Discussion

DISCUSSION

Plants are frequently exposed to a plethora of stress conditions such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. Salinity is a major environmental stress and is a substantial constraint to crop production. Plant growth is drastically reduced under increasing salt stress (for recent reviews see Hasegawa et al, 2000; Munns and Tester, 2008).

Chlorophyll plays an important role in harnessing solar energy for its utilization in photosynthesis. Chloroplast biogenesis is regulated by chlorophyll biosynthesis that is adversely affected by salt stress. As shown in Fig.1 salt sensitive cultivar PB1 (Fig.1 B) was more affected by salt stress than relatively resistant cultivar CSR10 (Fig.1 A). Chlorophyll and carotenoids contents were also reduced in response to increased NaCl stress in both the cultivars (Figs. 2 and 3), although the relative decrease in Chl and carotenoids contents were more in salt sensitive PB1 as compared to that of salt-tolerant CSR10. Chl a/b ratio increased in response to salt stress possibly due to less synthesis of Chlb (Fig. 2 D). Pérez-Tornero et al, 2009 have reported decrease in total chlorophyll in *Citrus macrophylla* in response to salt stress. In wheat chlorophyll contents decrease due to NaCl stress (Shafi et al, 2009). Chl and carotenoids contents decreased in halophyte *Cakile maritime* at extreme salt concentration (Debez et al, 2008).

Impact of salt-stress on ion homeostasis

To understand the molecular mechanism of resistance of CSR-10 to salt stress, concentrations of different salts in roots and shoot were analysed. Sodium contents increased in shoot as well as root in response to salt stress in both the cultivars (Fig.5). However, in salt-sensitive PB1, the shoots accumulated more Na^+ than that in CSR10 (Fig.5 B). However, CSR10 roots accumulated more sodium as comparared to that in PB1 (Fig.5 A). This shows that CSR10 sequesters Na^+ in roots. The K^+ content decreased due to stress in both the cultivars in shoot as well as root (Fig. 6) and the Ca^{2+} increased in the shoot (Fig.8 B). In salt-stressed rice Sengupta and Majumdar, 2009 observed reduced accumulation of Na^+ in shoots of salt-resistant rice cultivar and Jia et al, (2008) observed a decrease in K^+ contents of leaf and root tissues in respose to salt stress in plant species from Elemyus genus.

Na^+ disequilibrium is a primary consequence of salt stress and leads to catastrophic pathologies affecting cell survival, division, and growth (Hasegawa et al., 2000; Tester and Davenport, 2003; Zhu, 2003). Another consequence of Na^+ disequilibrium that is caused by salt stress is the detrimental effect of Na^+ on K^+ acquisition and nutrition. K^+ has essential functions in plant metabolism (e.g. charge balance, osmotic adjustment, and enzyme catalysis) and in growth and development (Maathuis and Sanders, 1996; Rigas et al., 2001; Elumalai et al., 2002). Na^+ in the soil solution disturbs K^+ homeostasis in plants presumably because the cytotoxic ion competes for binding sites in transport systems that mediate K^+ uptake (Epstein, 1961; Rains and Epstein, 1965; Niu et al., 1995; Hasegawa et al., 2000).

Effect of temperature stress on chlorophyll biosynthesis and the greening process

Inhibition of Chl biosynthesis by salinity stress was due to reduced synthesis of ALA (Fig. 10), the committed precursor of Chl. Unlike bacterial and animal systems, in plants ALA is synthesized from a five carbon compound glutamate. Accumulation of Glutamate -1-semialdehyde (GSA), an intermediate leading to ALA synthesis, was higher in salt-resistant CSR10 as compared to that in salt-sensitive PB1 cultivar (Fig. 9). The increased synthesis of GSA in CSR10 may be due to increased gene expression of *GluTR*, encoding Glutamyl-t-RNA reductase that converts Glutamyl-t-RNA to GSA. GluTR expression also increased in PB1 due to salt stress although the extent of increase was smaller than that in CSR10 (Fig. 21 A).

GSA is converted to ALA by Glutamate-1-semialdehyde aminotransferase coded by *GSAT*. Its expression also increased in CSR10 in response to salt stress leading to higher ALA synthesis (Fig. 21 A). In salt sensitive genotype PB1, its expression partially downregulated in response to salt stress. Results demonstrate that the maintenance of ALA biosynthesis in salt stressed condition is extremely important and plants upregulate the gene expression of ALA biosynthetic enzymes. The gene and protein expression of GSAT also increases in heat-stressed wheat and cucumber seedlings (Mohanty et al, 2006).

