Identification of the thiol induced transcripts, their functional characterization and role in crop improvement

By

Ashish Kumar Srivastava

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Recommendations of the Doctoral committee

As members of the Viva Voce Board, we certify that we have read the dissertation prepared by **Ashish Kumar Srivastava** entitled "**Identification of the thiol induced transcripts, their functional characterization and role in crop improvement**" and recommend that it may be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Chairman- (Dr. S. K. Apte) Date: 20/02/2012

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Member 2- (Dr. N.K. Ramaswamy) Date: 20/02/2012

External Examiner- (Prof. K. V. Rao) Date: 20/02/2012

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DECLARATION

I, hereby declare that the investigations presented in the thesis have been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree/diploma at this or any other Institution/University.

(Ashish Kumar Srivastava)

Dedicated to my parents

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Ashish Kumar Srivastava,
NABTD, BARC

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Synopsis

Preamble

In the current scenario of increasing interest for sustainable agriculture, soil salinity is considered as one of the major environmental constraints that reduce the crop yield worldwide. More than 800 million ha of land throughout the world are salt-affected which accounts for around 6% of the world's total land area. Besides, out of 1500 million ha of land farmed by dryland agriculture, 32 million ha (2%) are affected by salinity to varying degrees and out of the current 230 million ha of irrigated land, 45 million ha (20%) are salt affected (FAO, 2008). The problem of soil salinity is becoming severe day by day due to bad agricultural practices (Munns and Tester, 2008). Therefore, efforts are underway to increase and/or manage the salinity tolerance in plants either by genetic engineering or plant breeding. However, these approaches have not been much successful as far as consistency, reliability and visible effects at the field level are considered. This is mainly because of the multigenic and complex nature of salinity stress tolerance (Yamaguchi and Blumwald, 2005). Towards this endeavor, another approach is to use transcription factors in transgenic technology to activate a set of genes involved in salinity/drought stress tolerance. However, the limitation of this concept lies in the fact that one transcription factor may regulate several metabolic pathways and one metabolic pathway may need an orchestrated regulation from more than one transcriptional element. Thus, the development of stress tolerant transgenics using gene transfer approaches needs much more understanding of the plant stress tolerance and gene regulatory network systems.

Another important factor regulating the key processes in growth and development as well as stress tolerance is the cellular redox state. The maintenance of the redox homeostasis is collectively achieved by redox couples, antioxidant systems

(catalase and superoxide dismutase etc.) and other secondary metabolites (flavonoids, alkaloids, and carotenoids) (Dietz, 2008). In response to any external stimuli, plants modify their redox state and the extent of change is dependent on the nature of the stimulus itself, the dose, and exposure time to the tissue in question (Miller et al., 2009). On the basis of these facts, we have hypothesized that if the external application of any molecule can help the plants to maintain their redox homeostasis under stress conditions, it may enhance their stress tolerance potential. This concept is based upon the priming of the existing defense mechanisms and hence avoids any direct manipulation of the genome. Earlier in collaboration with Rajasthan Agriculture University, Bikaner (India), our lab had studied the effect of three thiol compounds viz. thiourea (TU), dithiothreitol (DTT) and thioglycollic acid (TGA) on the performance of wheat and mustard crops under salinity and drought affected fields of Rajasthan (India). The field data indicated that the pre-treatment of seeds and foliar spray of the seedlings at later stages with all these thiol compounds independently could increase the stress tolerance. More importantly, there was an enhancement in the crop productivity and among all the thiols tested, the performance of TU was found to be the best (Sahu and Singh, 1995; Sahu et al., 2005).

Thus, considering the efficiency of TU in alleviating the salinity stress and increasing crop yield, in the present thesis, studies were undertaken to investigate various molecular and biochemical responses involved in TU-mediated salinity-stress tolerance. Details of the above mentioned studies have been arranged into five chapters. Chapter-I: General introduction details about the scientific information available in the literature related to the present work. In Chapter-II, the materials and methodology are described. The differential effects of salt with/without TU at the seed

germination and seedling stages are described in Chapter III and IV, respectively. The Chapter V summarizes the present work and includes future perspectives.

Chapter I [General Introduction]

This is an introductory chapter that describes the literature related to the effects of salinity stress on plant and the possible mechanism(s) of salt-tolerance. The general information on TU and its effect on the biological system are also discussed.

The soil-salinity affects growth of crop plants and leads to poor yields. The primary effect of excess salinity is increase in the osmotic pressure of the soil solution which reduces the plant's ability to take up water. Besides osmotic effect, the excessive concentration of the salt may also prove toxic for the plants and/or may retard the absorption of other essential plant nutrients. Salinity stress, like many other abiotic or biotic stresses, has to be perceived for the activation of tolerance mechanisms. In plants, salinity stress can be sensed either at the outer or inner surface of the plasma membrane by a trans-membrane protein, or within the cytosol by various osmo-sensors. Once the signal of salinity stress is perceived, the level of abscissic acid gets enhanced triggering an array of responses that assist the plant in perceiving the exact nature and magnitude of the stress. One of the important changes that occur at the initial stage of stress perception is a transient increase in cytosolic free Ca^{2+} (Ca^{2+}_{cyt}) which arise because of the flux of Ca^{2+} into the cytosol, either from the external medium or from the sub cellular compartments. Such a change in the Ca^{2+}_{cyt} pattern in the form of its frequency, amplitude and shape enable the plant to generate stimulus-specific response. Besides calcium signalling, the redox homeostasis is yet another important process and it is now widely accepted that redox signals are the key regulators of plant response under stress. Apart from this, it has also been suggested that redox state can also act as a central point to regulate the

signal transduction pathway under different conditions. Any external stimulus, such as salinity, that causes oxidative stress may lead to a shift in the redox state towards the more oxidised condition. This can act as a cellular messenger, which in conjunction with the other mediators (calcium signature) transmits the signal to the nucleus for the transcription of regulatory genes required for the salt-tolerance.

In plants, the salinity-stress responses can be studied either at the seed germination or at the seedling stage. In general, the seed germination is considered as the most sensitive stage in the plant life cycle and is also characterized with some unique properties. For instance, the seed mitochondria play an important role in generating the hypoxic environment inside the seed which is required for the dormancy breakage. Any loss in the functioning of mitochondria in response to the salinity stress may lead to the reduction in the seed germination ability. At the seedling stage, the series of events leading to the perturbation of cellular metabolism under NaCl stress are suggested to be as follows: less water availability, stomata closure, altered gaseous exchange, inhibition of photosynthesis, effect on electron flow in electron transport chain (ETC) in chloroplast and mitochondria, increase in the production of reactive oxygen species (ROS) and disturbed status of adenine (ATP) and pyridine nucleotides (NADH, NADPH). Hence, the imposition of oxidative stress and altered energy reactions affect the general plant metabolism its growth and finally the yield. The extent of ROS accumulation and oxidative damage under salinity stress depends on the level of stress and the ability of plant to counteract it. Essentials of ROS detoxification during normal metabolism and particularly during stress, are low-molecular weight non-enzymatic [Ascorbate (ASC) and Glutathione (GSH)] and enzymatic [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) etc.] antioxidants.

Thiourea has a wide range of applications. In plants, the use of TU was first proposed as a dormancy breaking agent (Tukey and Carlson, 1945) and later on this effect was found to be correlated with its anti-catalase activity. This has led the TU to be adopted as a plant growth regulator. Until recently, there have been various reports where TU was used either for enhancing the seed germination ability or for enhancing the crop productivity under field conditions. Apart from this, TU is also used as an additive in fertilizers because of its anti-nitrification property. Another most important characteristic of TU is that it can scavenge different ROS (Kelner et al., 1990; Gao et al., 2008). Because of this property, the use of TU was also explored to protect animal cell lines from stress-induced oxidative stress. Lately, the industrial use of TU has also emerged, such as for the extraction/recovery of precious metals such as gold (Rodriguez et al., 2006) and silver (Wang et al., 2010) and for making the chemical sensors (Kumar et al., 2010).

Chapter II [Materials and Method]

This chapter gives details of the experimental materials and methods used for various studies. The entire study was performed on Indian mustard (*Brassica juncea* L. cv TM-2) seeds/seedlings. All the seed germination and seedling growth assays were performed under the controlled conditions with a 12 h light-dark photoperiod, 25±2°C temperature, 70% relative humidity and a light intensity of 140 µmol m^{-2} s^{-1}. The primary evidence to support the protective effect of TU was derived from the seed germination assay in which seeds were independently soaked under different treatments such as distilled water control, NaCl (1M), NaCl (1 M)+TU (6.5 mM) and TU alone (6.5 mM) for 6 h and then allowed to germinate under controlled condition for 2 d. To gain insight into the molecular mechanism behind the differential seed germination profile, the gene expression studies were performed by differential

display as well as microarray hybridization. The differential expression of transcripts was validated by the quantitative real-time RT-PCR. Wherever possible, the representative pathway was also analysed at the biochemical level to support the conclusion drawn on the basis of gene expression data. Isolation of mitochondria was performed by percoll-density gradient centrifugation. The mitochondrial ATPase activity was measured by estimating the phosphate (Pi) released in the reaction. The endogenous level of ABA was measured by competitive ELISA technique. The seeds were sown in pots with fine sterilized sand and grown for 10 d. Seedling were subjected independently to different treatment conditions, such as distilled water control, NaCl (350 mg/150 g sand), NaCl (350 mg/150 g sand)+ TU (5 mg/150 g sand) and TU alone (5 mg/150 g sand). On three consecutive days, the differential phenotype was monitored. This was related to the improved water uptake potential of roots (measured by the quantitative real-time RT-PCR of different aquaporin isoforms) as well as the better energetics and antioxidant potential in shoots. The level of H_2O_2 and superoxide radical was estimated by histochemical staining with DAB (Di-amino Benzidine) and NBT (Nitro Blue Tetrazolium), respectively, and was further validated by the biochemical methods. The extent of oxidative damage was quantified by measuring the MDA content. The % radical scavenging activity was also measured in terms of DPPH radical scavenging activity. The level of non-enzymatic antioxidants (ascorbate and glutathione) and the activity of enzymatic antioxidants (SOD, CAT, APX and GR) were estimated following the standard protocols. The ratio of ATP/ADP, NAD/NADH and NADP/NADPH was estimated by HPLC based technique. The Na^+ and K^+ ion contents were measured by single channel flame photometry.

The experiments were performed in a completely randomized design (CRD). One-way analysis of variance (ANOVA) was done with all data to confirm the variability of data and validity of results, and Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments at a particular duration using SPSS 10.0.

Chapter III

[Identification of the thiourea-modulated and salinity stress dependent transcripts in seeds of *Brassica juncea*]

This chapter deals with the studies at the stage of seed germination and has been divided in two sections; section I describes the identification of differential transcripts by differential display and the section II describes the genome-wide identification of the differential transcripts by microarray.

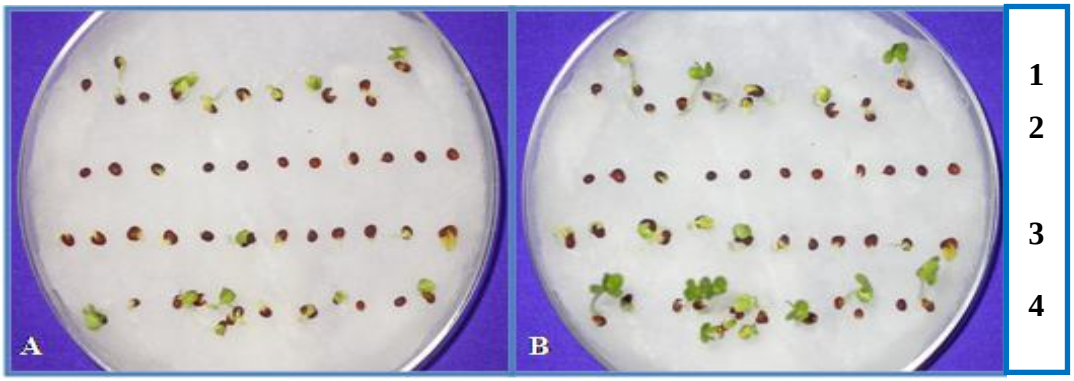
Section-A:

Differential display based approach.

The primary evidence in support of ameliorative effect of TU against NaCl stress was derived from the seed viability assay. The seeds soaked in NaCl (1 M) for 6 h exhibited drastic reduction in their germination ability and were not able to sprout even when subjected to control conditions (Fig. 1; Lane-2). However, seeds soaked for same time period in NaCl (1 M)+TU (6.5 mM), retained their germination ability to about control levels (Fig. 1; Lane-3). Seeds soaked in 6.5 mM germinated properly which indicated that TU itself has no negative effect on the germination process (Fig. 1; Lane-3). In order to identify the genes responsible for such response, the differential display was performed in seeds subjected to different treatments (as above) for 1 h. Unlike seed germination assay, here the soaking time was kept for only 1 h because of our interest to identify the regulatory genes affected under TU

treatment. The differential display experiment led to the identification of three transcripts whose expression was found to be differentially regulated in response to NaCl and NaCl+TU treatment. The sequence analysis of these transcripts confirmed the role of mitochondrial F_oF_1-ATP synthase (mtATPase) in TU-induced NaCl-tolerance.

The mitochondrial F_oF_1-ATP synthase is a multimeric enzyme, in which F_1 is a peripheral component and F_o is embedded in the hydrophobic region of the membrane. The F_1 structure consists of five subunits designated as $\alpha, \beta, \gamma, \delta$ and ε with decreasing order of their molecular weights having stoichiometries of $\alpha_3\ \beta_3\ \gamma_1\ \delta_1\varepsilon_1$. In order to validate the data of differential display and gain further insight, the expression level of all the five mtATPase subunits were monitored in seeds subjected to similar treatment condition by quantitative real-time PCR.



[Fig. 1: Differential germination response of *Brassica juncea* seeds subjected to different treatments for 6 h. Lanes marked as 1, 2, 3 and 4 represent seeds soaked in distilled water control, NaCl (1M), NaCl (1 M)+TU (6.5 mM) and TU alone (6.5 mM) treatments, respectively, for 6 h and then allowed to germinate under control condition. Section A and B represents the growth pattern after 1 and 2 d, respectively.]

The alpha subunit of mtATPase has a critical function with respect to the mitochondrial bioenergetics and its turnover might be involved in the control of mitochondrial activity during germination. In the present study, we found that it is downregulated in the seeds treated with NaCl for 1 h. This could be due to the negative impact of NaCl stress on mtATPase. Such a change during the early stage might have disturbed the mitochondrial homeostasis, which ultimately resulted in the poor seed germination. On the contrary, in NaCl+TU treatment the level of alpha subunit was close to that of control, which suggests the positive role of TU in maintaining the mitochondrial function under salinity stress. This was also reflected in terms of increased germination percentage and better growth pattern in NaCl+TU treatment (Fig. 1; Lane-3). The TU mediated upregulation of alpha subunit under NaCl stress, has not been reported so far and appears to be a novel response. Response of other mtATPase subunits was also found to be differential. For instance, the NaCl treatment for 1 h led to the upregulation of beta subunit while such an upregulation was not seen when TU was supplemented along with NaCl treatment. In case of gamma, delta and epsilon subunits also, we found that the presence of TU along with NaCl treatment reversed their expression profile seen under NaCl stress alone. The change in the expression level of different mtATPase subunits was also reflected in terms of total mtATPase activity which was decreased significantly in NaCl, however, it increased almost to the level of control in NaCl+TU treatment.

Thus the data obtained indicated that seeds have the *inbuilt* mechanism in the form of mitochondria that has to remain intact for proper germination under stress. The presence of salinity stress hampers the efficiency of various mitochondrial components, which leads to the poor germination and growth pattern. By contrast, these negative effects were reversed when TU was supplemented along with NaCl.

Section-B:

Microarray hybridization based approach.

The initial data of differential display indicated that TU exerted its effect at the transcriptional level. This presupposes that TU might affect the expression of various signalling and effector components involved in NaCl stress tolerance. To address this hypothesis, the genome wide transcript profiling was performed in seeds subjected to similar treatment conditions (as stated in Chapter-III/Section-A) for 1 h. Since, the Arabidopsis array is an established tool for the gene expression analysis of Brassica species (Hudson et al., 2007), Agilent based *Arabidopsis thaliana* microarray platform was used.

In TU alone treated seeds, 53 genes were down-regulated as compared to that of control. The functional analysis of these genes indicated that most of them were either transcription factors/heat shock proteins or associated with the responses such as calcium signaling, protein synthesis/degradation and antioxidant machinery. The decline in the expression level of genes involved in protein synthesis/degradation in TU alone treatment probably stabilized different cytosolic proteins and therefore, reducing the overall protein turnover. The TU mediated down-regulation of the genes for different heat shock (HSPs) and universal stress proteins (USPs) further indicated that the de novo synthesis of these proteins were not required. Besides, eight genes were also found to be up-regulated in TU alone treatment, which are associated with the cellular energetics system.

In NaCl-stressed seeds, a total of 83 down-regulated and 28 up-regulated genes were identified. Clustering analysis of these 111 differentially expressed genes resulted in the identification of 33 genes whose expression was modulated under NaCl+TU treatment. These genes were divided into cluster-1 and -2 consisting of 14

and 19 genes, respectively, depending on their down- or up-regulation in NaCl+TU, as compared to that of NaCl stress. These 33 genes were collectively denoted as TU-modulated and NaCl stress responsive genes. Of these, 27% genes encoded proteins with a putative regulatory function (transcription factors, hormone and calcium signaling), suggesting a significant modulation in regulatory networks in NaCl+TU treatment. Apart from that, 6% genes encoded proteins involved in ABA metabolism, 9% were related to the energy production and 12% of genes were found to be involved in storage and transport. Further, the quantitative real-time PCR analysis of the eight selected genes were performed at two time points (1 h and 6 h) to validate the microarray data and to monitor their temporal regulation. An induction in the expression level of most of the genes was observed as early as 1 h in NaCl+TU treatment. In contrast, upon NaCl treatment, almost similar level of induction was observed only at 6 h. Additionally, the data on redox state (in the terms of GSH:GSSG ratio), ABA level and antioxidant enzyme activities were also differential at both the time points in NaCl and NaCl+TU treatment. Another interesting feature associated with most of the TU modulated transcripts was that these were either directly related to calcium signaling or were the potential targets of the calmodulin/calceneurin B-like proteins. This suggested that the action of TU might be dependent upon calcium signaling. To confirm this, similar molecular and biochemical parameters were evaluated in response to $LaCl_3$ treatment (a calcium channel blocker) along with NaCl+TU. Under such conditions, the inhibition of TU mediated beneficial effects both at the molecular as well as the biochemical level was observed confirming the involvement of calcium signaling in TU mediated response.

Thus, these studies suggested an early activation of various regulatory and effector mechanisms resulting in the NaCl stress tolerance.

Chapter IV

[Thiourea dependent modulation of water balance, redox state and antioxidant system to alleviate NaCl-induced oxidative damage in seedlings of *Brassica juncea*]

This chapter relates to the various biochemical and molecular parameters to TU-mediated alleviation in the NaCl-stress induced damage in the seedlings of *Brassica juncea*. The chapter has been divided into two sections; section-I describes the short- and long-term effects of NaCl stress with/without TU on the expression level of aquaporins and its relation with water homeostasis in roots while the section-II describes the co-ordinated regulation of redox and antioxidant system in shoots.

Section-A: Studies on the temporal regulation of aquaporins and its impact on the water-balance in roots.

A primary effect of NaCl-stress is the creation of water deficit conditions for the plants due to generation of osmotic differences between cytoplasm and soil solution. Plants have evolved several mechanisms to cope up these effects, such as closing of the stomata, biosynthesis of compatible solutes and the regulation of membrane permeability and transport. Aquaporins constitute a large family of membrane proteins which facilitate the diffusion of water across cell membranes. They are classified into four groups, namely PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (nodulin-26 like intrinsic proteins) and SIPs (small intrinsic proteins); PIPs carry out the function of water uptake and transport and are further classified into two highly homologous subgroups, PIP1 (PIP1;1- PIP1;5) and PIP2 (PIP2;1 - PIP2;8). In recent years, the specific roles of different PIPs are being studied and the data indicated that the complex regulation of these PIPs is required to perform water uptake/transport from soil to root and to other

organs in a coordinated manner under normal and stressed conditions. In this perspective, investigations were made to see whether TU supplementation can modulate the expression profile of PIPs involved in the maintenance of the root water homeostasis. To investigate this concept, the temporal regulation [short (30-90 min) as well as long (1-3 d) term] of 13 PIP isoforms was studied in roots of *Brassica juncea* seedlings subjected to different treatments such as distilled water control, NaCl (350 mg/150 g sand), NaCl (350 mg/150 g sand)+TU (3 mg/150 g sand) and TU alone (3 mg/150 g sand). The roots were selected for the present study as it is the first organ to come in direct contact with stress. The data on expression profiling revealed that the level of most of the PIPs remained up-regulated under NaCl stress, till 90 min; however, in the long term, their levels were extremely downregulated as compared to that of control. The primary effect of NaCl stress would be an induction of significant osmotic stress and, therefore, an early increase in the expression level of PIPs could be considered as a typical stress response mechanism to support the maximum water uptake. In contrast to NaCl, under NaCl+TU treatment, expression level of PIPs exhibited a decrease in the expression at an early phase and this lower level was maintained till 3d of stress, except for the slight increase in the expression level during the 1 d after stress. On the 3rd d after stress, the extent of downregulation was much higher in NaCl than in NaCl+TU treatment. Although, an increase in the expression level of PIPs would enhance the water uptake from soil, this would also increase the symplastic water transport from roots to other tissues and *vice versa*. Hence, an upregulation of PIPs at an early stage might prove to be negative due to significant symplastic water movement resulting in lower water content while their downregulation might prove to be beneficial, particularly under stress conditions when there is a greater need to retain water.

Further, on the basis of temporal expression pattern observed in NaCl+TU treatment, the various PIP isoforms could be putatively classified into two broad categories. The first putative category, PIP-L (L denoting the loss of water from roots to other tissues) included PIP-1;3, PIP1;4, PIP2;1, PIP2;4, PIP2;6 and PIP2;7. The isoforms of PIP-L category showed a time dependent decrease in the expression level. On the contrary, isoforms of the second putative category, PIP-U (U denoting the uptake of water from outside), which included PIP-1;1, PIP-1;2, PIP-1;5, PIP-2;2, PIP-2;3, PIP-2;5 and PIP-2;8 showed time dependent increase in the expression level and at 90 min, their level approached towards that of control. Thus, on the basis of expression profile of aquaporins, differing in stress-specific and time-dependent manner, we could putatively assign specific functions to different PIP isoforms for the uptake or loss of water under salt stress.

PIPs of the putative PIP-L category showed a time dependent decrease in their expression level in NaCl+TU treatment. Of these, PIP1;4 demonstrated the maximum downregulation after 90 min (10-fold downregulated). This is in contrast to NaCl treatment where, at the same time, its level was slightly higher than that of control. This suggests the importance of PIP-1;4 in regulating the loss of water under stress. Further, the transcriptional modulation of various PIPs occurred in a coordinated manner in NaCl+TU treated roots. For example, at 30 min, the expression of PIP-2;2 was almost 2-fold lower in NaCl+TU as compared to that in NaCl treatment. However due to a time dependent increase in its expression, at 90 min, its level was almost similar in both NaCl and NaCl+TU treatment. PIP-2;2 is considered as the most abundant aquaporin isoforms contributing significantly towards soil water uptake. It thus proposes that initial downregulation of PIP2;2 presumably occurred to limit the

water loss during the early stage and assisted subsequent water uptake at the later stage in NaCl+TU treatment to maintain the water homeostasis.

Thus, the TU mediated regulation in the expression profile of different PIPs was found positive in maintaining the water status of roots under NaCl-stress.

Section-B: Thiourea mediated regulation in the redox state and antioxidant responses to reduce the NaCl-induced oxidative damage.

This section reports the ameliorative effect of TU treatment against NaCl-induced oxidative damage at the seedling stage. The preliminary analysis of differential phenotype of the seedlings under different treatments (as stated in Chapter-IV/Section-A) demonstrated that TU treatment itself did not impose any negative impact on growth and also its addition along with NaCl treatment helped the seedlings to maintain their growth pattern similar to that of control. The NaCl stress is known to cause various changes in the cellular metabolism such as the increased production of ROS and altered ratio of adenine (ATP/ADP) and pyridine (NADH, NADPH) nucleotides which are the main cause of NaCl-stress induced toxicity. In the present study, the increased level of different ROS (H_2O_2 and superoxide radical) and MDA content (an indicator of membrane damage) suggested the built-up of oxidative stress under NaCl stress; whereas the significant alleviation of the stress was noticed under NaCl+TU treatment. Apart from this, a time dependent decrease in the Na^+ ion accumulation and an increased potential to retain K^+ ion (as indicated by the Na^+/K^+ ratio) was also observed in NaCl+TU treatment as compared to that of NaCl. Under NaCl stress, significant reduction in the ratios of NADP/NADPH and NAD/NADH was observed which indicated the over-reduction of electron transport chain in chloroplasts and mitochondria, respectively. In contrast, no such impact was observed in NaCl+TU treatment as these ratios were maintained at par with that of control. The

glycolate oxidase (GO) mediated salvage pathway, for the removal of excess NADPH, was found to be stimulated significantly in NaCl+TU treatment as compared to NaCl. This might have further contributed towards the maintenance of proper NADP/NADPH ratio in NaCl+TU treatment. The ATP/ADP ratio was also declined to a higher level in NaCl+TU as compared to NaCl treatment due to a greater ATP consumption probably to support various energy consuming tolerance mechanisms such as exclusion/sequestration (a lower ion accumulation is observed in this study) of the ions and synthesis of different osmolytes etc.

The cellular redox state plays a critical role in maintaining the plant's potential to scavenge ROS. This is preferably regulated by glutathione (GSH) and ascorbate (ASC). The data of GSH and ASC showed that only the responses of GSH and GSH/GSSG were differential, with the significantly higher values being observed in NaCl+TU than in NaCl treatment. It thus appeared that a better response of GSH was responsible for the lower levels of ROS observed under NaCl+TU treatment. Additionally, the activity of SOD, which is considered as the first line of defence against the oxidative stress, was also increased significantly in NaCl+TU treatment as compared to that of NaCl. The product of SOD activity is H_2O_2, which is still toxic and must be eliminated either by CAT and/or APX. In NaCl+TU treatment, CAT activity was not increased significantly which could be due to the lower level of H_2O_2 that can be efficiently managed through non-enzymatic action of GSH and ASC. Under NaCl stress, the significant increase in the CAT activity appeared to be a compensatory mechanism to scavenge higher H_2O_2 levels.

The activities of enzyme associated with ASC-GSH cycle were almost unaffected under NaCl+TU treatment except for the MDHAR which activity was increased probably to support the regeneration of ASC being consumed in the non-

enzymatic detoxification of H_2O_2. In NaCl treatment, although the activities of APX, MDHAR and DHAR were significantly increased, the proper functioning of ASC-GSH cycle was hampered due to the lower availability of GSH. This finally resulted in the built-up of oxidative stress in NaCl treatment. The activity of ascorbate oxidase, an important player of oxidative stress signalling, was significantly increased at an early stage (1 d) in NaCl+TU, whereas in NaCl treatment, the comparable level of induction was observed only at 3 d. This suggested that TU treatment was helpful in the early perception of NaCl stress and hence the appropriate defence mechanism was induced to reduce the damage.

Chapter V

Major conclusion

The major findings of the present thesis are summarised as below:

1. The addition of TU protects the plants from the NaCl-stress induced damage. This was demonstrated both at the seed germination as well as the seedling stage.
2. The transcripts responsible for the differential response of seeds under NaCl stress with/without TU treatment were identified. The data obtained revealed the early and co-ordinated induction of ABA, redox and calcium based signalling and effector mechanism only in NaCl+TU treatment. TU was also shown to maintain the proper functioning of mitochondria. All these changes together help the seeds to cope with the NaCl-stress induced damage and thereby maintaining their viability and germination ability.
3. The ameliorative effect of TU at the seedling stage was also demonstrated. The TU treatment differentially modulated the expression of aquaporins in a time dependent manner. This was positive in maintaining comparatively better root-water status in NaCl+TU as compared to NaCl treatment. Further, the NaCl+TU

treatment was found to reduce the ROS load which helps the plants to maintain the reduced redox state more efficiently than in NaCl stress. This also curtailed the requirement to stimulate different antioxidants. Owing to this, plants were able to channel their energy towards Na^+-ion exclusion/avoidance resulting into the significant alleviation of NaCl-stress induced oxidative damage.

