher, 1969). Subsequently, an intramolecular aldol condendation of 5-keto-D-glucose-6-phosphate formed by the oxidation of D-glucose-6-phosphate by NAD^+ leads to the cyclization of the molecule and also explains NAD^+ requirement (Kiely and Sharman, 1975).

It has been found that *myo*-inososе-2, 1-phsophate as one of the intermediates, which arose from the cyclization of 5-ketoglucose 6-phosphate and that the rate of cyclization of 5-ketoglucose 6-phosphate was far greater than that of reduction of *myo*-inososе-2, 1-phosphate by NADH (Kiely *et al*., 1974).

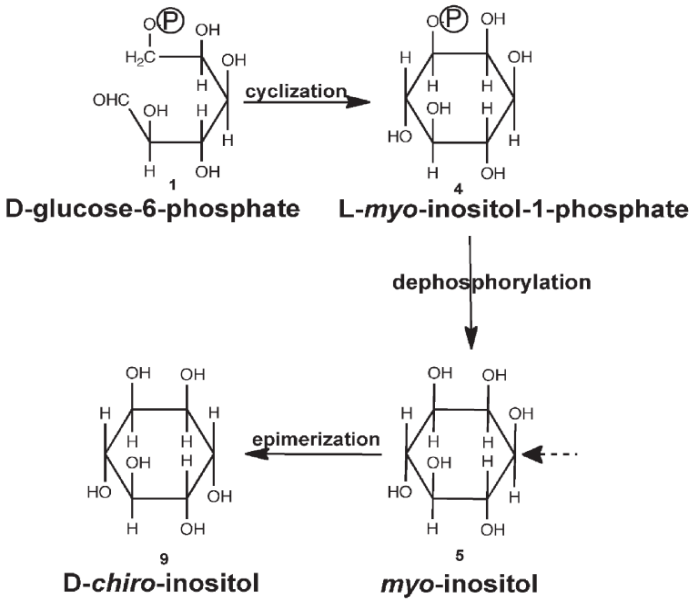


Fig-2.6 Conversion of D-glucose-6-phosphate to *myo*-inositol-1-phosphate by cyclization catalyzed by *myo*-inositol-1-phosphate synthase. *Myo*-inositol-1-phosphate is then dephosphorylated to *myo*-inositol by *myo*-nositol-1-phosphate phosphatase. *Myo*-inositol may be converted by epimerization to D-chiro-inositol.

In lily pollen and wheat germ, the mechanism of producing 1L-*myo*-inositol-1-phosphate (I-1-P) have been studied and it was found that I-1-P was produced *de-novo* by the involvement of MIPS as well as Mg^{2+} dependent ATP kinase respectively. Since MIPS is the sole pathway for biosynthesis of *myo*-inositol from hexose phosphate, the MI kinase pathway functions as salvage mechanism which supplies the I-1-P requirement of the plant cells from stored myo-inositol (Loewus *et al*., 1982).

Myo-inositol is synthesized through cyclization of glucose-6-phosphate molecule (Eisenberg, 1967), it could also be produced by cyclic synthesis (Agranoff *et al*., 1958; Paulus and Kennedy, 1960) and by the hydrolysis of phosphatidylinositol which makes its biosynthesis a unique process. In the synthetic and cyclic pathways, two different forms of compounds are produced, the L-enantiomer and D-enantiomer of *myo*-inositol

respectively though the intermediate, *myo*-inositol 1-phosphate is same in both pathways (Parthasarathy and Eisesnberg, 1986). Both these compounds are hydrolyzed by *myo*-inositol-1-phosphatase (Eisenberg, 1967) generating *myo*-inositol in the process. Thus *myo*-inositol-1-phosphatase (EC: 3.1.3.25) is crucial in the biosynthesis of free *myo*-inositol from inositol-1-phosphate (Nigou and Besra, 2002).

The *de-novo* generation of *myo*-inositol occurs by a universal mechanism that is conserved throughout the phylogenetic domain (Majumder *et al.*, 2003). L-*myo*-inositol-1-phosphate synthase (EC: 5.5.1.4) is a rate-limiting enzyme that catalyzes the first step in the biosynthesis of all *myo*-inositol containing compounds (Seelan *et al.*, 2009). It converts D-glucose-6-phosphate (G-6-P) to L-*myo*-inositol-1-phosphate (I-1-P). The phosphate moiety in I-1-P is subsequently removed by inositol monophosphatase 1 (IMPase) to produce free *myo*-inositol (Majumder and Biswas, 2006).

2.3. L-*myo*-Inositol-1-phosphate synthase from plant system

2.3.1. Occurrence of L-*myo*-Inositol-1-phosphate synthase in different plants

L-*myo*-inositol-1-phosphate has been detected from a number of genera across the divisions in plant kingdom (Table 2.1). Among algae, the enzyme has been detected in *Euglena gracilis*, *Chlorella vulgaris*, *Spirogyra maxima* and *Microspora willeana* while 7 day old culture *Euglena gracilis* produced noteworthy synthase activity (Dasgupta *et al.*, 1984). The enzyme has been purified to electrophoretic homogeneity from *Spirulina platensis* and *Euglena gracilis* (RayChoudhuri *et al.*, 1997). From fungi, inositol synthase activity was recorded in *Saccharomyces cerevisiae* (Donahue and Henry, 1981a), *Neurospora crassa* (Pina *et al.*, 1978), *Polyporus anthelminticus, Ganoderma lucidum, Irpex flavus*, *Agaricus compestris, Schizophyllum commune, Lentinus*

subnudus and *Scleroderma sp*. (Dasgupta *et al*., 1984). In the fungus *Cryptococcus neoformans* both the synthesis and catabolism of *myo*-inositol has been studied (Molina *et al.*, 1999).

Among bryophytes, e.g. *Lunularia sp., Targionia sp., Marchantia polymorpha, Dumortiera sp*, of the order Marchantiales, appreciable degrees of *myo*-inositol synthase (MIPS) were recorded. In *Marchantia polymorpha*, the synthase activity was found to be associated with the development of the reproductive structures. Similarly, MIPS activity was detected in *Marchantia nepalensis* in the reproductive part bearing thallus (Chhetri *et al*., 2006). The enzyme was also detected from from another liverwort, *Lunularia cruciata* (Chhetri *et al*., 2009). The presence of *myo*-inositol synthesizing activity in algae, fungi and bryophytes suggests a more ubiquitous occurrence of this cyclitol and a more general physiological significance of the same in the lower plant groups (Dasgupta *et al*., 1984). From the pteridophytes, inositol synthase activity was detected from the reproductive pinnules of *Diplopterygium glaucum* (Chhetri *et al*., 2006). The enzyme was also isolated from other pteridophytes lik *Azolla filiculoides* (Benaroya *et al*., 2004), *Lycopodium clavatum* and *Selaginella monospora* (Basak *et al*., 2012). Among gymnosperms, the activity of L-*myo*-inositol-1-phosphate synthase was reported from the pollen grains of *Pinus ponderosa* (Gumber *et al*., 1984). The enzyme was partially isolated from the leaves of *Taxus baccata* L. (Chhetri and Chiu, 2004). L-*myo*-inositol-1-phosphate synthase activity has been detected from a wide number of angiosperm genera viz., *Vigna radiata* (Majumder and Biswas, 1973b; Adhikari and Majumder, 1983), *Lemna gibba* (Ogunyemi *et al*., 1978), *Lilium longiflorum* (Sherman *et al*., 1981), *Citrus paradisi* (Abu-abied and Holland, 1994), *Arabidopsis thaliana*

(Johnson and Sussex, 1995), *Phaseolus vulgaris* (Johnson and Wang, 1996), *Thymus vulgaris* (Ray Choudhuri *et al.,* (1997), *Swertia bimaculata* (Chhetri *et al*., 2008) etc.

Most of the L-*myo*-inositol-1-phosphate synthase activity in plants is present in the soluble or cytosolic fractions of the cell. However, about 10% of phosphoinositides (membrane phospholipids) are present in the chloroplast of green plant (Imhoff and Bourdu, 1973), which requires an endogenous pool of *myo*-inositol for their synthesis. Chloroplast membrane is impermeable to the cyclitol (Wang and Nobel, 1971) and points towards the fact that the chloroplast may be the site for the synthesis of *myo*-inositol. Labelled glucose was incorporated into *myo*-inositol in chloroplast preparation from pea (Imhoff and Bourdu, 1973). Similarly chloroplastic inositol synthase has also been reported from *Euglena gracilis* (Loewus *et al*., 1986; Adhikari *et al*., 1987), *Vigna radiata* seedlings (5 to 7 days old) grown under alternate light/dark conditions (Adhikari *et al*., (1987) and *Diplopterygium glaucum* (Chhetri *et al*., 2006c). Moreover, plant species belonging to thallophytes, monocots and dicots contains both chloroplastic and cytosolic forms of *myo*-inositol synthase (Ray Choudhury *et al*., 1997). Tissue specific MIPS activity localized in the outer integumentary areas of developing embryo in soybean seeds have been detected using immunolocalization techniques.This enzyme activity was associated with oxalate crystal idioblasts (Cheira and Grabau, 2007). Expression of MIPS activity was also found in different organs e.g pollen grains, ovules, leaves and in different stages of seed development. The activity showed differential expression under heat and cold stress conditions suggesting its role in abiotic stress response (Abreu and Aragao, 2007). During embryogenesis of mungbean seeds, the expression of MIPS activity was found to be recorded 7-9 days after flowering (Wongkaew *et al*., 2010).

Table -2.1. Distribution of L-*myo*-inisitol-1-phosphate synthase in plant kingdom

Origin	Source	Tissue	Reported by
ALGAE	*Euglena gracilis*	Whole plant	Dasgupta *et al.,* (1984)
	Chlorella vulgaris	Whole plant	Dasgupta *et al*., (1984)
	Spirogyra maxima	Whole plant	Dasgupta *et al*., (1984)
	Microspora willeana	Whole plant	Dasgupta *et al.,* (1984)
	Spirulina platensis	Cultured cells	Ray Choudhuri *et al.,* (1997)
	Enteromorpha linza	Vegetative	Loewus & Loewus (1971)
FUNGI	*Saccharomyces cerevisiae*	Whole plant	Donahue & Henry (1981)
	Neurospora crassa	Whole plant	Pina *et al.* (1978)
	Polyporus anthelminticus	Whole plant	Dasgupta *et al.,*(1984)
	Ganoderma lucidum	Whole plant	Dasgupta *et al*., (1984)
	Irpex flavus	Whole plant	Dasgupta *et al*., (1984)
	Agaricus compestris	Whole plant	Dasgupta *et al*., (1984)
	Schizophyllum commune	Whole plant	Dasgupta *et al*., (1984)
	Lentinus subnudus	Whole plant	Dasgupta *et al*., (1984)
	Scleroderma sp.	Whole plant	Dasgupta *et al.,* (1984)
	Cryptococcus neoformans	Whole plant	Molina *et al*., (1999)
BRYOPHYTES	*Lunulariacruciata*	Whole plant,	Dasgupta *et al*., (1984)
	Lunularia cruciata	Reproductive thallus	Chhetri *et al.,* (2009)
	Targigonia sp.	Whole plant	Dasgupta *et al,* (1984)
	Marchantia polymorpha	Sex organs	Dasgupta *et al.,* (1984)
	Dumortiera sp.	Whole plant	Dasgupta *et al*., (1984)
	Marchantia nepalensis	Reproductive thallus	Chhetri *et al*., (2006)
	Brachymenium bryoides	Whole plant	Yonzone *et al.,* (2018)
PTERIDOPHYTES	*Diplopterygium glaucum*	Reporoductive pinnules	Chhetri *et al.,* (2006, 2007)
GYMNOSPERMS	*Pinus ponderosa*	Pollen grains	Gumber *et al*., (1984)
	Taxus baccata	Leaves	Chhetri and Chiu (2004)
ANGIOSPERMS	*Vigna radiata*	Seed	Majumder & Biswas (1973b)
	Acer pseudoplatanus	Cultured cells	Loewus & Loewus (1971)
	Lemna gibba	Whole plant	Ogunyemi *et al*., (1978)
	Lilium longiflorum	Pollen	Sherman *et al*., (1981)
	Phaseolus vulgaris	Embryo,root,leaf	Loewus & Loewus (1971)
	Oryza sativa	Callus	Johnson & Wang (1996)
	Oryza sativa	Leaves	Funkhouser & Loewus (1975)
	Hevea latex	Latex serum	Ray Choudhuri *et al* (1997)
	Vigna radiata	Leaves	Loewus *et al.,* (1986)
	Thymus vulgaris	Leaves	Ray Choudhuri *et al.,* (1997)
	Rosemarinus officinalis	Leaves	Loewus & Loewus (1971)
	Petroselinum crispum	Leaves	Loewus & Loewus (1971)
	Lemna perpusilla	Whole plant	Loewus & Loewus (1971)
	Salvia officinalis	Leaves	Loewus & Loewus (1971)
	Swertia bimaculata	Leaves	Chhetri *et al.,* 2008

2.3.2 Isolation and characterization of L-*myo*-inositol-1-phosphate synthase of plant origin

L-*myo*-inositol-1-phosphate synthase was purified and characterized from *Saccharomyces cerevisiae* by conventional enzyme purification techniques (Donahue and Henry, 1981b). The M_r of the enzyme determined by gel-filtration chromatography was 240 kDa with a subunit molecular weight of approximately 62 kDa. Some mutants of *Saccharomyces cerevisiae* auxotrophic for inositol which cannot synthesize inositol containing phospholipids exhibited 'inositol-less-death' when deprived of inositol (Culbertson and Henry, 1975). This may be due to the important role played by inositol lipids during the growth of the fungi (Strauss, 1958). Inositol synthase was also purified to homogeneity from another fungus, *Neurospora crassa* by Escamilla *et al*., (1982). The M_r estimated was 345 kDa and the subunit M_r was 59 kDa and the enzyme showed a hexameric structure which was stimulated by 10 mM $(NH_4)_2SO_4$ and also by 50 mM KCl. The same enzyme purified from *Acer pseudoplatanus* had M_r of about 150 kDa (Loewus and Loewus, 1971) while the enzyme from *Lemna gibba* had a M_r of about 135 kDa (Ogunyemi *et al*., 1978).

The enzyme was partially purified and characterized from a gymnosperm, *Pinus ponderosa* pollen grains (Gumber *et al*., 1984). The gymnospermic MIPS showed maximum activity at pH 7.25 to 7.75. The K_m for its substrate, D-glucose-6-phosphate was 0.33 mM. Inhibition by parachloromercurobenzoate and N-ethyl-maleimide and partial protection against this inhibition by G-6-P in the presence of NAD^+ suggested that there was a SH-group involvement at the substrate-binding site. Gymnospermic MIPS was also purified from the needles of *Taxus baccata* L. and the enzyme was

found to be highly stimulated by NH_4^+ , the V_{max} and K_m of the enzyme for its substrate, glucose-6-phosphate and NAD^+were 2.95mM and 1.05 mM respectively (Chhetri and Chiu, 2004). L-*myo*-inositol-1-phosphate synthase was isolated and partially purified from the alga, *Euglena gracilis* by Dasgupta *et al*., (1984). The enzyme exhibited total inhibition by SO_4^{2+}, a pH optimum of 7.5 and the K_m for G-6-P was 2.1 mM. Deduction of endogenous NAD^+, reduced the enzyme activity to about 30% suggesting the occurrence and important role played by NAD^+ in the enzyme from the experimental plant.