ALA is metabolized to protoporphyrin IX by a set of enzymes named ALA dehydratase (ALAD) that converts ALA into porphobilinogen (PBG), Porphobilinogen deaminase (PBGD), Uroporphyrinogen decarboxylase (UROD), Coproporphyrinogen oxidase (CPO) and Protoporphyrinogen oxidase (Protox) acting in series. The activity of ALAD that converts ALA to porphobilinogen was reduced in PB1 by salt stress whereas in CSR10 it was not substantially

affected (Fig. 12). Similarly, its gene expression was downregulated in PB1 although it was partially affected in CSR10 (Fig. 21 A). Similar to salt-stress, the ALAD cativity and *ALAD* expression decrease in response to heat and chill stress in in *cucumber* seedlings (Tiwari and Tripathy, 1998).

The PBGD activity declined due to salt stress in both the cultivars (Fig. 13). Salt-induced decline in its activity well correlates with the reduced gene expression of *PBGD* (Fig. 20 A). In temperature-stressed cucumber seedlings the PBGD activity is also reduced (Tiwari and Tripathy, 1998).

The UroD, the next enzyme in Chl biosynthetic pathway, decarboxylates uroporphyrinogen III to coproporphyrinogen III and its gene expression and protein abundance were downregulated in CSR10 and to a larger extent in PB1 (Fig. 18 B and 20 A). The next enzyme in the series is Coproporphyrinogen oxidase (Coprox) that converts coproporphyrinogen III to protoporphyrinogen IX. Its activity was downregulated both in CSR10 and PB1 cultivars (Fig. 14). In the same vein activity of Protoporphyrinogen oxidase (Protox) that remove six electrons from macrocycle is also downregulated in both of the cultivars in response of salt (Fig.15). Decreased Protox activity is due to its transcriptional and translational downregulation (Fig. 18 B and 21 A). Decline in enzymatic activities of coprox and protox was observed in cucumber seedlings in response to chill-stress by Tiwari and Tripathy, 1998. In heat stress coprox activity was not affected although protox activity increased (Tiwari and Tripathy, 1998).

Mg is inserted to the protoporphyrin ring by an ATP dependent reaction of Mg-chelatase. The latter consists of three subunits namely ChlH, ChlD and ChlI. In response to salt stress as compared to CSR10, the Mg-chelatase activity was severely down regulated in PB1 (Fig. 16). This was due to severe downregulation of gene expression and protein abundance of ChlI in PB1 cultivar (Fig. 18 B and 21 A). Similar decline in Mg-chelatase activity and ChlI expression was previously observed in cucumber and wheat in response to temperature-stress (Tiwari and Tripathy, 1998, Mohanty et al., 2006).

Protochlorophyllide (Pchlide) is converted to chlorophyllide (Chlide) by protochlorophyllide oxidoreductase (POR). The POR abundance substantially increased in response to salt stress in the salt resistant CSR10 cultivar and only partially increased in PB1 (Fig. 19). Due to higher POR abundance the POR activity, measured as percent

phototransformation of Pchlide to Chlide, was not substantially affected in CSR10 due to salt stress. However the POR activity was substantially declined in PB1 (Fig. 17). The *ChlP* that codes geranyl-geranyl reductase is responsible for phytol synthesis. It was partially downregulated in CSR10 and more severely in PB1 due to salt stresss treatment (Fig.18 B).

Chlorophyll a oxygenase (CAO) converts Chl a to Chl b, and its gene expression was severely downregulated in PB1 seedlings due to salt-stress (Fig. 20 A). The decreased CAO could reduce Chl b biosynthesis leading to increased Chl a/b ratio in both the cultivars (Fig. 2 D).

Shibata shift: Leaves of dark grown rice seedlings has a smaller emission fluorescence peak (77K) at 632 nm due to non-phototransformable Pchlide and a larger peak at 658 nm due to phototransformable Pchlide. The non-phototransformable Pchlide emitting at 632 nm is due to monomeric Pchlide complex or esterified Pchlide i.e. Protochlorphyll (Lindsten et al. 1988), which spontaneously dimerizes to form $(POR\text{-}Pchlide\text{-}NADPH)_2$. The short-wavelength, monomeric Pchlide is not flash-photoactive; instead it regenerates the long wavelength Pchlide forms (Schoefs and Franck 1993; He et al. 1994; Schoefs et al. 1994, 2000a, 2000b). The dimer has the absoption maximum at 638 nm and emission maximum at 645 nm (Ouazzani Chahdi et al. 1998; Lebedev and Timko 1999). The dimeric POR-Pchlide- NADPH complex further polymerizes to form 16-mer or larger aggregates of POR-Pchlide- NADPH complex i.e., $(POR\text{-}Pchlide\text{-}NADPH)_n$ having absorption maximum at 650 nm and emission maximum at 658 nm (Böddi et al. 1989; Wiktorsson et al. 1993) and is flash photoactive (Böddi et al., 1991). However, long-term illumination i.e., greater than minute usually converts non-active Pchlide to photo-active Pchlide.

Results demonstrate that results demonstrate that in etiolated rice seedlings, the phototransformable peak is substantially higher than the non-phototransformable peak demonstrating the presence of large aggregate of POR-Pchlide-NADPH ternary complexes.