Future work

The present study proposes the role of reduced redox state for the NaCl-stress perception and tolerance. The exact identity of such redox-sensitive and NaCl-stress responsive sensor needs to be explored. The results lead to postulation that TU treatment maintains this sensor in its active form (-SH state) so that the NaCl-stress can be perceived. Once perceived, the signal is transferred to the nucleus through calcium/ABA signalling which results in the induction of some common regulator required for the activation of different protective mechanisms. The preliminary analysis of promoter elements in TU modulated NaCl-stress responsive transcripts indicated that they can be co-regulated by the transcription factor, Dof-2. However, this needs to be explored further to confirm the hypothesis. In the absence of TU, the NaCl-sensor may get inactivated (-S-S- state) due to the induction of oxidative stress in the apoplastic region. Consequent to improper perception, the co-ordinated induction of different responses may not be achieved under NaCl stress. This could be the major reason of the differential phenotype of seeds and seedlings observed under NaCl and NaCl+TU treatment. Apart from this, the yet unexplored aspect of the study is the contribution due to the direct action of TU.

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CHAPTER 1

INTRODUCTION

Agricultural productivity in many parts of the world is challenged by multitude of stresses which includes biotic (plant pathogens) and abiotic (salinity, drought, extreme heat, cold and heavy metals) factors. Of all the abiotic stresses, salinity is one of the major factors severely affecting the crop yield leading to food insecurity for millions of people particularly in developing countries (FAO 2008; http://www.fao.org/ag/agl/agll/spush/). The research world-wide is therefore focused to design various strategies such as breeding (conventional and mutation breeding based approach); biotechnological (transgenic based approach) and agronomical (priming based approach) either for the development of salt-tolerant crops or for imparting salinity tolerance to available crop varieties. Traditional breeding methods have played a significant role in integrating favorable genes to induce stress tolerance in crops. However, such attempts have met with limited success due to the complexity of salt tolerance trait (Yamaguchi and Blumwald, 2005). The mutation breeding has not yet delivered the salt-tolerant genotypes, but has generated a number of salt-sensitive types which became useful genetic material for understanding mechanisms of salt tolerance (Tester and Davenport, 2003). The transgenic technology deals with alteration in the activity of one or two genes to achieve overall tolerance and has generated salt-tolerant lines in some crops. However none of the developed lines have been field tested and released commercially (Ashraf and Akram, 2009; Mittler and Blumwald, 2010).

In this context, the approach of using bioregulators that can enhance plant's ability to withstand stress exposure has gained considerable interest. This concept is based upon the priming of existing defense mechanisms and does not involve any genetic modification. The manifestation of stress induced damages is mediated through the

uncontrolled redox reactions and oxidative damage to functional macromolecules (Dietz, 2010). Thus, it can be hypothesized that if the external application of any molecule can restore the redox homeostasis then the stress tolerance of the plant can be enhanced. Previously our group evaluated the effects of three different sulphydryl compounds (–SH containing) viz. thiourea (TU), dithiothreitol (DTT) and thioglycollic acid (TGA), the well known redox modulators, on the performance of mustard and wheat crops under salt affected fields of Rajasthan (India). The field trial data showed that both pre-treatment of seeds and the foliar spray of the seedlings at later stages with above thiol compounds increased the stress tolerance and crop productivity (Sahu et al., 2005; Sahu et al., 2006). In order to understand the mechanism behind the increased crop yield in thiol sprayed plant, a preliminary experiment was carried out using ^{14}C-Sucrose, as a radiotracer. The data obtained confirmed that thiol treatment enhances the translocation of sucrose metabolites from source (leaves) to sink (pods) that ultimately results into the increase in crop productivity (Srivastava et al., 2008a). The application of TU as a "sulphydryl bioregulatory technology for crop improvement" is proposed and is being popularized at the state level in Rajasthan (India). Among three thiols used in the field studies, the use of TU was promoted because it is economical from the farmer's point of view. The promising impact of TU application observed at the field level in terms of enhanced stress tolerance and crop yield led us to investigate the basic mechanisms of thiourea mediated action. In the present thesis, different molecular and biochemical studies were performed in seeds as well as seedlings subjected to salt (NaCl) treatment with/without TU in Indian mustard (*Brassica juncea* (L.) Czern.). The results revealed that various components of salinity stress tolerance are modulated by TU treatment leading to the

improvement of growth phenotype. Thus, the present work has both basic and applied implications. The thesis not only addresses the mechanistic cues involved in salinity stress tolerance but also supports the concept of using TU for enhancing crop productivity under field conditions.

In this chapter, we have first discussed the general information about the soil salinity and the effect of salinity stress both at the whole plant level as well as at cellular and molecular level. The important salt tolerance mechanisms and different strategies for enhancing the salt tolerance in plants are also presented. The brief information about the plant system and TU are summarized. At the end, aims and objectives of the present thesis are listed.

1.1. Soil salinity-A general Introduction

Saline soil contains high concentrations of dissolved mineral salts, such as sodium chloride (NaCl), sodium sulphate (Na_2SO_4), sodium nitrate ($NaNO_3$), magnesium sulphate ($MgSO_4$), magnesium chloride ($MgCl_2$), potassium sulphate (K_2SO_4) and calcium carbonate ($CaCO_3$). Sodium chloride is the most soluble and abundant salt present in the soil and is mainly responsible for salinity related problems in higher plants. According to the FAO Land and Plant Nutrition Management Service (FAO, 2008), over 6% of the world's land area (400 million ha) is affected by salinity. Of this 400 million ha salt-affected area, 45 and 32 million ha land is associated with irrigated and dryland agriculture, respectively, and the remaining area is non-cultivated. In India, 6.73 million ha of land is affected by salinity and 25% of ground water used for irrigation is saline. According to an estimate, 11.7 million ha land in India is likely to be affected by salinity by the year 2025.

1.1.1. Measurement of soil salinity

Soil salinity is measured by its electrical conductivity. The SI unit of electrical conductivity (EC) is dS/m. Soil is defined as saline if its EC is 4 dS/m or more (USDA-ARS, 2008), which is equivalent to approximately 40mM NaCl and generates an osmotic pressure of approximately 0.2 MPa.

1.1.2. Causes of the Soil Salinity

The origin of soil-salinity could be either natural (primary salinization) or human-induced (secondary salinization). Primary salinity results from the accumulation of salts over long periods of time. It normally occurs by two natural processes. The first is the weathering of parent rock materials to release various types of soluble salts such as chlorides and to a lesser extent, sulphates and carbonates of sodium, calcium and magnesium. The second is the deposition of oceanic salts carried by wind and rain. The amount of salt stored in the soil varies with the soil type, being low for sandy soils and high for soils containing a high percentage of clay minerals. It also varies inversely with the average annual rainfall. Secondary salinization results from human activity that changes the hydrologic balance of the soil. The hydrologic balance is defined as the ratio of water applied to the soil (irrigation or rainfall) and water evaporated by the crops (transpiration). The most common causes for the hydrologic imbalance are (i) the land clearing and the replacement of perennial vegetation with annual crops and (ii) irrigation schemes using salt-rich irrigation water or having insufficient drainage. In perennial vegetation, the water used remains in balance with the rainfall. Their deep root further ensures that the water table resides much below the soil surface. Due to the clearing of perennial vegetation with annual crops, the irrigation frequency is increased providing

more water than what crops can use. The excess water raises water table that mobilizes the salts stored in subsoil region towards the root zone. Plants use the water and leave the salts behind. As the amount of salts removed by crops is negligible, salts keep on accumulating in the root zone and in due course the soil becomes saline.

1.2. Sodium transport in plants

The salinity stress tolerance is governed by three interdependent parameters such as Na^+ exclusion, tissue tolerance to Na^+ and osmotic tolerance (Munns and Tester, 2008). The central regulatory step behind all the three mechanisms of salinity tolerance is the transport of water and ions (primarily Na^+, K^+ and Cl^-) both from the soil solution to the roots as well as from roots to other plant parts. The mechanism for net delivery of Na^+ to the plants is depicted in Fig. 1.1. The entire process can be divided into four distinct components (Tester and Davenport, 2003):

1. Influx into cells in the outer half of the root.
2. Efflux back from these cells to the soil solution.
3. Efflux from cells in the inner half of the root to the xylem.
4. Influx back into these cells from the xylem before the transpiration stream delivers the Na^+ to the leaf blade.

1.2.1. Na^+ influx to the root across the plasma membrane

The main site of Na^+ entry in roots is still uncertain. The Na^+ ion enters into the root cells mainly by the passive transport, which is favored by the concentration and voltage gradient across the plasmamembrane (Cheeseman, 1982). However, Na^+ may also enter via transporters of the high-affinity K^+ transporter (HKT) family (Laurie et al., 2002). Apart from this, nonselective cation channels, such as cyclic nucleotide–gated

channels and ionotropic glutamate receptor–like channels are also involved in the uptake of Na^+ ion (Demidchik et al., 2002).

Most of the Na^+ that enters root cells in the outer part of the root is likely to be pumped back out again via plasmamembrane Na^+/H^+ antiporters. The identities of the genes encoding these Na^+ efflux proteins are uncertain. In Arabidopsis, the Na^+/H^+ antiporter activity has been demonstrated for Salt Overly Sensitive 1 (SOS1) protein (Qiu et al., 2003). Besides, the involvement of other mechanisms for Na^+ efflux, such as primary pumping by Na^+- translocating ATPases, is also proposed (Mennen et al., 1990). The Na^+ remaining in the roots can be either sequestered in vacuoles or transported to the shoot. Compartmentation in vacuoles is achieved by tonoplast Na^+/H^+ antiporters, such as Na^+/H^+ exchanger (NHX) family proteins in Arabidopsis (Pardo et al., 2006). Because of the passive leakage of Na^+ ion back to the cytosol from vacuoles (possibly via tonoplast nonselective cation channels), the constant re-sequestration of Na^+ into vacuoles is required. The constitutive overexpression of *NHX-1* (Apse et al., 1991) or the gene encoding the *Arabidopsis thaliana* vacuolar H^+-translocating pyrophosphatase (Gaxiola et al., 2001), which maintains the electrochemical potential gradient required for pumping of Na^+ into the vacuole, increases both tolerance to and accumulation of Na^+. Thus the efficient sequestration of Na^+ ion into the vacuole that reduces the effective concentration of ion in the cytosol might prove to be an effective strategy for enhancing the salt stress tolerance in crop plants.

1.2.2. Radial transport of Na^+ into the roots and its loading into the xylem

From the cytoplasm of epidermal/cortical cells of roots, Na^+ might move radially into the root stele through the symplast of consecutive cells. Symplastic transport denotes

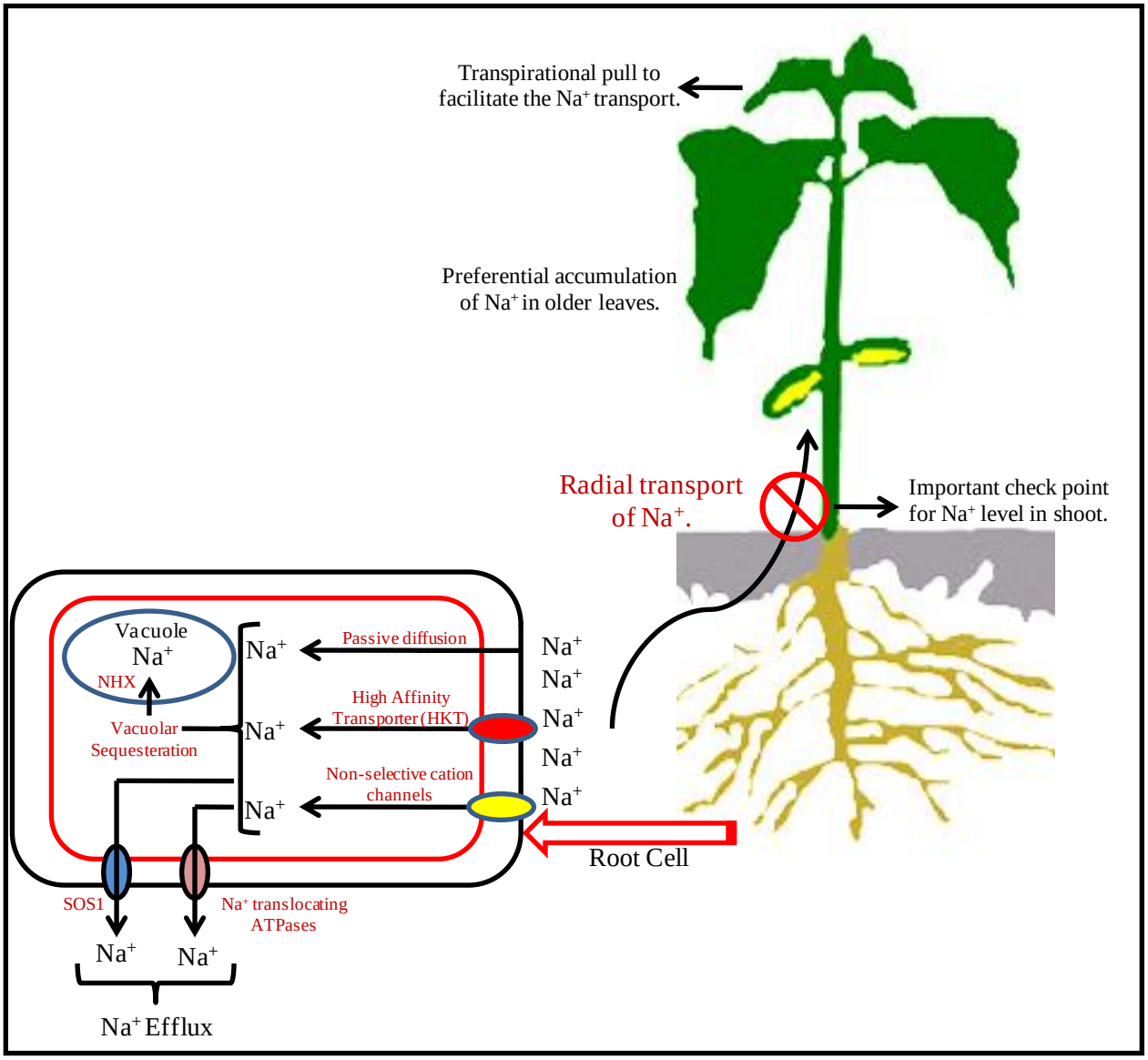


Fig. 1.1: The Na^+ transport system in plants.

the transport and distribution of molecules within the cytoplasm of nonvascular cells and the spreading of these molecules from cell to cell via plasmodesmata (Pickard, 2003). Not much is known specifically of radial transport of Na^+ in the symplast and whether the passage through plasmodesmata is sensed or regulated. From the symplast of stelar cells, Na^+ ions are loaded into the xylem. The plasmamembrane Na^+/H^+ antiporter and SOS1 of stelar cells could be involved in the efflux of Na^+ from root stelar cells into the xylem. An important strategy to control the shoot Na^+ concentration is to retrieve the Na^+ from the xylem. This acts as a check point for the root to shoot transport of Na^+ ion. In Arabidopsis root, AtHKT1;1 is involved in the retrieval of Na^+ from the xylem and their over-expression is shown to reduce the Na^+ level in shoot (Sunarpi et al., 2005; Davenport et al., 2007). Similar function for members of the closely related HKT1;5 gene family in rice (Ren et al., 2005) and wheat (Davenport et al., 2005) is also proposed.

1.2.3. Transport of Na^+ to the shoot and its partitioning

Na^+ transport from root to shoot in the xylem is mediated by the transpiration stream (De Boer and Volkov, 2003). The decrease in hydrostatic pressure as a result of the evaporation of water from the leaves generates a transpirational pull that drives the flow of water upwards through the xylem. Ions and nutrients dissolved in the water also get transported to the shoot with the water. Movement of Na^+ from the shoot through the phloem to the root is also proposed (Tester and Davenport, 2003); for instance, in lupin (Munns et al., 1988), *Trifolium alexandrium* (Winter, 1982) and maize (Qing et al. 2009) significant recirculation of Na^+ is proposed. The recirculation of Na^+ has also been linked with salinity tolerance in *Phragmites communis* (Matsushita and Matoh, 1991) and *Lycopersicon pennellii* (Perez-Alfocea et al., 2000). In the shoot, the highest

concentrations of Na^+ have been shown to accumulate in the oldest leaves in both monocot and dicots (Ghanem et al., 2009). As plants can shed the older leaves, the localization of high levels of Na^+ in them provides an additional strategy for the control of Na^+ in the plant (Yeo and Flowers, 1982).

1.3. Effect of salt stress on plant at the whole plant level

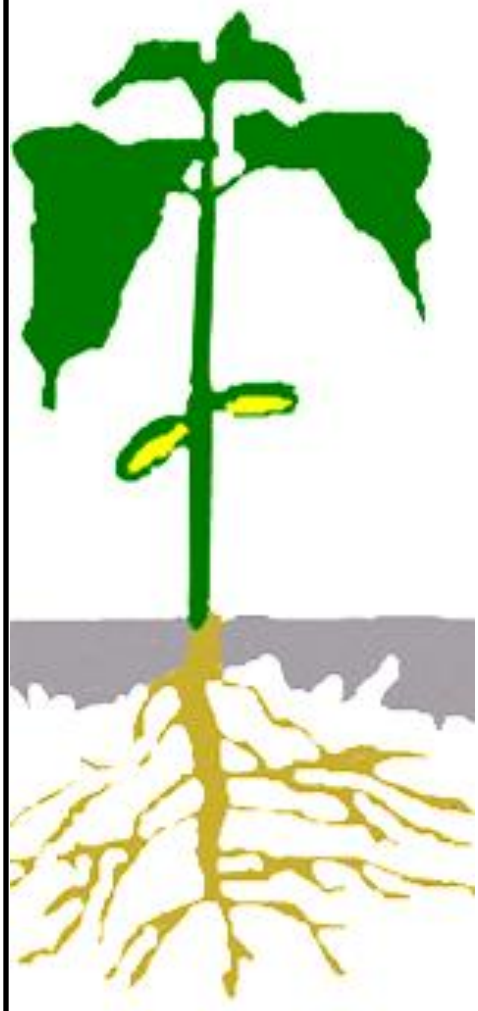
Salinity stress adversely affects the growth of plants. It causes poor and patchy stands of crops, uneven and stunted growth and poor yields. At the whole plant level, the effect of salt-stress is depicted in Fig. 1.2. The primary and rapid effect of excess salinity is that it increases the osmotic pressure of soil solution that renders water less available to plants. The secondary and relatively slower effect is the absorption of individual ions into the plants which may be toxic or may also retard the absorption of other essential plant nutrients. The first osmotic-specific phase of salt stress decreases the overall shoot growth rate, leaf expansion rate, emergence potential of the new leaves and development of lateral buds. In cereals, the major effect of salinity on total leaf area is a reduction in the number of tillers; while in dicotyledons, the major effect is the dramatic curtailing in the size of individual leaves or in the number of branches. In general, the shoot growth is more affected than that of the root. The decrease in the leaf area would decrease the water use by the plant and thus allows them to conserve soil moisture. The secondary ion-specific effect of salt stress is because of the accumulation of toxic ions. Such effects are more prominent in old leaves, which are no longer expanding and hence cannot dilute their salt concentration. This finally results in the death of old leaves. If the rate at which the old leaves die is greater than the rate at which new leaves are produced, the photosynthetic capacity of the plant will no longer be able to supply the carbohydrate

requirement of the young leaves, which further reduces the growth rate of plant. The high Na^+ level also disrupts the uptake of K^+ ion which is one of the essential and most abundant monovalent cations in cells (Taiz and Zeiger, 2006).

A significant genetic variation exists among species with respect to the osmotic and ionic phases of salt stress and an increased tolerance to both the components would enable a plant to grow better under salinity stress. For most species, Na^+ appears to reach a toxic concentration before Cl^- does, and so most studies have concentrated on Na^+ exclusion and the control of Na^+ transport within the plant. However for some species, such as soybean, citrus, and grapevine, Cl^- is considered to be the more toxic ion (Lauchli, 1984; Storey and Walker, 1999). The evidence for this is the association between the rate of Cl^- accumulation and the level of plant's salinity tolerance. This difference may arise because Na^+ is withheld so effectively in the roots and stems that little reaches to the leaves. Thus Cl^-, which continues to pass to the lamina, becomes the more significant toxic component of the saline solution.

1.4. Effect of salt stress on plant at the cellular and molecular level

In response to salt stress, the ability of plants to coordinate different metabolic processes gets disturbed leading to the formation of different types of reactive oxygen species (ROS) such as $^1O_2$ (singlet oxygen), H_2O_2 (hydrogen peroxide), $O_2^{\bullet-}$ (superoxide radical) and $HO^{\bullet}$ (hydroxyl radical) (Mittler, 2002). These ROS are toxic molecules and can cause oxidative damage to proteins, DNA and lipids (Apel and Hirt, 2004). The general physiology of the different ROS and the mechanism for their production in different cellular organelle are discussed below.



In shoots:

- ✓Reduction in the photosynthesis efficiency which decreases the overall shoot growth rate.
- ✓Reduction in the leaf area.
- ✓Death of the older leaves.

In roots:

- ✓Reduction in the water availability.
- ✓Less availability of the plant nutrients.

Fig. 1.2: Effect of salt stress at the whole plant level.

1.4.1. General chemistry of ROS

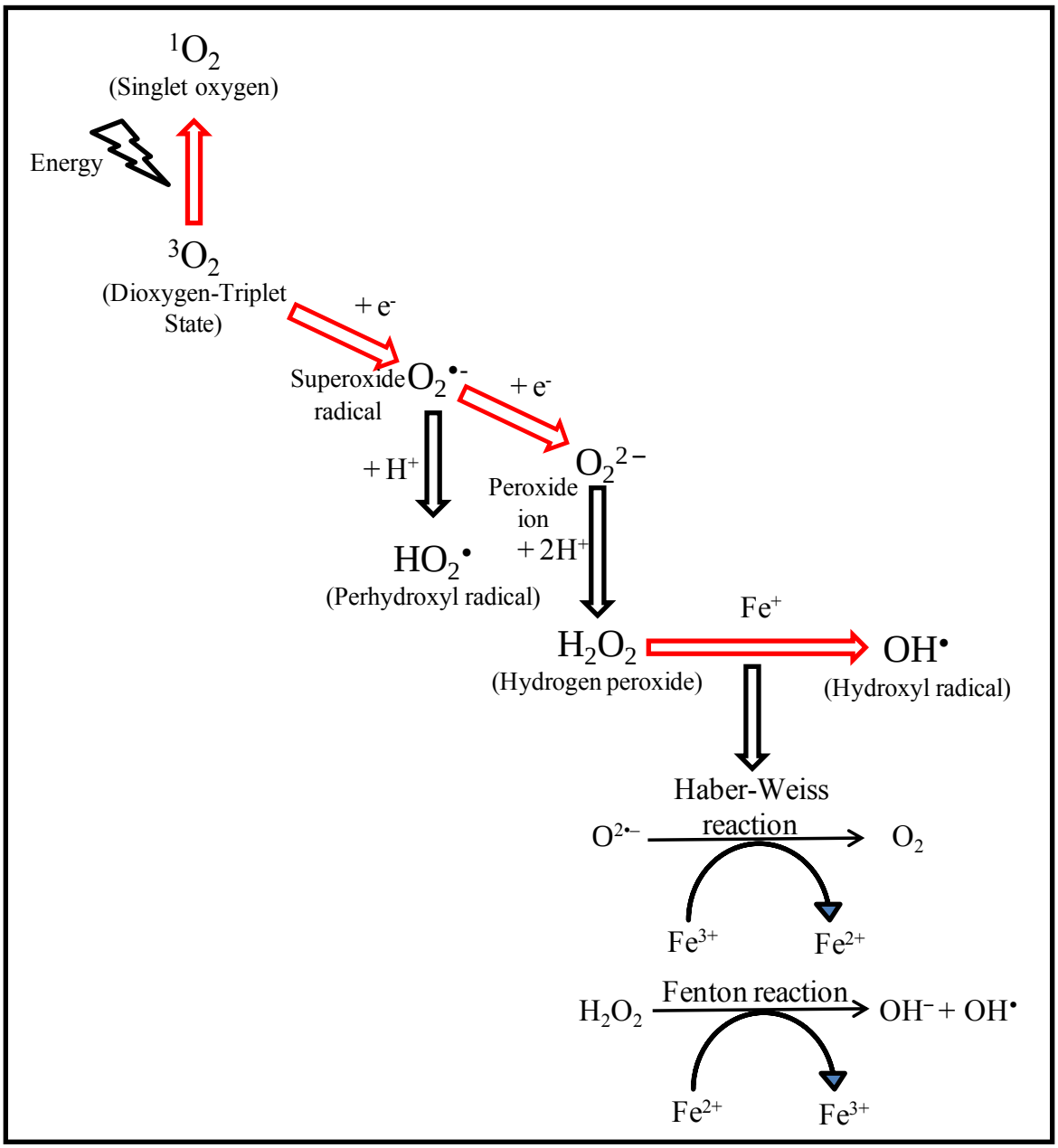
About 2.7 billion years ago, molecular oxygen (O_2) was introduced in our environment by O_2-evolving photosynthetic organisms and since then ROS have become the uninvited companions of aerobic life (Halliwell, 2006). It has been estimated that approximately 2% of O_2 consumed by plants is sidetracked to produce ROS in various subcellular compartments (Bhattachrjee, 2005). The O_2 molecule is a free radical, as it has two unpaired electrons that have the same spin quantum number. This spin restriction makes O_2 a preferred molecule to accept electrons leading to the generation of ROS. The process of generation of ROS as a reduction product of O_2 is depicted in Fig. 1.3.

The single electron reduction of O_2 results in the generation of the $O_2^{\bullet-}$. The dismutation of $O_2^{\bullet-}$ is unavoidable and results into the formation of H_2O_2. Furthermore, $O_2^{\bullet-}$ can also be protonated to form the $HO_2^{\bullet}$ (perhydroxyl radical). Additionally, in the presence of transition metals, such as copper and iron, further reactions take place, e.g. Haber-Weiss or Fenton-type reactions, giving rise to OH^- and $OH^{\bullet}$, which are the most reactive chemical species in the biological world. $^1O_2$ is another form of ROS in which an electron is elevated to a higher energy orbital, thereby freeing O_2 from its spin-restricted state. $^1O_2$ can be formed by photoexcitation of chlorophyll and its reaction with O_2.

1.4.2. Production of ROS in different cellular organelles

1.4.2.1. In Chloroplast

One of the early effects of salinity stress is the closure of stomata, so as to conserve the water. This causes the limitation in the CO_2 fixation, which is coupled with the over-reduction of the electron transport chain (ETC). In the over-reduced state of ETC, the electron flows from PS-I to O_2 and generates $O_2^{\bullet-}$. A membrane bound



1.3: Process of ROS generation.

copper/zinc superoxide dismutase (Cu/Zn SOD) in the vicinity of PS-I converts $O_2^{\bullet-}$ to H_2O_2. The process of the generation and dismutation of $O_2^{\bullet-}$ at the PS-I site is known as water-water cycle or Mehler reaction (Asada, 2006).

The singlet oxygen ($^1O_2$) is also generated at PS-II by excited triplet-state chlorophyll at the P_{680} reaction centre and in the light-harvesting complex when the ETC is over-reduced (Asada, 2006). The H_2O_2 produced in the Mehler reaction can positively regulate the formation of $^1O_2$ at PS-II. The H_2O_2 promotes the oxidation of quinone A (primary plastoquinone electron acceptor), which increases the photosynthetic electron transport flow and hence the level of $^1O_2$ is decreased (Asada, 2006; Moller et al., 2007). $^1O_2$ can cause lipid peroxidation and extensive tissue damage which may also lead to the growth inhibition and lethality (Lee et al., 2007; Triantaphylides et al., 2008). Controlling ROS production and scavenging in the chloroplast was shown to be essential for tolerance to drought and salinity in transgenic plants and in drought- or salinity-tolerant cultivars (Hernandez et al., 2000; Tseng et al., 2007). In contrast, deficiency in chloroplastic ROS-scavenging mechanisms enhances the stress sensitivity to both salinity and drought (Serrato et al., 2004).