L-*myo*-inositol-1-phosphate synthase had also been partially purified from chloroplasts of 6-day-old *Vigna radiata* seedlings. The enzyme exhibited its optimum activity at a pH of 7.5 to 7.75 and the presence of NH_4Cl (9 mM) caused a 2-fold stimulation of the enzyme activity. The chloroplastic MIPS showed K_m of 1.8 mM and 0.13 mM for G-6-P and NAD^+ respectively (Adhikari *et al.*, 1987). Cytosolic and chloroplastic forms of inositol synthase from *Euglena gracilis*, *Oryza sativa* and *Vigna radiata* have been isolated, purified and characterized. The enzyme from these sources differ only in the molecular mass of the chloroplastic and cytosolic native holoenzymes, which appeared to be due to the association of either three or four equal subunits constituting the holoenzyme (Ray Choudhuri *et al*., 1997). From pteridophytic source, MIPS have been isolated and characterizd for the first time from *Diplopterygeum glaucum*. The pteridphytic MIPS showed pH optima between 7.0 and 7.5 while the temperature maxima was 35^oC. The *Km* for its substrate G-6-P and co-factor NAD^+ were found to be 0.83mM and 0.44 mM respectively. Similarly, the V_{max} values were 1.42 and 1.8mM for G-6-P and NAD^+ respectively (Chhetri *et al*., 2006a, 2006b, 2006c).

2.4. Metabolism of inositol phosphates

2.4.1. Various *myo*-inositol phosphates in plants: From mono- to hexakisphosphate

Biosynthesis of phosphate derivatives of *myo*-inositol has been studied in quite details by several wokers (Loewus, 1968; Molinary and Hoffman-Ostenhof, 1968; Asada *et al*., 1969; Tanaka *et al*., 1976; Stephens and Irvine, 1990; Mandal and Biswas, 1970) and it has been reported that higher inositol phosphates are directly produced by the phosphorylation of lower inositol phosphates in germinating mung bean seeds. The enzyme, phosphoinositol kinase was found activated during the formation of seeds, but at a later stage of maturation it declines (Majumder *et al*., 1972). *Myo*-inositol monophosphate can be phosphorylated to higher derivatives upto IP_6 by the same enzyme in presence of ATP serving as the phosphate donor. The reaction kinetics of phosphoinositol kinase suggests that accumulation of *myo*-inositol phosphate is not significant in quantity during the biosynthesis of hexaphosphate. This observation is in consonance with the *in vivo* experiment where appreciable accumulation of other *myo*-inositol phosphates but IP_6 has been found. This inhibitor of phosphoinositol kinase accumulates during last stages of ripening and is destroyed or becomes ineffective during early hours of germination (Majumder and Biswas, 1973b).

Myo-inositol monophosphate is the precursor, from which *myo*-inositol hexaphosphate is biosynthesized, utilizing two enzymes, *myo*-inositol-1-phosphate synthase and *myo*-inositol kinase. The existence of inositol synthase in plant system has been well documented. The existence of inositol kinase has been reported from germinating mung bean seeds (Dietz and Albersheim, 1965) and the formation of *myo*-inositol-1-phosphate

from *myo*-inositol kinase was concluded to be the initial step during the biosynthesis of inositol hexakisphosphate in seeds (English *et al*., 1966).

In plants, different isomers of inositol monophosphate are found viz. I(1)P, I(2)P, I(3P) and I(4)P. The biosynthesis of I(3)P takes place by inositol synthase as well as by an inositol kinase. The hydrolysis of $I(1,4)P_2$ produced the two forms of inositol monophosphate, I(1)P and I(4)P (Joseph *et al*., 1989; Memon *et al*., 1989; Drobak *et al*., 1991; Martinoia *et al*., 1993). Furthermore, I(2)P, is formed as a consequecce of acid–phytase catalyzed hydrolysis of phytic acid (Cosgrove, 1980).

In plants, inositol bisphosphate (IP_2) are of different types of which $I(1,2)P_2$, $I(1,4)P_2$ and $I(4,5)P_2$ are formed by the hydrolysis of phytic acid while $I(1,4)P_2$ and $I(4,5)P_2$ are formed as a breakdown product of $I(1,4,5)P_3$. In addition, the action of PI-PLC on PI(4)P also produces $I(1,4)P_2$ (Murthy, 1996).

The interest in the study of inositol-triphosphate generated with the discovery of second messenger role played by inositol triphosphate, $I(1,4,5)P_3$ (Drobak *et al*., 1991; Martinoia *et al*., 1993). This triphosphate $I(1,4,5)P_3$, is formed by the action of PI-PLC on $PI(4,5)P_2$ (Huang *et al*., 1994). Beside $I(1,4,5)P_3$, other two triphosphates, $I(1,2,3)P_3$ and $I(1,2,6)P_3$ are also formed as the acid phytase catalyzed intermediate products of phytic acid (Cosgrove, 1980). The 1,4,5-triphosphate [$Ins1,4,5)P_3$] is metabolized to inositol 1,3,4,5-tetraphosphate [$Ins(1,3,4,5)P_4$] by a 3-kinase which subsequently by the inositolphosphate-5-phosphomonoesterase converted to three different inositol triphosphate isomers, $Ins(1,3,5)P_3$, $Ins(1,3,4)P_3$. $Ins(1,3,4)P_3$, which in turn gets

converted to inositol 3,4-bisphosphate by a different enzyme, inositol polyphosphate-1-phosphatase. This bisphosphate is further converted to inositol-3-phosphate. Similarly, the existence of Ins(1,2,3) P_3, Ins(3,4,6) P_3, Ins(2,4,5) P_3, Ins(3,5,6) P_3, Ins(1,2,6) P_3, Ins(1,5,6) P_3 etc in aleurone tissue in *S. polyrhiza* and *Arabidopsis* suggest that all these isomers are the products of Ins P_6 metabolism *in-vivo.* Action of kinase over I(1,4,5)P_3 produces inositol-tetrakisphosphates [I(1,4,5,6)P_4] in plants. Different other isomers of inositol-tetrakisphosphates viz., I(1,2,3,6)P_4, I(1,2,5,6)P_4 and D or L-I(1,2,3,4)P_4 are reported to be produced in pants by enzyme catalyzed hydrolysis of phytic acid (Barrientos *et al*., 1994). Ins(1,3,4,5)P_4, another inositol-tetraphosphate is produced by phosphorylation of Ins(1,4,5) P_3 by an enzyme β-kinase (Irvine,1999). Ins(1,3,4)P_3 may be converted to (1,3,4,5)P_4 or (1,3,4,6)P_4 by the action of Ins 1,3,4-triphosphate kinase-2 (Marathe *et al.*, 2018).

Myo-inositol (1,3,4,5,6) pentakisphosphate may be produced from I(1,4,5)P_3 (Stephens *et al*., 1988) by phosphorylating I(3,4,5,6)P_4, It was found that I(1,3,4,5)P_4, I(1,3,4,6)P_4 and I(1,3,4)P_3 are the intermediates in inositol pentakisphosphate synthesis pathway. Most probably, either I(3,4,5,6)P_4 was synthesized from I(1,4,5)P_3 by an unknown route or a second IP_4 might also act as an intermediate in the formation of IP_5 (Stephens *et al*., 1988).

Inositol hexakisphosphate or phytic acid is the major form of phosphorous accumulating in large amounts in the storage tissues and it may limit the availibity of minerals (Aggarwal *et al.*, 2018). 75% of the phosphate is stored as phytin in matured seeds and during germination this stored phytin is decationized and hydrolyzed by phytases for the

germination of embryo (Raboy, 2003). The biosynthesis of phytic acid (inositol hexakisphosphate) may take place either by the sequential phosphorylation of inositol by kinases (Majumder and Biswas, 1973a, 1973c; Biswas *et al.*, 1978; Igaue *et al*., 1980) or by the ultimate hydrolyis of sequential phosphorylation of an inositol derivative (Raboy, 1990; Drobak, 1992). In general, there are two $InsP_6$ generating pathways, a) a lipid- dependent pathway via PLC and b) a lipid-independent pathway via direct phosphorylation of MIPS- generated Ins3P (Raboy 2003; Stevenson-Paulik *et al.*, 2005) [Fig-2.7].

As per lipid dependent pathway, PtdIns(4,5)P_2 formed from *myo*-inositol is hydrolyzed by PLC generating Ins(1,4,5)P_3, which is phosphorylated into IP_6 in stepwise manner by IPP multikinase2(IPK2), inositolpolyphosphate 3-/5-/6-kinase, IPK1, inositol polyphosphate 2-kinase in sequence (Stevenson-Paulik *et al*., 2005; Sweetman *et al*., 2006; Murphy *et al*., 2008). Through the lipid-independent pathway the Ins3P formed by MIPS by the cyclization of G-6-P is directly phosphorylated to Ins(3,4)P_2 and further via Ins(3,4,6)P_3 to Ins(3,4,5,6)P_4 and Ins(1,3,4,5,6)P_5 and finally to $InsP_6$. This system describes the endogenous inositol phosphates in any plant tissues. This sequence differs from that reported in the slime mould *Dictyostelium discoideum* (Brearley and Hanke, 1996) where IP_6 synthesis is catalyzed by a series of soluble ATP dependent kinases.

Besides these pathways, other possible pathways are there in different plant organs and across species to generate IP_6. The mammalian cells seem to use the lipid-dependent pathway, to synthesize $InsP_6$ while in plants it may be synthesized by either pathways based on the tissue type (Irvine, 2003, 2005; York, 2006; Alcazar-Roman and Wente, 2008; Letchar *et al*., 2008; Shears, 2001, 2009).

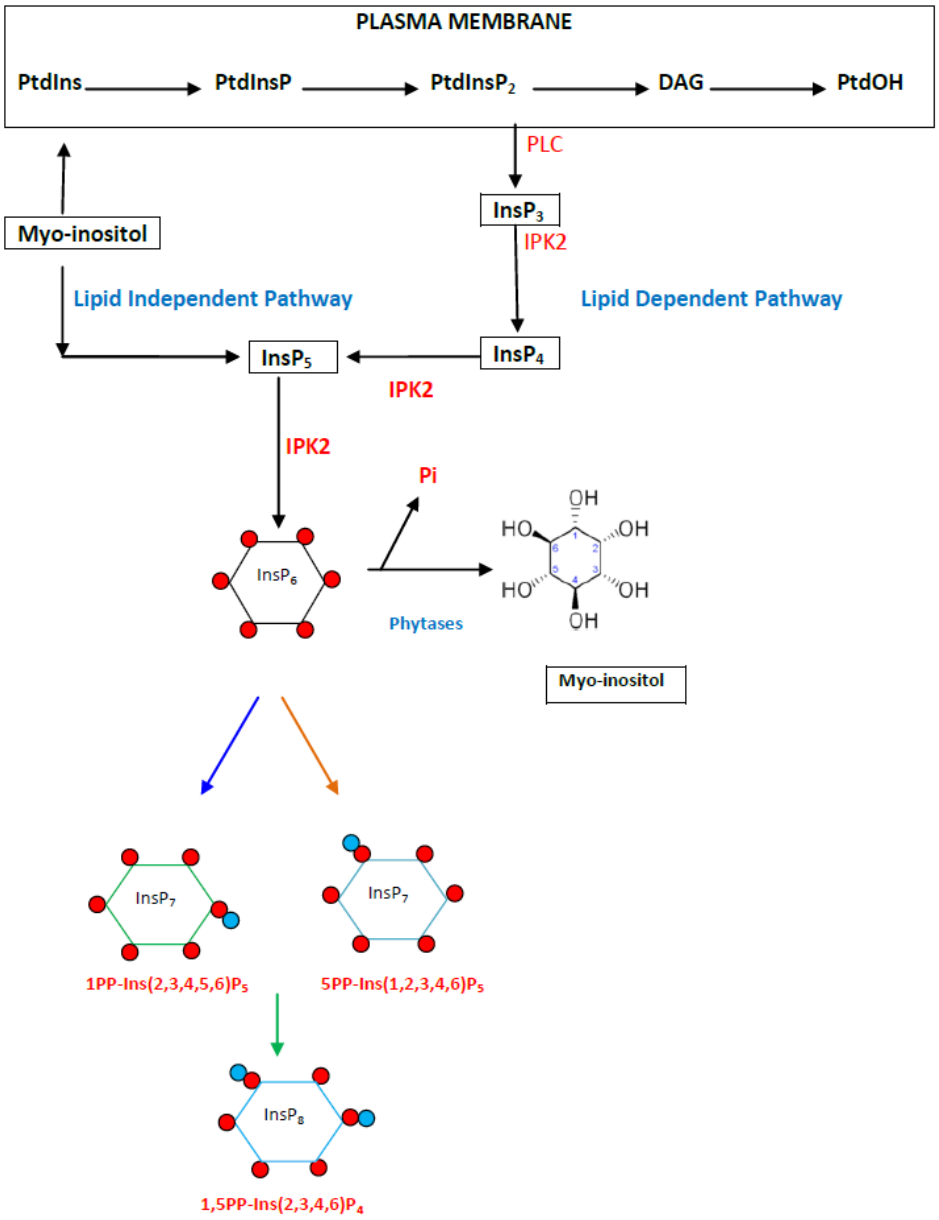


Fig-2.7 Pathway for the generation of $IP_{6,}$ other inositol phosphates, inositol pyrophsphate

2.4.2. Occurrence of other *myo*-inositol phosphates

Formation of inositol pyrophosphates, diphosphoinositol pentakisphosphate (PP-IP_5) and bis-diphosphoinositol tetrakisphosphate [$(PP)_2$-IP_4], containing seven and eight phosphates respectively in inositol ring indicates that even after the formation of $IP_{6,}$ it

may be metabolized to other moleculesis having higher number of phosphate groups. This phenomenon is supported by the discovery of an IP_6-kinase with a high affinity and specificity for IP_6 and the capacity to transfer a phosphate from PP-IP_5 to ADP to form ATP. This ATP synthase activity demonstrates a high phosphoryl group transfer potential for PP-IP_5 suggesting its physiological role (Voglmaier *et al.*, 1997). Pyrophosphate containing inositol molecules have been detected by Stephens *et al.*, (1993) from *Dictyostelium discoideum*, a slime mold. Inositol pyrophosphates are largely synthesized from $IP_{6,}$ generating IP_7 and IP_8. These IP_7 pyrophosphate also link to metabolism at the molecular (phosphate homoeostasis), cellular (energetic) and organismal (insulin signaling) levels (Wilson *et al*., 2013).