The flash-induced photo-transformation and Shibata shift leading to chloroplast biogenesis is substantially affected in salt-stressed samples. Upon flash illumination (0.2 sec) of leaves from control seedlings the phototransformable Pchlide peak at 658 nm emanating from large aggregates of polymeric POR-Pchlide-NADPH complexes almost disappeared due to photo-reduction of Pchlide to Chlide and a new peak appeared at 692 nm . Transformation of $Pchlide_{658}$ into $Chlide_{692}$ was previously observed by exposing the leaf primodia of common ash (*Fraxinus*

excelsior L.) and Hungarian ash (*Fraxinus angustifolia* Vahl.) (Solymosi and Böddi 2006) and that of Horse chestnut (*Aesculus hippocastanum*) (Solymosi et al. 2006) to white light flash of 10 sec.

After half an h of dark incubation following flash illumination of etiolated rice seedlinds (Five day old), a hump at 676 nm appears (shibata shift). However in present experimental conditions a complete blue shift to 676 nm was not observed and major peak remained at 692 nm. After 1h dark incubation after flash treatment lead to a complete blue shift at 684 nm (Fig.22). This 684 peak could be due to Chlorophyllide$_{684}$. After 1 h of dark incubation some amount of phototransformation pchlide (F658) were regenerated. Salt stress impairs shibata shift even after 30 min (Fig. 23). of dark incubation following flash illumination there was no blue shift was observed. Only after 1 h of dark incubation following flash illumination a small hump at 676 nm appeared and the major peak remained at 692 nm demonstrating the arrest of shibata shift by salt stress in CSR10 cultivar. During this 1 h of dark incubation following flash illumination protochlorophyllide which was regenerated accumulated in POR-Pchlide-NADPH monomeric form leading to the formation of non-phototransformable pchlide having fluorescence maximum at 632 nm. Similarly in PB1 after half an h of dark incubation following fash illumination of etiolated rice seedlinds (Five day old), a hump at 676 nm appears and major peak remained at 692 nm. After 1h dark incubation after flash treatment lead to a complete blue shift at 684 nm (Fig.24). Salt stress impairs shibata shift also in PB1 cultivar, after 30 min. of dark incubation following flash illumination there was no blue shift was observed (Fig.25). Only after 1 h of dark incubation following flash illumination a small hump at 676 nm appeared and the major peak remained at 692 nm demonstrating the arrest of shibata shift by salt stress. As in PB1 after 1 h of dark incubation following flash illumination hump appeared was not as prominent as it was in CSR10 so this suggests there is more arrest of shibata shift in PB1 due to salt stress than that in CSR10. There is no substantial regeneration of non-phototransformable Pchlide in PB1 as comparative to that in CSR10 after 1 h of dark incubation following red flash.

Overall, in response to salt-stress rice seedlings down-regulated Chl biosynthesis by regulating biochemical reactions, protein abundance and gene expression of several enzymes involved in tetrapyrrole synthesis. In the presence of salt, the salt-tolerant CSR-10 cultivar accumulated more Chl than that of salt-sensitive genotype PB1 by upregulating the gene expression and protein abundance of several Chl biosynthetic enzymes.

Impact of salt stress on photosynthesis

Chl a fluorescence is used as a signature to understand the interrelationship of structure and function of photosynthetic apparatus (Govindjee, 1995, 2004). Although the F_0 was not affected in CSR10 after 24 h of greening, it declined in PB1. However after 24 h and 72 h of greening in the presence of salt the F_0 value significantly declined in both the cultivars due to their reduced Chl contents (Fig. 26 A). The ratio of F_v/F_m is a measure of the functional status of PSII. It was not significantly declined after 24 h of greening in the presence of salt. After 48 h of greening in presence of salt, it declined in PB1 and was not affected in CSR10. However 72 h of greening it decreased in both of the cultivars suggesting that salt stress impairs quantum efficiency of PSII (Fig 26 C).

Electron transport rate (etr), photochemical quenching (qP) and quantum yield of PSII (ΦPSII) decreased with increasing salt stress and duration of salt stress exposure (Figs. 27-35). However, the rate of decline with duration and intensity of salt stress was more in salt-sensitive PB1 than salt-tolerant cltivar CSR10. Decline in etr in both the cultivars was observed both at low and high light intensites suggesting damage to the light harvesting complex as well as reaction centre.