1.4.2.2. In Mitochondria

Mitochondria are also known as a source of ROS production, although, they generate smaller amounts of ROS as compared with chloroplasts and peroxisomes (Foyer and Noctor, 2005a; Rhoads et al., 2006). In mitochondria, complex I and complex III in the respiratory ETC are the major sites of ROS production (Moller et al., 2007). Ubisemiquinone intermediate formed at complex I and III donates electrons to O_2 and generates $O_2^{\bullet-}$ that is, in turn, reduced to H_2O_2 (Raha and Robinson, 2000; Rhoads et al.,

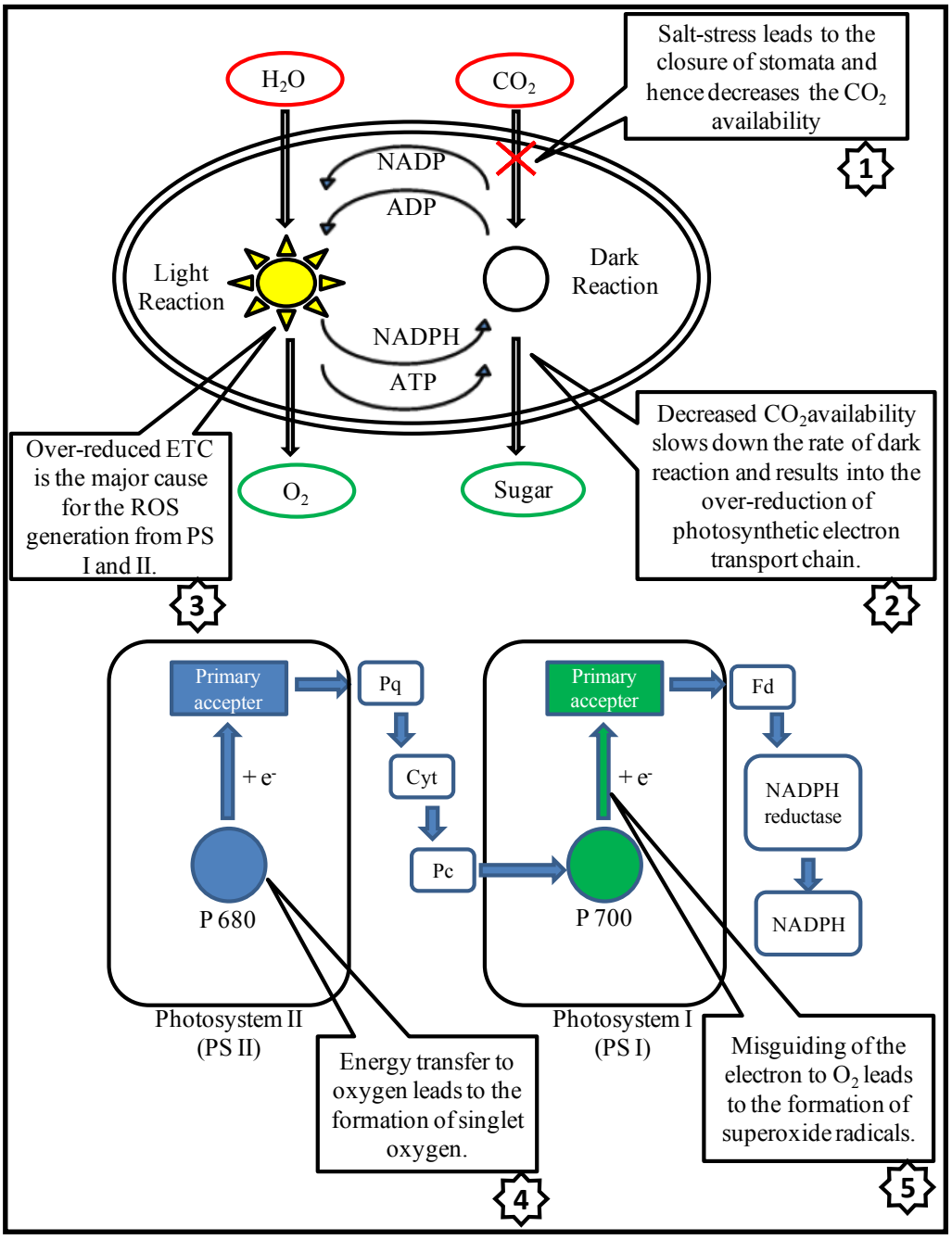


Fig. 1.4: Mechanism of ROS generation from chloroplast (Number represents the series of event under salt-stress).

2006). ROS production in the mitochondria has been shown to increase under abiotic stress conditions, especially drought and salinity (Bartoli et al., 2004; Pastore et al., 2007). Atkin and Macherel (2009) suggested that respiration rate increases under severe drought because the demand for ATP synthesis, causing enhanced production of ROS in the mitochondria.

Mitochondrial alternative oxidase (AOX) and manganese SOD (Mn-SOD) are the key enzymes that function to control the ROS production in mitochondria (Foyer and Noctor, 2005b). Recent studies demonstrated that Arabidopsis plants with loss of function in mitochondrial alternate oxidase (AOX) are sensitive to drought stress and exhibit altered expression of transcripts involved in antioxidant mechanisms in mitochondria (Giraud et al., 2008). In addition, a salt-tolerant tomato cultivar showed higher activity of Mn-SOD compared with a salt-sensitive cultivar during salinity (Mittova et al., 2003).

1.4.2.3. In Peroxisomes

Peroxisomes produce H_2O_2 and $O_2^{\bullet-}$ at high rates through several metabolic processes. Reduced water availability and stomatal closure decrease CO_2 to O_2 ratio in mesophyll cells and increase photorespiration and production of glycolate in chloroplasts. Oxidation of glycolate by glycolate oxidase in peroxisomes accounts for the majority of H_2O_2 production during photorespiration (Noctor et al., 2002). Catalases (CAT) and ascorbate peroxidases (APX) localized in peroxisomes are the major antioxidative enzymes that detoxify H_2O_2 (Mittler et al., 2004; Vandenabeele et al., 2004). Apart from this, $O_2^{\bullet-}$ is also generated by xanthine oxidase in the matrix of leaf peroxisomes.

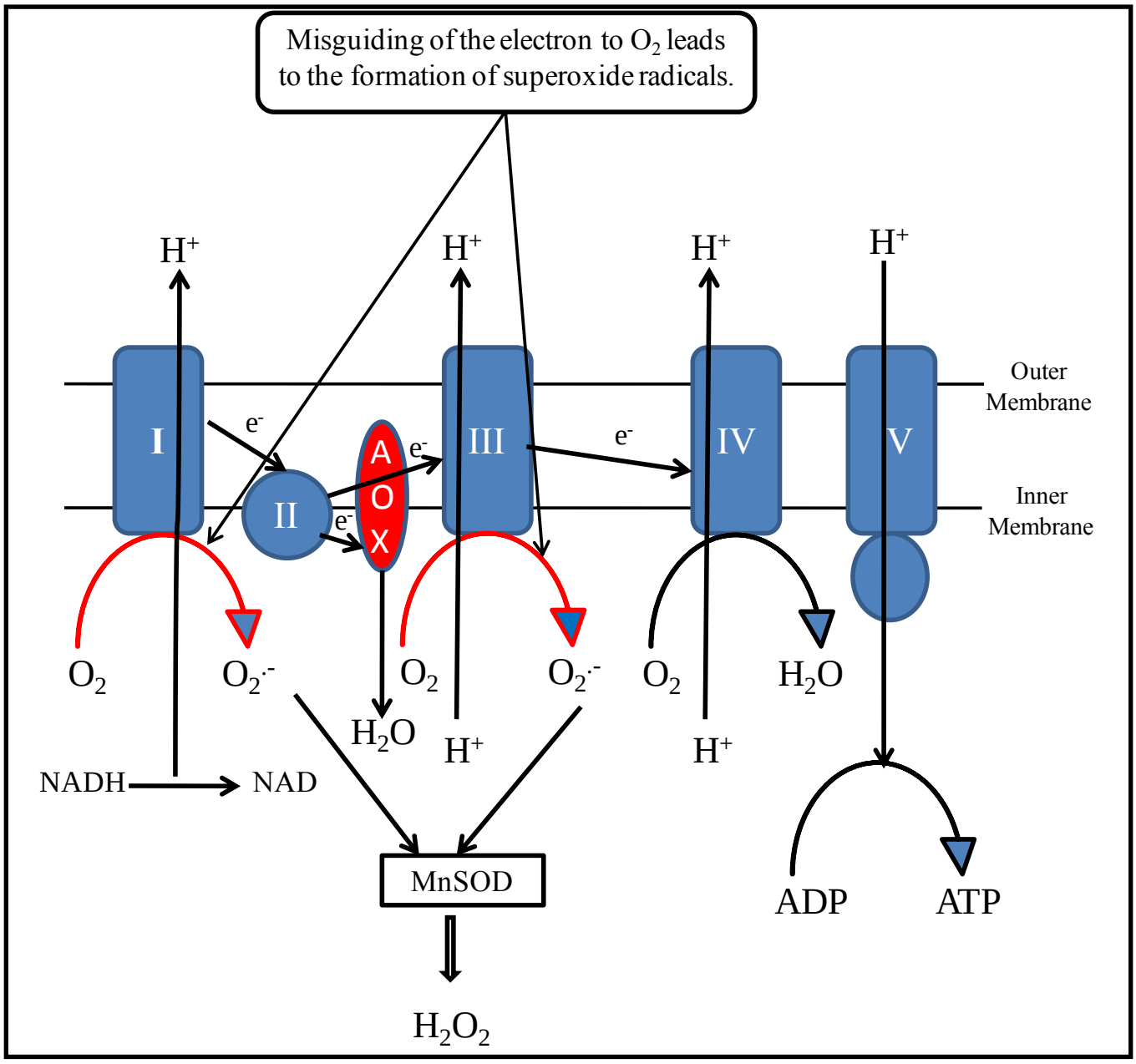


Fig. 1.5: Mechanism of ROS generation from mitochondria.

1.4.2.4. In Apoplast

Recent studies indicated that the apoplast is also an important site for H_2O_2 production under drought and salinity stresses (Hu et al., 2005; Jubany-Mari et al., 2009). The NADPH oxidases dependent generation of apoplastic ROS is required for abscissic acid (ABA)-induced stomatal closure (Torres and Dang, 2005). Other apoplastic ROS-forming enzymes are cell wall-associated oxidases and peroxidases and polyamine oxidases (Moschou et al., 2008).

1.5. Salinity stress perception and associated signaling

Plants sense salt stress through both ionic (Na^+) and osmotic stress signals. Excess Na^+ can be sensed either on the surface of the plasma membrane by a transmembrane protein or within the cell by membrane proteins or Na^+ sensitive enzymes (Zhu, 2003). Entry of Na^+ may cause membrane depolarization that activates calcium (Ca^{2+}) channels and thus generates Ca^{2+} oscillations, and signals salt stress (Sanders et al., 1999). Besides, the salinity-induced hyperosmotic stress may decrease the cell volume due to the retraction of the plasma membrane from the cell wall. This may be sensed by both stretch-activated channels and transmembrane protein kinases, such as two component histidine kinases and wall-associated kinases (Seki et al., 2002). Salinity stress also up-regulates the biosynthesis of the plant stress hormone ABA (Xiong and Zhu, 2003) and causes accumulation of ROS (Foyer and Noctor, 2005a, b). ABA and ROS mediated signaling also plays an important role in mediating the damage and repair processes under salinity stress. Thus, the perception of salt stress and induction of salt tolerant mechanisms require the co-ordinated action of calcium, ABA and ROS signaling mechanisms (Fig. 1.6).

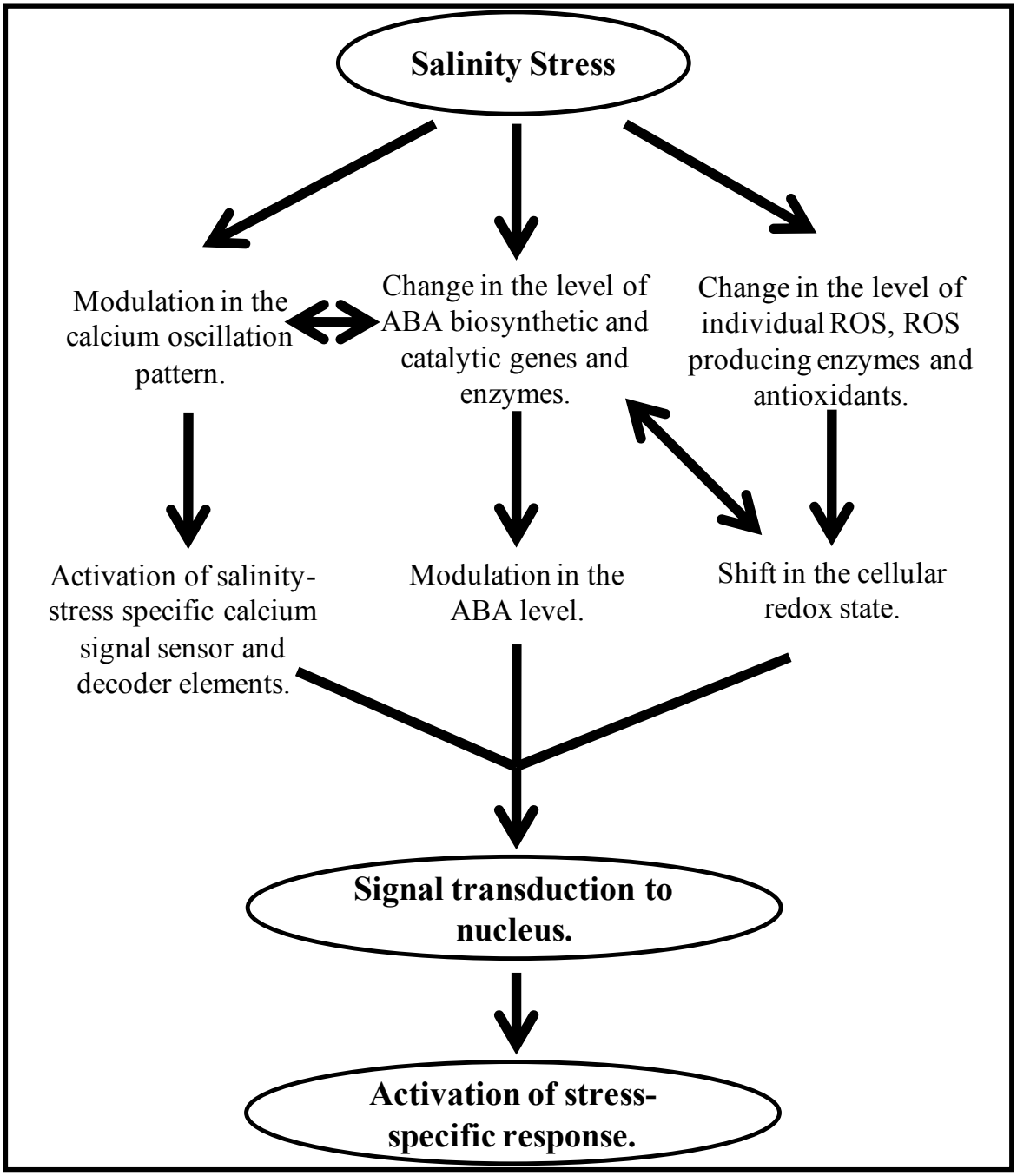


Fig. 1.6: Activation of different signaling mechanisms under salinity stress.

1.5.1. Calcium signaling under salt stress

In response to any external stress stimulus, such as salinity stress, one of the early responses is the change in the calcium signal in the form of transient increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) which arise because of the flux of Ca^{2+} into the cytosol, either from the external medium or from sub cellular compartments, where the concentration of Ca^{2+} is higher as compared to that of cytosol. The increase of $[Ca^{2+}]_{cyt}$ concentration led Webb et al., (1996) to formulate the concept of "Ca^{2+} signatures" which is defined as the repetitive oscillations or spiking of $[Ca^{2+}]_{cyt}$. The frequency (period), amplitude and shape (e.g. sinusoidal, square-wave) of Ca^{2+} signature is determined by the nature and magnitude of the stimulus. It is thought that stimulus specific temporal changes in $[Ca^{2+}]_{cyt}$ enable the Ca^{2+} ion to encode stimulus-specific information within this so-called calcium signature (Dodd et al., 2010). An additional level of regulation and specificity is achieved by a set of calcium binding toolkit, which includes the Ca^{2+}-binding proteins functioning as Ca^{2+} signal sensors (CBLs, calcineurin B-like proteins) and decoders (CIPKs, CBL-interacting protein kinases) that together relay the information encoded within calcium signatures. In Arabidopsis, 10 CBL-type calcium sensors and 26 CIPKs are reported and the signaling between SOS3 (CBL4)/SOS2(CIPK24) has been shown to specifically mediate the NaCl-stress response (Qiu et al., 2002). Thus, the co-ordinated induction of calcium signaling seems to be an important mechanism that determines the salinity stress perception.

A substantial progress has been made in understanding the SOS-pathway in Arabidopsis. As proposed by Zhu (2002), the increase in $[Ca^{2+}]_{cyt}$ under salinity stress is read by SOS3 (CBL-4; a Ca^{2+} sensor). The SOS3 protein interacts with a SOS2 (CIPK-

24; a Ca^{2+} decoder) protein kinase and the SOS3-SOS2 complex then activates the SOS1 protein (a plasma membrane Na^+/H^+ antiporter) and thereby re-establishing Na^+ homeostasis in cells. The interplay between Ca^{2+} signatures and Ca^{2+} sensor/decoder element thereby together helps in generating the stimulus specific response (Kudla et al., 2010). It has also been postulated that the SOS1 protein, which has a long C-terminal tail residing in the cytosol, might also be involved in the sensing of Na^+ (Shabala et al., 2005). Thus, as per this hypothesis, the Na^+ must enter into the cytosol to be sensed. However, for some halophytic plant it was shown that Na^+ entry into the cell is not necessary for $[Ca^{2+}]_{cyt}$ elevation (D'Onofrio et al., 2009).

1.5.2. ABA signaling under salinity stress

The perception of plant towards salinity stress is also affected by the phytohormone, ABA, which acts as an endogenous messenger for the regulation of different processes in plants such as, seed germination and developmental processes (Swamy and Smith, 1999; Xiong et al., 2002). The perception of salt stress is followed by the activation of complex signaling cascade including the generation of secondary signal molecules such as Ca^{2+}. The stress signal then transduces inside the nucleus to induce multiple stress responsive genes, the products of which ultimately lead to plant adaptation to stress tolerance. The stress-induced genes also include those of ABA biosynthetic pathway leading to an increase in ABA, which initiates the second round of signaling.

In plants, the ABA is synthesized from β-carotene through several enzymatic steps. The ABA biosynthetic genes have been cloned which includes zeathanxin epoxidase (Known as ABA1 in Arabidopsis), 9-cis-epoxycarotenoid dioxygenase (NCED), ABA aldehyde oxidase and LOS5 (also known as ABA3). The salinity-induced

activation of these genes is dependent upon the calcium based signaling. The accumulation of ABA can also stimulate the expression of ABA biosynthetic genes through calcium-signaling pathway in feedback mechanism and can also activate the ABA catabolic enzymes to degrade ABA. The main function of increased level of ABA appears to regulate the plant water homeostasis. This is also supported by the fact that several ABA deficient mutants of Arabidopsis (for example: *aba1* and *aba3*) have been reported to show severely reduced growth phenotype under water-deficient condition (Koornneef et al., 1998). Under salinity stress, ABA-dependent change in the gene expression is dependent upon the presence of cis-acting ABA-responsive elements (ABRE). Some of the important transcripts whose level is regulated by ABA are RD29A, RD22, COR15A, COR47 and P5CS.

1.5.3. Redox mediated signaling under salinity stress

The redox homeostasis is an important process and is now widely accepted that redox signals are the central regulators of plant metabolism, morphology and development. The redox state of the cell is governed by the level of individual ROS, ROS-producing enzymes, antioxidants, their oxidized forms, and/or oxidation/reduction states (Potters et al., 2010). Any external stimulus which causes the oxidative stress leads to the shift in the cellular redox status to a more oxidized state. Such a change in the cellular redox state acts as a cellular messenger that in conjunction with the mediators such as calcium transmits the signal to the nucleus for the activation of the stimulus specific response (Foyer and Noctor, 2005b). However, because of the complexity and cross talk in different signaling mechanisms, till date, no information is available which

deals with the impact of redox regulation on the calcium signaling pattern in plant under the changing environmental condition.

1.6. Salt tolerance mechanisms in plants

The perception of salinity stress led to the activation of different mechanisms required for the salt tolerance. Some of the important salt-tolerant mechanisms are as below.

1.6.1. Detoxification of ROS

The salinity stress led to the generation of reactive oxygen species (ROS; refer to section 1.4.2.) that can damage various cellular components and affect redox biology of the plants (Miller et al., 2009). The extent of ROS accumulation and oxidative damage under salinity depends on the level of stress and the ability of plants to counteract it. The ROS detoxification mechanisms in plants mainly includes low-molecular weight non-enzymatic [ascorbate (ASC) and glutathione (GSH)] and enzymatic [superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11)] antioxidants (Miller et al., 2009). The importance of these ROS regulating mechanisms has been manifested by their presence in various cellular compartments and also by various gain- and loss-of-function mutants and transgenic lines (Tseng et al., 2007; Ashraf, 2009). Superoxide dismutase is the major scavenger of superoxide radicals ($O_2^{\bullet-}$) and its enzymatic action results in the formation of hydrogen peroxide (H_2O_2), which is then regulated by CAT and various classes of peroxidases. The detoxification of H_2O_2 by APX in ASC-GSH cycle utilizes ASC as an electron donor, which gets oxidized to dehydroascorbate (DHA) in the process. The regeneration of DHA to ASC is performed by monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and

dehydroascorbate reductase (DHAR; EC 1.8.5.1), utilizing reduced glutathione (GSH) as a reductant, which, in turn, gets converted to oxidized glutathione (GSSG). The final reaction of the cycle is catalyzed by glutathione reductase (GR; EC 1.6.4.2) leading to conversion of GSSG back to GSH. In addition, both ASC and GSH may also directly quench ROS (Noctor, 2006).

1.6.2. Enhanced water uptake and synthesis of compatible solutes

A primary effect of salinity stress is the creation of water deficit conditions for the plants due to generation of osmotic differences between cytoplasm and soil solution (refer to section: 1.3.1). Plants have evolved different mechanisms to cope with such effects such as improved water uptake and synthesis of compatible solutes. The water uptake is facilitated by a large family of membrane proteins called "Aquaporins" (Maurel and Chrispeels, 2001). In Arabidopsis, 35 aquaporin isoforms are present and are classified into four distinct categories on the basis of their subcellular localizations (Luu and Maurel, 2005). The most abundant aquaporins are tonoplast intrinsic protein (TIP) and plasma membrane intrinsic protein (PIP) which are present on the vacuolar and plasma membranes, respectively. The PIP class is further subdivided into two homology subgroups, PIP1 and PIP2, which in Arabidopsis comprise five and eight members, respectively. Nodulin intrinsic proteins (NIPs) are another aquaporin and are expressed in the peribacteroid membrane of symbiotic N_2-fixing root nodules. NIPs also occur in non-leguminous plant species where their subcellular localization is not known. The fourth class of plant aquaporins comprises the small basic intrinsic proteins (SIPs), with three homologues in Arabidopsis. Their function and localization are still unknown. In response to salinity stress, plants adjust their water balance by changing the aquaporin

gene expression, their protein abundance, subcellular relocalization and channel gating by reversible phosphorylation or by intracellular protons (Luu and Maurel, 2005). The emergence of novel mechanisms of regulation by hetero-tetramer formation (Fetter et al., 2004); reactive oxygen species (Henzler et al., 2004) and pH (Fischer and Kaldenhoff, 2008) is also proposed. The water deficit condition generated under salinity stress can also be avoided by the synthesis of the compatible solutes such as proline and glycine betaine (GB). These are low molecular weight compounds and are nontoxic at high cellular concentrations. They protect plants from stress through different ways such as cellular osmotic adjustment, detoxification of ROS, protection of membrane integrity, and stabilization of enzymes/proteins. Furthermore, because some of these solutes also protect cellular components from dehydration injury, they are also termed as osmoprotectants (Ashraf and Foolad, 2007).

1.7. Strategies for increasing the salt-tolerance in crops

In order to improve the crop yield, various efforts are underway to increase the salt tolerance of crop plants. The basis of all these efforts is to improve the plant's acclimation efficiency so that they can perform better at the onset of stress. The plant acclimation process depends upon the activation of cascades of molecular events involved in stress sensing followed by signal transduction and the expression of stress-related effector genes and metabolites. The process of plant adaptation is complex and involves the coordinated induction of more than one gene. The major approaches to develop the salt-tolerant plant can be discussed under the following heads:

1. The natural genetic variations/resources can be used, either through direct selection in stressful environments or through conventional plant breeding or mutation breeding based approach, for the development of tolerant plants.
2. The salt-tolerant transgenic plants can also be developed with the altered expression level of novel/existing genes that affect either the salt stress perception or tolerance.
3. The external application of some bio-regulatory molecules can also improve the *built in* defense mechanisms of plants and lead to enhanced tolerance.

1.7.1 Plant breeding based approach

Plant breeding approaches include the exploitation of natural genetic variations either through direct selection in stressful environments or through the mapping of quantitative trait loci (QTLs – regions of a genome that are associated with the variation of a quantitative trait of interest) (Flowers, 2004) and subsequent marker-assisted selection (MAS).

The direct selection of superior salt-tolerant genotypes under field conditions is hindered because of the fact that salinity tolerance is influenced by various environmental factors (Richards, 1996). There is also evidence supporting the notion that salt tolerance is a complex trait involving the function of many genes (Flowers, 2004). Salt tolerance in plants also appears to be a developmentally regulated process and the tolerance of one stage might be different with that at other stages. For example, the QTLs associated with salt tolerance at the germination stage in barley (Mano and Takeda, 1997), tomato (Foolad, 2004) and Arabidopsis (Quesada et al., 2002) were different from those QTLs associated with salt tolerance at the early stage of growth. The plants selected by their

ability to germinate at high salinity did not display similar salt tolerance during vegetative growth. The development of molecular biology techniques has enabled the development of DNA markers that can be used to identify QTLs. The use of QTLs has improved the efficiency of selection for the salinity tolerance. QTLs and marker-assisted selection provide several advantages over direct phenotypic screening, particularly because of the development of PCR-based methodologies which can be used to screen the population.

Although considerable progress was made during the 20th century to improve the crop yield and quality through conventional breeding, very few crops have been reported to show improved resistance against salinity stress at the field level. For example, some lines/cultivars of alfalfa (*Medicago sativa* L.) such as AZ-GermSalt II (Dobrenz et al., 1989), AZ-90NDC-ST (Johnson et al., 1991), AZ-97MEC and AZ-97MEC-ST (Al-Doss and Smith, 1998) were tested under natural field conditions for salinity tolerance. Similarly, two salt-tolerant lines/cultivars of bread wheat (*Triticum aestivum* L.) such as S24 (Ashraf and O'Leary, 1996) and KRL1-4 (Hollington, 2000) were evaluated on natural salt-affected soils. These few examples depict that there has been a limited success in producing salt-tolerant cultivars of different potential crops using the conventional breeding approach. The main problem with the conventional plant breeders is to have the low magnitude of genetically based variation in the gene pools of most crop species. Thus, one cannot expect substantial improvements in crop salt tolerance. Under such circumstances, it is advisable to utilize salt-tolerant wild relatives of crop plants as a source of genes for crop improvement for enhanced salt tolerance. However, transferring the salt-tolerant genes from wild relatives to domesticated crops is not so easy because of

the reproductive barriers (Ashraf et al., 2008). The induced mutation breeding is an alternative approach and has led to the development of superior crop varieties (Kharkwal and Shu, 2009). More than 3000 mutant cultivars of crop plants with significantly improved attributes such as disease and stress resistance, increased yield and improved quality are released worldwide (D'Souza et al., 2009). The limitation of breeding based approach is that they are highly time-consuming and labor-intensive and undesirable character often appears along with the desirable ones.