The identification of inositol pyrophosphates (inositol with diphosphate groups), with different arrangement of phosphate, diphosphoinositol pentaphosphate [IP_7 or PP-IP_5 or $(PP)_2$- $InsP_3$] (Draskovic *et al.*, 2008; Wilson *et al.*, 2013) and two diphosphate and four monophosphates [IP_8 or $(PP)_2$-IP_4] (Shears, 2009) or with one triphosphate and five monophosphates [IP_8 or (PPP)-IP_5] with seven and eight phosphate groups attached to the six-carbon inositol ring, thus possessing one and two pyrophosphate moieties respectively indicates that IP_6 is the precursor of these pyrophosphates (Draskovic *et al.*, 2008). Inositol pyrophosphates are largely synthesized from IP_6 generating IP_7 and IP_8. Two isomeric forms of IP_8 has been presented in mammalian cells, $[(1,5)PP]_2$-IP_4 and [$(PP)_2$-IP_4] but the plant *D. discoideum* contains $[(5,6)PP]_2$-IP_4 isomers of IP_8. Therefore, taking into account the diverse substrates and the formation of pyrophosphate moiety at different positions of the inositol ring, inositol pyrophosphate

have the potential to become a very large family of molecule (Losito, 2009; Wilson et al., 2013).

2.5. Signalling in plants through inositol phosphates

2.5.1. Signal transduction in plant cells

Signalling pathway in general commences with the binding of the effecter molecules (i.e. growth factor, hormone, neurotransmitters etc) to its cognate receptor on the cell surface. This binding activates a protein kinase and enzymes that leads to the activation of signalling molecules, eventually resulting in the transcription of specific genes (Barik, 1996). Many signal transduction processes occurs when plants are challenged with environmental stresses. Thus low/high temperature, drought and high salinity are very complex stimuli that possesses many different yet related attributes, each of which may provide the plant cell with quite different information for example, low temperature may immediately result in mechanical constraints, changes in activities of macromolecules and reduced osmotic potential in the cellular milieu. High salinity includes both an ionic (chemical) and an osmotic (physical) component. The multiplicity of information embedded in abiotic stress signals underlines the complexity of stress signaling.

On the basis of this multiplicity, it is unlikely that there is only one sensor that perceives the stress condition and controls all subsequent signalling. Rather a single sensor might only regulate branches of the signalling cascade that are initiated by one aspect of the stress condition. For example, low temperature is known to change membrane fluidity (Murate and Los, 1997). A sensor defeacting this change would initiate a signalling

cascade control initiated by an intracellular protein whose conformation/activity is directly altered by low temperature. Thus, there may be multiple primary sensors that perceive the initial stress signal.

Secondary signals (i.e. hormones and second messengers) can initiate another cascade of signalling events, which can differ from the primary signalling in time (i.e. lag behind) and in space (e.g. the signals may diffuse within or among cells, and their receptors may be in different sub-cellular locations from the primary sensors). These secondary signals may also differ in specificity from primary stimuli, may be shared by different stress pathways, and may underline the interaction among signalling pathways for different stresses and stress cross-protection. Therefore, one primary stress may activate multiple signalling pathways differing in time, space and outputs. These pathways may connect or interact with one another using shared components generating interwtined networks. Arachidonic acid, inositol 1.4.5-triphosphate (IP_3) and 1.2-diacylglycerol (DAG) are three different messenger molecules known to be produced from phosphoinositides. All three different messenger molecules signal differently. Arachidonate is oxygenated to form other signaling mediators like IP_3 which mobilize Ca^{2+} from its intracellular storage and DAG which performs the function of an essential cofactor of protein kinase-C (Majerus *et al.*, 1986).

2.5.2. Inositol triphosphate and calcium signalling

One early response to low temperature, drought and salinity stress in plant cells is a transient increase in cytosolic Ca^{2+}, derived from internal stores (Sanders *et al*., 1999; Knight, 2000). In the cascade of events Ca^{2+} plays a pivotal role for the conversion of an external stimulus into biological responses in plant cells (Ranjeva *et al.*, 1998). Internal

Ca^{2+}, release is controlled by ligand sensitive Ca^{2+} channels. It is thus crucial to modulate the entry of external calcium through the plasma membrane and the release of Ca^{2+} from intracellular stores to maintain its cytosolic concentration. Ca^{2+} is released from its intracellular storage which is accompanied by the breakdown of phosphatidylinositol 4.5-bisphosphate to release IP_3. The role of IP_3 coupled to the intracellular Ca^{2+} mobilization was determined from a preparation of saponin permeabilized hepatocytes. Ca^{2+} was released from non-mitochondrial vesicular store and the induction of Ca^{2+} release by IP_3 along with its kinetics and properties strongly suggests that this compound is a second messenger (Joseph *et al*., 1984).

Inositol polyphosphates, cyclic ADP ribose and nicotinic acid, adenine dinucleotide phosphate has been found to be able to induce Ca^{2+} release in plant cells and in general and guard cells in particular (Schroeder *et al*., 2001). An important feature of the role of Ca^{2+} as a signalling molecule is the presence of repetitive Ca^{2+} transients. These transients may be generated both by first round of second messengers and by other signalling molecules such as ABA that may themselves be produced as Ca^{2+} signals may have quite different signalling consequences and therefore, physiological meaning.

In plant cells, there is more than one membrane that serves as a major compartment for the sequestration and storage of Ca^{2+} with steep electrochemical gradients. Plant vascular membranes (tonoplast) possess an electrogenic H^+ pumping-ATPase (inwardly directed) that energize for the Ca^{2+} accumulation via a H^+/ Ca^{2+} antiport mechanism (Schumaker, 1986). ER may also serve as an intracellular calcium store and act as an alternative source of mobile calcium within the same cell. At least 3μM Ca^{2+}

concentration has been measured within isolated ER vesicles from aleurone cells (Bush *et al.*, 1989). Franklin-Tong *et al*., (1996) showed that Ca^{2+} waves in pollen tubes are triggered by photolysis of caged $InsP_3$ which is initiated in the nuclear-RER. Therefore there is some evidence that calcium release may occur from the ER in response to $InsP_3$ (Martinec *et al*., 2000, Choi *et al.,* 2018).

Calcium is responsible for signal transduction cascades which manifests as different physiological responses in plantss (Gilroy *et al*., 1993). In plant cells, though all the elements of calcium based transduction system are there still its detection is difficult. The IP_3 based signaling system is detectable only during the development of guard cells, pulvini and stem epidermal cells that are constantly responding to stimuli (Trewavas and Gilroy, 1991). Abscisic acid activates the channels allowing Ca^{2+} influx to the cytosol through the plasma membrane (Schroeder and Hagiwara, 1990). Free Ca^{2+} is a major structural component of the cell wall in plants and the concentration of free Ca^{2+} in the cell wall regulates growth, development and physiological responses of plants to external stimuli (Trewavas and Gilroy, 1991).

IP_3 acts to mobilize calcium from intracellular stores and constitutes an IP_3/Ca^{2+} pathway. Thus IP_3 plays a central role in the second messenger system by its ability to release Ca^{2+} (Berridge, 1987). Besides mobilization of internal calcium through IP_3, the concentration of Ca^{2+} may also increase due to the influx of external calcium. Both IP_3 and IP_4 (inositol 1,3,4,5-tretrakisphosphate) may be responsible in the entry of external calcium (Berridge and Irvine, 1989). It is known that the calcium directly flows into the cytosol through channels in the plasma membrane. However, it may also ener into the ER before entering the cytosol. The entry of external Ca^{2+} only happens when the

internal pool of Ca^{2+} is emptied. Therefore, the entry of external Ca^{2+} is coupled to the IP_3 mediated emptying of Ca^{2+} from its storage.

Fig-2.8 describes a general scheme to explain the events from the binding of ligand to its receptor leading to the formation of IP_3, DAG etc. and release of of Ca^{2+} from intracellular storage to the cytosol (Karp, 2013).

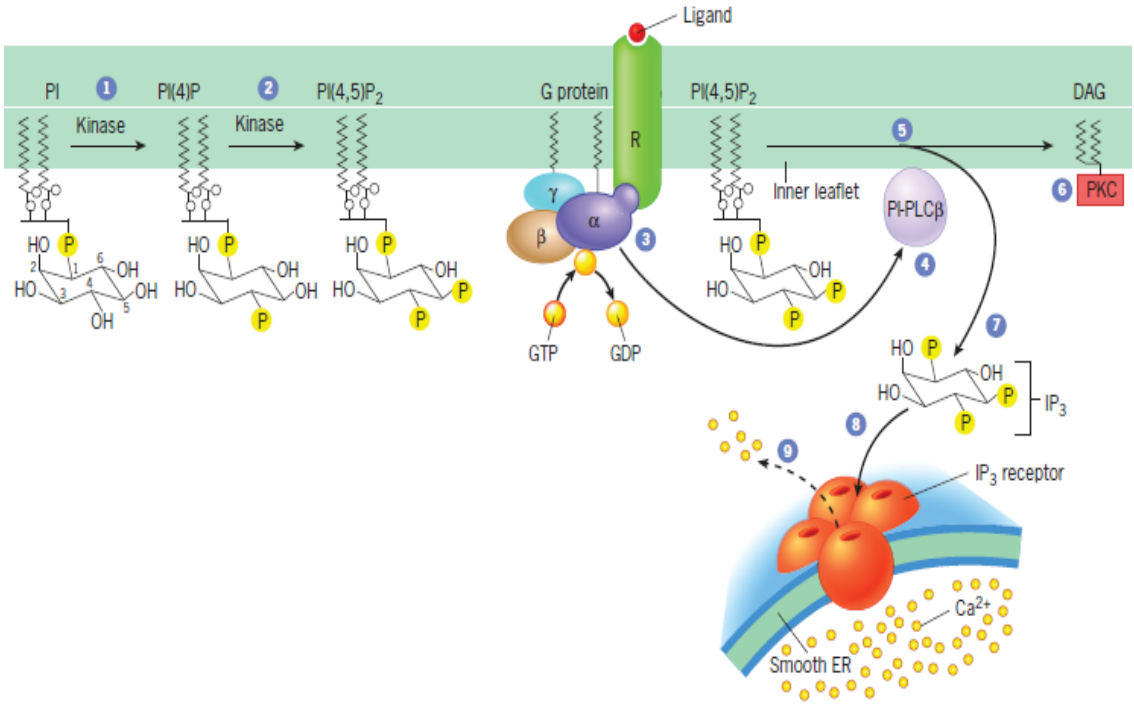


Fig-2.8 Signalling events caused by ligand induced activation of PI in the cell membrabe.Formation of PIP by the addition of phosphate group to PI by kinase (1); formation of PIP_2 by the addition of phosphate group to PIP by another kinase (2); activation of G-protein (3); activation ofe PI-specific phospholipase-C (4); breakdown of PIP_2 to IP_3 and DAG by PI-specific phospholipase-C (5); activation of protein kinase-C (PKC) in the membrane by DAG (6); release of IP_3 in the cytosol (7); binding of IP_3 to its receptor and Ca^{2+} channel in the SER membrane (8) and release of Ca^{2+} in the cytosol (9).

Drobak and Ferguson (1985) first indicated that Ins $(1,4,5)P_3$ was able to release Ca^{2+} from intercellular stores in plant cells. This release of Ca^{2+} was highly dependent on the

extra vesicular Ca^{2+} activity, which is accompanied by an inward flux of K^+, and the release has been shown to be sensitive to K^+ channel blockers (Canut *et al*., 1985). Gilroy *et al*., 1990 showed a direct evidence for Ins(1,4,5)P_3 -induced Ca^{2+} release in stomatal guard cells.

Besides Ins(1,4,5)P_3, there are other triphosphates (Ins P_3 species) namely Ins(1,3,4)P_3, Ins(1,5,6)P_3 and Ins(2,4,5)P_3, which may execute Ca^{2+} mobilization from microsomes/ vacuoles as well as liposomes to some extent *in-vitro*. For Ca^{2+} release and its mobilization, the prime requirement is the ability of any InsP_3 to bind to its receptor (InsP_3R). In plants the receptor consists of four subunits like its animal counterparts and it binds to both Ins(1,4,5)P_3 and Ins (2,4,5)P_3 with little difference in their affinities. However, the nature of the conformational changes in the InsP_3R induced by Ins(1,4,5)P_3 and Ins (2,4,5)P_3 are different. This conformational change is involved in a membrane-spanning helical domain in InsP_3R, which is required for its property as a Ca^{2+} channel. Ins(1,4,5)P_3 is about four times more effective than Ins(2,4,5)P_3 in Ca^{2+} mobilization from microsomes/ vacuoles in mung bean while Ins(1,3,4)P_3/Ins(1,5,6)P_3 cause an insignificant release of Ca^{2+} because of their poor interactions with the receptor (Gupta *et al*., 1997).

In plants, the level of intracellular I(1,4,5) P_3 increases in resposnse to a variety of extracellular stimuli such as light, pathogens, cell fusion, H_2O_2 and ethanol (Drobak, 1992; Chapman, 1998; Stevenson *et al*., 2000). Transient formation of IP_3 was observed in winter oil-seed rape leaf discs, 30 minutes after exposure to freezing temperature (Smolenska-Sym and Karpareska, 1996). Transient and long term increase in IP_3 was

also seen in *Arabidopsis* cell suspension culture exposed to cold temperature at $0^{o}C$ (Ruelland *et al*., 2002).

Chages in IP_3 level in response to hyperosmotic shock and salinity were also observed in *A. thaliana* cultured cells (T87 cells) [Taji *et al*., 2006]. A rapid and momentary increase in Ca^{+2} within a few seconds of exposure to osmotic stress has suggested that cytoplasmic increase in Ca^{+2} level in response to hyperosmotic stress is due to influx of extracellular stores (Knight *et al*., 1997).

IP_3 formation in plants has also been found to be influenced by phytohormones. Abscissic acid is involved in the plant adaptation to different environmental stress conditions. Endogenous ABA level increases significantly in many plants under water deficit conditions resulting in the tolerance to water stress (Finkelstein *et al.*, 2002; Himmelbach *et al*., 2003). ABA is responsible for inducing the stomatal closure (Schroeder *et al*., 2003). Microinjection of IP_3 in guard cells has been shown to induce stomatal closure (Gilroy *et al*., 1990). A sudden increase in IP_3 level in guard cells has been found in *Vicia faba* in response to ABA treatment (Lee *et al*., 1996). ABA induced closure of stomata under dehydaration conditions is triggered for optimization of loss of water vapour (Hethrington, 2001).

In *Phaseolus coccineus* L. leaf pulvinus, inositol triphosphate [$Ins(1,4,5)P_3$] level increases during darkness (blue light). IP_3 is the second messenger in the PI signalling cascade which increases intracellular Ca^{2+} that in turn increases membrane conductance to close K^+ channel. This intracellular mobilization of Ca^{2+} causes cells to shrink. While

in red light, the second messenger, inositol triphosphate [Ins(1,4,5)P_3] level was decreased so the mobilization of Ca^{2+} was also decreased thereby opening K^+ channel. Therefore, it indicates that in *Phaseolus coccineus*, increased inositol triphosphate production in dark leads to Ca^{2+} mobilization with the shrinking of the cells while light induces swelling the cell by the extracellular Ca^{2+} in the extensor protoplast. Therefore, this movement of the ions in and out of the cell changes turgor of the motor cells within the pulvinus which consequently leads to leaf movement (Morse *et al*., 1987, Cote, 1995; Mayer *et al*., 1997).