Non-photochemical quenching (qN) increased in response to salt stress in both of the cultivars although it was higher in salt sensitive PB1 (Figs. 36-38). The major component of qN is due to a regulatory mechanism, called qE, which results in thermal dissipation of excess absorbed light energy in the light-harvesting antenna of PSII. qE is induced by low pH of thylakoid lumen that is generated by photosynthetic electron transport in excess light. Low thylakoid lumen pH that inducees qE has two roles. One is the pH dependent activation of a lumen localized violaxanthin de-epoxidase (VDE) that catalyzes conversion of violaxanthin to zeaxanthin via the initermediate antheraxanthine (Demmig-Adams and Adams, 1996). Zeaxanthin is necessary for qE in plants. In limiting light intensity zeaxanthine epoxidase (ZE) converts zeaxanthin back to violaxanthin. This light dependent interconversion is known as xanthophylls cycle (Yamamoto et al, 1999). The second role of low lumen pH is driving protonation of one or more PSII protein involved in qE (Horton and Ruban, 1992). The characterization of *Arabidopsis thaliana* mutant that lack qE has revealed that qE requires PsbS in addition to low lumen pH. The *npq1* and *npq2* mutants were defective in VDE and ZE respectively (Niyogi et al, 1998) whereas *npq4* mutants which lack qE and delta-A535 have

normal xanthophylls cycle. The *npq*4 mutation was mapped to chromosome 1 and ultimately have shown to affect the gene encoding PsbS (Li *et al*, 2000). Although the mechanism of qE remains unresolved, increase in qN suggests increased operation of xanthophyll cycle leading to excess qE quenching and loss of excitation energy as heat to protect the membrane from photodamage.

Increased qN in PB1 in response to salt-stress suggests that it is not able to dissipate the pH gradient created across the thylakoid membrane probably due to the damage to the photophosphorylation apparatus (CF_0 and CF_1). The proteomics data clearly suggest the down-regulation of ATP synthase CF_1 beta subunit. Low thylakoid lumen pH would activate VDE converting violaxanthine to zeazaxanthine responsible for increased qE or qN quenching

In Sorghum, maximum quantum yield of photosystem II (Fv/Fm), photochemical quenching coefficient (qP) and electron transport rate (ETR) significantly decreased, but non-photochemical quenching (qN) increased substantially under saline conditions (Netondo et al., 2004). In *Spirulina platensis* cells salinity stress induced a decrease in oxygen evolution activity, which correlated with the decrease in the quantum yield of PSII electron transport (Φ PSII) and induced an increase in non-photochemical quenching (qN) and a decrease in photochemical quenching (qP) (Lu & Vonshak, 2002). Similar decrease in etr and increase in non-photochemical quenching was shown by Moradi and Ismail, 2007 in salt tolerant rice cultivars IR651 and IR632 and in salt sensitive cultivar IR29.

Due to salt stress whole chain electron transport rate (H_2O to MV) (Fig. 39) and PD supported partial electron transport of PSII (H_2O to PD) (Fig. 41) were severely impaired in both CSR10 and PB1 cultivars. However, the CSR10 had reduced loss of electron transport activity as compared to that of PB1. PSI was only partially affected by salt stress (Fig.40).

Salt stress is known to inhibit PSII and/or PSI activities in cyanobacteria (Allakhverdiev and Murata, 2008), *Spirulina platensis* (Lu & Vonshak, 2002), *Arabidopsis thaliana* (Stepien and Johnson, 2009), and several other plants (Bongi and Loreto, 1989; Mishra et al., 1991; Masojidek & Hall, 1992; Baelkhodja et al., 1994; Everard et al., 1994). The results of salinity stress on PSII photochemistry are conflicting. Some studies have indicated that salt stress has no effects on PSII (Robinson et al., 1983; Brugnoli and Bjorkman, 1992; Morales et al., 1992; Abdia et al., 1999).

To ascertain if the inhibition of PSII reaction was due to reduction in quantum yield of PSII or in the light saturated electron transport rate or both, the rate of PSII reaction as a function of light intensity was measured in thylakoid membranes isolated from control and salt treated rice seedlings after 72 h of greening (Fig. 42 and 43). Dependence of PSII activity on light intensity showed typical saturation kinetics. Both the initial slope at limiting light intensities as well as light saturated electron transport was affected in salt treated seedlings of CSR10 cultivars (Fig. 42). As compared to control, the percent inhibition of PSII reaction in salt-treated thylakoids was almost constant (nearly 45 %) at all the light intensities used (Fig.42). Both Vmax and quauntum efficiency of PSII were reduced almost by 45 %, suggesting that inhibition of Vmax and quantum efficiency of PSII was predominantly due to damage to the reaction centre.

In Pusa Basmati 1 (PB1) both the initial slope at limiting light intensities as well as well as light saturated electron transport rates were affected in salt treated seedlings (Fig.43). As compared to control, the percent inhibition of PSII in salt stressed thylakoids was almost 50 % at higher intensity and was more pronounced at lower intensities (≥60 %). The quantum efficiency of PSII was reduced by almost 50 % and V_{max} by almost 75 %. This suggests that inhibition of V_{max} and quantum efficiency of PSII was due to both, damage to the reaction centre as well as antenna complex. The estimated ETR measured from the leaves of salt-stressed rice seedlings was also affected both at low and high light intensities.