1.7.2. Transgenic technology based approach

Transgenic plants are those plants that have been genetically altered by inserting desired genes directly into a plant cell. Such plants are special in a way that they are developed from a single plant cell. Transgenic plants have been developed through different genetic engineering techniques to contain desirable traits. Despite a number of social, political and legal concerns, many countries are now allowing transgenic crop production in conjunction with their conventional crop production. According to the International Service for the Acquisition of Agro-Biotech Applications (ISAAA), the top five countries growing transgenic crops in 2010 were USA, Brazil, Argentina, India and Canada (ISAAA, 2010). For improving the salt tolerance trait in various crops through transgenic approach, plant biologists have focused on genes that encode: ion transport proteins, compatible organic solutes, antioxidants, heat-shock and late embryogenesis abundant proteins, and transcription factors for gene regulation (Ashraf et al., 2008).

As discussed earlier (refer to Chapter-1/Section 1.2), the overall plant salt-tolerance can be managed by altering the levels of different types of proton channels and antiporters. Bao-Yan et al. (2008) has generated transgenic lines of Arabidopsis using

MsNHX1 from alfalfa (*Medicago sativa*) and shown considerable improvement in seed germination under saline conditions. Verma et al. (2007) have shown about 81% improvement in shoot and root lengths in transgenic rice line containing *PgNHX1* from *Pennisetum glaucum* under salt stress.

Plants synthesize different organic solutes such as proline, trehalose, sucrose and glycinebetaine (GB) which act as compatible solutes and maintain the cellular osmoticum under salinity stress (Rhodes and Hanson, 1993). Genes encoding key enzymes of GB biosynthesis have been engineered in plants such as Arabidopsis (Huang et al., 2000), cabbage (Bhattacharya et al., 2004), rice (Sakamoto et al., 1998), and mustard (Prasad et al., 2000) to increase the salt-tolerance. The over-expression of gene for Δ1-pyrroline-5-carboxylate synthetase (P5CS) enzyme, involved in proline synthesis, has been performed in rice (Su and Wu, 2004), potato (Hmida-Sayari et al., 2005), wheat (Sawahel and Hassan, 2002) and tobacco (Hong et al., 2000). Indeed, all transgenic lines accumulated proline many fold higher times than that of wild type and showed increased tolerance under saline conditions.

The plant salt-tolerance can also be increased by over-expressing the genes of antioxidant system. For example, Ushimaru et al. (2006) have shown that the expression of rice *DHAR* in transgenic arabidopsis enhanced the degree of salt tolerance measured as germination ability under salt stress. Different types of SODs were also over-expressed in plants such as rice (Tanaka et al., 1999), tobacco (Badawi et al., 2004) and arabidopsis (Wang et al., 2004) and marked improvement in the salt tolerance was observed. Genes for glutathione S-transferase (Roxas et al., 2000) and CAT (Al-Taweel et al., 2007) were also engineered in tobacco to demonstrate increased salt tolerance.

Another viable approach for enhancing the salt-tolerance is based on the transfer of transcription factors such as DREB1A (Kasuga *et al.,* 1999); CBF4 (Haake et al., 2002); OSISAP1 (Mukhopadhyay et al., 2004); NAC (Hu et al., 2006) and AP37 (Oh et al., 2009) to activate a set of genes involved in salinity/drought stress tolerance. However, the limitation of this concept lies in the fact that one transcription factor may regulate several metabolic pathways and one metabolic pathway may need an orchestrated regulation from more than one transcriptional element. Thus, the development of stress tolerant transgenics using gene transfer approaches needs much more understanding of the plant stress tolerance and gene regulatory network systems.

1.7.3. Priming based approach for enhanced salt tolerance

In contrast with the breeding and transgenic approaches which rely upon the changes at the genome level, priming is an entirely different strategy where various kinds of regulatory molecules are used externally to strengthen the *built in* defense system of the plant (Ashraf et al., 2008). Different researchers have used wide range of priming agents for enhancing the plant salt tolerance, such as salt (Patade et al., 2009); proline (BenAhmed et al., 2011) and GB (Ali and Ashraf, 2011); putrescine (Quinet et al., 2010); salicylic acid (Arfan et al., 2007; Khan et al., 2010); β-aminobutyric acid (Jakab et al., 2005); calcium (Horie et al., 2006) and silicon (Liang et al., 2007). The exact mechanism of priming induced salt-tolerance is dependent upon the nature of priming agent. In general, the priming treatment is known to activate signaling components or transcription factors required for the salt stress perception and tolerance. Bruce et al. (2007) proposed that priming agents may also impart epigenetic changes that would enable plants to better tolerate a stress during the subsequent stress exposure. An entirely different mechanism is

proposed for calcium mediated priming where it is proposed that Ca^{2+}-induced the reduction of cell surface negativity and hence reduces the uptake of Na^{+} ion (Kinraide, 1998). Thus, the priming based strategy is a slightly different concept for enhancing the salt tolerance and the primed plants generally do not suffer from metabolic imbalances which are normally seen when transgenic lines are grown under the field condition.

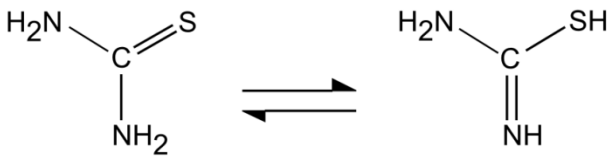
1.8. About the plant system: *Brassica juncea*

The genus Brassica is one of 521 genera in the tribe Brassicaceae belonging to the crucifer family and is the economically most important genus containing 37 different species. The Indian mustard [*Brassica juncea* (n = 18)] is an amphidiploid species derived from interspecific crosses between *Brassica nigra* [n = 8] and *Brassica rapa* [*n* = 10]. India is an important oil-seed crop and produces around 5 million tones of mustard annually. It stands 3rd among all the mustard producing countries and contributes 11% of the world's total production (http://www.crnindia.com/commodity/MR.html). In India, Rajasthan and Uttar Pradesh are the major mustard producing states and together contribute over 50% of the total Indian production. The leaves, seeds and stem of Indian mustard are used in Indian cuisine. Mustard oil is used as cooking oil especially in northern India. It is also a folk remedy for arthritis, foot ache, lumbago, and rheumatism. Brassica is used to remove heavy metals from the soil in hazardous waste sites because it has a higher tolerance for these substances and stores the heavy metals in its cells.

1.9. Basic information of thiourea

Thiourea (TU) is an organosulfur compound with formula $SC(NH_2)_2$. It is structurally similar to urea except that oxygen atom is replaced by a sulfur atom. The

properties of urea and thiourea differ significantly. TU is a planar molecule and occurs in two tautomeric forms as shown below.



Thione form Thiol form

TU has a wide range of applications. In plants, the use of TU was first proposed as a dormancy breaking agent (Tukey and Carlson, 1945) and later on this effect was correlated with anti-catalase activity (Hendricks and Taylorson, 1975). This led TU adopted as a plant growth regulator (Gul and Weber, 1998). Until recently, there have been various reports where TU was used either for enhancing the seed germination ability or for enhancing the crop productivity under field conditions (Sahu and Singh, 1995; Sahu et al., 2005; Sahu et al., 2006; Garg et al., 2006; Ile et al., 2006; Shahba et al., 2008). Thiourea is also used as an additive in fertilizers because of its antinitrification property (Grant and Wu, 2008). Another characteristic of TU is that it can scavenge different ROS (Kelner et al., 1990; Gao et al., 2008) and hence, it is also used to protect animal cell lines towards different carcinogenic agents and to reduce the frequency of chromosomal aberrations, cytotoxic and genotoxic effects (Whiteman and Halliwell, 1997; Araujo et al., 2001; Saeed et al., 2010; Gateva et al., 2010). The industrial use of TU has also emerged for extraction/recovery of precious metals such as gold (Rodriguez et al., 2006) and silver (Wang et al., 2010) and for making the chemical sensors (Kumar et al., 2010).

1.10. Aims and Objectives

The aim of the present thesis was to study the thiol mediated changes at cellular and whole plant level, which are responsible for the crop improvement under salinity stress. Since previous studies from our laboratory demonstrated that among various sulphydryl compounds tested, TU was most effective and economical in enhancing the crop productivity, therefore the present thesis work was focused on revealing the underlying mechanisms of TU mediated salt (NaCl) tolerance in Indian mustard (*Brassica juncea* (L.) Czern.). The objectives of the present thesis are listed below:

- Differential display and microarray based expression profiling of TU modulated and NaCl stress dependent transcripts in seeds.
- Investigation of the TU potential for regulating the water homeostasis under NaCl stress in roots.
- Studies on the TU mediated regulation of redox and antioxidant mechanisms in under NaCl stress in shoots.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

Sodium chloride, thiourea, dimethyl sulphoxide (DMSO), thiobarbituric acid (TBA), Tris base, HEPES buffer, ethylene diamine tetra acetic acid (EDTA), glutathione (GSH), diethyl pyrocarbonate (DEPC), SYBR Green 2X master mix, 2X PCR ready mix, sodiumdodecylsulphate (SDS), 2-mercaptoethanol (2-ME), sodium sarcosinate, phenylmethanesulphonylfluoride (PMSF), agarose, hydrogen peroxide, NADPH, triton X-100, ethidium bromide, Murashige and Skoog medium, ampicillin, Tri reagent and protenase K were purchased from Sigma Chemical Company, USA. Plant RNeasy kit and Gel purification kit were procured from the Qiagen. The dNTPs mix was obtained from Roche Molecular Bio chemicals, Germany. The Oligo dT-primer was purchased from Novagen, USA. The first strand reverse transcription-polymerase chain reaction (RT-PCR) kit was procured from Invitrogen, USA. The RNA image kit was obtained from GenHunter Corporation, USA. The TA cloning kit was obtained from MBI Fermentas, USA. Dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular probes, USA. The radio-labelled α^{32}P-dATP was procured from board of radiation and isotope technology (BRIT) India. The gene specific primers for RT-PCR were custom synthesized from Metabion International through the local agents. The reagent solutions were prepared in nanopure water from a Millipore Milli-Q system just before the use. All the other chemicals with maximum available purity were purchased from reputed local manufacturers/suppliers.

2.2. Plant material

The entire study was performed on Indian mustard, *Brassica juncea* (L.) Czern. var. TM-2.

2.3. Stress induction and thiol treatment

Seeds were surface sterilized with 30% ethanol for 3 min and washed thoroughly with sterile distilled water to remove the traces of ethanol. The differential germination response of seeds was assessed under the above treatments. Seeds were subjected to different treatments such as distilled water (D); NaCl (1 M); NaCl (1 M)+TU (6.5 mM) and TU (6.5 mM) for 6 h and then spread on a single Petriplate in separate lanes. Seeds were allowed to germinate in the presence of distilled water for 2 d under a 12-h light/dark cycle at 25±2°C and 70% humidity. A comparative germination percentage was followed under similar conditions for 2 d. For differential display and microarray analysis, surface sterilized seeds were subjected to different treatments (as mentioned above) for 1 h and then used for RNA isolation. For quantitative real time RT-PCR and biochemical analyses, two time points (1 h and 6 h) were used. For these analyses, surface sterilized seeds were given different treatments [DW; NaCl (1 M); NaCl (1 M)+TU (6.5 mM); TU (6.5 mM) and NaCl (1 M)+TU (6.5 mM)+$LaCl_3$ (5 mM)].

For seedling stage experiments, surface sterilized seeds were sown in plastic pots (10 X 15 cm) containing thoroughly washed and sterilized fine sand. All the pots were kept in dark for 1 d and then transferred to light (a 12 h photoperiod) with a day/night temperature of 25±2°C and relative humidity of 70%. After germination, seedlings were allowed to grow under distilled water till 6 d and then water was changed with ½ strength MS medium (Murashige and Skoog, 1962). After 10 d of germination, NaCl (350 mg/150 g sand); NaCl (350 mg/150 g sand)+TU (5 mg/150 g sand) and TU (5 mg/150 g sand) treatments (prepared in ½ strength MS) were independently given to the first three sets and remaining one set was maintained as control. Comparative growth pattern of the

seedling was monitored at the end of 3rd day of treatment. The different biochemical parameters were measured in shoots at all the three consecutive days after stress. The expression profiling of the genes was performed in the roots subjected to similar treatments for short (30, 60 and 90 min) as well as long (1, 2, and 3 d) time point. Two different concentrations of NaCl were selected for seedlings (350 mg/150 g sand) and seeds (1 M) considering the variable time point (long and short, respectively) used in these experiments and diffusion limitations of seeds due to the presence of seed coat.

2.4. Methods for Molecular studies

2.4.1. Isolation of total RNA from seeds

Obtaining high quality, intact RNA is the first and often the most critical step in expression profiling technique such as differential display and microarray. All the pipette tips, eppendorf tubes and glassware used for RNA isolation were treated with DEPC to remove RNase contamination. Total RNA from seeds was extracted using TRI-reagent (Sigma, T 9424), as per the manufacturer's instructions. The RNA was further purified using RNeasy Plant Mini Kit (Qiagen-74903). The total RNA in sample was quantified by measuring the absorbance at 260 nm. Measuring absorbance ratio of 260 nm and 280 nm suggests the purity of RNA. The RNA free from protein and DNA contamination gives the ratio of A_{260}/A_{280} in between 1.8 to 2. The integrity of RNA was further validated using an Agilent 2100 Bioanalyzer. The RNA integrity number (Schroeder et al., 2006) was developed using a RIN software algorithm and a value greater than eight was treated as a quality control for the RNA to be subjected for molecular analysis.

2.4.2. Differential display

2.4.2.1. Production of differential transcripts

DNA-free total RNA (2 μg) was reverse transcribed using oligo-dT primer. The cDNA obtained was subjected to differential display using RNA image kit (Gen Hunter, Tm), as per the manufacturers protocol. Details of the primers used for differential display is mentioned in Table 1. During amplification, radioactive labeling was achieved by adding α^{32}P-dATP. After amplification, the products were run on a 6% denaturing polyacrylamide gel. After complete run, the gel was dried on a Whatman 3 MM filter paper and autoradiogram was developed. Once differentially induced bands were identified from the autoradiogram, the hyperfilm was aligned with the dried gel and bands of interest were cut from the gel. The DNA from the dried gel was eluted by heating it with 100 μl of water at 94ºC for 20 min. To prevent the picking of any false positive band all the amplifications were conducted thrice and only those bands were selected which showed similar pattern in all the three independent experiments. One parallel DNA control was also maintained for every treatment along with the corresponding test reaction. The band detected in the test lane similar to corresponding band present in the DNA control was not considered for further analyses.

2.4.2.2. Re-amplification and cloning of differential transcripts

The DNA eluted from differential bands was subjected to re-amplification using the same set of the anchored and arbitrary primers used for the differential display. A portion of the reamplified DNA was run on a 6% polyacrylamide gel to make sure that amplification had taken place. This gel was used to quantify the amount of DNA and its size. After quantification, each of the differential transcripts was cloned in pTZ57R/T (MBI, Fermentas), as per manufacturer's instructions.

2.4.2.3. Sequencing and data analysis

Differential products were sequenced using an automated capillary sequencer. Sequence alignment and homology searches were performed with Blast N search algorithm in gene bank nr database (Altschul et al., 1990).

2.4.3. Microarray analysis

2.4.3.1. Microarray hybridization and data analysis

DNA-free total RNA was used to prepare the cy-3/cy-5 labeled complementary RNA (cRNA) using Agilent Low RNA Input Linear Amplification Kit, as per the manufacturer's guidelines. The amplification reaction has been optimized to be linear and not introduce bias of the abundant mRNA species over the rare mRNA populations. The labeled cy3-/cy-5 cRNA was purified using the Agilent cRNA cleanup module. The quantification of the labeled cRNA was performed using a Nanodrop spectrophotometer and the profile of amplified RNA was checked using Agilent 2100 Bioanalyzer. An equal amount of labeled cRNA was used for each RNA sample. All hybridizations were performed with 4X44K Arabidopsis Array Slide. Hybridization and washing steps were carried out using Agilent gene expression hybridization kit. For each microarray experiment, samples were processed in triplicate with independent dye swap labeling. Separate images for each hybridization were acquired using DNA microarray scanner (Agilent Technologies) by simultaneous two-color scanning at 5 and 10 micron resolution. Image analysis was performed using Agilent feature extraction software.

Expression data were evaluated using Agilent data analysis software in which the parameters were set as follows: false discovery rate: 5%; data response type: paired data; data in log scale: $\log_2$; number of permutations: 1000; imputation engine: K-Nearest neighbor imputer. A cutoff value of 0.8 fold (for both up- and -downregulation) and a P

value of 0.01 were used to select a subset of significant genes for further analysis. Gene clustering analysis was performed with GeneSpring version 6.1 (Silicon Genetics, CA).

2.4.3.2. Identifying Biological Functions of Differentially Expressed Genes

Using tools on the TAIR website (www.arabidopsis.org/index.jsp), the differentially expressed genes were categorized in terms of their biological function. Expected frequencies for each category were calculated based on the entire database of annotated Arabidopsis genes using Nick's classification superviewer tool (Provert and Zhu, 2003).

2.4.4. Real-time quantitative RT-PCR

2.4.4.1. Primer designing and concentration optimization

All the primers used for the SyBr green real-time RT-PCR were obtained from the *Arabidopsis thaliana* RT-PCR primer pair database (Han and Kim, 2006). Primers were obtained from Metabion International (www.metabion.com). Before using, the specificity of all the primers was confirmed by sequence analysis of RT-PCR amplicons. Primer optimal concentrations were determined with serial dilutions of cDNA obtained from 10 μg of RNA. For all the genes analyzed, 12.5 pmoles of the primer were used in 25 μL of PCR reaction mix.

2.4.4.1. cDNA synthesis, quantitative real-time PCR and data analysis

For quantitative RT-PCR, all the primer sets were tested by real time PCR on a dilution series of cDNA (1:2; 1:4; 1:8; 1:16; 1:32 and 1:64) derived from first strand cDNA mix, independently from the different treatments in triplicate, and primer efficiencies were calculated using REST-384 version 2 software (Pfaffl et al., 2002). The primer sets displayed comparable amplification efficiencies (1.9–2.1) and generated

single, specific PCR products was used as a quality control parameter for the primer pairs to be used for real-time PCR.

For cDNA synthesis, 2-5 μg of DNA free total RNA from different samples were taken and subjected to reverse transcription reaction (RT+) with oligo dT primer using Super Script First Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA), as per the manufacturers protocol. Parallel control reactions (RT-; in which reverse transcriptase enzyme was replaced with water) were also conducted for all RNA samples. Both RT+ and RT- samples were compared for all the primer sets by real time quantitative RT-PCR to confirm that no DNA contamination is present in any of the samples. Real time PCR was carried out using the Rotor Gene 6600 (Corbett Life Science, www.corbettlifescience.com). Reactions were set up by combining 10 μl of SyBr green PCR reaction mix (Sigma; S 4320) with 2.5 μl of 1 to 20 diluted cDNA templates, 1.5 μl each of forward and reverse primer (10 mM each), and 4.5 μl of PCR grade water (Sigma W1754). For gene expression analyses, the following PCR protocols were followed: 95ºC for 15 min; 40 cycles of 94ºC for 20 sec, 55ºC for 30 sec, and 72ºC for 30 sec, sample read; 72ºC for 10 min; and melting curve analysis. The global minimum was subtracted for baseline correction. The threshold line was adjusted to be above early cycle background fluorescence. Data on the threshold cycle (CT) at which the fluorescent intensity of each sample first increased above background levels was collected, and was normalized to actin levels (which showed very little expression variation among different treatments). The efficiency adjusted relative expression ratios were calculated using REST-384 version 2 software. All the reactions were carried out with three biological and three technical replicates.

At the end of each PCR run, a melting curve was also generated and analyzed with the dissociation curve software built into the Corbett rotor gene 6600 (Corbett Life Science). The melt curve obtained depends on the GC/AT ratio and the overall length of the amplicon. This analysis allowed products to be distinguished from one another and can identify primer dimers or other erroneous dsDNA (Ririe et al., 1997).

2.5. Methods for biochemical studies

2.5.1. Measurement of total protein

The total protein content was measured following the method of Lowry et al. (1951). Four reagents (A-D) were used for the procedure:

A = sodium potassium tartrate (2%)

B = copper sulphate (1%)

C = sodium carbonate (2%) in 0.1 N NaOH

D = Folin and Ciocalteau's Phenol reagent diluted with distilled water in 1:1 ratio

Reagent A (1 ml) and B (1 ml) were added to reagent C to make 100 ml. Five ml of this solution was added to the protein extract (0.5 ml) and kept for 10-15 min at 30ºC. Reagent D (0.5 ml) was finally added and thoroughly mixed. After 30 min the absorbance was recorded at 700 nm. Bovine serum albumin was used as a standard. Protein content of the plants is expressed as mg g^{-1} FW.

2.5.2. Measurement of mitochondrial ATPase (mtATPase) activity

2.5.2.1. Isolation of mitochondria

Isolation of mitochondria was performed using percoll-density gradient centrifugation. The tissue was ground in 1:3 ratio in chilled homogenization buffer (0.3 M sorbitol, 1mM EDTA, 2.5 mM DTT, 25 mM HEPES-NaOH pH 7.5 and 0.4 mM

PMSF). The content was filtered through four layers of cheese cloth and filtrate was centrifuged at 13,000 g for 15 min at 4°C. The pellet was resuspended in 25 ml of suspension buffer (0.3 M sorbitol, 1 mM DTT, 20 mM HEPES-NaOH pH-7.5 and 0.2 mg/ml BSA) and then centrifuged at 1,500 g for 10 min. The supernatant was collected and then centrifuged at 13,000 g for 20 min. Pellet is resuspended in minimum amount of the suspension buffer, layered above a Percoll cushion (28% Percoll, 0.3 M sorbitol and 20 mM HEPES-NaOH pH-7.5) and ultracentrifuged (Beckman SW60 rotor) at 40,000 g for 80 min at 4°C. The centrifugation resulted in the formation of a white hazy mitochondrial ring in the middle of the Percoll cushion. The ring is carefully separated and washed twice with wash solution (0.3 M sorbitol, 1mM DTT and 20 mM HEPES-NaOH, pH-7.5) at 25 000 g for 20 min. This step is required to remove the residual Percoll. The final mitochondria pellet was suspended in the minimum volume of suspension medium (same as wash solution plus BSA at 0.2 mg/mL). The mitochondria was stored at -80°C until use. The purity of mitochondrial fraction was assayed by measuring the activity of succinate dehydrogenase (E.C.1.3.9.9) activity that is considered as a mitochondrial marker (Gopalkrishnan and Rao, 2006). Sample (50 μL) was mixed with 950 μL substrate solution [potassium phosphate buffer-pH-7 (55 mM), 2,4-iodophenyl-3,4-nitrophenyl-3,4-nitrophenyl-5-phenyl tetrazolium chloride (INT, 0.11%), sodium succinate (55 mM), sucrose (25 mM)] and incubated for 38°C for 20 min. The reaction was terminated by the addition of 1 mL of 10% trichloroacetic acid. Ethyl acetate (2 mL) was added in the solution to extract the color in the organic phase. The color intensity was measured at 420 nM. In the blank tube, the INT was replaced with distilled water.

2.5.2.2. Measurement of ATPase (E.C.3.6.1.3) activity

Activity of mtATPase was measured by the method described by Gallagher and Leonard (1982). The reaction mix contained 0.3 mL of substrate solution [ATP (6 mM), Tris-MES-pH-9 (30 mM), $MgSO_4$ (3 mM), KCL (50 mM)] and 0.2 mM of sample. The content was thoroughly mixed and incubated at 37°C for 30 min. The reaction was terminated by the addition of 10% SDS and the solution was centrifuged at 3000 g for 15 min. In the supernatant, 100 μL molybdate reagent [ammonium molybdate (20.22 mM), Concentrate H_2SO_4 (3 N)] and 40 μL of ANSA reagent (1-amino-2-naphthol-4-sulfonic acid) were added. The reaction mix was incubated at room temperature for 10 min and then the absorbance was recorded at 660 nM. In the blank tube, the substrate solution was replaced with the distilled water.

2.5.3. Biochemical, histochemical and fluorescence based detection of ROS in leaf segments

For estimation of hydrogen peroxide (H_2O_2) levels, plant samples were homogenized in 0.5% (w/v) trichloroacetic acid (TCA) in an ice bath and centrifuged at 14,000 g for 15 min at 4°C. For H_2O_2 determination, 0.5 ml of supernatant was mixed with 0.5 ml 100 mM potassium phosphate buffer (pH 7.0) and 1 mL of freshly prepared 1 M potassium iodide. Reaction was allowed to develop for 1 h in dark and absorbance was measured at 390 nm (Alexieva et al., 2001). The amount of H_2O_2 was calculated from a standard curve prepared using the known concentrations of H_2O_2. The rate of superoxide radicals ($O_2^{\bullet -}$) production was measured following the method of Chaitanya and Naithani (1994). About 500 mg of fresh plant samples were homogenized under N_2 atmosphere in cold (0−4°C) in 100 mM sodium phosphate buffer (pH 7.2) containing 10 mM sodium

azide to inhibit SOD activity. After centrifugation at 22,000 g at 4°C for 20 min, level of $O_2^{\bullet-}$ was measured in the supernatant by its capacity to reduce nitroblue tetrazolium (NBT). The level of $O_2^{\bullet-}$ was measured by analyzing the reduction of NBT (ε=12.8 mM^{-1} cm^{-1}). The reaction mixture (1 mL) contained 100 mM sodium phosphate buffer (pH 7.8), 0.05% (w/v) NBT, 10 mM sodium azide and 0.2 mL of extract. Absorbance was measured at 580 nm at 0 min and 60 min. The level of $O_2^{\bullet-}$ is expressed as increase in absorbance per min per gram fresh weight.

In vivo H_2O_2 accumulation was analyzed using 3,3′-diaminobenzidine (DAB) according to Schraudner et al. (1998) and $O_2^{\bullet-}$ were detected by NBT staining according to Jabs et al. (1996) as per the procedure given in Wohlgemuth et al. (2002). Control reactions included 10 mM $MnCl_2$, a highly effective $O_2^{\bullet-}$ dismutating catalyst agent. Cotyledons leaves were immersed in 20–30 mL staining solution (0·1% (w/v) DAB, 10 mM 2-(N-morpholino) ethanesulphonic acid (MES), pH 6·5, or 0·1% (w/v) NBT, 10 mM sodium azide, 50 mM potassium phosphate, pH 6·4) in a desiccator. Infiltration was carried out by building up a vacuum (~100–150 mbar, for about 5 min) and releasing it two or three times until the leaves were completely infiltrated. Incomplete infiltration can lead to NBT staining in the non-infiltrated areas. The incubation was done for 45 min under lab light (for DAB) or for 20 min in the dark (for NBT, absolutely necessary). Leaves were destained with 96% (v/v) ethanol, under heating at 40°C. DAB staining was visualized as a red-brown color, NBT staining by blue formazan formation. Stained segments were mounted on a glass slide, and photograph was taken.

For the measurement of total ROS load, samples were infiltrated with 17 µM 2',7'-dichlorodihydrofluorescein diacetate acetyl ester (DCFDA, Molecular Probes). After

1 h of infiltration, different treatments were given independently and then ROS produced were measured by monitoring the fluorescence intensity with fluorescence microscope (Carl Zeiss Microscope, Germany) at 495-nm excitation and 500- to 550-nm emission wavelengths. The fluorescence of DCFDA increases because of the ROS mediated oxidation of dye to dichlorofluorescein.