2.5.3. Involvement of IP_4, IP_5 and IP_6 in cell signalling.

It is believed that IP_4 controls the transfer of calcium between intracellular pools (Irvine *et al.*, 1988). A specific high affinity receptor for IP_4 has been isolated suggesting that this polyphosphate may have a messenger function (Hawthorne, 1996). IP_4 increases intracellular calcium concentration, provided calcium-mobilizing IP_3 is present. It is believed that this molecule controls the transfer of calcium between intracellular pools (Irvine *et al*., 1988) and stimulates nuclear calcium influx (Theibert *et al*., 1997). Signalling functions attributed to both IP_5 and IP_6 including Ca^{2+} uptake, neurotransmitter release and modulation of desensitization in agonist-stimulated cells have also been postulated (Menniti *et al*., 1993; Sasakawa *et al*., 1995). $InsP_6$ also acts as a signaling molecule (Lemitiri-Chlich *et al*., 2000, 2003). $InsP_6$ which is formed rapidly in response to ABA was found to be about 100 times more potent in releasing Ca^{2+} than $InsP_3$.

The enzyme inositol 5' phosphatase removes a 5' phosphate from several potential second messengers and thus terminates the signal transduction events. A gene encoding

inositol 5' phosphatase activity (At5PTase1) has been described from *Arabidopsis.* When expressed transiently in *Arabidopsis* leaf tissue or ectopically in transgenic plants, At5Ptase1 causes the hydrolysis of $I(1,4,5)P_3$ and $I(1,3,4,5)P_4$ but the enzyme does not hydrolyze I-1-P, $I(1,4)P_2$ or $PI(4,5)P_2$. The multiplicity of At5PTases indicates that these enzymes may have different substrate specificities and play different roles in signal termination (Berdy *et al*., 2001).

Inositol 1,2,3,4,5,6 hexakisphosphate (IP_6) is the most abundant inositol phosphate in cells. This molecule is involved in ABA signal transduction in guard cells and thus plays role in osmostic stress response. IP_6 is also involved in environmental stress response and in *Schizosaccharomyces pombe*, the level of IP_6 increases more than threefold in response to hyperosmotic stress (Ongusaha *et al*., 1998). Increase in IP_6 level in plants in response to dehydration leads to disruption in the activity of ion channels in guard cells. In the guard cells of *Solanum tuberosum* the IP_6 level increased more than five fold with the treatment of ABA. Here IP_6 mimics the effects of ABA that inhibits plasmalemma inward K^+ channel in the guard cells in a Ca^{+2} dependent manner (Lemitiri-Chlich *et al*., 2000). Release of IP_6 in guard cell protoplasts of *Vicia faba* has been shown to induce transient increase in Ca^{+2}. IP_6 did not affect the Ca^{2+} permeable channels in plasma membrane and that it could inhibit the plasmalemma inward K^+ channel without external Ca^{2+} suggests that IP_6 induced transient Ca^{2+} increase within guard cells was not due to external influx of Ca^{2+} but due to the release of Ca^{2+} from internal stores. Therefore, it may be concluded that IP_6 functions as a signaling mediator in ABA signal transuction in guard cells triggering Ca^{2+} release that inhibits inward K^+ channels (Lemitiri-Chlich *et al*., 2003).

2.6. Signalling in plants through phosphoinositides

2.6.1. Occurrence of phosphoinositide system in plants

Phosphoinositides in plants was first reported by Boss and Massel (1985). Since then, a number of reports have appeared indicating the presence of the compound in whole plants and the tissue cultured cells (Lehle, 1990). The occurrence of polyphosphoinositides in plant cells is now an established fact (Strasser *et al*., 1986; Heim and Wagner, 1986; Morse *et al*., 1987; Drobak *et al*., 1988). In plants, plasma membrane is the enriched source of PIP and PIP_2. The membrane-localized phosphoinositides were separated from the pulvinar extract of *Samanea saman*, and the ratio of PI, PIP and PIP_2 was found to be 32: 8:1 (Morse *et al*., 1987). The main phospholipid precursor $PI(4,5)P_2$ has been identified in plant tissues and green algal cells. Kinases that catalyze the sysnthesis of this lipid from phosphatidylinositol have been demonstrated and phosphoinositide specific phospholipase-C which hydrolyze $PI(4,5)P_2$ to yield IP_3 and DAG have been reported (Cote and Crain, 1993).

It has been reported that the plants have the same set of polyphosphoinositols as those of animals (Irvine *et al*., 1989). However, there is a difference between phosphoinositides of higher plants and animal origin in their fatty acid composition. The main fatty acids of plant inositol lipids are palmitic acid, linoleic acid and linolenic acid (Mudd, 1980; Helsper *et al*., 1987). However, there is no conclusive evidence that arachidonic acid is also a component in the higher plants (Lehle, 1990).

The enzyme, phospholipase C (PLC) is responsible for the agonist induced hydrolysis of PIP_2 (phosphatidylinositol 4,5-bisphosphate) to give two important molecules IP_3

(inositol 1,4,5-triphosphate) which acts as a second messenger and DAG. PLC activities have been detected from different plant species namely celery (Irvine *et al*., 1980; McMurray and Irvine, 1988), pollen tubes of *Lilium* (Helsper *et al*., 1987) and soybean and bush beans (Pfaffmann *et al*., 1987), green alga, *Dunaliella salina* (Einspahr *et al*., 1989), *Arabidopsis thaliana* (Hunt *et al*., 2004; Tasma *et al*., 2008, Gaude *et al*., 2008, Munnik and Testerink, 2009), tomato (Vossen *et al*., 2010), potato and rice (Singh *et al*., 2013). PLC activities from all the plant species was stimulated by Ca^{+2} in contrast to the enzymes from animal sources.

The activity of protein kinase C (PKC) is to phosphorylate different proteins. It is important in the sense that one of its substrates is IP_3-phosphatase that terminates the second messenger function of IP_3 (Connolly *et al*., 1986). PKC function in plants has been reported from zucchini (Schafer *et al*., 1985), *Amaranthus tricolor* (Elliot and Skinner, 1986) and *Neurospora crassa* (Favre and Turian, 1987).

Diacylglycerol kinase is another enzyme, which converts DAG into phosphatidic acid (PA) for which the *Arabidopsis thaliana* genome encodes seven genes (Mueller *et al*., 2002; Arisz *et al*., 2009). During the conversion, phosphoinositide synthesis may be initiated while also controlling the intracellular concentration of DAG. A membrane associated DAG-kinase of plant origin has been isolated. This enzyme having a 51 kDa protein has been identified which requires divalent cations but are completely devoid of activity in absence of phospholipid (Wissing *et al*., 1989). PA can also be generated via a separate, PLD signaling pathway (Munnik and Vermeer, 2010). PA which is produced can be further phosphorylated to diacylglycerol pyrophosphate (DGPP), which is

catalyzed by a so- called PA kinase (PAK). This enzyme and DGPP are both implicated in osmotic stress signalling (Schooten *et al*., 2006). The presence of *scyllo*-inositol containing phosphatidylinositol in the plant cells points towards the involvement of this compound in the phosphoinositide and inositol phosphate metabolism in the plant system (Kinnard *et al*., 1995).

$PI(4,5)P_2$ is an important signal mediator in stomatal opening and it is synthesized by the enzyme PIP(5)K4. When irradiated with white light, the level of $PI(4,5)P_2$ increases more at the plasma membrane than in the cytosol of guard cells possibly due to light induced increase in biosyntheisis or decresse in hydrolysis of $PI(4,5)P_2$. The synthesis of $PI(4,5)P_2$ is the rate limiting step in the plant phosphoinositide pathway (Im *et al*., 2007). Another hydrolysis product of $PI(4,5)P_2$ is IP_3 which is already famous for its role on stomatal closing (Gilroy *et al*., 1990, Blatt *et al*., 1990). It is clear that PIP5K4 is responsible for the synthesis of $PI(4,5)P_2$ and sufficient $PI(4,5)P_2$ must be there in the guard cell plasma membrane for stomatal opening to happen (Lee *et al*., 2007). PI-PLC has already been implicatd in the ABA signal transuction in guard cells (Lee *et al*., 2003)

After the discovery of PI system in plants, there have been many studies on its role in stress tolerance and stress signalling (Cho *et al*., 1993; Pical *et al*., 1999; Drobak and Watkins, 2000; Dewald *et al*., 2001; Perera *et al*., 2004; Zonia and Munnik, 2006; Konig *et al*., 2007; Leshem *et al*., 2007; Nishizawa *et al*., 2008; Wang *et al*., 2008; Yang *et al*., 2008; Zhu *et al*., 2009; Munnik and Vermeer, 2010).

2.6.2. Phospholipase-C (PLC) in plants

Phosphoinositide specific phospholipase C (PI-PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) with the production of two important molecules, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). These two molecules are second messengers that can activate protein kinase C and trigger Ca^{2+} release respectively. Second messenger IP_3 which is produced, is a soluble compound in the cytosol that triggers transient increases in the cytosolic Ca^{2+} level, and a lipid diacylglycerol (DAG) stays within the plasma membrane and activates PKC (Berridge, 1993). It is evident that IP_3 mediated Ca^{2+} release occurs in plant cell (Drobak 1992, 1993; Cote and Crain, 1993, 1994).

The presence of a PtdIns-specific phosphodiesterase (phospholipase C, PLC) in plant tissues was reported by Irvine *et al*., (1980) who found a soluble form of the enzyme in celery stems capable of hydrolysing PtdIns, the resulting products being Ins1P and cyclic Ins(1:2)P. Pfaffmann *et al*., (1987) found a PtdIns-specific PLC activity in plant stems with 90% of the activity being in a soluble form and the remainder associated with membranes. Melin *et al*., (1987) has reported plasma membrane associated PLC activity capable of hydrolysing all three phosphoinositides. This enzyme had a 5-20 times greater activity towards PtdIns4P and PtdIns(4,5)P_2 than the PtdIns. There are two main groups of PLC in plants PLC type I and II. PLC type I is predominantly soluble (cytosolic/ vacuolar) which is reported by Irvine *et al.*,(1980), has a clear preference for PtdIns over PtdIns4P and PtdIn(4,5)P_2 and requires millmolar concentration of Ca^{2+} for full activity, while PLC type II is predominantly associated with the plasma membrane,

which shows marked preference for polyphosphoinositides versus PtsIns and is fully activated by low µM concentration of Ca^{2+} (Drobak,1992).

Molecular cloning of cDNA encoding multiple PI-PLC isoforms have been reported from *Arabidopsis thaliana* (Yamamoto *et al.*, 1995; Hirayama *et al*., 1995, 1997) and *Glycine max* (Shi *et al.*, 1995). *Arabidopsis thaliana* contains nine PLCs (Roeber and Pical 2002; Munnik and Testerink, 2009), but out of nine, only seven are likely to be catalytically active (Hunt *et al.*, 2004; Tasma *et al.*, 2008). *Arabidopsis thaliana* contains six of them, which are called NPCs for non specific PLC (Gaude *et al.*, 2008). Similarly, tomato contains six (Vossen *et al*., 2010), rice contains nine (Singh *et al*., 2013), and potato contains three phosphoinositide-specific phospholipase C isoforms.

PLC in eukaryotes are classified into six different subfamilies, PLCβ,γ,ε,π,δ and ζ (Munnik and Testerink, 2009). Out of these six different subfamilies, PLCβ isoform is activated by the heterotrimeric regulatory GTP-binding protein belonging to the Gq subfamily (Wu *et al.*, 1993), while in *Dunaliella salina* , the plasma membrane polyphosphoinositide-specific PLC is activated by 100 µM –GTPγS over a range of free Ca^{2+} concentration . Evidence in favour of involvement of G-protein in PLC activation has also been presented by Dillenschneider *et al.*, (1986) and it was who found that guanine nucleotide stimulated the release of inositol phosphates from ^{3}H-inositol labelled membranes isolated from cultured sycamore cells.

Environmental signals controls the aperture of stomatal pore by activiating guard cell signalling pathways that result in alterations to guard cell turgor (Schroeder *et al*.,

2001). The control of guard stomatal opening is mediated by ABA which acts by increasing cytosolic Ca^{2+} concentration (Schroeder and Hagiwara, 1990; Blatt, 2000). ABA may generate the increased level of guard cell Ca^{2+} concentration by the involvement of phosphoinositide specific phospholipase-C (Gilroy, 1990; Lee *et al.*, 1996, Staxen *et al*., 1999). It is believed that in addition of IP_3 and possibly IP_6 (Lemitiri-Chlieh *et al*., 2000) may act as Ca^{2+} mobillising compound in guard cell ABA signaling (MacRobbie, 2000). On the other hand, PLD has also been found to be involved in ABA induced stomatal closure (Sang *et al*., 2001), which is interestingly independend to elevation in Ca^{2+} level (Jacob *et al.*, 1999). In ABA induced guard cell signalling, one of the six different mechanisms may be utilized for the increase in cytosolic Ca^{2+} concentration which in turn is dependent on ABA concentration (MacRobbie, 2000). In this view, it may be safely concluded that guard cell ABA signaling pathway is characterized by flexible connections rather than being hardwired (Lee *et al.*, 2003).

2.6.3. Phosphoinositide signalling during osmotic stress

Osmotic stress is a sudden change in the solute concentration around a cell causing a rapid change in the movement of water across its cell membrane. There are two types of osmotic stress: hypo and hyperosmotic stresses. All organisms have mechanisms to respond to osmotic shock depending on the nature and level of stress with sensors and signal transduction networks providing information to the cell about the osmolarity of its surroundings, these signals activate responses to deal with extreme conditions and

thus re-establish the osmotic balance. Different phosphoinositide isomers have been identified in plant cells which help to maintain osmotic equilibrium, (Munnik, 2010).

Plants have evolved several mechanisms to respond to changes in the extracellular osmotic potential. Sudden shifts of extracellular osmotic gradients induce dynamic changes in ion fluxes across the plasma membrane as an early osmoregulatory response (Schroeder and Hagiwarra 1989; Ivashikina *et al.*, 2001). Osmoregulatory ion fluxes are also regulated by specific inositol polyphosphate signal and by PI(4,5)P_2 dependent phospholipase C (PLC) signalling. Osmotically triggered PI(3,5)P_2 responses have been reported from *Chlamydomonas*, tobacco pollen tube, epidermal strips of pea leaves and suspension cultured cells of tomato and alfalfa (Meijer *et al.*, 1999; Zanig and Munnik, 2004). In *Arabidopsis thaliana* PtdIns(4,5)P_2 levels was increased by 8-25 times when cells are subjected to hyperosmotic stress. PtdIns(4,5)P_2 produced is then hydrolyzed to Ins(1,4,5)P_3 and diacylglycerol. These two second messengers rapidly increases with the increased salinity and hyperosmotic stress in *Arabidopsis thaliana* cells and induces Ca^{2+} release from the internal stores. Besides this, they actively participate to regulate the dynamics of the actin cytoskeleton through the interaction with actin binding proteins and activate protein kinase C and PI-PLC. A new-3-phosphorylated phosphoinositides phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2] was also identified in *Arabidopsis* and yeast. This [PtdIns(3,5)P_2] also proposed to play a role in membrane trafficking in the endosomal/lysosomal system by regulating the fission of endolysomal subcompartments, which is accelerated by hyperosmotic stress (Dove *et al*., 2009).