These conclusions are further supported from immunoblot analysis where the reaction centre proteins D_1 and D_2 and oxygen evolving protein 33 (OEC33) and antenna protein LHCPII were highly degraded in PB1 whereas in CSR10 they were partially affected (Fig 48 A). This also explains the reason why the damage to PSII is higher in PB1 than CSR10 at saturating as well as limiting light intensities. Whole chain electron transport was also affected more in PB1 as comparative to CSR10. This could be due to higher loss of other components of electron transport chain such as cyt b, cyt f, Rieske iron-sulfur centre protein and subunit IV of cytbf complex, PSI subunits III and V (Fig. 49 and 50). In the same vein gene expression of PsbA and PsbD and PetD coding for D1 and D2 reaction centre proteins and subunit IV respectively were also severely affected in PB1 as comparaed to that in CSR10 (Fig. 51).

Electron micrograph reveals a larger disorganization of grana stacking of thylakoid membranes in PB1 as comparative to that of CSR10. Starch granules completely disappeared due to 200 mm NaCl stress treatment in chloroplast suggesting loss of carbohydrate accumulation due to inhibition of photosynthesis (Fig. 52 and 53).

Room temperature emission fluorescence spectrum of thylakoids membrane have a peak at 684 nm due to PSII while excited at 440 nm having 8 nm slit width that produce a actinic illumination. Thylakoids membrane isolated from CSR10 and PB1 had a reduced fluorescence emission at 684 nm originating from PSII due to the loss of variable fluorescence (Fig. 44). Gross perturbation of thylakoid membranes usually induces a difference in fluorescence spectral properties at low temperature (77K). The low temperature fluorescence spectra have a peak at 685 nm (F_{685}) and at 695 nm (F_{695}) emissions which mostly originates from PSII CP43 and CP47 respectively (Govindjee, 1995, 2004) and a large F_{735} peak that originates from mostly from PSI (Mullet et al, 1980). If LHCP1 is removed from PSI by detergent treatment the inner antenna of reaction centre I (RCI) fluoresces at 722 nm (Kung et al, 1984)). Isolated LHCI fluoresces at around 735-740 nm (Haworth et al, 1983). Thus it is apparent that inner antenna of RCI emits F_{722} and LHC emits F_{735} (Briantais et al, 1986). In the absence of Mg^{2+} thylakoid membranes isolated from control samples of CSR10 had a F686/F740 ratio of 1.14 (Fig. 45). Due to salt stress the F686/F740 ratio increased to 1.37 due to decrease of PSI fluorescence. Similarly, in PB1 in the absence of Mg^{2+} the F686/F740 ratio is 1.095 (Fig. 46) and due to salt-stress the ratio increases to 1.5 due to decline in PSI fluorescence. This demonstrates degradation of PSI due to salt stress was much higher in salt sensitive cultivar PB1 as comparaed to that of salt resistant CSR10. This was further supported by PSI activity measurement where the PSI activity was reduced in more in PB1 as compared to CSR10.

In the presence of Mg^{2+} due to grana stacking LHCPII migrates closer to PSII and preferentially gives energy to PSII. Therefore, fluorescence due to PSII increases and that due to PSI decreases leading to increase in ratio of PSII/PSI fluorescence. In control samples of CSR10 in the prescence of Mg^{2+} the F686/F740 ratio increased to 1.9 from 1.147 (in the absence of Mg^{2+}). In salt stressed samples in the presence of Mg^{2+} F686/F740 ratio increased from 1.37 (in the absence of Mg^{2+}) to 2.083. This clearly demonstrates that grana stacking is substantially reduced in salt stressed samples due to loss of LHCPII. Similarly in in PB1 in the presence of Mg^{2+} in control samples F686/F740 ratio increased to 2.006 from 1.095 (in the absence of Mg^{2+}).

In salt stressed samples in the presence of Mg^{2+} F686/F740 ratio increased from 1.4703 (in the absence of Mg^{2+}) to 2.211. This suggests more reduced grana stacking in PB1 due to loss of LHCPII. Reduced grana stacking are further supported by larger decrease of LHCPII protein in salt-sensitive PB1 as compared to salt tolerant CSR10 (Fig 48 A).