2.5.4. Measurement of the DPPH-Radical Scavenging Activity

DPPH is a stable free radical and has a violet color in radical form. In the presence of a reducing substance/antioxidant, DPPH radical (1,1-diphenyl-2-picrylhydrazyl) changes to reduced form (1,1-diphenyl-2-picrylhydrazine) with the loss of violet color. For the measurement of DPPH radical scavenging activity, 200 mg leaf material was homogenized at 4ºC in 2.0 mL of absolute ethanol. Aliquot (0.5 mL) was mixed with 0.25 mL of 0.5 mM DPPH ethanol solution and 0.5 mL of 100 mM acetate buffer (pH 5.5) and the absorbance was measured at 517 nm after 30 min (Kang and Saltveit, 2002). The scavenging of DPPH radicals in percentage terms was calculated by the equation: radical scavenging activity (%RSA) = $(1 - A_1/A_0) \times 100\%$. A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of extract.

2.5.5. Measurement of Lipid peroxidation

Oxidative damage to the membrane lipids was estimated by analyzing the content of total thiobarbituric acid reactive substances (TBARS), expressed as equivalents of malondialdehyde (MDA). During lipid peroxidation membrane lipids break down into number of products such as hydro peroxides, alkadienal, malonaldehyde, 4- hydroxy nonenal (4HNE) etc. The TBARS method is based on the principle that malondialdehyde forms a 1:2 adduct with thiobarbituric acid i.e. one molecule of malonaldehyde and two

molecules of TBA to form a red malonaldehyde-TBA adduct that can be quantitatively estimated either by spectrophotometrically (532 nm) or by fluorimetry. The absorbance at 532 nm is not specific to TBA chromogen but some other products of lipid peroxidation like 2, 4 alkadienals also react with TBA to show strong absorption at 532 nm. In order to emphasis the lack of specificity, the values obtained in the test are commonly described as TBARS (TBA reactive substances). The amount of MDA was calculated as described by Hodges et al. (1999). Plant tissue samples were homogenized with 80:20 (v:v) ethanol:water in a ratio of 1:25 (g FW:ml) followed by centrifugation at 3000 g for 10 min. A 1 ml aliquot of sample was added to a test tube with 1 ml of either (i) –TBA solution comprised of 20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene, or (ii) +TBA solution containing the above plus 0.65% TBA. Samples were then mixed vigorously, heated at 95°C for 25 min, cooled, and centrifuged at 3000 *g* for 10 min. Absorbances were read at 440 nm, 532 nm, and 600 nm. MDA equivalents were calculated as per the formula given in Hodges et al. (1999).

2.5.6. Assay of oxidases [Glycolate oxidase (GO) and Ascorbate oxidase (AO)]

Plant samples were homogenized in 20 mM HEPES (pH 7.0) containing 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 2.5% polyvinylpyrrolidone (PVP) and 10% glycerol under chilled conditions. Homogenate was squeezed through four layers of cheese cloth and centrifuged at 12,000 *g* for 15 min at 4°C. Ascorbate oxidase (AO; EC 1.10.3.3), was determined by measuring the decrease in absorbance at 265 nm due to ASC oxidation (ε=2.8 mM^{-1} cm^{-1}) in the reaction mixture containing 66 mM potassium phosphate buffer (pH 6.0) and 170 μM ASC (Yamamoto et al., 2005). Glycolate oxidase (GO) (1.1.3.15) activity was assayed by following the

method of Baker and Tolbert (1966). Reaction mixture (1 mL) contained 100 mM potassium phosphate buffer (pH 7.8), 40 mM sodium glycolate, 100 mM L-cysteine, 100 mM phenylhydrazine, 1 mM FMN and a suitable aliquot of enzyme extract. The increase in absorbance due to formation of glyoxylate-phenylhydrazone was monitored at 324 nm and the activity was calculated using the extinction coefficient of 17 mM^{-1} cm^{-1}.

2.5.7. Determination of adenine and pyridine nucleotides

The analysis of adenine and pyridine nucleotides was performed by high performance liquid chromatography (HPLC). Samples (100 mg) were subjected to either acid extraction using 0.6 M perchloric acid (for measurement of ATP, ADP, NADP and NAD) or alkaline extraction using 0.5 M potassium hydroxide (for measurement of NADPH and NADH). The extract was centrifuged at 14,000 g at 4ºC for 10 min followed by neutralization with either 0.5 M KOH or 1 M KH_2PO_4, respectively and re-centrifugation at 14,000 g at 4ºC for 10 min to remove the precipitate. Supernatant was then filtered using 0.22 µm syringe filters and used for the assay. HPLC conditions were standardized following Caruso et al. (2004) with some modifications. The mobile phase consisted of 0.1 M KH_2PO_4 solution at pH 6.0 (Buffer A) and a 0.1 M KH_2PO_4 solution at pH 6.0, containing 10% (v/v) of CH_3OH (Buffer B). All buffer solutions, after preparation and pH adjustment, were filtered through a 0.22-µm Millipore filter. The chromatographic conditions were as follows: 8 min at 100% of buffer A, 7 min at up to 25% of buffer B, 2.5 min at up to 90% of buffer B, 2.5 min at up to 100% of buffer B, held for 7 min at 100% B, 5 min at up to 100% Buffer A and held for 8 min at 100% buffer B to restore the initial condition. The flow rate was 1 ml/min and detection was performed at 254 nm (Waters 996, PDA detector). Separation was performed on a 10 µm

C18 analytical column (250 x 4.6 mm) equipped with a guard column. The peaks were identified using the standard samples. The analytical recovery was tested by adding a known amount of standard compound prior to extraction and recovery was found to be 94% to 100% for different compounds. The data was analyzed using Empower software.

2.5.8. Assay of antioxidant enzymes

For the assay of antioxidant enzymes, plant samples (500 mg) were homogenized in 100 mM chilled potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM PMSF and 1% polyvinyl pyrrolidone (w/v) at 4ºC. Homogenate was squeezed through four layers of cheese cloth and the extract thus obtained was centrifuged at 15,000 g for 15 min at 4ºC. The supernatant was used to measure the enzymatic activities. The activities of superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; EC 1.11.1.6) and glutathione reductase (GR; EC 1.6.4.2) were measured following the methods of Beauchamp and Fridovich (1971), Nakano and Asada (1981), Aebi (1974) and Smith et al. (1988), respectively as described previously (Srivastava et al., 2006). The reaction mixture for SOD activity assay contained 40 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and a suitable aliquot of enzyme extract. After the reaction under light for 15 min, the absorbance was taken at 560 nm. One unit of activity is the amount of protein required to inhibit 50% initial reduction of NBT under light. Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was assayed by the formation of ASC at 265 nm (ε = 14 mM^{-1} cm^{-1}) in a reaction mixture containing 0.1 M Na–phosphate buffer (pH 6.2), 2 mM GSH, 1 mM dehydroascorbic acid (DHA) and enzyme extract (De Tullio et al., 1998). Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was assayed by

monitoring NADPH oxidation at 340 nm (ε = 6.2 mM^{-1} cm^{-1}) in a reaction mixture containing 0.1 mM NADPH, 2.5 mM ASC, 50 mM Na-phosphate buffer (pH 7.6) and enzyme extract. The reaction was started by the addition of 4 units of ascorbate oxidase (Drazkiewicz et al., 2003).

2.5.9 Estimation of redox couples [Ascorbate/dehydroascorbate (ASC/DHA) and reduced/oxidized glutathione (GSH/GSSG) ratios]

The level of reduced (GSH) and oxidized (GSSG) glutathione was determined fluorometrically using o–phthaldialdehyde (OPT) as fluorophore (Hissin and Hilf, 1976). Liquid nitrogen ground plant samples (500 mg) were homogenized in 3.75 ml of 0.1 M phosphate EDTA buffer (pH 8.0) and 1.0 ml of 25% meta-phosphoric acid. The homogenate was centrifuged at 20,000 g for 20 min at 4ºC and supernatants obtained were used for the estimation of GSH and GSSG. The following reaction procedure was followed.

GSH Assay

0.5 ml supernatant

4.5 ml buffer (pH 8.0)

↓ Mix

100 µl of mixture

1.8 ml buffer (pH 8.0)

100 µl OPT solution

↓ Incubation at room temperature for exactly 15 min

Fluorescence determined at 420 nm

GSSG Assay

0.5 ml supernatant

200 µl NEM (0.04 M)

↓ Incubation at room temperature for exactly 20-30 min

Add 4.3 ml NaOH (0.1 N)

↓ Mix

100 µl of mixture

1.8 ml NaOH (0.1 N)

100 µl OPT solution

↓ Incubation at room temperature for exactly 15 min

Fluorescence determined at 420 nm

(excitation at 350 nm) (excitation at 350 nm)

The known concentrations of GSH (L-glutathione reduced, Sigma) and GSSG (L-glutathione oxidized, Sigma) were used to prepare a standard curve for calculating the GSH and GSSG levels. The content of GSH and GSSG is expressed as μmol g^{-1} FW.

The level of total, reduced and oxidized ascorbate (ASC) contents in plants was measured following the protocol of Gillespie and Ainsworth (2007). Plant samples (50 mg) were homogenized in 1 ml 6% trichloroacetic acid (TCA) under chilled conditions and centrifuged at 13,000 *g* for 5 min at 4°C. To 200 μL of sample, 100 μL 75 mM phosphate buffer (pH 7.0) was added. In total ASC tubes, 100 μL 10 mM dithiothreitol (DTT) was added and incubated for 10 min at room temperature to reduce the pool of oxidized ASC. Then, 100 μL 0.5% N-ethylmaleimide was added to remove excess DTT. For the reaction, 500 μL 10% TCA, 400 μL 43% orthophosphoric acid, 400 μL 4% 2,2'-bipyridyl and 200 μL 3% $FeCl_3$ were added to all tubes. After incubation at 37°C for 1 h, absorbance was measured at 525 nm. The level of dehydroascorbate (DHA) was calculated by subtracting ASC values from total ASC.

2.5.10. Measurement of abscisic acid (ABA) content

Plant samples (200–300 mg FW) from different treatments were collected, weighed and immediately frozen in liquid nitrogen, thawed and extracted with distilled water (tissue : water ratio 1 : 20, w/v) for 16 h at 4ºC in the dark. A competitive immunoassay-based quantitative determination of the ABA content was performed in crude aqueous extracts using the Phytodetek ABA test kit, following the procedure described by the manufacturer (Idetek, Sunnyvale, USA).

2.5.11. Measurement of Na^+ and K^+ ion content

For ion analysis, the plant samples were oven dried for a minimum of 72 h at 80°C to 85°C. On achieving constant dry weight, the tissue (100 mg) was kept in 3 mL of concentrated HNO_3 for 72 h at room temperature. All samples were then heated at 120°C for 1 h and subsequently at 180°C till dryness. The residue was then taken up in 10 mL milli-Q water and the level of Na^+ and K^+ ions was estimated using a single-channel flame photometer (Digital Flame Analyzer model 2655-00; Cole-Parmer). In each analytical batch, reagent blanks and spiked samples were included in the acid digestion to assess the accuracy of the chemical analysis. The recovery of spike was 94–95% (n=6). The 100 μM and 40 μM solutions of NaCl and KCl were used as reference standards during analysis.

2.6. ^{31}P NMR for the measurement of vacuolar pH of root

Proton decoupled ^{31}P-NMR spectrum of roots (approximately 10g fresh weight) was recorded at 20ºC on 500 MHz NMR spectrometer (Bruker). The 10-mm multinuclear broadband probe was tuned at 202.404 MHz and the data acquisition was performed at 70º pulse with 0.6 s relaxation delay and a sweep width of 20,000 Hz. A broadband decoupling was applied using the Waltz sequence; the signal was digitized at 4K data points zero-filled to 8K, and processed with a 5-Hz line broadening. Spectra were referenced to methylene diphosphonic acid (pH 8.9). To assign the resonance of inorganic phosphate and soluble phosphate-containing compounds to specific peaks observed on in vivo ^{31}P-NMR spectra, the spectra of perchloric acid extracts prepared from the samples frozen immediately after *in vivo* analyses were compared with the spectra of standard solutions of known compounds using spiking method. Intracellular

pH values were estimated from the chemical shift of the cytosolic and vacuolar Pi pools using standard curve.

2.7. Isolation of plant protoplast and fluorescence microscopy experiment

The protoplast isolation from root was performed using the protoplast isolation kit (Bioworld, USA). The root tissue (500 mg) was cut into fine pieces and then incubated with enzyme solution containing 200 mg and 40 mg of cellulose and macerozyme, respectively in 20 ml of enzyme buffer. The solution was kept for shaking (100 rpm) for 3-5 h at 25ºC. The protoplast formation was monitored under microscope using 10 µl of suspension. Once the protoplasts are released, then the entire suspension was allowed to settle for 10 min in a centrifuge tube and then the supernatant was transferred into another tube and centrifuged at 300 g for 10 min at 4ºC. The protoplast pellet was washed twice with wash buffer to remove the traces of enzyme.

The isolated protoplast was incubated with FITC-TU (6.5 mM) for 1 h and then washed twice with the wash buffer to remove the unbound fluorescence. The protoplast is now dissolved in minimum volume of 11% mannitol and then mounted on glass slide. The fluorescence microscope (Carl Zeiss Microscope, Germany) data was recorded at 495-nm excitation and 500- to 550-nm emission wavelengths.

CHAPTER 3

Identification of the thiourea-modulated and salinity stress dependent transcripts in seeds of *Brassica juncea*

Section I: Differential display based approach for the identification of thiourea modulated transcripts.

3.1. Introduction

The cellular redox state is one of the key factors that regulate various plant processes such as growth, development and stress tolerance (refer to Chapter-1\Section-1.5.3). The maintenance of the redox homeostasis is collectively achieved by redox pairs (e.g. GSH/GSSG), antioxidant systems (catalase and superoxide dismutase etc.) and other secondary metabolites (flavonoids, alkaloids, and carotenoids) (Dietz, 2008). In response to any external stimuli, plants modify their redox state and the extent of change is dependent on the nature and dose of the stimulus (Miller et al., 2009). On this basis, it is hypothesized that if external application of any molecule can help plants to maintain their redox homeostasis under stress conditions, it may enhance their stress tolerance potential. In order to study the redox-regulated components of salinity stress tolerance, the transcript profiling was performed in seeds subjected to NaCl stress with/without TU treatment. The preliminary data on the differential transcriptome was obtained by doing the differential display and the data obtained showed that mitochondrial ATPases (F_0F_1-ATP synthase) play an important role in TU induced salt tolerance (Srivastava et al., 2009).

Mitochondrial F_oF_1-ATP synthase is a multimeric enzyme, in which F_1 is a peripheral component located on the face of inner membrane carrying the catalytic sites for ATP synthesis/hydrolysis (Sane et al., 1996). The other component F_o is embedded in the hydrophobic region of the membrane so as to constitute a proton channel (Pedersen et al., 2000). Prokaryotes and eukaryotes have the same F_1 structures, but somewhat

different F_0 structure (Boyer, 1997; Kinosita et al., 1998). The F_1 structure consists of five subunits designated α, β, γ, δ and ε with decreasing order of their molecular weights having stoichiometries of $\alpha_3\ \beta_3\ \gamma_1\ \delta_1\varepsilon_1$. Both of the two major subunits, α and β, contain a nucleotide binding site and play essential roles in the catalytic function of the F_1. The amino acid sequences of these two major subunits are not only highly conserved during evolution but also related to each other. The other subunits (γ, δ and ε) are considered as minor subunits (Amzel and Pedersen, 1983).

Thus, understanding the involvement of mtATPase is of interest for revealing the molecular components of TU mediated stress tolerance. The data will also be helpful for deciphering the integrative function of various ATP synthase subunits under salt stress.

3.2. Results

3.2.1. Differential germination ability of seeds in response to different treatments

Differential germination response of seeds under different treatments is shown in Fig. 3.1. At 1 d, seeds soaked in distilled water control and TU showed 100% germination with fully developed radical and cotyledons. Seeds soaked in NaCl showed only 18% germination. Seeds soaked in NaCl+TU showed 100% germination but their growth was reduced (Fig. 3.1-A). At 2 d, the growth pattern of TU soaked seeds was better than any other treatments. In NaCl soaked seeds, the growth was completely inhibited while in NaCl+TU treatment, the growth pattern was comparable to that of control (Fig. 3.1 B).

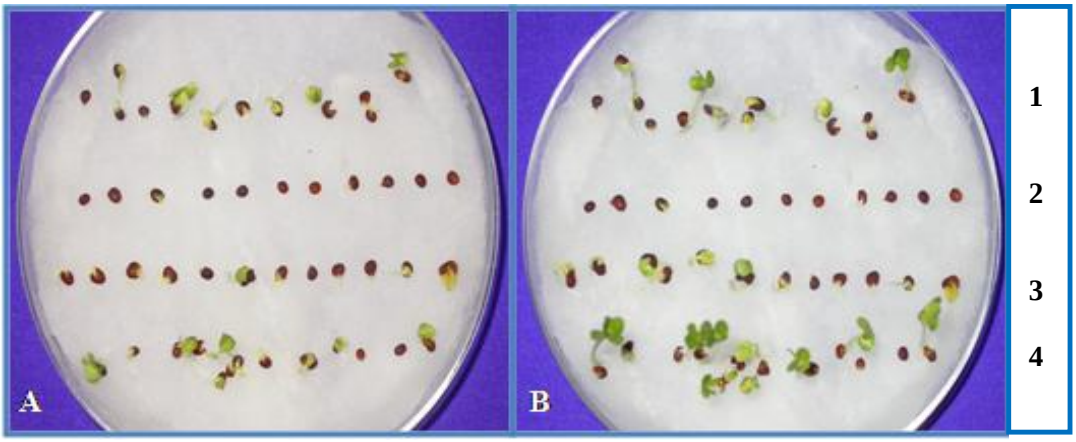


Fig. 3.1: Differential germination response of *Brassica juncea* seeds given different treatments for 6 h. Lanes marked as 1, 2, 3 and 4 represent seeds soaked in distilled water (Control); NaCl (1M); NaCl (1M)+TU (6.5 mM) and TU (6.5 mM) respectively for 6 h and then allowed to germinate under control conditions. Section A and B shows the growth pattern after 1 and 2 days respectively.

3.2.2. Identification of the differentially induced set of genes by differential display

The DNA-free total RNA (Fig. 3.2-A) was isolated from four different seed samples [D (Control); N (NaCl); NT (NaCl+TU) and T (TU)] and was subjected to differential display using the commercially available set of anchored and arbitrary primers from GenHunter Corporation (USA). The sequence of anchored and arbitrary primers are aagctttttttttttg and aagcttcgactgt, respectively.

3.2.3. Identification of salinity dependent thiourea induced genes

The radiolabelled amplified fragments from all the four samples were run onto the denaturing polyacrylamide gel and then autoradiogram was developed. The autoradiogram was analyzed and fragments showing differential intensity in between the samples were marked. Out of the total cDNA bands (around 150) obtained, eight were selected on the basis of their presence only in NaCl+TU and not in NaCl treatment (Fig. 3.2-B). Out of eight, three bands did not give amplification due to the insufficient DNA and other two bands gave small sized poorly defined bands. Therefore, these five bands were not taken for further analysis. However remaining three cDNA, which gave well defined single band, were cloned into pTZ57R/T. Size of the cloned cDNA fragments ranged from 150-350 bp. The detailed description of all the three cDNA clones is given in Table 3.1.

3.2.4. Sequencing and identification of cDNA clones

The results of the NCBI blast (Altschul et al., 1990) are summarized in Table 3.2. Out of three, two were associated with the different subunit composition of the mitochondrial ATPase. This provided us the evidence for the involvement of mtATPase in the protective effect of TU under salinity stress.

3.2.5. Expression profiling of mtATPase subunits in *Brassica juncea* seeds

The expression profiling of various mtATPase subunits was performed by quantitative real time RT-PCR. The details of the primers used are given in Table 3.3. Treatment of NaCl for 1 h led to the downregulation of alpha and delta subunits by 0.931 and 1.409 fold respectively as compared to that of control. Under similar condition, beta, gamma and epsilon subunits were upregulated by 0.999, 1.027 and 0.944 fold respectively. When TU was provided along with NaCl, changes in the expression profile were observed. In NaCl+TU treatment, except beta all other subunits like alpha, gamma, delta and epsilon subunits were upregulated by 0.103, 0.221, 0.597 and 0.141 fold respectively; while beta subunit was 0.808 fold downregulated as compared to that of control. No significant change in the expression pattern of any mtATPase subunit was observed in TU alone treatment (Fig. 3.3).

3.2.6. Effect of thiourea on mtATPase activity

The mtATPase activity was decreased by 28% in response to NaCl as compared to control. However, in NaCl+TU treatment, the decrease was limited to only 6% with respect to control. In TU alone treatment the mtATPase activity was comparable to that of control (Fig. 3.4).

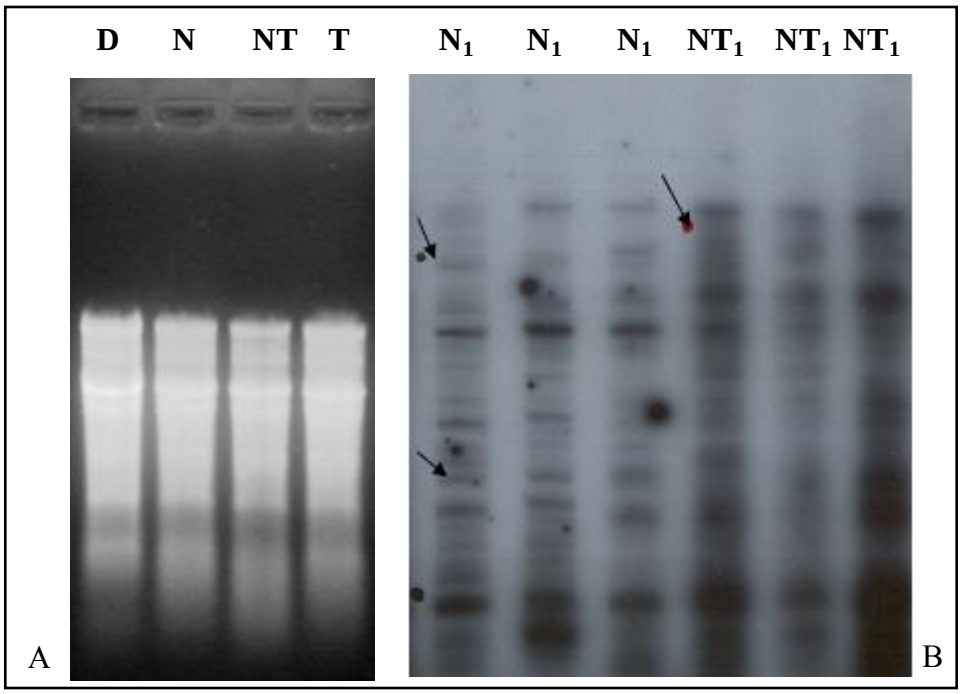


Fig. 3.2: Isolation of RNA from *Brassica juncea* seeds. The panel-A represents DNA-free total RNA isolated from seeds subjected to different treatments for 1 h. Lanes marked as D, N, NT and T denotes distilled water (Control); NaCl (1M); NaCl (1M)+TU (6.5 mM) and TU (6.5 mM), respectively. Panel-B represents the portion of autoradiogram showing the differential expression profile in NaCl and NaCl+TU treated seeds. Three independent amplification reactions were carried out for each treatment (N_1, N_2 and N_3 for NaCl and NT_1, NT_2 and NT_3 for NaCl+TU) and loaded side by side. Arrows represents the cDNA bands showing differential regulation among the treatments.

Table-3.1: Details of the differential display data.

Clone	Accession Number	Size of PCR fragment (bp)	Source	Nature of expression
DD1 (AJR001)	EE111313	290	Seed	Present in all the treatment except NaCl.
DD2 (AJR002)	EE111314	137	Seed	Present in all the treatment except NaCl.
DD3 (AJR003)	EE191543	337	Seed	Absent in all the treatment but present in NaCl.

Table-3.2: Sequence analysis of the fragments showing the differential expression.

Clone	Description of the sequence showing maximum homology with the query sequence				
	Accession Number	E-Value	% identity	Score	Description
DD1 (EE111313)	NM_126747.2	2e-135	98	489	Arabidopsis thaliana ATP synthase alpha chain, mitochondrial, putative (AT2G07698) mRNA, complete cds
DD2 (EE111314)	EE464322.1	1e-43	92	183	Brassica napus seed coat BNSCS2CT Brassica napus cDNA
DD3 (EE191543)	AJ271468.1	3e-45	100	190	Arabidopsis thaliana mRNA for mitochondrial F1 ATP synthase beta subunit (p_beta gene)

Table-3.3: Details of the gene-specific primers used for quantitative real-time PCR.

Subunits for mtATPase	Primer sequence (5'→3')	Amplicon Size (bp)
Alpha	Forward-ggcaggggcttgatcaggaattag	349
	Reverse-gcgggaacatccacaatagatccag	
Beta	Forward-gggcgtgtgtgtcaggttattgg	340
	Reverse-ctggagcgtctctgtgaatgggta	
Gamma	Forward-ggactttggcagccatttactgcac	142
	Reverse-acagagccctgctcactttaacgac	
Delta	Forward-tgccgaacccactaagaacttcctc	149
	Reverse-ggggaagcggaatgactgtggta	
Epsilon	Forward-aacatctgtgcgaatatcgtgagga	143
	Reverse-tcaggtgtgtctgaccgcaaaac	
Actin	Forward-ctcctgccatgtatgtcgctatcc	155
	Reverse-aaggtccaaacgcagaatagcatgt	

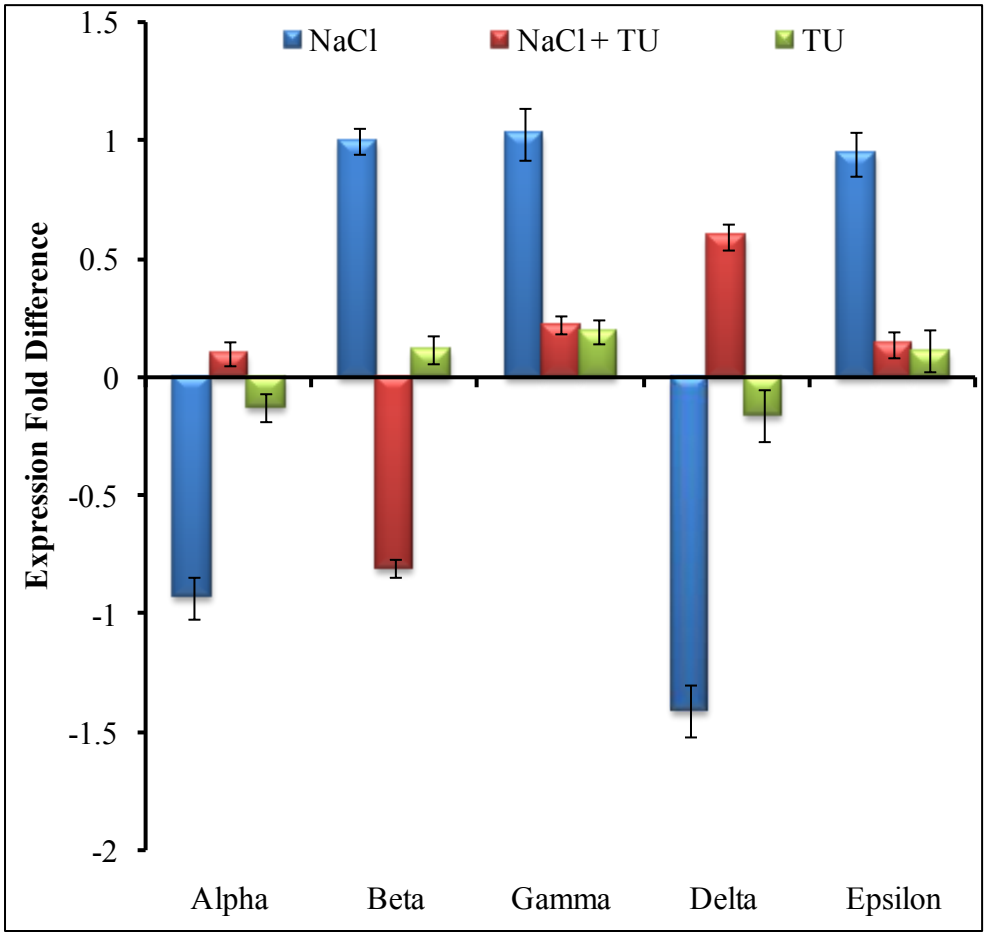


Fig. 3.3: Integrated expression profile of various mtATPase subunits in 1 h soaked seeds. Relative expression ratio plot represents the expression level of various subunits in seeds given NaCl, NaCl+TU and TU treatment for 1 h. The base line expression (0 Value on y-axis) represents the expression level of distilled water control. The values represent the mean of three technical and two biological replicates. The differences in the mean were found to be statistically significant at $p<0.01$, in one way anova test.