Hyperosmotic stress induces increase in the levels of $PI(4,5)P_2$ in plant cells (Pical *et al.*, 1999; Takahashi *et al.*, 2001; Dewald, 2001) and in some cases it is correlated with an increase in IP_3 (Drobak and Watkins, 2000; Takashashi *et al.*, 2001) which may have a role in mobilizing cytosolic Ca^{2+} (Knight *et al*., 1997; Kiegle *et al.*, 2000). However, the studies of Lemitri-Chlich *et al.*, (2000, 2003) suggest that higher order inositol polyphosphates may induce the mobilization of cytosolic Ca^{2+}. Under hyperosmotic stress yeast cells induces the production of $PI(3,5)P_2$ which is supposed to play a role in membrane trafficking in the endosomal/lysosomal system. $PI(3,5)P_2$ regulated the fission of endosomal subcompartments which is accelerated in yeast in response to hyperosmotic stress in different cells (Leshem *et al.*, 2007; Zonia and Munnik, 2008). Plant cells rapidly responds to hyperosmotic stress with a battery of signals including PI(5)P, $PI(3,5)P_2$, $PI(4,5)P_2$, PA (phsphatidic acid), DGPP and IP_3 (Xiong *et al.*, 2002, Meijer and Munnik, 2003). These signals have been identified in a number of different plant species and tissues either singly or in combination and appear to be correlated with the severeity of the stress (Zonia and Munnik, 2006). The varieties of signals elicited by hyperosmotic stress indicate the osmotic status of the plant cells and are critical for the survival strategies of the plants.

In *Chlamydomona*s, different phospholipid signaling pathways were stimulated by the degree of severity of stress. The PtdOH generated in response to a strong hyperosmotic signal appears to be an outcome from activation of PLC-DGK and PLD pathways. Hyperosmotic stress also increased the production of numerous phosphinoside phosphates (Munnik and Meijer, 2000). Increase in $PI(4,5)P_2$ occurred at the expense of

PI(4)P and not PI(5)P suggesting that PI(3,5)P_2 may be synthesized from PI(5)P (Meijer *et al.*, 2001).

But during hypo-osmotic stress, PLD activity was rapidly stimulated and induces increase in PA with change in Cl^- anion flux across the plasma membrane (Teodoro *et al.*, 1998; Barber-Brygoo *et al.*, 2000; Shabata *et al.*, 2000). An evidence of phosphoinositide mediated responses to hypotonic stress comes from experiments with the green alga, *Dunaliella salina*, a species that survives in salinities between 0.05 and 0.5M. On long term basis, the plant maintains osmotic equilibrium by adjusting glycerol levls. In the short term, rapid changes in ion flux and cell volume protects the cells. Under hypotonic conditions, in *D. salina* PI(4,5)P_2 is rapidly hydrolyzed as seen in the decrease in radioactive phosphates in PI(4,5)P_2 and PI(4) (Einspahr *et al.*, 1988). In plants, incresed Ca^{2+} level have been implicated in cell responses to low osmolarity (Okazaki and Tazawa, 1990). In green algae, phosphoinositide turnover have been found to trigger Ca^{2+} mobilization, which mediates responses to hypotonic stress (Cote *et al*., 1996). *Laniprothamnium succinum* responds to hypotonic stress with an influx of Cl^- (Okazaki and Iwasaki, 1992) which depolarizes the plasma membrane and triggers K^+ efflux through depolarization activated K^+ channels. The efflux of K^+ and Cl^- decreases cytosolic osmotic concentration and balances the environmental osmotic strength. These changes in ion permeability may be triggered by Ca^{2+} mobilization because they are preceeded by an increase in cytoplasmic Ca^{2+} levels (Okazaki and Iwasaki, 1992).

2.6.4. Signalling in plants by wounding

In natural habitats plants are exposed to frequent stress by herbivory, insect attack, pathogen attack, physical injury or mechanical wounding etc. Therefore plants develop systemic defence responses as well as express several sets of defence-related genes that are involved in healing damaged tissues (Lawton and Lamb, 1987; Brederode *et al.*, 1991; Memelink *et al.*, 1993). These genes are activated through signalling pathways that include various protein kinases (Seo *et al.*, 1995; Mizoguchi *et al.*, 1996; Bogre *et al.*, 1997; Zhang and Klessig, 1998a). Plant responses to wounding are diverse and many plant species demonstrate a systemic increase in phosphatidic acid (PA) and lysophospholipid levels. In the wounded tomato leaf, phosphatidic acid increased approximately four fold within five minutes whereas lysophosphatidylcholine and lysophosphatidylethanol-amine increased two fold within 15 min of wounding. Similarly phosphatidic acid levels were increased upon wounding in broad bean, soybean, sunflower and pepper seedlings (Lee 1997). In soybean (*Glycine max* L), phosphatidic acid (PA) acts as a second messenger in wound signalling, as levels of PA increases rapidly and transiently at the wound site (Lee *et al.*, 1997).

Mitogen-activated protein kinase (MAPKs) may also be important in wound signal transduction in plants. Two different MAPKs, WIPK (wound induced protein kinase) and SIPK (Salicylic acid-induced protein kinase) are activated in tobacco plants after wounding (Zhang and Klessing 1998a). The kinase activity and mRNA levels of WIPK (tobacco) and its orthologs from alfalfa (SAMK) and *Arabidopsis* (*A*tMPK3) increase upon mechanical stress (Seo *et al.*, 1995; Mizoguchi *et al.*, 1996; Bogre *et al.*, 1997). Similar to tobacco SIPK, the alfalfa and *Arabidopsis* orthologs SIMK and AtMPK6 are also activated in response to wounding (Zhang and Klessing, 1998b; Romeir *et al.*,

1999). In soybean (*Glycine max*.L), wounding activates the SIMK- like MAPK. The wound activation of the MAPK is inhibited when PA production is suppressed and exogenously applied PA specifically activates the MAPK in soybean cells. This suggests that PA participates as a second messenger in wound signal transduction by activating a specific MAPK cascade in soybean (*Glycine max*.L) (Lee *et al.*, 2001).

In *Arabidopsis*, wound signalling is implicated by various biochemical signals, including jasmonic acid (JA), salicylic acid (SA), auxin and Ca^{2+}. The levels of inositol 1,4,5 triphosphate ($InsP_3$) increased four to five fold within 30 min of mechanical wounding accompanied by transient decrease in the precursor lipids PtdIns, PtdIns4P and PtdIns(4,5)P_2, in *Arabidopsis.* Jasmonic acid (JA), formation is necessary for the production of wounding-induced $InsP_3$ signals. Exogenous methyl-JA may cause increased production of $InsP_3$ in wounded *Arabidopsis.* Similar increased levels of $InsP_3$ in *Arabidopsis* rosettes treated with sorbitol have been observed, suggesting that $InsP_3$ may have been formed upon release of endogenous JA (Lobler and Lee, 1998; Stenzel *et al.*, 2003).

Induction of various defence-related genes induced by wounding suggests the role of phosphoinositides in the regulation of defence gene expression. Growth of herbivorous caterpillars was increased on plants with attenuated phosphoinositide signalling. These results establish the involvement of the phosphoinositide system in signal-transduction events leading to the induction of defence responses after mechanical wounding in *Arabidopsis* leaves (Mosblech *et al.*, 2008).

2.6.5. Phosphoinositide signalling in response to pathogen attack

The phosphoinositides are minor phospholipids present in all eukaryotic cells. They are storage forms of messenger molecules that transmit signals across the cell membrane and evoke responses to extracellular agonists. The phosphoinositides break down to liberate messenger molecules in response to occupancy of receptors by specific agonists. All three phosphoinositides are degraded by Phospholipase C (PLC) to form IP_3 and DAG that are messenger molecules serving different functions.

Plants are constantly challenged by pathogens such as bacteria or fungi and become a major threat to plants. Therefore, to cope with the challenges, sophisticated defence mechanisms are erected that resulted in a highly structured plant immune system (Wirthmueller *et al.*, 2013). There are two components of plant immunity: a pathogen-associated molecular pattern triggered immunity (PTI) and an effector triggered immunity (ETI) (Ruelland *et al.*, 2014). A PTI is activated upon recognition of receptor (PRR), whereas a more specialised ETI is responsible for recognition of race-specific pathogen effectors by nucleotide-binding to leucine-rich repeat (NB-LRR) proteins (Kumar and Mysore, 2013). Both PTI and ETI acts simultaneously, which leads to an expression of defence genes and facultatively, to a hypersensitive cell death response (HR) that restricts pathogen propagation at the infection site.

According to Canonne *et al*., (2011), plant phospholipases are essential components for PTI-response. In tomato cells, a significant increase of PA production, implicating both PLD and PLC/DGK pathway was observed after 30 min of treatment with Chitosan, a polysaccharide elicitor (Raho *et al.*, 2011). Similarly, a rapid accumulation of

lysophosphatidylcholine by the activation of PLA2 in cultured cells of California poppy elicited with a yeast glycoprotein was reported (Viehweger *et al*., 2002). These findings suggest that each phospholipase has its specific role in plant immunity. Similar implication of lipid signalling to ETI response is also evident. In *Arabidopsis thaliana* high accumulation of PA has been observed in response to wounding and thus may play a role in biotic stress signalling necessary for resistance to herbivore attack (Bargmann *et al.*, 2009).

In rice cells, the application of exogenous PA or (DAG) could mimic the effects of the N-acetylchitooligosaccharide elicitor as suggested by the triggering of ROS generation or expression of defence-related genes (Yamaguchi *et al.*, 2005), while in tomato PA application is sufficient to induce the accumulation of phytoalexin (Wang *et al*., 2013). Therefore, PA acts as an active molecule in the immune response.

Several types of phospholipases in plant immunity have been participated at the molecular level also. A role in *Arabidopsis thaliana* resistance to powdery mildew fungus (*Blumer graminis* f sp *Hordei*) has been specifically ascribed to PLDδ that accumulated in the plasma membrane near the site of fungal attack (Pinosa *et al.*, 2013). Analysis of PLDδ deficient plants demonstrated a declining resistance to fungal spore's penetration. The chitin induced expression of defence genes was also retarded in pldδ mutant. In pepper, silencing of the CaPLP1 gene, encoding a pPLA2, activity resulted in loss of resistance to *Xanthomonas campestris* pv. *vesicatoria* (Kim *et al.*, 2014). The expression of CaPLP1 *in A. thaliana* (under the CaMV 35S promoter) has rendered plants more tolerant to the pathogen infection. This effect was accompanied by an

increased ROS generation and an enhanced expression of several defence-related genes upon infection (Kim *et al.*, 2014). In contrast, the activity of some of the plant phospholipases seems to favour the resting state of plant defences. Suppression of PLDˇ1 expression in rice resulted in the activation of defence like reactions that include accumulation of ROS and phytoalexins and expression of defence-related genes, contributing to elevated resistance to pathogenic fungi. These defence reactions are thus constitutively repressed by a basal PLDˇ1 activity (Yamaguchi *et al.*, 2009).

2.7. Molecular biology of MIPS gene

2.7.1. Evolution of MIPS gene

Duplication of gene is one of the key driving forces in the evolution of gene and important features of genomic architecture of living organisms including plants. Gene duplication which is followed by its divergence and adoptive specialization of the pre-existing genes may have given diversity to plants (Zhang 2003; Flagel and Wendel, 2009). Therefore, gene duplication followed by functional divergence possibly results in two alternative evolutionary fates i) Neo-functionalization, where one copy acquires an entirely new function whereas the other copy maintains the original function. ii) Sub-functionalization, where each duplicate gene copy adopts part of the task of their parental gene (Ohno, 1970; Jenesen, 1976; Orgel, 1977; Hughes, 1994; Nowak *et al*., 1997). Among these two evolutionary fates, subfunctionalization is reported as a more prevalent outcome in nature because gene duplicate diverge mostly through the partitioning of gene expression and it can also take place at the protein function level leading to functional specialization when one of the duplicated gene becomes better at

performing one of the original functions of the progenitor gene (Hughes, 1994; Gu *et al.*, 2002; Zhang *et al.*, 2002; Conant and Wolfe, 2008).

Structural and functional genomics along with bioinformatics throws ample light in explaining the evolution of genes across divergent phylogenetic groups among prokaryotes and eukaryotes. Biosynthesis of iositol has been considered as an evolutionarily conserved pathway. Free inositol occupies the central position in inositol metabolism because this free inositol can be chanellized to various metabolic routes and produce different inositol derivatives (Fig 2.9)

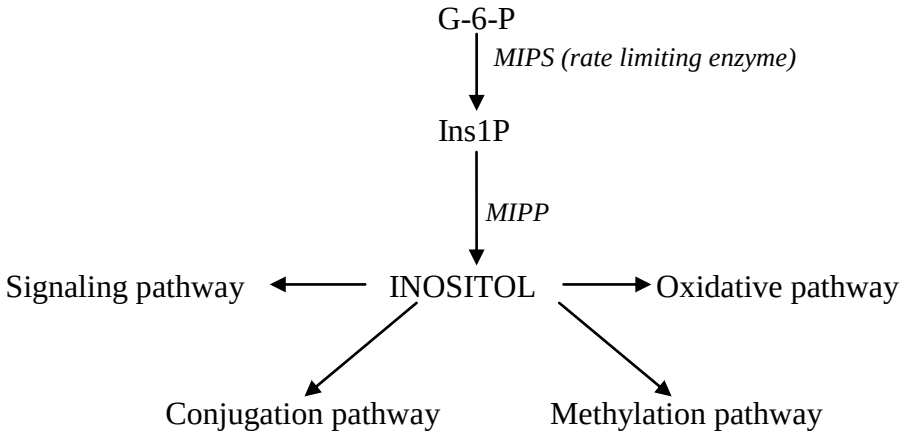


Fig-2.9 Outline of inositol biosynthesis and its utilization in other pathways

This free inositol and its derivatives have acquired different functions over the course of evolution e.g. inositol containing phospholipids are the important constituents of many archaea. With the emergence and diversification of eukaryotes, inositol is shown to be involved in growth regulation, membrane biogenesis, hormone regulation, signal transduction, pathogen resistance, stress adaptation etc. (Loewus and Murthy, 2000;

Stevenson *et al.*, 2000; Michael, 2008). MIPS enzyme is considered to be an ancient protein/gene. Few higher plants and algae are reported to have cytosolic and chloroplastic form of MIPS. However, the enzymatic and biochemical properties of these two forms do not differ significantly between each other. Since the identification of first structural gene for MIPS (*INO1*) from a fungus (Donahue and Henry, 1981a; many MIPS genes has been reported from various sources including prokaryotes and eukaryotes. Majumder *et al*., (2003) have highlighted the evolution and diversification of MIPS in which prokaryotic MIPS protein sequences were quite divergent among themselves and significantly distinct than any other known eukaryotic sequences. In contrast, the eukaryotic MIPS sequences show remarkable similarities among each other. In the biological kingdom, diverse organisms present an overall evolutionary divergence of the MIPS sequences to produce a phylogenetic tree. Therefore, one close subgroup constitutes higher plants and the other subgroup in the eukaryotic cluster constitutes fungi and other organisms.