Salt-stress and reactive oxygen species

Oxidative stress is one of the various influences caused by salt-stress (Ashraf, 2009; Noreen et al., 2009; Sekmen et al, 2007). It has been well established that to counter-act salt-induced oxidative stress, plants generate different types of enzymatic and non-enzymatic antioxidants (Gupta et al., 2005). Commonly known enzymatic antioxidative enzymes are superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase and glutathione reductase (GR), whereas non-enzymatic antioxidants are glutathione (GSH), ascorbate (AsA), carotenoids and tocopherols (Gupta et al., 2005). In the present investigation cellular superoxide dismutase (SOD) enzymatic activity increased in response to salt stress in both the cultivars (Fig. 54). However, the relative increase in SOD enzymatic activity with respect to control was more in PB1 than CSR10 (Fig.54). This is correlated with increased gene expression of SOD in response to salt stress in both the cultivars (Fig. 60). Activity of different isoforms of SOD was seen by native in-gel assay. As shown in Fig. 55, three SOD isoforms were observed clearly which were identified by H_2O_2 inhibition studies. H_2O_2 completely inhibits Fe-SOD and partially Cu-Zn-SOD (Asada et al, 1974; Seng et al, 2004). In in-gel assay Mn-SOD, Fe-SOD and Cu-Zn-SOD and all the three isoforms upregulated in salt stress. Cu-Zn-SOD was responsible for most of the enzymatic activity. Takemura et al, 2002 have shown an increase in Cu-Zn-SOD transcripts level in *Brugiera gymnorrhiza* in response to salinity stress. Wang et al, 2004 have shown an increase in Mn-SOD and Fe-SOD isofoms in native gel assay in *Suaeda salsa* in response to salinity stress.

Ascorbate peroxides (APX) detoxifies H_2O_2 using ascorbate as electron donor (Nakano and Asada, 1981). Ascorbate peroxidase activity also increased in response to salt stress in both of the cultivars. At higher salt stress relative increase in activity was higher in PB1 (Fig.56). Gene expression of *OsAPx2* coding for one of the cytosolic form of ascorbate peroxidase in rice increased in both of the cultivars (Fig. 60). Parida *et al.* 2004 have shown an increase in ascorbate peroxidase activity in *Brugiera gymnorrhiza* in response to salinity stress.

Glutathione reductase is one of the enzyme operating in Haliwel-Asada pathway of detoxification of superoxide and is needed for regeneration of glutathione. Its activity was partially increased by salt stress in both of the cultivars after 24 h of greening (Fig.57). Gene expression of *GRase*, coding for glutathione reuctase increased in response to salt stress (Fig.60). Similar increase in enzymatic activity was observed by Wang *et al*, 2008 in *Philatus popularis* in response to salt stress.

Catalse is a heme-containing enzyme that detoxifies H_2O_2 by catalyzing its conversion into water and molecular oxygen. Catalase activity increased in response to salt stress in both the cultivars (Fig. 58). Similar increase in catalase activity was observed by Takemura *et al.* 2000 in *Brugiera gymnorrhiza* in response to salinity stress. However the relative increases with respect to control was more in PB1. Catalase activity decreased with time of light exposure. Transcript level of gene *CatA* encoding cytosolic isoform increased in rice seedlings in response to salt stress in both the cultivars (Fig 60). Antioxidative response of catalase to salt stress was confirmed by native in gel assays as shown in Fig.59. All the three isoform observed in in-gel assays were upregulated in response to salt stress. Isoform I was responsible for most of catalase enzymatic activities. Increase of antioxidative enzymes activities in response to salt stress as seen by spectrophotometric as well as in gel assays are well correlated (Kim et al., 2005).

MDA is an index of membrane lipid peroxidation (Mandhania et al. 2006; Xiao et al, 2009). MDA production increased in response to salinity as well as time of stress (light exposure) in both the cultivars (Fg.61). However the relative production of MDA is higher in PB1 as comparative to CSR10 in response to salt stress. Higher MDA production in PB1 than CSR10 during salt stress are correlated with increased degradation of photosynthetic proteins and loss of photosynthetic functions (Fig. 61). Similar increase in MDA production was shown by Moradi and Ismail, 2007 in salt tolerant rice cultivars IR651 and IR632 and in salt sensitive cultivar IR29 in response to salt stress. Increase in MDA contents were also reported by Siegal et al, 1982 in *Cucumis, Brassica* and other seedlings in response to salinity stress.

H_2O_2 contents decreases in response to salt stress in both of the cultivars (Fig. 62). Decrease in H_2O_2 is probably due to increased activities of antioxidative enzymes such as catalase, ascorbate peroxidase, glutathione reducatse in response to salt stress. (Noreen and Ashraf, 2009; Kim et al, 2005).

Tocopherols, known collectively as vitamin E, are lipid soluble antioxidants synthesized by plants and other photosynthetic organisms (Grusak, 1999; Traber and Sies, 1996). Tocopherols are thought to have a number of important functions in higher plants, including protection of chloroplasts from photooxidative damage (Eenennaam et al., 2003). α- tocopherols are known to reduce reactive oxygen species (ROS) levels (mainly $^1O_2$ and $OH^·$) in photosynthetic membranes and limit the extent of lipid peroxidation by reducing lipid peroxyl radicals to their corresponding hydroperoxides (Maeda et al. 2005, Havaux et al. 2005). In the present study both α and γ tocopherols contents decreased in response to salt stress. Ratio of α to γ increased in response to salt stress (Fig. 63). Decrease in tocopherols levels may induce cell signaling response by producing phytohormones to protect plants from stress (Munne-Bosch et al, 2007).