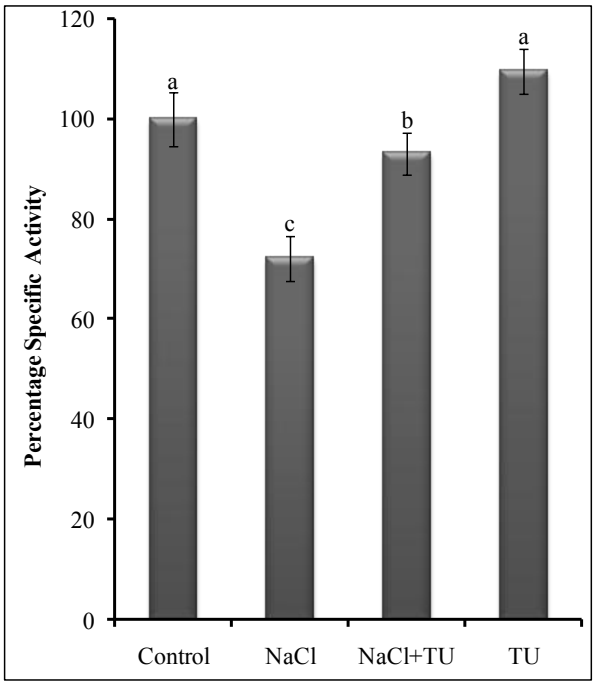


Fig. 3.4: Differential activity of mtATPase. The differential mtATPase activity is represented in terms of the percentage specific activity with reference to control (D). The N, NT and T represent NaCl, NaCl+TU and TU treatment respectively. The values represent the mean±S.D. of six technical and three biological replicates. The difference in the mean was statistically significant (P <0.01).

3.3. DISCUSSION

Seeds of higher plants are known to be desiccation tolerant and their mitochondria exhibit unique properties (Macherel et al., 2007). Its respiration starts after few minutes of imbibition and affects the process of germination in several ways. For instance during imbibition, the energy production by seed mitochondria helps to cope up with desiccation. This is dependent upon the mechanism of ATP production by ATP synthase. Another important role of mitochondria is to generate the hypoxic environment which is essential for proper seed germination. Hence, decrease in the integrity and performance of mitochondria during early stage of germination can hamper the above mentioned processes. In turn, stress conditions lead to decrease in germination and yield. However, if this can be taken care through the application of any external stimuli or effector molecule which can help in maintaining mitochondrial functions under stress, it may maintain the seed viability under stress. Our preliminary studies at the field level have indicated that the application of TU can reduce the crop loss under stress (Sahu et al., 2005; Sahu et al., 2006). In the present experiment, the molecular mechanism of TU action in *Brassica juncea* seed is investigated. The genome sequence of *Arabidopsis thaliana* was helpful in doing the expression analysis in *Brassica juncea* because of the close similarity in the gene sequence between the two species (Girke et al., 2000; Hudson et al., 2007).

Application of TU was found to be effective in maintaining the functions of seed mitochondria under NaCl stress. The primary evidence in support of this was derived from the seed viability assay (Fig. 3.1). Differential display of seeds exposed to various treatments for 1 h was performed to identify differentially induced transcripts. The

technique was chosen owing to its high sensitivity and capability of simultaneous comparison of multiple RNA samples (Liang and Pardee 1992; Landrum, 2006). The soaking time was kept only 1 h because of our interest in identifying the regulatory genes associated with TU treatment. Out of total 150 transcripts obtained, eight were found to be differentially expressed. Among the eight differentially expressed transcripts, three was identified as subunits of mtATPase. Considering that mtATPase has some role in TU-induced salt tolerance, expression of all the five subunits viz., alpha, beta, gamma, delta and epsilon was monitored using RT-PCR.

As integrity and functioning of mitochondria is very important, the modulated expression of its various subunits points towards an important regulatory mechanism, which is controlled by TU application. Alpha subunit of mtATPase has a critical function with respect to the mitochondrial bioenergetics and its turnover might be involved in the control of mitochondrial activity during germination (Rajjou et al., 2004). In the present study, the expression level of alpha subunit was downregulated in seeds treated with NaCl for 1 h (Fig. 3.3). This could be because of the increased generation of the reactive oxygen species or change in the Na^+/K^+ ratio associated with Na^+ toxicity (Sairam and Tyagi, 2004; Arora et al., 2002). Stress caused due to hydrogen peroxide exposure has been shown to damage subunits of ATP synthase of mitochondria (Sweetlove et al., 2002). Such a change during the early stage might have disturbed the mitochondrial homeostasis, which ultimately resulted in the poor seed germination. On the contrary, in NaCl+TU treatment the level of alpha subunit was close to that of control which suggests the positive role of TU in maintaining the mitochondrial function under salinity stress (Fig. 3.3). This was also reflected in terms of increased germination percentage and better

growth pattern in NaCl+TU treatment (Fig. 3.1; Lane-3). Since TU is a thiol compound, it changes the redox state of the system in such a way that the harmful effects of salinity stress are neutralized (Buchanan et al., 1994). The TU mediated upregulation of alpha subunit under salinity stress, has not been reported so far and appears to be a novel response. Some kind of a dual role for this subunit has been suggested in the animal system assigning it a heat shock protein status (Luis et al., 1990). The alpha subunit in plants may also perform the dual function. Thus, its induction would lead not only to the restoration of the mitochondrial function but also impart stress tolerance to the plants. In the recent study, Zhang et al., (2006) identified a salt-responsive gene from rice and found it to be a subunit of ATP synthase. Its over- expression in tobacco made the transgenics more tolerant towards salinity stress.

Response of mtATPase subunits shows considerable variation under different environmental stress and with one plant to other. Previously, it has been shown that mtATPase subunits are the major sites of damage under stress (Hamilton et al., 2001). In *Arabidopsis thaliana* cells, the expression levels of both alpha and beta subunits were significantly decreased under oxidative stress (Sweetlove et al., 2002). Under aluminum stress, ATPase activity increased significantly, however the level of alpha subunit remained the same (Hamilton et al., 2001). In the present study, NaCl treatment for 1 h to the seeds of *Brassica juncea* led to the upregulation of beta subunit while such an upregulation was not seen in NaCl+TU treatment (Fig. 3.3). In case of gamma, delta and epsilon subunits, the presence of TU along with NaCl treatment reversed their expression profile seen under salinity stress (Fig. 3.3). In case of *Oryza sativa*, NaCl stress leads to increase in the expression of delta subunit (Zhang et al., 2006) while in *Brassica juncea*

this subunit was downregulated under NaCl stress (Fig. 3.3). Such an integrated regulation in the transcript level of these subunits in response to TU treatment is likely to impose significant flux restrictions on the tricarboxylic acid cycle and electron transport, thereby limiting the synthesis of ATP (Sweetlove et al., 2002). This may be one of the mechanisms by which TU treatment reduces the extent of salinity induced oxidative stress.

Activity of mtATPase was also assayed to see how it is regulated by the change in the expression profile of various subunits. Results showed that mtATPase activity decreased in NaCl, however, it approached to the level of control in NaCl+TU treatment (Fig. 3.4). The increased ATP production in NaCl+TU treatment might support the energy demanding processes associated with the salt tolerance. Such a contrasting observation where TU changes the expression profile of various subunits of mtATPase subunits in an integrated manner (Fig. 3.3) with an overall consequence of increase in mtATPase activity (Fig. 3.4) indicates that the activity of mtATPase may possibly be regulated at the post translational level as well (Hamilton et al., 2001).

3.4. CONCLUSION

Mitochondria play an important role in maintaining the seed germination under stress. The differential regulation in the expression profile of various mtATPase subunits was observed in NaCl and NaCl+TU treatment. Such a response was positive in maintaining the higher mtATPase activity in NaCl+TU as compared to that in NaCl treatment. This is ultimately reflected in the form of proper germination and growth pattern in NaCl+TU treated seeds. This regulation of mtATPase activity constitutes to one of the mechanisms by which TU imparts salt tolerance.

Section II: Microarray hybridization based approach for the identification of thiourea modulated transcripts.

3.5. Introduction

The differential display data indicated that the redox-sensitive molecule like thiourea (TU) has its effect at the molecular level and its exogenous supplementation reduces the damage in seeds subjected to NaCl stress (refer to Chapter-3\Section-1). In order to identify the total repertoire of genes whose expression level is regulated by the redox state, the microarray analysis was performed in seeds subjected to NaCl stress with/without TU. The TU was used as a chemical probe to decipher the redox regulatory steps which plays an important role in determining the salt-stress tolerance. Since, the Arabidopsis array is an established tool for the gene expression analysis of Brassica species because of their close phylogenetic relationship (Hudson et al., 2007), the Agilent based *Arabidopsis thaliana* microarray platform was used.

3.6. Results

3.6.1. Global analysis of early TU-responsive gene expression in seeds

The complete microarray data which represent the average ratios of normalized signals in $\log_2$ scale calculated from three independent replicates is available under the accession number of E-MEXP-1903 in EBI microarray database (Mukherjee *et al.,* 2005). In TU alone treated seeds, 53 genes were down-regulated as compared to that of control (Table. 3.4). The functional analysis of these genes indicated that most of them are either transcription factors/heat shock proteins or associated with the responses such as calcium signaling, protein synthesis/degradation and antioxidant machinery. A total of eight genes

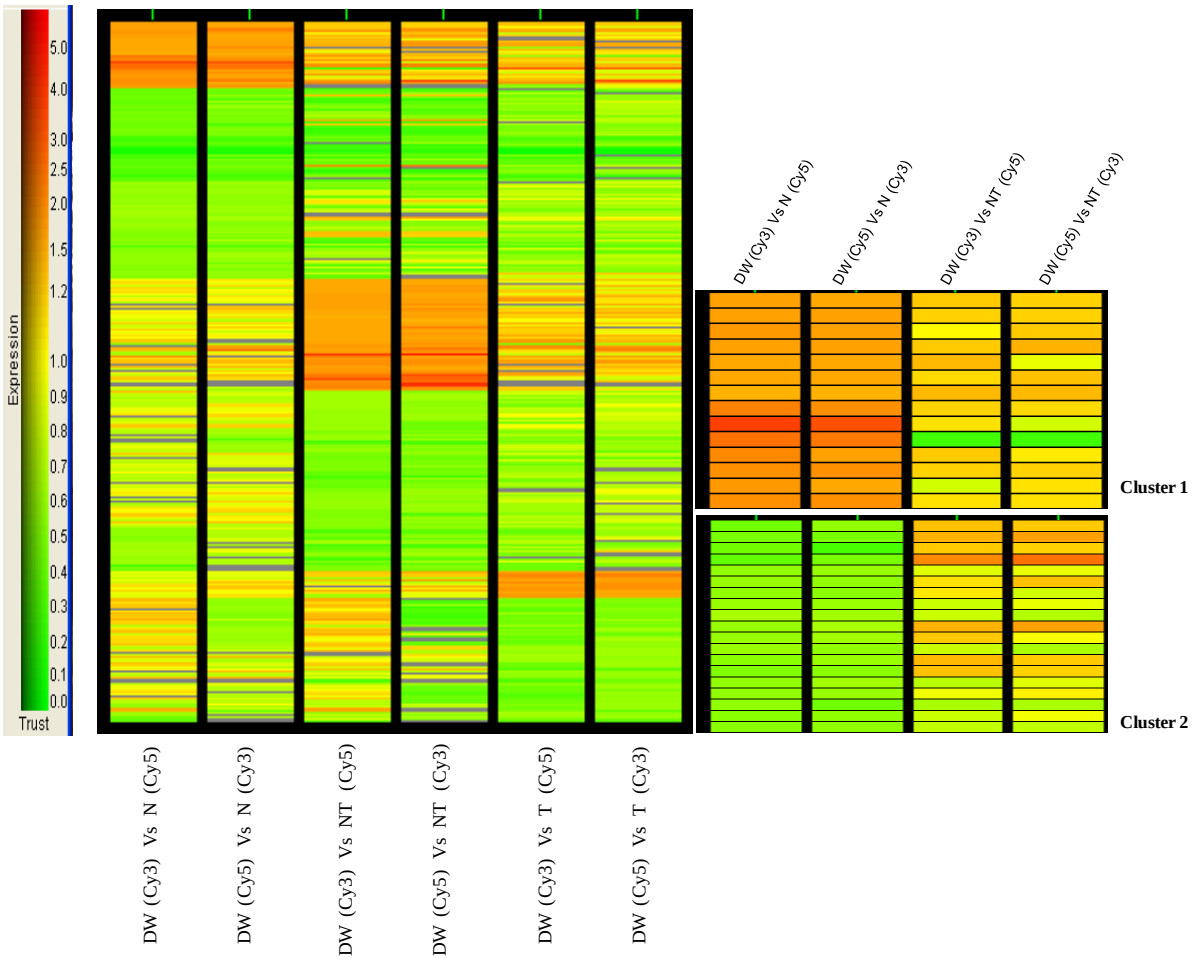


Fig. 3.5: Microarray analysis in *Brassica juncea* seeds. An overview of microarray analyses (DW Vs. N; DW Vs. NT and DW Vs. T) is represented. Each experiment was performed in triplicate and the average ratios of normalized signals in $\log_2$ scale were calculated from three independent replicate experiments using GENESPRING software. The DW, N, NT and T represent distilled water control, NaCl (1 M), NaCl (1 M)+TU (6.5 mM) and TU (6.5 mM) treatment, respectively. Cluster 1 and 2 represents 19 and 14 genes which were down and up regulated respectively in NaCl but gets modulated under NaCl+TU treatment. The attached trust represent the scale of expression fold difference (green and red color represent the down and up regulation, respectively).

Table 3.4: List of the genes downregulated in response to TU treatment.

S. No.	ID	Description	D Vs. T
Transcription Factor, Kinases and Transporters			
1.	At1g74100	SOT16 (Sulfotransferase 16)	-3.93
2.	At3g29350	AHP2 (Histidine-containing phosphotransmitter-2)	-1.44
3.	At5g59820	ZAT12 (Responsive to high light)	-1.13
4.	At5g53590	Auxin-responsive family protein	-1.12
5.	At1g06225	CLE3 (CLAVATA3/ESR-RELATED 3); receptor binding	-1.09
6.	At4g17920	Zinc finger (C3HC4-type RING finger) family protein	-1.09
7.	At5g61600	Ethylene-responsive element-binding family protein	-1.09
8.	At1g23740	Oxidoreductase, zinc-binding dehydrogenase family protein	-1.09
9.	At1g72430	Auxin-responsive protein-related	-1.04
10.	At3g49530	Arabidopsis NAC domain containing protein 62; transcription factor	-0.92
11.	At2g19170	SLP3; serine-type peptidase	-0.86
12.	At4g31800	ATWRKY18_WRKY18; Transcription factor	-0.86
13.	At5g03720	HSFA3_AT-HSFA3; DNA binding / Transcription factor	-0.84
14.	At3g55980	ATSZF1 (Salt-Inducible Zinc Finger 1)	-0.83
15.	At3g16210	F-box family protein	-0.77
16.	At4g25950	VATG3 (Vacuolar ATP Synthase G3)	-0.76
17.	At5g28080	WNK9; kinase/ Protein kinase	-0.74
18.	At2g27050	EIL1 (Ethylene-Insensitive3-Like 1); Transcription factor	-0.73
19.	At2g24840	AGL61 (Agamous-like 61); Transcription factor	-0.69
20.	At2g23030	SNRK2.9 (SNF1-related protein kinase 2.9)	-0.68
21.	At5g59340	WOX2 (Wuschel related homeobox 2); Transcription factor	-0.63
Protective genes			
22.	At3g09640	APX2 (Ascorbate peroxidase 2)	-1.64
23.	At4g10270	Wound-responsive family protein	-1.16
24.	At1g20620	CAT3 (Catalase 3)	-1.1
25.	At1g59860	17.6 kDa Class I Heat shock protein (HSP17.6A-CI)	-1.04
26.	At1g63940	Monodehydroascorbate reductase	-0.92
27.	At1g69920	ATGSTU12 (Glutathione S-transferase TAU 12)	-0.76
28.	At3g13310	DNAJ heat shock N-terminal domain-containing protein	-0.73
29.	At3g53990	Universal stress protein (USP) family protein	-0.72
30.	At5g59720	HSP18.2 (Heat shock protein 18.2)	-0.66
31.	At2g29500	17.6 kDa class I small Heat shock protein (HSP17.6B-CI)	-0.64
Protein synthesis/Degradation			
32.	At1g73480	Hydrolase, alpha/beta fold family protein	-2.26
33.	At5g14980	Esterase/lipase/thioesterase family protein	-1.27
34.	At5g64660	U-box domain-containing protein	-1.2
35.	At2g07715	Ribosomal protein L2, putative	-1.07
36.	At3g18740	60S ribosomal protein L30 (RPL30C)	-0.88
37.	At1g66160	U-box domain-containing protein	-0.86
38.	At4g27140	2S seed storage protein 1 / NWMU1-2S albumin 1	-0.84
39.	At3g18080	BGLU44 (B-S GLUCOSIDASE 44)	-0.79

S. No.	ID	Description	D Vs. T
Calcium signaling related			
40.	At1g21550	Calcium-binding protein, putative	-1.16
41.	At1g01140	CIPK9 (CBL-INTERACTING PROTEIN KINASE 9)	-1.09
42.	At5g25110	CIPK25 (CBL-INTERACTING PROTEIN KINASE 25)	-1.08
43.	At3g63380	Calcium-transporting ATPase/ putative (ACA12)	-1
44.	At2g41410	Calmodulin, putative	-0.91
Molecular Function Unknown			
45.	At4g38060	Unknown protein	-1.37
46.	At3g07900	Unknown protein	-1.14
47.	At2g07708	Unknown protein	-1.1
48.	At2g07772	Unknown protein	-1.1
49.	At1g62840	Unknown protein	-1.01
50.	At1g27330	Unknown protein	-1.01
51.	At3g60990	Unknown protein	-0.99
52.	At1g33055	Unknown protein	-0.87
53.	At5g65300	Unknown protein	-0.71

Table 3.5: List of the genes upregulated in response to TU treatment.

S. No.	ID	Description	D Vs. T
1.	At1g13340	Oxygen-evolving enhancer protein	1.09
2.	At5g44360	Aldo/keto reductase family protein	0.91
3.	At5g02380	AHA3; ATPase/ hydrogen-exporting ATPase	0.84
4.	At5g13440	ATOPT6 (Oligopeptide transporter 1)	0.83
5.	At1g17710	FAD-binding domain-containing protein	0.82
6.	At5g56000	Ubiquinol-cytochrome C reductase iron-sulfur subunit	0.74
7.	At1g32470	MT2B (METALLOTHIONEIN 2B); copper ion binding	0.70
8.	At1g10650	Unknown protein	0.65

Table 3.6: List of genes upregulated under NaCl but differentially regulated in NaCl+TU treatment.

S. No.	ID	Annotation	Fold D vs. N	Fold D vs. NT
1.	At3g54050	Fructose-1,6-bisphosphatase, putative	1.97	-0.09
2.	At1g04560	AWPM-19-like membrane family protein	1.89	0.14
3.	At5g63290	Coproporphyrinogen oxidase-related	1.8	-0.08
4.	At2g36420	Unknown protein	1.78	0.12
5.	At2g15580	Zinc finger (C3HC4-type RING finger) family protein	1.74	-0.09
6.	At2g29090	CYP707A2 (cytochrome P450, family 707, subfamily A, polypeptide 2)	1.70	0.23
7.	At5g66940	Dof-type zinc finger domain-containing protein	1.65	-0.27
8.	At4g19170	NCED4 (9-cis-epoxycarotenoid dioxygenase)	1.64	-0.15
9.	At4g36040	DNAJ heat shock N-terminal domain-containing protein (J11)	1.58	0.26
10.	At1g20630	CAT1 (catalase 1); catalase	1.35	0.37
11.	At1g60690	Aldo/keto reductase family protein	1.3	-2.01
12.	At2g37770	Aldo/keto reductase family protein	1.3	-2.01
13.	At5g64290	DCT/DIT2.1 (Dicarboxylate transport)	0.74	0.19
14.	At4g33550	Lipid transfer protein (LTP)	0.61	-0.09

Table 3.7: List of genes downregulated under NaCl but differentially regulated in NaCl+TU treatment.

S. No.	ID	Annotation	Fold D vs. N	Fold D vs. NT
1.	At5g61600	Ethylene-responsive element-binding family protein	-2.46	0.24
2.	At4g36220	FAH1; ferulate 5-hydroxylase	-2.0	0.98
3.	At2g41410	Calmodulin, putative	-1.85	1.2
4.	At2g37040	PAL1; phenylalanine ammonia-lyase	-1.78	1.13
5.	At5g53590	Auxin-responsive family protein	-1.6	1.49
6.	At2g16060	AHB1 (*Arabidopsis* hemoglobin 1)	-1.2	1.1
7.	At5g44120	CRA1 (CRUCIFERINA); nutrient reservoir	-1.04	0.6
8.	At4g02380	Senescence-associated gene 21 (SAG21)	-1.01	1.2
9.	At5g62480	ATGSTU9; glutathione transferase	-0.9	2.30
10.	At1g20620	CAT3 (Catalase 3); catalase	-0.8	1.35
11.	At2g06050	OPR3 (OPDA-reductase 3)	-0.8	1.3
12.	At4g05010	F-box family protein	-0.8	1.02
13.	AT2G07698	ATP synthase alpha subunit, mitochondrial	-1.159	0.52
14.	At3g13320	CAX2 (Cation exchanger 2)	-0.7	1.02
15.	At3g22910	Calcium-transporting ATPase, plasma membrane-type, putative / Ca(2+)-ATPase, putative (ACA13)	-0.7	1.4
16.	At3g29350	AHP2 (Histidine-containing phosphotransmitter 2)	-0.7	1.85
17.	At3g59970	MTHFR1; methylenetetrahydrofolate reductase	-0.70	1.81
18.	At3g61440	ARATH; (BETA-substituted Ala synthase 3;1)	-0.7	0.8
19.	At5g64310	AGP1 (Arabinogalactan-protein 1)	-0.67	1.63

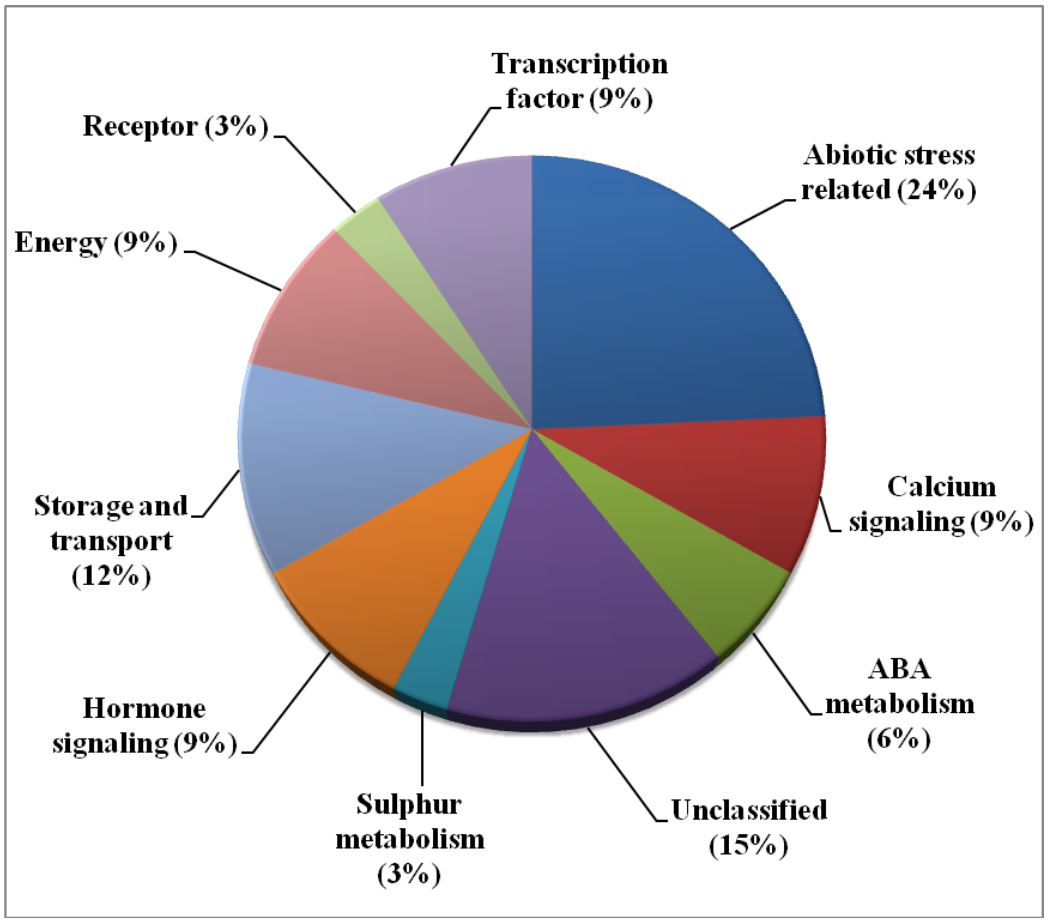


Fig. 3.6: Functional classification of TU modulated and salinity stress responsive genes. Genes were classified according to the function of the protein that they encode (Provart and Zhu, 2003; www.arabidopsis.org).

were found to be up-regulated in TU alone treatment, which are associated with the cellular energetics system (Table. 3.5).

In NaCl treated seeds, overall 83 down-regulated and 28 up-regulated genes were identified. Clustering analysis of these 111 differentially expressed genes resulted in the identification of 33 genes whose expression was modulated upon TU supplementation to NaCl. These genes were divided into cluster 1 and 2 (Fig. 3.5) of 14 (Table. 3.6) and 19 (Table. 3.7) genes, respectively depending on their down-regulation or up-regulation in NaCl+TU as compared to that of NaCl stress. These 33 TU modulated and salinity stress responsive genes were then functionally classified (Fig. 3.6). A total of 27% genes encoded proteins with a putative regulatory function (transcription factors, hormone and calcium signaling), suggesting a large adjustment in regulatory networks in response to TU treatment under salinity stress, 6% genes encoded proteins involved in ABA metabolism, 9% were related to the energy production and 12% of genes were found to be involved in storage and transport.

3.6.2. Quantitative real-time PCR validation of selected genes under different treatments

The quantitative real-time PCR of the eight selected TU modulated and salinity responsive genes was performed to validate the results of microarray (Fig. 3.7-A). The details of the gene specific primers used are given in Table 3.8. The pattern of the gene expression obtained under different treatments by real-time PCR correlated well with the data of microarray experiments (as determined by Pearson correlation coefficients). However, the values of $\log_2$ ratio obtained by real-time PCR were generally higher than

Table 3.8: Details of the primers used for the quantitative real-time PCR.