The phylogenetic tree constructed on the basis of multiple alignments of MIPS gene shows clear segregation of these genes into monocotyledons and dicotyledons. Therefore, a divergence of MIPS gene happened after the divergence of monocots and dicots. Alignments of the DNA sequence of the gene encoding plant MIPS revealed remarkable evolutionary conservation. The presence of conserved sequences and conservative changes observed in a wide range of organisms indicate the central role that enzyme plays in biological systems (Majumder *et al*., 2003). The evolution of the MIPS gene is more diverse and complex among the prokaryotes than amongst the eukaryotes. More than 80 genes homologous to *INO1* have been reported till date and it

has been found that a core catalytic structure is conserved across evolutionarily divergent taxa (Majee and Kaur, 2011).

2.7.2 Identification of *INO1*gene from *Saccharomyces*

Culbertson and Henry (1975) have reported several mutants of *Saccharomyces cerevisiae*, which lack the enzyme MIPS. These mutants are auxotrophic for inositol and are therefore, conditionally defective in the synthesis of inositol containing phospholipids. These mutants have been categorized into 10 different genetic complementation groups termed ino-1 through ino-10 of which the *INO1* locus has been identified as the structural gene for inositol synthase (Donahue and Henry, 1981a). Other loci have been considered to have regulatory function (Culbertson and Henry, 1975). Of these, special mentions may be made of the mutants of ino-5 class, which has been designated as the model organism for the proposed 'rescue-synthesis' of *myo*-inositol (Majumder, 1981). Almost all of these mutants exhibited 'inositol-less-death' when deprived of inositol. Inositol requiring mutants of some other fungi also exhibit this property (Strauss, 1958). The fact that 'inositol-less-death' is observed in a variety of fungal mutants and strains suggests that the phenomenon is basically related to the role of inositol containing lipids during the growth of fungi in media.

The enzyme MIPS (*myo*-inositol-1-phosphate synthase) is a large multimer (240 kDa) consisting of identical subunits of 62 kDa. The gene *INO1*, encoding the enzyme *myo*-inositol-1-phosphate synthase from *Saccharomyces cerevisiae* was isolated by genetic complementation. The *INO1* gene was fully regulated when its gene was located extrachromosomally on the autonomously replicating plasmid. The cloned sequence was shown to complement two independent INO alleles (*ino1-5* and *ino1-13*). Out of

these two, *ino1-5* fail to make any material that is cross reactive with antibody to the wild type inositol-1-phosphate synthase (Klig and Henry, 1984). Of all the inositol auxotrophs isolated, approximately 70% are shown to be alleles of the *ino1* locus, the structural gene for inositol-1-phosphate synthase. Alleles of two other loci, *ino2* and *ino4* comprise 9% of total mutants, with the remainder representing unique loci or complementation group. The expression of the *INO1* gene from *Saccharomyces cerevisiae* is regulated by factors that affect phospholipid synthesis (Hirsch and Henry, 1986) but are not regulated by the products of the *INO2*, *INO4* and *OPI1* (Graves and Henry, 1999), however, *INO2* expression in *Saccharomyces cerevisiae* is controlled by positive and negative promoter elements and an upstream ORF (Eijnhamer, 2001). Sequence analysis shows that *INO1-100* had a 239bp deletion in the INO protein. It was constitutive and independent of these regulators (Swifts and McGraw, 1995).

2.7.3. Occurrence of *INO1* in other plants and microbes

Molecular characterization of inositol synthase structural genes was undertaken from several organisms (Table-2.2) which revealed the biochemistry governing inositol synthase regulation (Majumder *et al.*, 1997). In the aquatic angiosperm, *Spirodela polyrrhiza*, inositol synthase expression was upregulated due to ABA induced morphogenetic response (Smart and Fleming, 1993). The synthase gene transcript increases in response to ABA treatment, accompanied by 3-fold increase in free *myo*-inositol. This is followed by sequential increase in inositol phosphates and in the accumulation of IP_6 (Flores and Smart, 2000). Hitz *et al.*, (2002) studied a mutation in soybean (*Glycine max* L. Merr.) involving a single base change in the third base of the codon that encodes the amino acid residue 396 of the peptide of a seed expressed MIPS

gene. The base substitution causes the residue 396 to change from lysine to asparagine and the change decreases the seed expressed MIPS activity by about 90%. With the decrease in this enzyme activity, the synthesis of *myo*-inositol hexakisphosphate also decreases while the level of inorganic phosphate increases.

In response to salinity stress, the inositol synthase expression is upregulated in *Mesenbryanthemum crystallinum*, (Ishitani *et al*., 1996). A cDNA encoding In *Arabidopsis thaliana* MIPS was produced by Johnson (1994). The expression of the corresponding gene was found to be regulated by inositol concentration (Johnson and Sussex, 1995). The homology of cDNA between *S. polyrrhiza* and *A. thaliana* sequences indicate a high degree of conservation in synthase gene (Johnson, 1994). Yoshida *et al.*, (1999) reported a cDNA clone, *pRINO1* from rice (*O. sativa*), that is highly homologous to the inositol synthase from yeast. The full-length *pRINO1* cDNA sequence revealed an ORF of 510 amino acids. Homology studies revealed an approximate 50% identity of this *INO1* gene to that from *S. cerevisiae* and 86-88% identity with those from *S. polyrrhiza, B. napus, A. thaliana* and *P. vulgaris* (Yoshida *et al*., 1999). It was also found that under the influence of either sucrose or ABA, a higher level of *pRINO1* expression was found to occur in the cultured cells, from scutellum of mature rice seeds. When the said culture was treated with both sucrose and ABA together, a much higher level of gene expression occurred suggesting a synergistic induction of the inositol synthase gene (Yoshida *et al*., 2002).

In case of *Candida albicans*, the sequence of inositol biosynthetic gene, *CaINO1* has been determined. Subsequently, the largest ORF was found to have a coding sequence

of 1560 base pairs, corresponding to a protein of 521 amino acids. The deduced amino acid sequence of *C. albicans INO1* gene product with its homolog in *S. cerevisiae* shows 64% identity and 77% similarity (Klig *et al*., 1994). Similarly, a monocotyledonous resurrection plant, *Xerophyta viscosa* expresses an *INO1* gene termed *XINO1* having striking homology (70-99%) with a numbers of *INO1* genes from other plant sources. This *XINO1* gene is catalytically active at lower range of temperature between 10 ^{o}C and 40^{o}C as opposed to the *RINO1* gene-product making this protein a unique among all MIPS proteins reported so far (Majee *et al*., 2005).

Table-2.2: List of some important organisms from which *INO1* gene homologs have been identified, cloned and sequenced

Organism	**Reference**
Aeropyrum pernix	Majumder *et al.,* 2003
Actinidia arguta	Vizzotto and Falchi, 2016
Actinidia eriantha	Vizzotto and Falchi, 2016
Actinidia rufa	Vizzotto and Falchi, 2016
Actinidia deliciosa	Vizzotto and Falchi, 2016
Anopheles gambii	Majumder *et al.,* 2003
Aster tripolium	Majumder *et al.,* 2003
Avena sativa	Majumder *et al*., 2003
Avicennia marina	Majumder *et al.,* 2003
Arabidopsis thaliana	Johnson(1994);Johnson and Sussex(1995); Johnson and Burk(1995)
Archaeoglobus fulgidus	Chen *et al*., (2000)
Brassica juncea	Majee *et al*., (2004); Das Chatterjee *et al.,* (2006)
Brachypodium distachyon	Nussbaumer *et al.,* (2013)
Candida albicans	Klig *et al*., (1994)
Cicer arietinum	Ishitani *et al.,* (1996); Boominathan *et al.,* (1996)
Citrus paradisi	Abu Abied and Holland (1994)
Drosophila melanogaster	Park *et al.,* (2000)
Eleusine coracana	Reddy (2013)
Entamoeba gracilis	Lohia *et al*., (1999)
Entamoeba histolytica	Lohia *et al*., (1999)
Euglena gracilis	RayChaudhuri *et al.,* (1997)
Glycine max	Hegeman *et al*., (2001); Chiera and Grabou (2007)
Homo sapiens	Ju *et al.,* (2004)
Hordeum vulgare	Larson and Kaboy (1999)

Ipomoea batatas	Zhai *et al.*, (2015)
Jatropha curcas	Wang *et al.*, (2011)
Medicago falcata	Tan *et al.*, (2013)
Medicago sativa	Tan *et al.*, (2013)
Medicago truncatula	Nussbaumer *et al.*, (2013)
Mesenbryanthemum crystallinum	Ishitani *et al.*, (1996)
Mycobacterium tuberculosis	Bachhawat and Mande (1999)
Nicotiana tobacum	Hara *et al.*, (2000)
Neurospora crassa	Mewes *et al.*, (2002)
Oryza sativa	Yoshida *et al*., (1999)
Passiflora caerulea	Abreu and Aragao (2007)
Passiflora coccinea	Abreu and Aragao (2007)
Passiflora edulis	Abreu and Aragao (2007)
Passiflora eichleriana	Abreu and Aragao (2007)
Passiflora nitida	Abreu and Aragao (2007)
Phaseolus vulgaris	Johnson and Wang (1996); Lackey *et al.*, (2003)
Porteresia caorctata	Majee *et al.*, 2004
Ricinus communis L.	Wei *et al.*, (2010)
Saccharomyces cerevisiae	Donahue and Henry(1981); Klig and Henry (1984); Johnson and Henry (1989)
Solanum tuberosum	Keller *et al.*, (1998)
Solanum lycopersicum	Keller *et al.*, (1998), Nussbaumer *et al.*, (2013)
Sorghum bicolor	Nussbaumer *et al.*, (2013)
Sesamum indicum	Chun *et al.*, (2003)
Spartina alterniflora	Joshi *et al.*, (2013)
Spartina alterniflora	Joshi *et al.*, (2013)
Spirodela polyrrhiza	Katsoulou *et al.*, (1996); Smart and Fleming (1993)
Spirulina platensis	RayChaudhuri *et al.*, (1997)
Synechocystis sp	Chatterjee *et al.*, (2004)
Triticum aestivum	Ma *et al.*, (2013)
Xerophyta viscosa	Majee *et al*., 2005
Zea mays	Carson and Raboy (1999)

The *Archaeoglobus fulgidus* inositol synthase gene was cloned and overexpressed in *E. coli*. The native enzyme presented a tetrameric structure of approximately 168 ± 4 kDa with four equal subunits of 44 kDa each. The K_m for G-6-P and NAD^+ were 0.12 ± 0.04 mM and 5.1 ± 0.9 µM respectively (Chen *et al*., 2000).

The *INO1* gene has also been identified, cloned and sequenced from *Mycobacterium tuberculosis*. The *INO1* mutation in yeast can be functionally compensated by *INO1* of this microbe and this gene revealed a distinct class of inositol synthase. The 367 residue-long enzyme from *Mycobacterium tuberculosis* is considerably shorter than the eukaryotic analogues which are at least 500 –550 residue long (Bachhawat and Mande, 1999).

INO1 gene from the *Entamoeba histolytica* has also been cloned and sequenced. The *INO1* gene encodes a 514 amino acid ORF with a molecular mass of 57.44 kDa. *INO1* cDNA clones from *E. histolytica* have been sequenced and its comparison with genomic sequence confirms that INO1 ORF contains no introns. The purified inositol synthase from *E. histolytica* was hybridized with polyclonal antibody raised against cytosolic inositol synthase from *E. gracilis* and and analysed. This pointed towards the structural similarities of inositol synthase across species (Lohia *et al*., 1999).

Eukaryotes use ATP dependent chromatin remodelling complexes to regulate gene expression. Inositol polyphosphates can modulate the activities of several chromatin remodelling complexes in vivo. Mutations in genes encoding inositol polyphosphate kinases that produce IP_4, IP_5 and IP_6 impair transcription in vivo. These results provide a link between inositol polyphosphates, chromatin remodelling and gene expression (Shen *et al*., 2003).

2.7.4. Expression of MIPS at different locations within cells and organs

MIPS, the rate limiting enzyme has been reported from various sources and MIPS-coding sequences have been cloned and characterized from widely different organisms

including plants (Majumder *et al.*, 2003). Expression of the enzyme has been detected in different organs as well as cell organelles. Two forms of MIPS enzyme – chloroplastic forms in some plants and algae and cytosolic form have been reported (Adhikari *et al.*, 1987; RayChaudhury *et al*., 1997; Hait *et al.*, 2002; Chhetri *et al.*, 2006c). Since MIPS enzyme is isolated as a soluble enzyme, it is expected that inositol phosphate biosynthesis is largely restricted to the cytosol. Cytosolic and plastidic MIPS activity has been localized in *Pisum sativum* (Imhoff and Bourdu, 1973), *Vigna radiata* and *Euglena gracilis* (Adhikari *et al.*, 1987), *Citrus paradisi* (Abu-abied, 1994), *Arabidopsis thaliana* (Johnson and Sussex, 1995), *M. crystallinum* (Ishitani *et al.*, 1996), *Phaseolus vulgaris* (Johnson and Wang, 1996), *Oryza stiva* (Ray Chaudhury and Majumder, 1996; Hait *et al.*, 2002), *Zea mays* and *Hordeum vulgare* (Keller *et al.*, 1998), *Brassica napus* (Larson and Raboy, 1999), and *Diplopterygium glaucum* (Chhetri *et al*., 2006a,c). Cytosolic MIPS has been isolated and characterized from many species of plants across the phylogetic divisions (Table-2.1)

Interestingly, in the developing organ of *Phaseolus vulgaris*, MIPS is expressed in several membrane-bound organelles such as plasma membranes, plastids, mitochondria, endoplasmic reticulum, nuclei as well as cell wall of beans (Lackey *et al.*, 2003). Multiple isoforms of MIPS enzyme often reflects the number of subcellular compartments in which the same catalytic reaction is required. Therefore, the detection of the number of MIPS isoforms reflects the distribution of the enzyme to different other cellular compartments in addition to the cytosol and chloroplast (Johnson and Wang, 1996). The *Phaseolus* gene contains upstream ORFs interspersed with consensus RNA splice sites that predict five transit peptides, each with a high probability of

directing the enzyme to different cellular compartments, including the nucleus, thylakoid membranes of chloroplast and microbodies.

MIPS transcript was discovered in the scutellum and aleurone layers of rice embryos by Yoshida *et al.*, (1999). Soybean (*Glycine max*) represents four MIPS genes: in flowers, leaves, roots and germinating cotyledons (Hegeman *et al.*, 2001; Chappelle *et al.*, 2006). Seven homologous MIPS sequence were mapped on different chromosomes in *Zea mays* (Larson and Raboy, 1999). In sesame (*Sesamum indicum*) two or three different sequences were expressed in leaves, stems, roots and developing seeds (Chun *et al*., 2003), but in *Passiflora edulis* MIPS was expressed in seeds (Emanuel, 2007). In developing *Arabidopsis* three isoforms of the MIPS gene have been localized in the cytosol of the endosperms (Mitsuhashi *et al.*, 2008).