OsDREB2 encodes for a transcription factor which binds and causes drought tolerance when overexpressed in rice (Chen et al, 2008, Dubouzet et al, 2003). OsCIPK15 a calcineurin B-like protein-interacting protein kinases which is induced by salt stress and causes salt tolerance when overexpressed (Xiang et al, 2007). OsRab7 encodes for protein involved in vasicular transport and is differentially regulated by various stress like salt, drought and cold (Nahm et al, 2003). Gene expression of all above genes induced by salt stress in both of the cultivars (Fig. 64). This suggests their role in regulating salt tolerance and plant defence from salt stress.

Salt-induced changes in soluble and thylakoid proteome of rice

Owing to their inherent genetic make-up, the response of the halophytes to environmental stress is expected to be different in comparison to the glycophytes. A meaningful comparison of the two types requires an assessment of the factors responsible for such differential response at the physiological and proteome level. Such consideration has earlier prompted comparative studies between Arabidopsis and Thellungiella, its halophytic counterpart (M'rah et al. 2007; Kant et al. 2006; Ghars et al. 2008; Volkov et al. 2004).

Partially salt tolerant CSR10 can be used as model for understanding the response of abiotic stress tolerance of rice. Keeping this in view, the present study was undertaken in salt tolerant genotype CSR10 and salt sensitive genotype PB1 for knowing comparative proteomic responses of two cultivars to salt stress. In response to salt stress many proteins were

differentially expressed. Proteomic studies were done for differential expression of thylakoids proteins and PEG fractionated soluble proteins from leaves of rice seedlings in response to salt stress.

Thylakoids proteins:

As seen from 2-Dimensional gel-electrophoresis pattern of thylakoid protein, most of the expressed proteins were distributed in acid area. Few differentially expressed proteins from thylakoids proteins were identified Proteome analysis of thylakoids proteins shows that many proteins are differentially regulated due to salt stress in CSR10 (Fig. 69 and 70) such as upregulation of ATP synthase CF_1 beta subunit in response to salt stress suggests its role in energy regulation and stress tolerance (Table.1).

Soluble proteins:

Total 35 proteins spots were given for MALDI-TOF/TOF analysis. Out of 35, 17 proteins were identified and assigned functions remaining protein were either hypothetical or unknown proteins (Figs 67-70 and Tabble 2. 3. 4). Proteins identified in such attempt have the potential to serve as important salt-tolerance factors and their expression alteration may contribute in the stress-biology of *Oryza sativa* L. The interpretations of the regulation pattern observed for some proteins upregulated or downregulated under salinity stress are discussed as follows:

Malate dehydrogenase: Identified from spot no 209, it was down regulated in CSR10 owing to salt-stress. It has a NAD binding domain. Malate dehydrogenases (MDH) is glycosomal and mitochondrial; member of the family of NAD-dependent 2-hydroxycarboxylate dehydrogenases. MDH is one of the key enzymes in the citric acid cycle, facilitating both the conversion of malate to oxaloacetate and replenishing levels of oxalacetate by reductive carboxylation of pyruvate. Members of this family are localized to the glycosome and mitochondria.

Dehydration responsive protein: The protein was identified from spot no. 219 and upregulated due to salt-stress in both the cultivars. However, it was highly upregulated in CSR10 than that in PB1. There are various types of dehydration responsive proteins in plants. The DREB/CBF family of proteins contains two subclasses, DREB1/CBF and DREB2, which are induced by cold and dehydration, respectively, to express various genes involved in stress tolerance (Shinozaki and Yamaguchi- Shinozaki 2000). Upregulation of this protein suggests its role in stress

tolerance. Proteins of this family share a Rossmann-fold NAD(P)H/NAD(P)$^{(+)}$ binding (NADB) domain. The NADB domain is found in numerous dehydrogenases of metabolic pathways such as glycolysis, and many other redox enzymes.

Homeobox protein: This protein was identified by spot no 204 and down regulated in salt stress. This protein is nuclear encoded and functions in transcriptional regulation. It has homeodomain; DNA binding domains involved in the transcriptional regulation of key eukaryotic developmental ocesses; may bind to DNA as monomers or as homo- and/or heterodimers, in a sequence-specific manner. This may be involved in salt stress induced transcriptional inhibition.

Mitogen activated protein kinase: The protein identified from spot no. 202 and upregulated in response to salt stress. Member of this family have Serine/Threonine protein kinases, catalytic domain. This is a phosphotransferases of the serine or threonine-specific kinase subfamily. The enzymatic activity of these protein kinases is controlled by phosphorylation of specific residues in the activation segment of the catalytic domain, sometimes combined with reversible conformational changes in the C-terminal autoregulatory tail. Upregulation of this protein suggests its role in regulating salt stress response. Mutants in Fusarium proliferatum of Fphog1, an orthologue of the Saccharomyces cerevisiae hog1 MAPK gene showed increased sensitivity towards different abiotic stressors including UV-irradiation, heat, salt, osmotic and hydrogen peroxide treatments (Adam et al., 2008).