Name of the gene	Primer sequence (5'→3')	Amplicon Size (bp)
FAH-1	Forward-ggctcgtgaaggcccgtaatgat	310
	Reverse-tcctcggggctccgtaataactcc	
Ca^{2+} ATPase	Forward-ggacacactcggtgcattggctt	413
	Reverse -tgcgatggcaatgcaaactccc	
CYP 707A2	Forward-catggggttgccttacatcggaga	279
	Reverse -accagccgcttaagggtagaatgg	
GST-9	Forward -cattcgcgagtccttacagcaaaag	588
	Reverse-tgaccacgctggtttcgtttatagc	
CAT-3	Forward-catcgccttggaccgaattatttgc	319
	Reverse -tgagacgtggctccgatagaatctc	
CAT-1	Forward-caccgtcttggaccaaactatctgc	303
	Reverse -aagcgcttcaacaaaacgcttcac	
EREBP	Forward-ggcttgggacttacgacactgcc	320
	Reverse -cctgctcccacatcccaaaaccc	
CAX-2	Forward-tcttcccggctgttcttcactacac	471
	Reverse -ggaccgcaaacatggaaatctgg	
Actin	Forward-ctcctgccatgtatgtcgctatcc	155
	Reverse-aaggtccaaacgcagaatagcatgt	

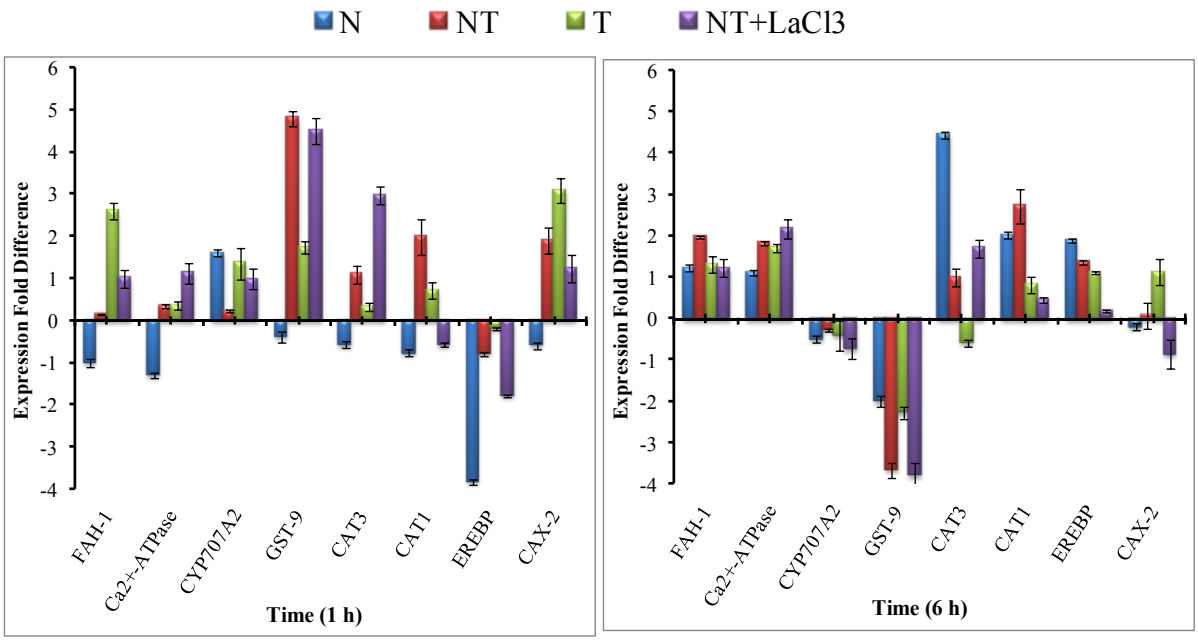


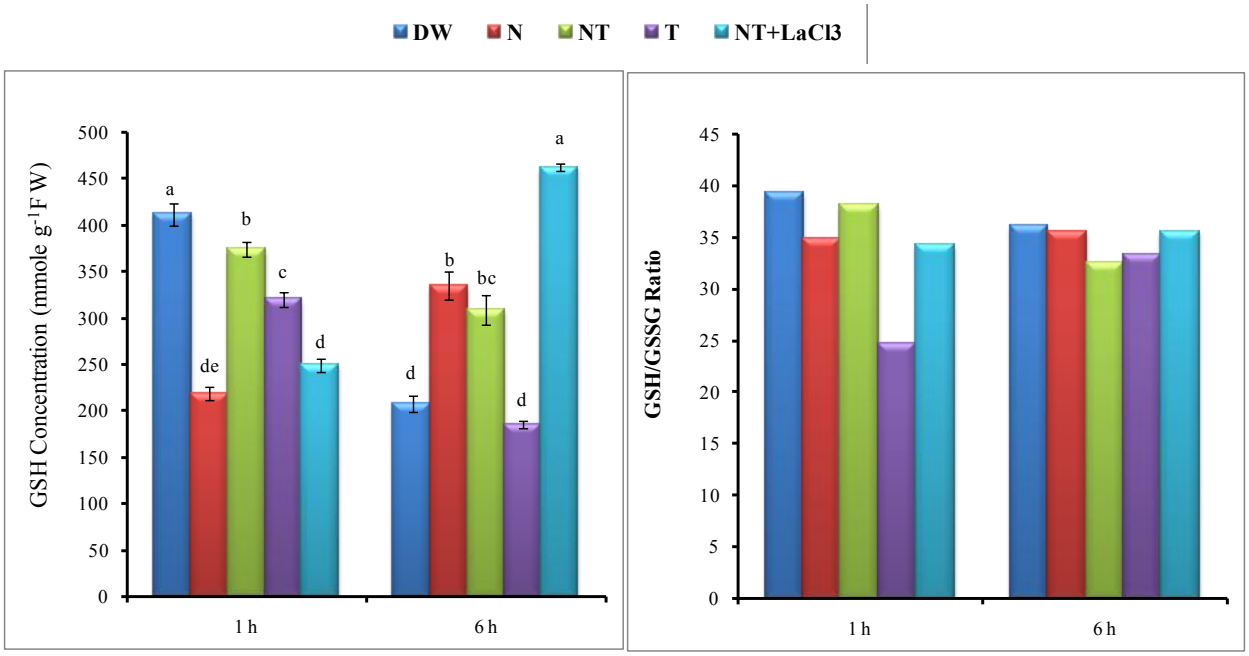
Fig. 3.7: Real-time PCR validation of the selected TU modulated salinity responsive genes. The expression level of the selected TU responsive genes was measured by quantitative real time PCR in seeds subjected to different treatments for 1 h (Panel-A) and 6 h (Panel-B) respectively. The N, NT, T and NT+$LaCl_3$ represent different treatments such as NaCl (1 M), NaCl (1 M)+TU (6.5 mM), TU (6.5 mM) and NaCl (1 M)+TU (6.5 mM)+$LaCl_3$ (5 mM), respectively. All real time PCR results were normalized to that of the actin in log_2 scale ratio as compared to that of distilled water control. The data represent average of three biological and three technical replicates (±S.D.). The differences in the mean were found to be statistically significant at $P<0.05$, in one way ANOVA test.

those obtained by microarray analysis. This observation may be attributed to the saturation of fluorescent signals in the microarray. Out of the eight genes analyzed by real-time PCR, the $\log_2$ ratio of CAT1 was not correlated with that obtained from the microarray. This could be due to the differential mRNA splicing that can affect the transcript levels detected at different regions of mRNA by two independent techniques (real-time and microarray).

Apart from microarray data validation, real-time PCR analysis was also performed to study the temporal regulation of same set of genes. The data obtained indicated that the transcripts were under dynamic regulation. At an early phase of imbibition (1 h), expression level of most of the genes was higher in NaCl+TU while lower in NaCl, as compared to control (Fig. 3.7-A). An increase in the expression level of most of the genes in NaCl treatment as compared to control was achieved only at 6 h (Fig. 3.7-B). At 6 h, the CYP707A2 and GST-9 were found to be down-regulated in all the treatments. Such a temporal regulation in the expression profile was not observed in TU alone treated seeds. In TU treated seeds, the expression of all genes was either at the level of control or up-regulated at both time points except CAT-1, whose expression decreased at 6 h.

3.6.3. Redox state regulation in seeds under different treatments

At 1 h, the level of GSH decreased by 47% in NaCl treatment in comparison to control, while the decline was 9% and 23% only in NaCl+TU and TU treatments, respectively. At 6 h, the level of GSH was at par to control in TU treatment; while it increased in NaCl and NaCl+TU treatments as compared to control (Fig. 3.8-A). In NaCl+TU treatment, the GSH:GSSG ratio was close to control at 1 h, but was 11% lower



A B

Fig. 3.8: Measurement of GSH and GSH/GSSG ratio. The level of GSH (A) and GSH/GSSG ratio (B) was measured in seeds subjected to different treatments for 1 and 6 h. The DW, N, NT, T and NT+$LaCl_3$ represents different treatment such as distilled water control, NaCl (1 M), NaCl (1 M)+TU (6.5 mM), TU (6.5 mM) and NaCl (1 M)+TU (6.5 mM)+$LaCl_3$ (5 mM), respectively. All the values represent the mean±S.D. of six technical and three biological replicates. The differences in the mean was found to be statistically significant at P <0.05, in one way ANOVA test. Different letters indicate significantly different values at a particular duration (DMRT, P <0.05).

than control at 6 h. In contrast, in NaCl treatment, an initial 12% decrease was seen in the GSH:GSSG ratio at 1 h as compared to control that was maintained till 6 h (Fig. 3.8-B).

3.6.4. Endogenous ABA level in seeds under different treatments

The level of ABA was only slightly increased in 1 h of NaCl treatment, while in NaCl+TU and TU treated seeds, it was increased significantly by 3- and 2.6-fold, respectively as compared to control (Fig. 3.9-A). At 6 h, the level of ABA was close to control in NaCl treatment, while it was 20% lower than control in NaCl+TU treatment. However, in TU treatment, ABA level remained 43% higher than control at 6 h (Fig. 3.9-B).

3.6.5. Activities of Catalase, PAL and GST in seeds under different treatment

The activity of catalase and PAL decreased significantly in NaCl treated seeds both at 1 h and 6 h as compared to that of control. In contrast, in NaCl+TU and TU treatments, catalase (Fig. 3.10-A) and PAL (Fig. 3.10-B) activities were either increased higher or remained close to control, except for 1 h TU treatment. The activity of GST decreased significantly as compared to control in NaCl treatment for 1 h, whereas at this time point, NaCl+TU and TU treated seeds showed an increased GST activity. At 6 h, the GST activity decreased in all treatments, except TU treated seeds, which still showed about 11% higher activity than control (Fig. 3.10-C).

3.6.6. Effect of calcium channel blocker on the functioning of thiourea

To reveal whether the functioning of TU is dependent upon $[Ca^{2+}]_{cyt}$ transient burst, $LaCl_3$ (specific Ca^{2+} channel blocker) was provided, along with NaCl+TU treatment. Out of the eight selected TU responsive genes (whose expression level was measured by real time-PCR), the expression profile of three of them, such as CAT1,

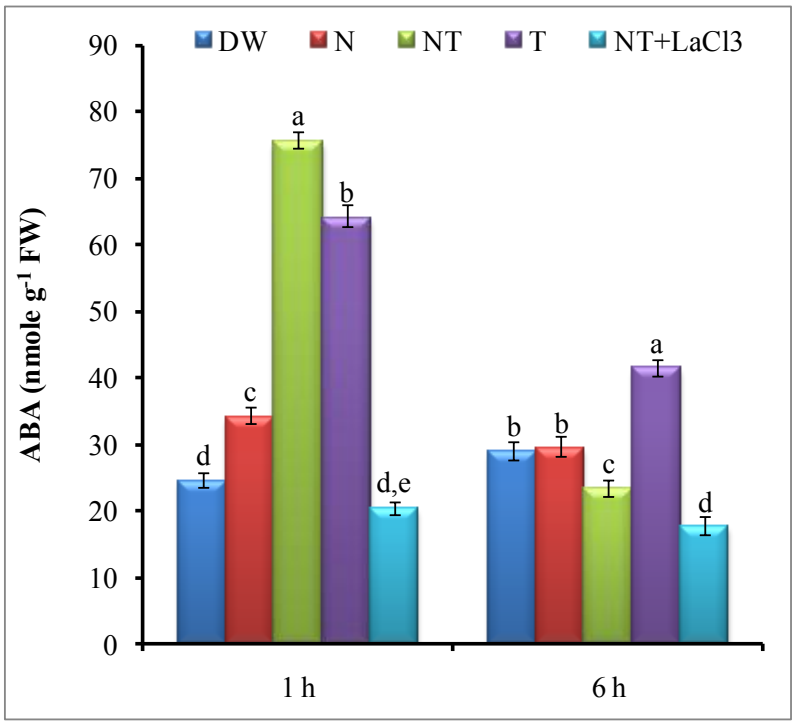


Fig. 3.9: Measurement of the ABA content. The endogenous level of ABA was measured in seeds subjected to different treatments for 1 and 6 h. The DW, N, NT, T and NT+$LaCl_3$ represents different treatment such as distilled water control, NaCl (1 M), NaCl (1 M)+TU (6.5 mM), TU (6.5 mM) and NaCl (1 M)+TU (6.5 mM)+$LaCl_3$ (5 mM), respectively. All the values represent the mean±S.D. of six technical and three biological replicates. The differences in the mean was found to be statistically significant at P <0.05, in one way ANOVA test. Different letters indicate significantly different values at a particular duration (DMRT, P <0.05).

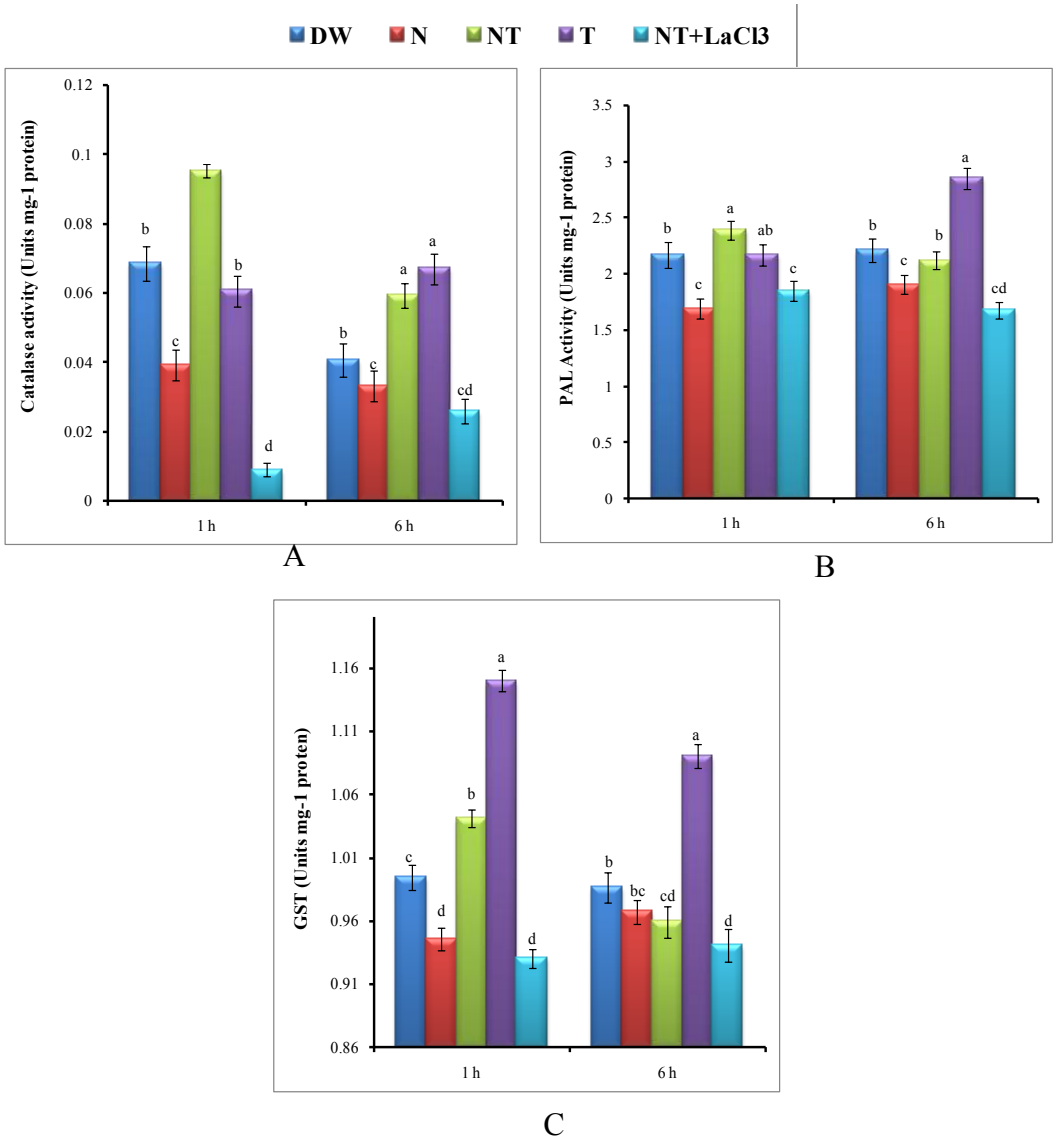


Fig. 3.10: Measurement of enzyme activities. The activities of catalase (A); PAL (B) and GST (C) were measured in seeds subjected to different treatments for 1 and 6 h. The DW, N, NT, T and NT+$LaCl_3$ represents different treatment such as distilled water control, NaCl (1 M), NaCl (1 M)+TU (6.5 mM), TU (6.5 mM) and NaCl (1 M)+TU (6.5 mM)+$LaCl_3$ (5 mM), respectively. All the values represent the mean±S.D. of six technical and three biological replicates. The differences in the mean was found to be statistically significant at P <0.05, in one way ANOVA test. Different letters indicate significantly different values at a particular duration (DMRT, P <0.05).

EREBP and CAX-2, was reversed when $LaCl_3$ was added to NaCl+TU treatment (Fig. 3.7-A and B). In addition, the synthesis of GSH was also slightly affected by $LaCl_3$ in the initial 1 h (Fig. 3.8-A), however the ratio of GSH/GSSG remained unaffected (Fig. 3.8-B). Another critical change was no alteration in the ABA level under NaCl+TU treatment upon $LaCl_3$ addition (Fig. 3.9). Further, the $LaCl_3$ treatment was also found to substantially inhibit the increase in the activity of catalase (Fig. 3.10-A), PAL (Fig. 3.10-B) and GST (Fig. 3.10-C), which were otherwise increased under NaCl+TU treatment.

3.7. DISCUSSION

In the present study, the mechanism of TU-mediated stress tolerance has been analyzed in Indian mustard (*Brassica juncea* L.) through the identification of differentially modulated transcripts using microarray followed by real-time PCR and biochemical assays of selected representative pathways. The approach to use high degree of salinity stress was based on previous studies, which demonstrated that TU supplementation was able to alleviate the damage even at supra-optimal levels (1 M NaCl) so that the viability of seeds was maintained (refer to Chapter-3\Section-3.2.1; Srivastava et al., 2009). Hence, it was intriguing to investigate various signaling and effector processes regulated by TU within an hour of treatment that allowed the seeds to maintain their viability and ability to tolerate the stress.

The genome wide transcriptome analysis was performed at a short duration of 1 h in Brassica seeds. The result of control vs. TU treatment showed that 53 genes were down-regulated (Table 3.4), while only 8 genes were up-regulated (Table. 3.5). However under similar conditions, the redox state (measured in terms of GSH:GSSG ratio) was lower than control that could be attributed to the decreased synthesis of GSH due to

exogenous supply of a –SH containing compound (TU). Therefore, TU-regulated transcripts could be categorized as potential redox-sensitive genes. TU mediated decline observed in the expression level of genes under the category of protein synthesis/degradation might cause a decrease in the overall protein turnover. This suggests that TU changes the redox state that helps in stabilizing different cytosolic proteins and hence reducing the need for overall protein turnover. The TU mediated down-regulation of the genes for different heat shock (HSPs) and universal stress proteins (USPs) further indicated that TU treated seeds did not require their *de novo* synthesis. Shelton et al., (2005) reported that the mammalian Hsp70 can be activated by the GST mediated glutathionylation. The higher expression of GST (Fig. 3.7-A; Table. 3.4) and increased GST activity (Fig. 3.10-C) together support the proposition that TU treatment prevented the degradation of HSPs and other proteins through their increased glutathionylation. A decrease in the expression level of various protective genes like ascorbate peroxidase, monodehydroascorbate reductase and catalase was also observed. This might be ascribed to TU mediated direct scavenging of ROS (Kelner et al., 1990; Gao et al., 2008). A set of transcription factors, such as AP-2/EREBB, NAC, WRKY and WOX-2, the potential upstream signal transducers i.e. kinases as well as a few genes related to calcium signaling, were also down-regulated in response to TU alone treatment pointing towards the importance of signaling mechanisms under changed redox state. However, a more detailed analysis is required to clarify how the different components interact with each other to regulate the downstream responses. Among the up-regulated genes, OPT6 is known to transport glutathione derivatives and to function in stress resistance responses (Cagnac et al., 2004). Oxygen-evolving enhancer (OEE) protein has

been demonstrated to possess thioredoxin activity (Heide et al., 2004). These results suggest that redox mediated stress tolerance was improved under TU alone treatment. An increase in the expression level of ATPase and ubiquinol-cytochrome C reductase suggests an improvement in energy metabolism to induce changes in redox state and to achieve the regulation of signaling components. Thus, TU alone treatment led to a decline in various HSPs, USPs and other stress responsive genes through complex signaling due to an improved redox state, however phenotypically, plants were comparable to that of control (refer to Chapter-4\Section-4.2.1; Fig. 4.1). Thus, the emphasis was not on the TU alone responses, but to investigate how NaCl stress was ameliorated upon TU supplementation and to find out the components involved.

The transcript profile data revealed that the expression of total 33 genes was differentially regulated in NaCl and NaCl+TU treated seeds (Fig. 3.5; Cluster- 1 and 2). These genes were together denoted as "TU modulated salinity stress responsive genes". Further, real-time PCR analysis of the selected genes at two time points demonstrated their dynamic regulation. An induction in the expression level of most of the genes was achieved as early as 1 h in NaCl+TU treatment. In contrast, upon NaCl treatment, almost similar level of induction in the expression of various genes was observed only at 6 h (Fig. 3.7). Similarly, the data of GSH:GSSG ratio also depicted the differential change in redox state under NaCl and NaCl+TU treatment at 1 h and 6 h (Fig. 3.8). These results suggest that in NaCl+TU treated seed, there was an early sensing of the stress and the induction of different mechanisms in a co-ordinated manner, as compared to that in NaCl-treated seeds. This early responsiveness in the presence of TU might be responsible

to maintain the seed germination ability even under the high degree of salinity stress (Srivastava et al., 2009).

In plants, as the salinity stress signal is perceived, the level of ABA gets enhanced triggering an array of responses including an increase in cytosolic calcium (Jakab et al., 2005). In the present study, the expression of two ABA metabolism genes, such as 9-cis-epoxycarotenoid dioxygenase-4 (NCED4), a regulatory enzyme of ABA biosynthesis (Qin and Zeevart, 1999), and cytochrome P450 sub family gene (CYP707 A2), a key enzyme of ABA catabolism (Saito et al., 2004), was modulated in response to TU treatment. This indicated the active involvement of ABA in TU mediated response. To confirm this, time dependent measurement of endogenous ABA level was performed in seeds under different treatments. A significant increase in the level of ABA as early as 1 h after imbibition in NaCl+TU as well as TU treatment was observed, which strengthens the proposition that TU mediated effects are ABA-dependent. With time, the level of ABA was found to decrease in all the treatments which is required to facilitate the process of seed germination (Penfield and King, 2009). In response to an increase in the level of ABA, other signaling mechanisms are initiated, such as the change in calcium signature that assist plants adapt under salinity stress. Calcium signature is a condition specific phenomenon and is governed by the coordinated function of different genes viz., calmodulin, calcium transporting ATPase and calcium exchanger (Knight et al., 1997). The level of such genes was found to be modulated in TU (Table. 3.4 and 3.5) and NaCl+TU (Table. 3.6 and 3.7) treated seeds, which suggested that TU mediated signaling changes in the process of stress amelioration also involved modulations in calcium signature pattern.

There are evidences for the role of ABA in triggering the production of H_2O_2 (Xing et al., 2008). In addition, the salinity stress itself induces oxidative stress (Borsani et al., 2001). In this context, the role of catalases becomes indispensible for efficiently scavenging the H_2O_2 (Willekens et al., 1997). They are encoded by a small multigene family consisting of CAT1, CAT2 and CAT3 (Frugoli et al., 1996). CAT1 is known to be regulated in an ABA dependent manner and is mainly involved in regulating the H_2O_2 dependent signaling; while CAT3 is regulated by an unknown mechanism and is mainly associated with the scavenging of excess H_2O_2. As compared to NaCl, early induction observed in the transcript level of CAT1 and CAT3 (Table. 3.6 and 3.7) as well as in the activity of catalase (Fig. 3.10-A) suggest change in the early involvement of catalase-mediated signaling and antioxidant defense in NaCl+TU treated seeds. Besides, TU treatment also modulated the expression of genes related to hormonal signaling. In NaCl+TU treated seeds, the expression level of an auxin responsive gene (Aux/IAA), an AHP family gene (AHP2; a mediater of cytokinin signaling), OPR-3 (12-oxo-phytodienoic acid reductase; a mediater of jasmonate biosynthesis and signaling) as well as EREB (ethylene responsive element binding gene) was up-regulated, whereas in NaCl treated seeds, all these genes were down-regulated. These results imply that TU may coordinately regulate auxin, cytokinins, ethylene and jasmonate mediated signaling to impart the stress tolerance as well as to allow the plants to maintain their normal growth.

In the course of stress management, phenylpropanoid signaling pathway also plays an important role in the complex but integrated signaling network. Phenylalanine ammonia-lyase (PAL) catalyzes the first step of the phenylpropanoid pathway. The NaCl+TU treated seeds showed an increased expression of one of the *PAL* genes, *PAL1*

(Anterola and Lewis, 2002) and a homologue of *PAL*, *FAH1* (Ferulate 5-hydroxylases; Costa et al., 2003) as well as a higher PAL activity (both at 1 h and 6 h) than that observed under NaCl treatment. Both molecular and biochemical data, thus, indicate that TU treatment might lead to an accumulation of major flavonoids, which would assist in decreasing the extent of damage in NaCl+TU treated seeds. The salinity stress also leads to the generation of various metabolic byproducts that need to be detoxified in order to maintain the cellular homeostasis. This is achieved via their conjugation with glutathione by the activity of glutathione-S-transferases (GSTs) (Wagnor et al., 2002). In this direction, increased expression and activity of GSTs, during an early time of 1 h, indicated the stimulation of effective detoxification of any stress-induced byproducts, such as lipid peroxides from membrane damage, in NaCl+TU treatment as compared to NaCl treatment.

An interesting feature associated with most of the TU modulated transcripts was that they were either directly related to calcium signaling or were the potential targets of the calmodulin/calceneurin B-like proteins (Luan et al., 2002). This suggests that the action of TU might be dependent upon the process of calcium signaling. To confirm this, various molecular and biochemical responses were studied in response to $LaCl_3$ addition (a calcium channel blocker) to NaCl+TU. The Ca^{2+}-permeable channels are required to generate the $[Ca^{2+}]_{cyt}$ burst which is necessary for the activation of different cellular responses (Kaplan et al., 2006). Under $LaCl_3$ supplementation along with NaCl+TU treatment, the expression profile of few genes such as (CAT-1; EREBP and CAX-2) got reversed as compared to their expression in the absence of $LaCl_3$ (Fig. 7). Besides, the TU mediated increase in the level of ABA (Fig. 9) and in the activities of various

enzymes, such as catalase, PAL and GST (Fig. 10) were also not observed in presence of $LaCl_3$. However, the redox state remained more or less unaltered (Fig. 8). Thus, these findings together indicate that the effect of TU might be dependent upon the cytosolic Ca^{+2} burst *per se*.

3.8. CONCLUSION

The results imply that upon stress imposition, TU modulates various signaling pathways such as redox, calcium and ABA leading to activation of a yet unidentified "common alarm signal". This in turn coordinately regulates different processes to increase the capacity to combat the oxidative stress and to detoxify stress-induced accumulation of any byproducts which together helps the seeds to maintain their viability under salinity stress.

CHAPTER 4

Thiourea dependent modulation of water balance, redox state and antioxidant system to alleviate NaCl-induced oxidative damage in seedlings of *Brassica juncea*

Section I: Studies on the temporal regulation of aquaporins and its impact on the water-balance in roots of *Brassica juncea* seedlings.