The three isoforms of MIPS gene, in the *Arabidopsis* genome are AtMIPS1, AtMIPS2, AtMIPS3 which are predominantly localized to the cytosol within the seed endosperms, with a small amount of MIPS protein in the embryo in the developing seeds (Mitsuhashi *et al.*, 2008). Besides other physiological processes, these AtMIPS gene are also involved in InP_6 synthesis via inositol or phosphatidylinositol synthase.

In *Glycine max*, the MIPS gene, GmMIPS-1 is expressed during the early stages of seed development, in maternal tissue (seed coat) and then transported to the developing embryo i.e. a distinct localization pattern of GmMIPS-1 occurred on one site of the micropyle (Gomes *et al.*, 2005). The localization of the RINO 1 transcript, in developing rice embryo has been detected at the apex of the embryo and then in the scutellum and aleurone layer (Yoshida *et al.*, 1999). Unlike rice, GmMIPS-1 expression

was first detected in the maternal tissues (primary inositol supply for the early embryo) and then in the embryo. Even within the embryo, expression was first observed in the radical rather than the apical regions. Therefore, this localization of GmMIPS-1 in soybean is vitally important for the initial development stages of the embryo.

In chickpea, two divergent MIPS genes- *CaMIPS1* and *CaMIPS2* have been differentially expressed. Both the genes show an overall similar structure consisting of 9 introns and 10 exons. Both the genes (*CaMIPS1* and *CaMIPS2*) showed high similarity (>85%) sequences in their coding regions. Both *CaMIPS1* and *CaMIPS2* encodes functional MIPS enzyme involved in the biosynthesis of inositol in chickpea (Kaur *et al.*, 2008). *CaMIPS1* transcript was found in root, shoot, leaves and flowers in equal amount but was not found in seed, whereas *CaMIPS2* transcript was found in all examined tissues including seeds, suggesting a key role of *CaMIPS2* in phytic acid biosynthesis in seed along with other aspect of inositol metabolism in chickpea. Therefore, these results indicated that there may be a differential regulation of two different genes in different organs to coordinate inositol metabolism in relation to cellular growth.

In rice seeds, phytin is mainly stored in protein bodies as spherical inclusions called globoids (Lott *et al.*, 1995). Localization of globoids or phytin containing particles corresponded well with the accumulation pattern of the *RINO1* transcript. In mature seeds of rice, globoids are observed in most of the embryo tissues and aleurone layer (Wade and Lott, 1997). These two tissues are the ones in which the *RINO1* transcript accumulated most abundantly. These result demonstrated a close relationship between the *RINO* transcript and globoid accumulation, suggesting that I(1)P synthase plays an

important role in phytin biosynthesis in developing seeds of rice. In addition, the apical meristem also accumulated the *RINO1* transcript during early embryogenesis. *RINO1* transcript could be detected in very young leaves of 3-week old plants (Koyama and Yoshida, unpublished data). It has been reported that the apical cells of the rice embryo, at the end of the globular stage are vacuolated and are the probable precursor of the scutellum. The accumulation of the *RINO* transcript in the apical region of the embryo 2DAA is likely to indicate the region expected to form the scutellum.

In view of the aforesaid studies, it is imperative that the research centered on *myo*-inositol and the principal enzyme for its biosynthesis, MIPS is of utmost importance for its various roles in plant systems.

2.7.5. Molecular cloning and overexpression of INO1 gene

The gene encoding *myo*-inositol-1-phosphate synthase was isolated from many plants including Yeast (Klig and Henry, 1984) and *Spirodela polyrrhiza* and as well as in *Citrus paradisi* (Abu-Abied, 1994). A full length cDNA clone from *Citrus paradisii* (Duncan grapefruit) have been isolated which expressed differentially in different developmental stages of citrus seedlings. The deduced amino acid sequence of *INO1* shows 84% identity to the deduced amino sequence of the *Spirodela* enzyme, where tur1 (cDNA) code for a protein which is highly homologous to yeast inositol synthase. The gene *CINO1* from *Citrus paradisi, tur1* from *Spirodela polyrrhiza* and *INO1* from yeast are highly homologous, all encoding for *myo*-inositol-1-phosphate synthase (Abu-Abied, 1994). Similarly, full length MIPS gene (1818bp) from immature embryo of

finger millet (*Eleusine coracana*) designated as *EcMIPS* was cloned which showed a high homology with maize and rice MIPS gene (Reddy, 2013).

The resurrection plant, *Xerophyta viscosa* expresses *INO1* gene termed *XINO1* having striking homology with a number of *INO1* gene from other plant sources. The *INO1* gene from *X. viscosa* was cloned from a cDNA library based on mRNA isolated during cold stress. This *Xerophyta* MIPS, *XINO1* is almost identical to *RINO1* proteins sequences except for only two- three amino acids residues. Overexpression of the *XINO1* was achieved and the bacterially expressed *XINO1* protein was turned out to be active even in a lower temperature of 10^{o}C in striking contrast to the corresponding RINO1 protein having more restricted higher temperature optimum. The *XINO1* gene from *Xerophyta* was catalytically active at lower temperature range between 10^{o}- 40^{o}C than the *RINO1* gene product, which was the first such report from any resurrection plant (Majee, 2005).

There were four isoforms of MIPS gene in *Glycine max* designated as *GmMIPS1*, *GmMIPS2, GmMIPS3* and *GmMIPS4* and one of these MIPS cDNA was shown to express mainly in developing seeds (Hegeman *et al.,* 2001; Chappell *et al.,* 2006), specially in the outer integumentary layer during early seed development (Chiera and Grabau, 2007). A full length *GmMIPS* cDNA with an open reading frame (ORF) of 1533bp has been analyzed which was predicted to encode a polypeptide of 510 amino acid with a 61bp 5′-UTR and 173bp 3′-UTR. Maximum expression of *GmMIPS* was seen in developing seeds and a much lower expression in other vegetative tissues like leaves, flower, roots and stems, where mainly *GmMIPS2, GmMIPS3* and *GmMIPS4*

shows a relatively higher transcript levels (Chappell *et al.*, 2006). Expression of all four isoforms expression confirmed the role of *GmMIPS* isoform in generating L-*myo*-inositol-1-phosphate as a substrate for phytic acid biosynthesis. Therefore, down-regulation of *GmMIPS* using a seed specific promoter may have a great potential for developing of low phytate soybean without affecting the inositol metabolism in other tissues (Kumari, 2013). Similarly, the grass halophyte (*Spartina alterniflora*) encodes *myo*-inositol-1-phosphate synthase MIPS in *Arabidopsis thaliana* and overexpresses as *SaINO1*gene. This *SaINO1* gene is a stress-responsive gene and its constitutive overexpression in *Arabidopsis* provides significantly improved tolerance to salt stress during germination and seedling growth (Joshi *et al.*, 2013) while sweet potato [*Ipomoea batatas* (L) Lam.] expresses *IbMIPS1* gene which is induced by NaCl, polyethylene glycol (PEG), abscisic acid (ABA) and stem nematode. Overexpression of *IbMIPS1* from sweet potato significantly enhances stem nematode resistance besides salt and drought tolerance in transgenic sweet potato by increasing the inositol and proline content and enhancing the rate of photosynthesis. Therefore, overexpression of IbMIPS1 gene has the potential for use to improve the resistance to biotic as well as abiotic stresses in plants (Fei-Bing, 2015; Zhai, 2016).

Jatropha curcas also expresses cDNA encoding *myo*-inositol-1-phosphate synthase (*JcMIPS*) involved in response to abiotic stress (abscisic acid, drought and low temperature). This *JcMIPS* transcript is highly present in seeds and leaf tissues, but at low levels in stem and flower tissues (Wang, 2011). *Ricinus communis* L. also expresses a MIPS cDNA designated as *rcMIPS* with full length of 1669 bp and contains 1533 bp ORF. This *rcMIPS* ORF encodes 510 amino acid residues, which corresponds to a

polypeptide with a molecular mass of approximately 56 KDa. This gene shows homology to the extent of 88.6% with *Zea mays*, 89% with *Arabidopsis*, 91.6% with *Vigna radiata* and 91.4% with *Xerophyta viscosa*. Under abiotic stress conditions, *rcMIPS* gene activity was significantly increased in leaves, stems, and roots of which the expression in levels were higher than those in the stems and roots. This result indicates that the expression of *rcMIPS i*n *R. communis* enhances defensive mechanism against drought stress (Wei *et al.*, 2010). A cDNA encoding *myo*-inositol-1-phosphate synthase from *Sesamum indicum* L. Seeds (*SeMIPS1* cDNA) has been cloned and its functional expression pattern was analyzed in association with salinity stress during seed germination. The *SeMIPS1* cDNA shows deduced amino acid sequences indicating high identity and similarity with other MIPS and the presence of highly conserved functional regions essential for MIPS activity such as co-factor binding and catalysis of the reaction (Majumder *et al.*, 1997; Bachhawat and Mande, 1999). Salt stress during sesame seed germination has an adverse influence on the transcription of *SeMIPS1*. The transcript levels proportionally reduced with the duration of exposure to salinity and the initiation of germination was also collaterally reduced with the increase in salt concentration. This result suggests that expression of *SeMIPS1* is downregulated by salt stress during sesame seed germination (Chun, 2003).

2.7.6. Regulation of INO1 gene

The biosysnthesis of *myo*-inositol is a highly regulated process in *Saccharomyces cerevisiae*. The sysnthesis is mediated by its main biosynthetic enzyme, MIPS which is a repressible cytoplasmic enzyme. In yeast, MIPP is the gene product of the *INO1* gene (Donahue and Henry, 1981), but several other genes influence the action of INO1 gene

(Klig and Henry, 1984). MIPS expression is prevented by *ino2* and *ino4* mutations which are not linked to INO1 gene. In addition, *opi1*, *opi2* and *opi4* mutations results in making the enzyme constitutive (Greenberg *et al.*, 1982). It has also been found that MIPS was regulated by the exogenous inositol and the addition of 50µM *myo*-inositol in the growth medium decreases the specific activity of MIPS by over 50 fold (Culbertson *et al.*, 1976). Cloned *INO1* gene located in the *ino1* cells on a high copy number plasmid extrachromosomally was fully regulated. Thus, positioning of the gene in its normal chromosomal location is not necessary for full regulation of of MIPS (Klig and Henry, 1984). Majority of the mutants lacking the capacity to synthesize MIPS have been found to be the alleles of the *INO1* locus which codes for a 62kDa subunit of MIPS (Donahue and Henry, 1981a). The *opi1, opi2* and *opi4* mutations are recessive and unlinked to *INO1*. It may be suggested that all three gene products are components of a single repressor molecule or maybe, one of them form the repressor and the others helps in either synthesis or activity of the same. One more mutant, *opi3* which does not synthesize MIPS constitutively, but affects phospholipid biosynthesis (Greenberg *et al.*, 1982).

Genetic manipulation of *INO1* gene may disrupt the cellular *myo*-inositol supply thereby affecting various physiological processes. In a study, two methods, based on gene deletion and antisense strategy were used to generate mutants of *Disctystelium discoideum*. These mutants are inositol auxotrophs and they show inability to live by phagocytosis of bacteria which is their exclusive source of nutrition. Tranformation of wild type *D. discoideum* cells (AX2) resulted in three independent transformants by gene disruption (*Ddino1 Δ1* - *Ddino1 Δ3*) and 18 independent transformants by antisense mutagenesis (*Ddino1 as1* - *Ddino1 as18*). These mutants are inositol

auxotrops and they lose their viability when deprived of inositol for longer than 24 hrs. Under inositol deficient conditions, the mutants produced by both the strategies (*Ddino1 Δ1, Ddino1 as1*) accumulate a substance, 2,3-BPG (2,3-bisphosphoglycerate) which is not a primary inositol metabolite (Fischbach *et al.*, 2006). Increase in the level of 2,3BPG may be the result of homoeostasis between IP_3 breakdown and the limited breakdown of PIP_2 or it may be due to delayed degradation of 2,3-BPG in response to *myo*-inositol addition. A link between glycolysis and *myo*-inositol metabolism (Shi *et al*., 2005) may explain the accumulation of BPG as it is essential for the activity of phosphoglycerate mutase in all eukaryotic cells (Fischbach *et al*., 2006).

On analysis of *Arabidopsis thaliana* genome it was found that it carries three isoforms of MIPS, of which MIPS1 seems to be the main player in MI biosysnthesis because mips1 have drastically reduced MI content (Donahue *et al.*, 2010). Analysis of MIPS1 promoter activity in the mips1 mutant background or by addition of MI to the growth medium indicates that MIPS1 protein itself is a positive regulaltor of MIPS1 transcription. MIPS1 recruitment on the MIPS1 promoter is required to interact with histone methyltransferase, ATXR5/6 to expedite DNA methylation and thereby allowing normal transcription. Addition of MI in the growth medium did not restore the activity of the MIPS1 promoter in the mips1 mutant suggesting that the MIPS1 protein itself, but not its catalytic activity, is required for this regulation. During pathogen attack, MIPS1 is downregulated to prevent pathogen proliferation. MIPS1 is released from chromatin to allow ATXR5/6 dependent silencing of MIPS1. Thus, in plants MIPS1 has emerged as a protein that connects cellular metabolism with chromatin functions (Latrasse *et al*., 2013).

Mycobacterium tuberculosis utilizes inositol to produce mycothiol (Fahey, 2001) which is important in protecting the bacteria from ROS and electrophilic toxins (Newton *et al*., 2000). Antisense oligonucleotides (ODNs) which inhibit gene expression are used against human pathogens (Lisziewicz *et al*., 1992). However, ODNs cannot pass through the mycobacterial cell envelope. On the contrary, phosphoorothioate modified oligonucleotides (PS-ODNs) are easily taken up by these bacterial cells (Harth *et al*., 2002). In *M. tuberculosis* MIPS specific PS-ODNs inhibits the expression of ino1mRNA, the production of mycothiol, the proliferation of bacteria and enhances its susceptibity to antibiotics (Li *et al*., 2007).

Reduring the phytic acid content from soybean seeds becomes important in the light that 60-80% of total seed phytate is stored in soybean seeds in the form of phytate which is not readily digestible to the non-ruminant animals, reducing its nutritional value. Induced mutagenesis has been one approach of reducing MIPS encoding gene expression in plants. A single recessive mutation in soybean by changing just one amino acid residue of MIPS gne decresed the specfic activity of seed expressed MIPS enzyme and decreased phytic acid level (Hitz *et al*., 2002). However, complete deletion of gene expression has not been possible due to the presence of multiple genes encoding MIPS in plant genome. Conversely, RNA interference technolaogy has been successful in generating post-transciptional silence to quasi-undetectable levels of mRNA transcripts (Scherer and Rossi, 2003). By this technique multiple copies of the MIPS encoding gene present in soybean genome may be knocked down as these genes have high degree of sequence similarity (Nunes *et al.*, 2006). Therefore, RNAi technology has been used in soybean (*Glycine max*) in order to silence the *myo*-inositol-1-phosphate synthase

(*GmMIPS*) gene, which resulted in a drastic reduction (up to 94.5%) of phytate content in the developed transgenic lines, improved phosphorous availability and inhibited seed germination demonstrating an important correlation between *GmMIPS* gene expression and seed development (Nunes *et al.*, 2006).