Putative protein kinase: It was identified from spot no. 216 and is upregulated by salt-stress. This protein belongs to PTKc: Protein Tyrosine Kinase (PTK) family, catalytic domain. This PTKc family is part of a larger superfamily that includes the catalytic domains of protein serine/threonine kinases, RIO kinases, aminoglycoside phosphotransferase, choline kinase, and phosphoinositide 3-kinase (PI3K). PTKs catalyze the transfer of the gamma-phosphoryl group from ATP to tyrosine (tyr) residues in protein substrates. They can be classified into receptor and non-receptor tyr kinases. PTKs play important roles in many cellular processes including, lymphocyte activation, epithelium growth and maintenance, metabolism control, organogenesis regulation, survival, proliferation, differentiation, migration, adhesion, motility, and morphogenesis. Receptor tyr kinases (RTKs) are integral membrane proteins which contain an

extracellular ligand-binding region, a transmembrane segment, and an intracellular tyr kinase domain.

Fructose bisphosphate aldolase chloroplast precursor: This protein was identified from spot no. 214 and upregulated in response to salt stress. It has a functional role in metabolism. Fructose-1,6-bisphosphate aldolase. The enzyme catalyzes the cleavage of fructose 1, 6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP).This protein upregulation in salt stress suggests its role in carbohydrate synthesis which to compensate loss to salt stress by plants.

Calcinurin B like proein 5: This protein was identified from spot no. 309 and its expression induced by salt stress in PB1. It is a Ca^{2+}-binding protein (EF-Hand superfamily) and have role in Signal transduction mechanisms / Cytoskeleton / Cell division and chromosome partitioning. This protein is possibly involved in Ca^{2+} mediated signaling in response to salt stress.

Cytochrome c oxidase subunit 6b-1: This protein was identified from spot no 208 and downregulated under salt stress. Cytochrome c oxidase (CcO), the terminal oxidase in the respiratory chains of eukaryotes and most bacteria, is a multi-chain transmembrane protein located in the inner membrane of mitochondria and the cell membrane of prokaryotes. It catalyzes the reduction of O2 and simultaneously pumps protons across the membrane. The number of subunits varies from three to five in bacteria and up to 13 in mammalian mitochondria. Subunits I, II, and III of mammalian CcO are encoded within the mitochondrial genome and the remaining 10 subunits are encoded within the nuclear genome. Found only in eukaryotes, subunit VIb is one of three mammalian subunits that lacks a transmembrane region. It is located on the cytosolic side of the membrane and helps form the dimer interface with the corresponding subunit on the other monomer complex. This protein involved in energy regulation and regulating proton gradient across the membrane.

Ankyrin repeat domain protein: This protein was identified from spot no. 213 and down regulated under salt stress. This protein has ankyrin repeats; ankyrin repeats mediate protein-protein interactions in very diverse families of proteins. The number of ANK repeats in a protein can range from 2 to over 20 (ankyrins, for example). ANK repeats may occur in combinations with other types of domains. The structural repeat unit contains two antiparallel helices and a

beta-hairpin, repeats are stacked in a superhelical arrangement; this alignment contains 4 consecutive repeats.

Putative amine oxidase: This protein was identified from spot no. 218 and upregulated in salt stress. This protein is NAD/FAD dependent oxidoreductase. It is involved in oxidation reactions.

As salt stress affects whole cellular mechanism of plants and also differentially expressed proteins lie in acid area, while global pattern of proteins largely remained unaltered. Proteome analysis of PEG-fractionated soluble proteins shows differential regulation of proteins involved in many cellular processes like cell signaling, metabolism, energy balance, antioxidative defence, structural organization and stress tolerance. Upregulation of putative mitogen activated kinase kinase alpha, putative protein kinase, putative indole 3 acetic acid regulated protein, calcineurin B like protein in salt stress suggests their role in cell signaling during salt stress. Upregulation of formin like protein 2 suggests its role in regulating structural organization during salt stress. Upregulation of fructose bisphosphate aldolase, amine oxidase, putative cytochrome p 450, ribulose bisphosphate carboxylase suggests their role in energy and carbon metabolism. A new protein synthesized in response to salt-stress is the putative transcription repressor HOTR. It may have a role in stress-induced transcriptional regulation. It is interesting to note that a putative retrtransposon-like protein identified from spot 205 from soluble protein is down regulated in CSR10 and upregulated in PB1 during salt stress (Table 2, 3, 4). Differential regulation of proteins in the salt-sensitive and salt-tolerant genotypes suggest that plants adjust their protein concentrations to combat salt stress.