RNAi mediated silencing of MIPS gene has also been used in in indica rice cultivar in order to generate low phytate rice. Here, MIPS gene was downregulated by using seed specific promoter, oleosin 18 (Ole18). The rice *INO1* (*RINO1*) cDNA was expressed under the control of Ole18 promoter directing the phytic acid biosynthesis in seeds. The transgenic lines revealed a reduction in phytate levels (reduced by 68%), along with an increased phosphorous content and the plant displayed normal phenotype (Kuwano *et al.*, 2009). Low phytate rice was also achieved by RNAi mediated seed specific silencing of inositol 1,3,4,5,6-pentakisphosphate-2-kinase gene (IPK1), which catalyzes the last step of phytic acid biosynthesis in rice. The transgenic rice revealed 3.85 fold downregulation in IPK1 transcripts in T4 seeds which also showed a significant reduction in phytate levels and a concomitant increase in the amount of inorganic phosphate. IPK1 gene silencing in rice seeds also provide 1.8 fold more iron in the endosperm and do not show any negative effect on seed germination. Thus, silencing of IPK1 gene reduces seed phytate levels without compromising on the growth and development of transgenic rice plants (Ali *et al.*, 2013). RNAi mediated downregulation of IPK1 also led to the development of low phytic acid containing wheat (Aggarwal *et al.*, 2018)

2.8. Role of *myo*-inositol and its metabolites in abiotic stress tolerance:

2.8.1. Stress response

Many plants possess an intrinsic ability to tolerate and survive under adverse environmental conditions (Knight and Knight, 2001). Phosphatidylinositol 4,5 bisphosphate (PIP_2) levels is increased during osmotic stress as found in *Arabidopsis* cells (Pical *et al.*, 1999; Wald *et al.*, 2001) and the time frame for the increase correlates with changes in cytosolic Ca^{2+} levels. The stress hormone ABA also elicits transient increases in IP_3 levels in *Vicia faba* guard cell protoplasts (Lee *et al.*, 1996) and in *Arabidopsis* seedlings (Sanchez and Chu, 2001; Xiong *et al.*, 2001). Transient increases in IP_3 were found in plants upon exposure to light, pathogen, gravity, anoxia or several plant hormones (Munnik *et al.*, 1998; Stevenson *et al*., 2000).

Phospholipase D (PLD) is activated and lead to transient increases in PA levels in plants during drought and hyperosmolarity (Frank *et al.*, 2000, Munnik *et al.*, 2000, Katagiri *et al*., 2001). PLD appears to be activated by osmotic stress through a G-protein (Frank *et al.*, 2000; Katagiri *et al.*, 2001). However, excess PLD activity may have a negative impact on plant stress tolerance. Drought stress induced PLD activities were found to be higher in drought sensitive than in drought tolerant cultivars of cowpea (Maarouf *et al*., 1999) suggesting that al high PLD activity may jeopardize membrane integrity. Consistent with this notion, *Arabidopsis* plants deficient in PLD was found to be more tolerant to freezing stress.

Two component histidine kinases, cyanobacterium histidine kinase HiK33 (Suzuki *et al*., 2000) and the *Bacillus sublitis* histidine kinase Desk (Aguilar *et al.*, 2001) are thermosensors that regulate desaturase gene expression in response to temperature downshifts. In the genome of *Arabidopsis thaliana*, several putative two component histidine kinase have been identified (Urau *et al.*, 2000).

Cold treatment activates some mitogen activated protein kinase (MAPK) by post translational modification in *Arabidopsis* and in *alfalfa* (*Medicago sativa*, Jonak *et al*., 1996). In yeast and in animals MAPK pathways that are activated by receptor sensors such as protein tyrosine kinases, G-protein- coupled receptors and two component histidine kinase, etc. are responsible for the production of compatible osmolytes and antioxidants. Similarly *Arabidopsis histidine* kinase AtHK1 can complement mutations in the yeast two component histidine kinase sensors SLN1 and therefore, may be involved in osmotic stress signal transduction in plants.

Ca^{2+} dependent protein kinase ($CDPK_5$) are serine threonine protein kinase with a C-terminal calmodulin like domain may be involved in abiotic stress signaling (Huang *et al*., 2000). In rice plant $CDPK_5$ was activated by cold treatment (Martin and Busconi, 2001). In addition overexpression of C_5CDPK_7 resulted in increased cold and osmotic stress tolerance in rice (Saijo *et al*., 2000). Thus $CDPK_5$ somehow play roles in the development of stress tolerance. A CDPK was also activated in response to pathogen infection (Romeis *et al*., 2000). Thus $CDPK_5$ somehow play roles in the development of stress tolerance.

2.8.2. Osmolyte accumulation during stress

Accumulation of osmolytes leads to osmotic adjustment which is an important mechanism to tide over the changing environment. Osmolytes help in osmotic adjustment of the cells and also protects the cells and macromolecules by maintaining membrane integrity, preventing protein degradation and protecting against oxidative damage by scavenging free radicals and lowering the T_m value of nucleic acids (Crowe *et al*., 1987; Nomura *et al.,* 1995). Osmolytes not only helps in the osmotic adjustment in the cellular milieu but also acts as scavengers of reactive oxygen species. The presence of D-ononitol and *myo*-inositol are the potential protectants of enzymes and membranes from damage by reactive oxygen species (Sheveleva *et al.*, 1997). Methylated inositols were found to be comparatively more effective quenchers of ROS as compared to glycine-betaine and the like (Orthen *et al*., 1994).

The plant species which are constantly exposed to saline conditions accumulates the cyclic sugar alcohols pinitol and ononitol (Paul and Cockburn, 1989). Thus *Mesembryanthemum crystallinum* accumulates these comounds when subjected to such stress (Bartels and Nelson, 1994). The upregulation of inositol biosynthesis by subjecting plants to salinity stress may be exploited for the enhanced production of *myo*-inositol from glucose-6-phosphate (RayChaudhuri and Majumder, 1996).

2.8.3. Role of *myo*-inositol and its derivatives in temperature Stress

Stress tolerant plants generally possesses a high levels of stress-related metabolites under normal growth conditions and/or accumulate large amounts of protective metabolites under unfavourable conditions, indicating that their metabolism is prepared for adverse growth conditions.

Under freezing conditions, the presence of cyclitol and low molecular weight compounds all together function as cryoprotective solutes, which diminishes the mechanical stress by decreasing the osmotic potential thus reducing the freeze induced shrinkage and accumulation of cryoprotectans prevents this alteration by balancing the concentration of cryotoxic substance during ice formation. Cyclitols like pinitol, quebrachitol, quercitol, O-methyl-*muco*-inositol which have been found to be accumulated at low temperature (Diamantoglou, 1974; Ericsson, 1979; Popp *et al.*, 1997).

In mistletoe (*Viscum album)* more than 25% of its dry matter is occupied by the cyclitols during winter (Richter, 1989). Similarly enhanced storage of cyclitols in the living bark tissue and buds has been found in a number of tree species during the onset of cold season (Poop and Smirnoff, 1995; Popp *et al*., 1997). Even, the transcription of the enzyme *myo*-inositol-o-methyl-transferase (a key enzyme on the biochemical pathway to ononitol and pinitol biosynthesis) has been induced in the Mediterranean species *M.crystallium* when the plant was exposed to 4^{o}C for 78 hr. Similarly, accumulation of pinitol in chickpea in the thylakoid membrane functions as cryoprotective solutes (Orthen, 2000). Proline and raffinose also play a crucial role of compatible solutes in *Arabidopsis* in freezing tolerance (Hannah *et al.*, 2006; Korn *et al.*, 2010).

MIPS-encoding gene cloned from *Passiflora edulis f. flavicarpa* called *PeMIPS1* is differentially transcribed during cold and heat stress, suggesting that it is important for

the environmental stress response. This result correlates with ecological adaptation of yellow passion fruit, a typical species adapted to tropical and subtropical environments that endures winter chills for short periods without injury (Abreu, 2007).

2.8.4 Drought Tolerance

Plants develop complex and dynamic systems like various physiological, morphological, biochemical, and metabolic approaches to respond to water deficit and to adapt to drought conditions (Vincent *et al.*, 2007; Ahuja *et al.*, 2010; Saidi *et al.*, 2011; Walbot *et al*., 2011).

Plants respond to drought stress by the synthesis of different metabolites including polyols in ripe olive fruit, (Martinelli, 2013), grape berry (Conde *et al.*, 2014) etc. Six different polyols (mannitol, sorbitol, galactitol, *myo*-inositol, glycerol and dulcitol) were significantly accumulated in the pulp of grape berries in responses to water deficit. *Myo*-inositol was the most abundant of the quantified polyols in mature leaves and tissues which helps the plant to cope with water deficit, either directly as an osmolyte or indirectly as a precursor of galactinol and raffinose family oligosaccharides (Conde *et al.*, 2014). Though *myo*-inositol is used as an osmolyte like other sugar alcohols, it also functions as a precausor of many metabolites involved in abiotic stress (Kaur *et al*., 2013). *Myo*-inositol is also closely related to the accumulation of RFOs and further stress tolerance (Elsayed *et al*., 2014). There is a close relationship between the metabolism of *myo*-inositol and RFO and the yield performance of maize under drought stress. Galactinol synthase (GolS EC 2.4.1.123) is the key enzyme that catalyzes to produce galactinol from *myo*-inositol. Therefore, galactinol synthase (GolS) has been

assumed to be the key regulatory factor in RFO biosynthesis (Taji *et al.*, 2002; Kerner *et al.*, 2004; Sengupta *et al.*, 2015).

There are seven GolS-related genes in the *Arabidopsis thaliana* and named as *At*GolS1,2,3,4,5,6 and 7 respectively. Among these, *At*GolS1 is the drought responsive gene, which mainly functions in drought stress tolerance (Taji *et al.*, 2002). Similarly, GolS1 from *Boea hygromenic*a enhanced drought tolerance (Wang *et al.*, 2009). The model plant *Ajuga reptans* expresses two distinct GolS, ArGolS1 and ArGolS2 which regulates RFO metabolism (Sprenger and Keller, 2000). RFOs have long been suggested to act as anti-stress agent in both generative and vegetative tissues (Taji *et al.*, 2002; Pennycooke *et al.*, 2003). *Xv*GolS gene encoding galactinol synthase was also identified in the leaves of *Xerophyta viscosa.* This gene shows negative correlation between RFO accumulation and *myo*-inositol depletion which was reversed after rehydration. This suggest that *myo*-ionsitol is channelled into RFO synthesis during water deficit and channelled back to metabolic pathway during rehydration to repair desiccation-induced damages (Peter *et al.*, 2007).

In addition to GolS, MIPS also control the levels of galactinol and raffinose because it controls the production of *myo*-inositol, the galactinol precursor. Therefore, both GolS and MIPS are proved to play important roles in drought–stress tolerance (Taji *et al.*, 2002; Evers *et al.*, 2010).

2.8.5 Salinity stress tolerance

Salinity tolerance is correlated with the increased accumulation of polyols such as mannitol, sorbitol, *myo*-inositol and its methylated derivatives (Loewus and Dickinson, 1982). The pathway from glucose-6-phosphate (G-6-P) to *myo*-inositol-1-phosphate (Ins1-P) and *myo*-inositol is essential for the synthesis of various other cyclic polyols like ononitol, pinitol etc. *Myo*-inositol-O-methyltransferase (*IMT1*) gene has been isolated from *Porteresia coarctata* Roxb. (*PcIMT1*) is implicated in the synthesis of pinitol that in turn enhances salinity stress (Sengupta, 2008). In *Porteresia coarctata* Roxb.(takeoka) (wild halophytic rice) salt-tolerant MIPS gene coded by *PcINO1* and *PcIMT1* generates *myo*-inositol and pinitol respectively even at high salt concentration and so that the inositol pool in the plant is well maintained during salinity stress.

The same gene encoding *myo*-inositol-O-methyltransferase, (*IMT1)* has also been isolated from the facultative halophyte, *Mesembryanthemum crystallinum* (Ishitani *et al.*, 1996). *Mesembyanthemum crystallinum* responds to osmotic stress by shifting from C_3 photosynthesis to crassulacean acid metabolism (CAM). Under salinity stress conditions, the plant also switches on the biosynthesis of a putative osmoprotectant, pinitol. Under salt stress, pinitol did indeed increase reflecting the induction of the gene. Also, low levels of pinitol have been observed previously in unstressed leaf tissue from mature plants (Vernon and Bohnert, 1992). In *Mesembryanthemum crystallinum* sodium uptake is facilitated by the phloem translocation of *myo*-inositol from root to leaves under salt stress. And a 10 fold increase in *myo*-inositol and ononitol was found with large increase in sodium following stress. Therefore *myo*-inositol plays a direct role in

sodium uptake and long distance transport. Thus, besides *myo*-inositol serving as a substrate, it also promotes as a leaf to root signal by sodium uptake (Nelson *et al.*, 1999)

In salt tolerant *Lycopersicon esculentum* plants, *myo*-inositol constituted two-thirds of the soluble carbohydrates in leaves while the most tolerant genotypes was found to have highest, the normal cultivar intermediate while the sensitive genotypes the lowest level of MI after treatment with salt. Thus, *myo*-inositol content is implicated to have an additive effect in salt tolerance in tomato (Sacher and Staples, 1985). *Chiro*-inositol was also isolated and identified in leaves and root tissues of *Limonium* species (Liu, 2009) for salt adaptation. *Chiro*-inositol was found in other halophytes too such as *Aegialitis annulata* L. Gmelinii and *L. latifolium* (Popp, 1984; Murakeozz *et al.*, 2002; Gagneul *et al.*, 2007). *Chiro*-inositol and *myo*-inositol are phloem transportable; therefore, these polyols are readily translocated from leaves to roots. This type of translocation facilitates sodium uptake through xylem to leaves leading to a transition from a non-tolerant to salinity-tolerant state (Nelson *et al.*, 1999).

Rice (*Oryza sativa*) is sensitive to salinity at the seedling stage and becomes tolerant at the vegetative phase and very susceptible at the reproductive phase (Anbumalarmathi, 2013). Therefore, this stress tolerance is a quantitative trait which is controlled by enhanced activity of chloroplastic form of *myo*-inositol-1-phosphate synthase noticeable only in chloroplast from light grown plants (Raychandhuri, 2002).

Overexpression of *PcMIPS* isolated from halophytic rice, *Porteresia coarctata* enhanced tolerance to salt in transgenic *Brassica juncea* and rice (Chatterjee *et al*.,

2006). It caused tolerance to salt and oxidative stresses in transgenic *Brassica juncea* and increased the seed survival rate under salt and dehydration stresses in transgenic tobacco plants (Goswami *et al.*, 2014). Similarly, overexpression of synthase gene from *Medicago falcata*, *MfMIPS1* improved resistance to chilling, drought and salt stress in transgenic tobacco plants (Tan *et al*., 2013). In the same way, overexpression of the gene IbMIPS1 from *Ipomoea batatas* L. enhances salt tolerance by regulating the expression of salt stress responsive genes, increasing the contents of inositol and proline and enhancing the rate of photosynthesis. Therefore, *IbMIPS1* gene has the potential to be used to improve salt tolerance in sweet potato and other plants (Fei-bing, 2015).