4.1. Introduction

The redox state is considered as an important parameter that regulates the cellular response under stress and it is believed that if the reduced redox state is maintained, the extent of stress induced damage can be minimized (refer to Chapter-1\Section-1.5.3; Mittler, 2002). In this regard, our earlier studies established that the application of TU in *Brassica juncea* maintains the redox state and seeds soaked in TU modulate different signalling and effector components of salinity stress tolerance (refer to Chapter-3\Section-II; Srivastava et al., 2010a).

A primary effect of salinity stress is the creation of water deficit conditions for the plants due to generation of osmotic differences between cytoplasm and soil solution (refer to Chapter-1\Section-1.3.1). Plants have evolved several mechanisms to cope up these effects (refer to Chapter-1\Section-1.6.2). Aquaporins constitute a large family of membrane proteins showing high isoform multiplicity, which facilitate diffusion of water and small neutral solutes across cell membranes (Maurel and Chrispeels, 2001). They are classified into four groups, namely PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (nodulin-26 like intrinsic proteins) and SIPs (small intrinsic proteins) with a total of 35 homologues (Johanson et al., 2001); however different aquaporins have non-redundant functions (Javot et al., 2003). PIPs carry out the function of water uptake and transport and are further classified into two highly homologous subgroups, PIP1 (PIP1;1- PIP1;5) and PIP2 (PIP2;1 - PIP2;8). In recent years, the specific roles of different PIPs are being studied in plants to understand the

mechanisms of regulation of water movement into/outside and within plants. Aquaporin isoforms that are up- or down-regulated by drought and salt stress have been pinpointed in Arabidopsis, rice, barley, and maize (Jang et al., 2004; Kawasaki et al., 2001). Some studies demonstrated that suppression of PIPs reduced water stress tolerance in plants, while overexpression was found to be beneficial (Siefritz et al., 2002; Qiuju et al., 2005). On the other hand, studies showed that an increased expression level of PIPs decreased plants' tolerance to the stress (Aharon et al., 2003; Jang et al., 2007). Hence there is still a lot of debate on specific roles of PIPs. Nevertheless, studies do demonstrate that a complex regulation of different PIPs is required to perform water uptake/transport/loss from soil to root and to other organs in a coordinated manner under normal and stressed conditions (refer to Chapter-1\Section-1.6.2; Luu and Maurel, 2005). Considering the importance of water homeostasis in whole plant physiology, the present study was undertaken to study the differential expression profile of PIPs in *Brassica juncea* roots subjected to NaCl stress with/without TU to reveal the role of redox state in the regulation of water homeostasis.

4.2. Results

4.2.1. Differential growth response of the seedlings under different treatments

In order to demonstrate the effect of thiourea, the differential phenotype was measured in the seedlings subjected to various treatments such as distilled water (control); NaCl (350 mg/150 g of sand); NaCl (350 mg/150 g of sand)+TU (3 mg/150 g of sand) and TU (3 mg/150 g of sand) for 3 d. The data revealed differential growth pattern under different treatments. The stressed phenotype (NaCl) was characterized by

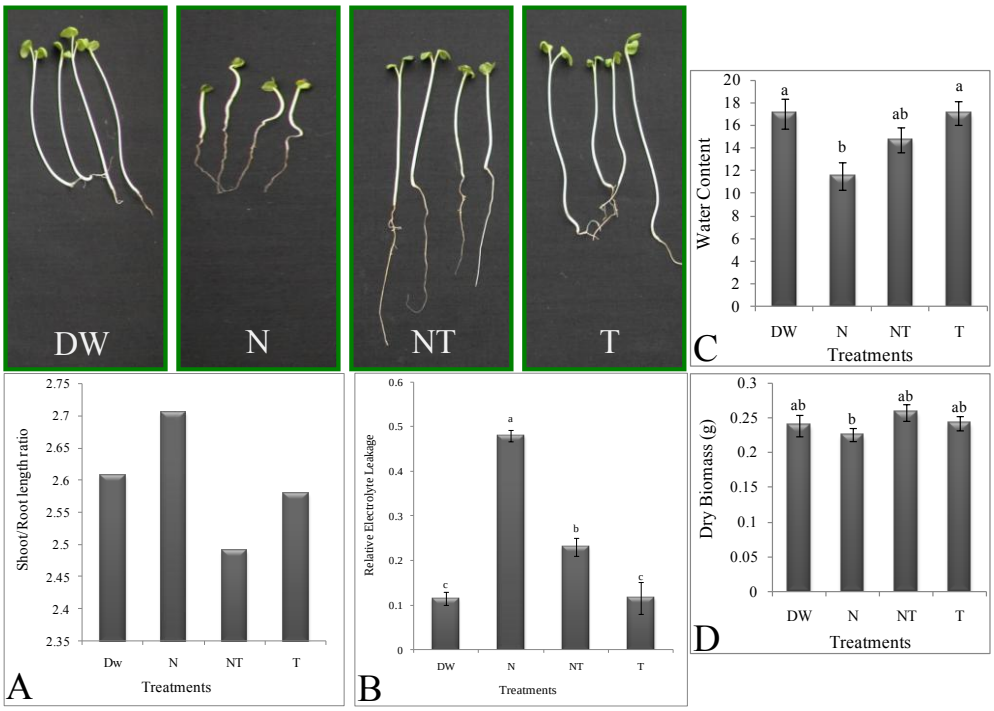


Fig. 4.1: Differential growth pattern of the seedlings under different treatments. The differential phenotype was observed in 10 d old *Brassica juncea* seedlings given different treatments for 3 d. Panel marked as DW, N, NT and T represents their growth pattern under distilled water, NaCl (350 mg/150 g of sand), NaCl (350 mg/150 g of sand)+TU (3 mg/150 g of sand) and TU (3 mg/150 g of sand) treatment respectively. The measurement of shoot/root length ratio (A); relative electrolyte leakage (B); water content (C) and dry biomass (D) was also performed in the same set of seedlings. The values represent the mean±S.D. of six technical and two biological replicates. The difference in mean value is found to be statistically significant in one-way ANOVA (p<0.01). Different letters indicate significantly different values at a particular duration (DMRT, $P < 0.05$).

diminutive growth; shoot/root length ratio increased by 4% while water content and dry biomass decreased considerably as compared to that of control. In NaCl+TU treatment, an increase in root length of the seedlings was observed and hence the shoot/root length ratio decreased by almost 5% as compared to the control. Besides, water content and dry biomass were also found almost at par with that of control (Fig. 4.1-A, C & D). All these data collectively indicated the potential role of TU in ameliorating the effect of salinity stress. These observations were also validated by measuring the relative electrolyte leakage, which was about 2-fold higher in NaCl than in NaCl+TU (Fig. 1-B). The different growth parameters of TU alone treatment were comparable to that of control (Fig. 4.1).

4.2.2 Measurement of ROS accumulation and intracellular pH in roots

In order to estimate the NaCl induced oxidative stress, the ROS accumulation was quantified by H_2DCFDA staining. The NaCl stress resulted in significant increase in ROS accumulation all along the axis of root. In contrast, the level of ROS in NaCl+TU and TU treatment was similar to that of control even after 90 min of treatment (Fig. 4.2-A). The typical time dependent change in root vacuolar pH under different treatments is represented in fig. 4.2-B. Under control and TU alone treatment, there was no significant time dependent change in chemical shift indicating a constant vacuolar pH. In NaCl treated roots, at 75 min, the vacuolar Pi signal shifted to downfield (~0.108 ppm change in chemical shift) which indicated vacuolar alkalization. After 75 min, it was not possible to measure the chemical shift because of the peak broadening which could be attributed to the leakage of Pi in different compartments under the severe NaCl stress. In contrast in NaCl+TU treated roots, although a transient change in the vacuolar pH towards alkalinity

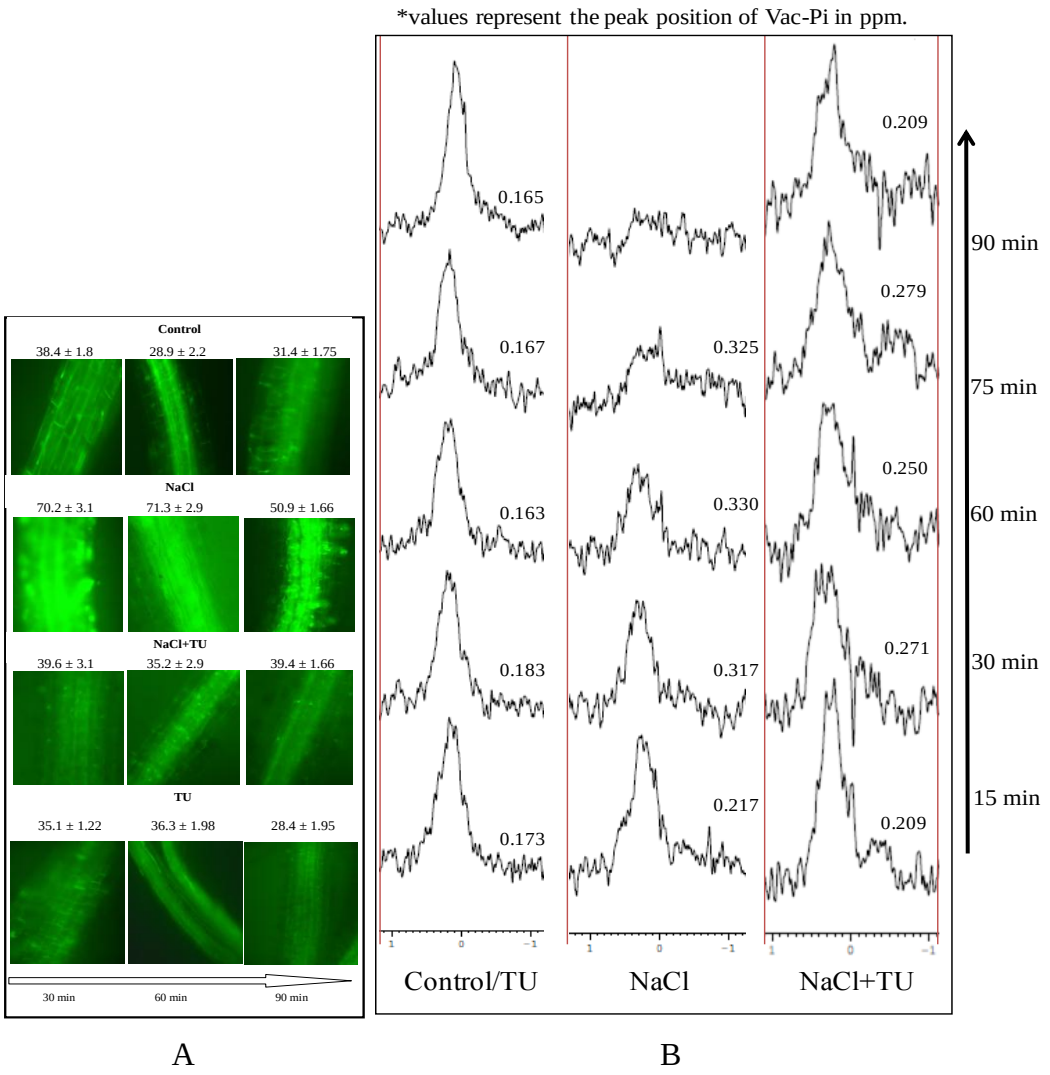


Figure 4.2: Measurement of ROS accumulation and vacuolar pH in roots. The 10 d old seedlings of *Brassica juncea* were subjected to different treatments such as distilled water control, NaCl, NaCl+TU and TU alone and then roots were harvested for the measurement of ROS accumulation [A; at different time point ranging from 30-90 min]. The numbers above the image represented the quantification of DCFDA staining with Image J 1.42q (W. Rasband) software in arbitrary units (mean ± SE). The measurement of vacuolar pH (B) was performed by *in vivo* ^{31}P-NMR; the chemical shift of the peak corresponding to the vacuolar Pi is represented along with each peak.

was observed at 45 minutes, almost complete recovery towards control pH value was observed in 90 min.

4.2.3. Short-term expression profiling of different aquaporin isoforms under salt treatment in roots

The temporal regulation of 13 PIP isoforms was studied by analyzing their expression level using quantitative real-time PCR. The details of the gene specific primers are mentioned in Table 4.1. In NaCl treated roots, at 30 min, the expression level of all isoforms of PIP-1 subgroup was increased with the maximum being observed for PIP-1;1 (3.8-fold upregulation as compared to control). Although the expression level of these isoforms showed a time dependent decrease, their level was found either above or close to that of control even at 90 min of stress treatment (Fig. 4.3). Unlike PIP-1 subgroup, the isoforms PIP-2 subgroup showed an opposite trend with significant downregulation at 30 min followed by an increase in expression with increase in time. At 90 min, significant upregulation was observed in PIP-2 isoforms except PIP-2;1, PIP-2;2 and PIP2;8. Unlike NaCl, in NaCl+TU treatment, all isoforms of both PIP-1 and PIP-2 subgroups were found to be downregulated on all time points (Fig. 4.3). However, on the basis of their temporal expression pattern, they could be putatively classified into two broad categories. The first putative category, PIP-L (L denoting the loss of water from roots to other tissues) included PIP-1;3, PIP1;4, PIP2;1, PIP2;4, PIP2;6 and PIP2;7. The isoforms of PIP-L category showed a time dependent decrease in the expression level and at 90 min, the maximum downregulation was observed. On the contrary, isoforms of the second putative category, PIP-U (U denoting

Table 4.1. Gene-specific primers used for the quantitative real-time PCR analysis of various PIP isoforms.

Gene Name	Primer sequence (5'→ 3')	Amplicon size
PIP1;1	Forward-tagcccggaagctgtcgcttacta	331
	Reverse-cctgtgccagtgattgggatggt	
PIP1;2	Forward-ccagcggttacgttcggtttgtt	305
	Reverse-ccgatagggagcggtgctagaat	
PIP1;3	Forward-tctggtttgggtgctgagataatcg	318
	Reverse-tggaatggctctgatgacaagttgg	
PIP1;4	Forward-ccctgctgtaacattcggtctgttc	355
	Reverse-ggtcccggtgatcggtattgttg	
PIP1;5	Forward-tgtggtggctcatggttacacaaag	216
	Reverse-ctggccgggttaatgccagttc	
PIP2;1	Forward-gccaccagctccgtttattgatg	345
	Reverse-gcacccaaacactgagcgattatg	
PIP2;2	Forward-tgattagggcggtgctttacatgg	462
	Reverse-tggaacctgaagcccttaggaca	
PIP2;3	Forward-attagggcggtgctttacatggtg	456
	Reverse-gcctgaggcccttagaacgaattg	
PIP2;4	Forward-ggcggcgtaggaatattgggtattg	292
	Reverse-caccgagtccggtacctttgttg	
PIP2;5	Forward-ctggtgggcatattaatccggcagt	458
	Reverse-acccacccagaatatccaatgatgg	
PIP2;6	Forward-tcggcctcctaggcatctcttg	360
	Reverse-agtcacgggcatttcgcttaggg	
PIP2;7	Forward-aacccaccatggaaaagactacgtg	301
	Reverse-ggccaagaacagaccgaaagtca	
PIP2;8	Forward-ctttggtggcatgatctttgtcctc	251
	Reverse-cagttccggtgctataaccatcagc	
Actin[†]	Forward-ctcctgccatgtatgtcgctatcc	155
	Reverse-aaggtccaaacgcagaatagcatgt	

	NaCl			NaCl+TU			TU		
	30 min	60 min	90 min	30 min	60 min	90 min	30 min	60 min	90 min
PIP1;1	3.876	0.997	0.594	-1.437	-1.18	-0.322	-0.037	0.115	-0.322
PIP1;2	2.837	0.257	0.057	-2.252	-2.175	-1.485	-0.025	0.165	0.451
PIP1;3	2.445	1.943	1.622	-0.79	-0.935	-1.544	-0.79	-0.987	-0.445
PIP1;4	1.289	0.788	0.893	-3.437	-5.678	-10.265	-0.337	-0.688	-0.256
PIP1;5	1.018	-0.27	0.065	-3.676	-2.556	-2.547	-0.026	-0.459	-0.541
PIP2;1	-1.911	-0.35	-0.033	-1.029	-1.59	-2.312	1.02	1.42	1.301
PIP2;2	-1.743	-0.901	-1.661	-3.065	-1.487	-1.762	-0.19	-0.466	-0.267
PIP2;3	-0.428	1.522	1.474	-1.846	-1.716	-1.645	-0.899	-0.752	-0.651
PIP2;4	-0.438	0.966	0.017	-0.862	-0.963	-2.55	-0.926	-0.652	-0.559
PIP2;5	-2.012	0.879	1.321	-1.715	-0.634	-0.034	1.11	1.641	1.043
PIP2;6	-0.477	1.737	2.147	-1.055	-1.291	-1.825	-0.055	1.211	0.925
PIP2;7	-0.4	1.235	2.385	-0.998	-1.155	-1.658	0.882	1.112	1.584
PIP2;8	-2.487	-1.482	-0.638	-2.824	-1.277	-1.17	1.621	1.211	1.141

Figure 4.3: Quantitative real-time PCR of various PIPs under short-term NaCl stress in roots of *Brassica juncea*. The 10 d old seedlings were subjected to different treatments such as distilled water control, NaCl, NaCl+TU and TU alone and then roots were harvested at different time points of 30, 60 and 90 min for the quantitative real-time PCR. The entire data is expressed in the terms of expression fold difference as compared to that of respective control. The green and red color designates the up and downregulated gene respectively, and the color intensity is directly proportional to the fold difference. The values represent the means value of three technical and three biological replicates and the differences in the mean values were found to be statistically significant at P, 0.01 (one-way ANOVA).

	NaCl			NaCl+TU			TU		
	Day-1	Day-2	Day-3	Day-1	Day-2	Day-3	Day-1	Day-2	Day-3
PIP1;1	-3.42	-5.99	-8.99	-0.18	-1.12	-2.15	-0.86	0.43	-2.23
PIP1;2	-2.22	-6.44	-8.28	-0.02	-1.8	-1.55	-1.23	0.18	-3.01
PIP1;3	-1.37	-4.56	-7.1	0.95	0.46	-0.96	-0.33	-0.39	-0.67
PIP1;4	-0.22	-2.15	-5.08	1.19	0.66	-1.6	-0.53	-0.55	0.92
PIP1;5	-5.53	1.8	-5.45	0.96	0.27	-0.98	-0.84	-0.07	0.39
PIP2;1	-7.84	-0.66	-8.8	-0.36	-0.31	-3.95	-0.93	1.16	-2.99
PIP2;2	-9.26	-2.27	-10.07	-0.24	-0.71	-3.21	-0.28	1.52	-2.49
PIP2;3	-2.62	-5.28	-7.74	-0.6	-0.83	-2.8	-0.92	0.47	-2.22
PIP2;4	-1.71	-1.77	-10.17	0.88	1.2	-2.91	0.54	0.57	-3.03
PIP2;5	0.44	-1.7	-6.86	2.93	1.25	-2.35	-1.77	-0.39	-0.36
PIP2;6	7.57	-1.81	-4.49	2.6	2.44	-0.98	0.61	1.18	6.17
PIP2;7	-0.12	-0.23	-3.1	0.66	1.02	-0.84	0.45	-0.76	3.71
PIP2;8	0.32	-5.31	-7.57	0.5	0.71	-2.38	-0.32	0.39	-4.51

Figure 4.4: Quantitative real-time PCR of various PIPs under long-term NaCl stress in roots of *Brassica juncea*. The 10 d old seedlings were subjected to different treatments such as distilled water control, NaCl, NaCl+TU and TU alone and then roots were harvested at different time points of 1, 2 and 3 day for the quantitative real-time PCR. The entire data is expressed in the terms of expression fold difference as compared to that of respective control. The green and red color designates the up and downregulated gene respectively, and the color intensity is directly proportional to the fold difference. The values represent the means value of three technical and three biological replicates and the differences in the mean values were found to be statistically significant at P, 0.01 (one-way ANOVA).

the uptake of water from outside), which included PIP-1;1, PIP-1;2, PIP-1;5, PIP-2;2, PIP-2;3, PIP-2;5 and PIP-2;8 showed time dependent increase in the expression level and at 90 min, their level approached towards that of control. Thus, on the basis of expression profile of aquaporins, differing in stress-specific and time-dependent manner, the putative functions to different PIP isoforms for the uptake or loss of water under salt stress could be assigned.

4.2.4. Long-term expression profiling of different aquaporin isoforms under salt treatment in roots

The expression profiling of all the 13 PIPs was also performed for longer time point (from 1-3 d) in roots subjected to different treatments. The data obtained indicated that, at 3 d time point, the expression level of all isoforms of PIP-1 and 2 subgroups was downregulated in both NaCl and NaCl+TU treatment; however, the extent of downregulation in NaCl was much more than that of NaCl+TU treatment. At 1 and 2 d, the level of most of the PIP isoforms was down- and upregulated in NaCl and NaCl+TU treatments, respectively (Fig. 4.4). In contrast with short-tern, the long term study does not revealed any differential expression pattern in aquaporin isoform associated with putative PIP–L and –U category.

4.3. Discussion

Water constitutes one of the most important requirements for the survival and growth of plants and hence water homeostasis demands highly coordinated regulation under normal and stressed conditions. In order to study the possible involvement of water balance in thiourea (TU) mediated NaCl-stress tolerance, the expression profile of 13 PIP isoforms was studied in roots of *Brassica juncea*. The study was performed on roots as it

is the first organ which comes in direct contact with the stress. The data obtained showed that the expression level of most of the PIPs (of both PIP-1 and PIP-2 subgroups) remained up-regulated under NaCl stress. The primary effect of NaCl stress would be an induction of significant osmotic stress and, therefore, an early increase in the expression level of PIPs could be considered as a typical stress response mechanism to support the maximum water uptake (Jang et al., 2004). However, 30% decrease in the WRA was found. In contrast to NaCl, under NaCl+TU treatment, expression level of different PIPs decreased on all time points. This was accompanied by only 11% decline in roots WRA. These contrasting results might be explained by the fact that although an increased expression of PIPs would enhance water uptake from soil, this would also increase the symplastic water transport from roots to other tissues and *vice versa* (Siefritz et al., 2002). Hence, an upregulation of PIPs might prove to be negative due to significant symplastic water movement resulting in lower root WRA while their downregulation might prove to be beneficial, particularly under stress conditions when there is a greater need to retain water (Aharon et al., 2003). Similarly, Katsuhara et al. (2003) found that overexpression of a barley aquaporin gene (PIP2;1) increased root hydraulic conductivity in transgenic rice (*Oryza sativa*) plants but reduced root and shoot growth under salt stress. It is a proven fact that water uptake from roots is regulated by the rate of transpiration and that the rate of water uptake controls the stomatal density and hence the transpiration (Aharon et al., 2003). Thus, the higher level of aquaporins would not only facilitate more water uptake but might also positively enhance the rate of transpiration and, hence would interfere with plants's response to reduce transpiration to avoid water loss. On the contrary, decreased expression level of aquaporins might augment the signal

to reduce the rate of transpiration in addition to a decline in symplastic water movement. However, this needs to be evaluated further.

PIPs of the putative PIP-L category showed a time dependent decrease in their expression level in NaCl+TU treatment. Of these, PIP1;4 demonstrated the maximum downregulation after 90 min (10-fold downregulated). This is in contrast with NaCl treatment where, at the same time, its level was slightly higher than that of control. This suggests the importance of PIP-1;4 in regulating the loss of water under stress. In a previous study, transgenic Arabidopsis plant over-expressing PIP1;4 were found to have retarded germination and growth as compared to that of wild-type plants under water deficit condition (Jang et al., 2007). Further, the transcriptional modulation of various PIPs occurred in a coordinated manner in NaCl+TU treated roots. For example, at 30 min, the expression of PIP-2;2 was almost 2-fold lower in NaCl+TU as compared to that in NaCl treatment. However due to time dependent increase in its expression, at 90 min, its level was almost similar in both NaCl and NaCl+TU treatment. PIP-2;2 is considered as the most abundant aquaporin isoform contributing significantly towards soil water uptake (Javot et al., 2003). The initial downregulation of PIP2;2 occurred to limit the water loss during the early stage and assisted subsequent water uptake at the later stage in NaCl+TU treatment to maintain the water homeostasis. This is further validated by the higher expression level of different PIPs under long-term treatment in NaCl+TU as compared to that of NaCl stress (Fig. 4.4). The TU mediated coordinated transcriptional downregulation restricted the soil water uptake as well as the symplastic water transport at an early stage (till 90 min) and at the same time allowed the plants to uptake water at the later stage (till 3 d). This could be an important defense mechanism to prevent the fast

wilting under high degree of salt stress. Since, the earlier results showed that TU treatment restricts the stress induced change in the redox homeostasis (refer to Chapter-3\Section-II; Srivastava et al., 2010a), the TU mediated regulation of different aquaporin isoform can thus also be visualized as a redox dependent phenomenon.

Plant water homeostasis and regulation of aquaporins have been suggested to be closely linked to the production of ROS (Foyer and Noctor, 2005b); hence the extent of ROS accumulation was measured using DCFDA to see whether an improved water status under TU supplementation was also associated with reduced ROS levels. As evident in fig. 4.2-A, the TU treatment was positive in controlling the extent of ROS production under stress. The root cell water permeability is also regulated by the pH gradient between neutral/slightly basic cytoplasm (pH: 7.2) and acidic vacuole (pH: 4.9). The disturbance in the ΔpH due to the vacuolar alkalinization and cytosolic acidosis (due to the catalytic activity of tonoplast Na^+/H^+ antiporters; refer to Chapter-1\Section-1.2.1) is another factor that limits the root water uptake (Tournaire-Roux et al., 2003). The data of *in vivo* NMR showed that at 30 min, vacuolar alkalinization occurred both in NaCl and NaCl+TU treated roots that might have resulted into cytosolic acidosis and negatively affected the root water uptake through the gating effect on aquaporins (Fischer and Kaldenhoff, 2008). However, the continuous time dependent increase in the extent of vacuolar alkalinization indicates the uncontrolled gating effect of aquaporins in NaCl as compared to that in NaCl+TU, where ΔpH was found to get recovered towards control with time. At 90 min, the loss of NMR signal also indicated about the loss of function of tonoplast in roots under NaCl treatment. Thus, apart from regulating the aquaporins

expression at the transcriptional level, TU treatment also positively controlled their gating indirectly through maintenance of ΔpH gradients.

4.4. CONCLUSION

The various events that occur upon TU supplementation would be the TU mediated control of ROS production that signals to downregulate the aquaporins in a coordinated manner to reduce the loss of water under NaCl stress. The maintenance of ΔpH between cytosol and vacuole was another factor involved in the control of water homeostasis upon TU supplementation under NaCl stress. However, further study in this direction is needed to pinpoint how the TU mediated regulation of aquaporins occurred at the genetic and biochemical level and to find the exact role of each PIP isoform in the integrated function of water homeostasis.

Section II: Thiourea mediated regulation in the redox state and antioxidant responses to reduce the NaCl-induced oxidative damage.

4.5. Introduction

One of the major effects of salinity stress is uncontrolled production of different ROS (refer to Chapter-1\Section-1.4.2). The ROS produced might change the redox state towards the more oxidized end which proves to be unfavorable for plant growth (Mittler, 2002; Pfannschmidt et al., 2009). Another facet of ROS is that they are also involved in the signaling mechanisms (Miller et al., 2009). Thus, proper understanding of both the components of oxidative metabolism that result in ROS production (prooxidant) and dismutation (antioxidant) is important. The disturbance in the balance between pro- and antioxidant processes are the major reason behind the NaCl-stress induced oxidative damage. Thiourea (TU), a redox active molecule, can coordinate these processes to prevent the increase in ROS level. This might prove positive in reducing the oxidative damage under stress. In order to study this, different redox and antioxidative responses were studied in *Brassica juncea* shoots subjected to NaCl stress with/without TU treatment.

4.6. Results

4.6.1. The level of ROS, lipid peroxidation and radical scavenging activity under different treatments

The biochemical analysis showed that the levels of superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) increased significantly in NaCl treatment as compared to control, whereas their levels remained, in general, close to control values in NaCl+TU and TU alone treatments (Fig. 4.5-A and B). These results were also validated by the

histochemical staining for $O_2^{\bullet-}$ and H_2O_2 (Fig. 4.6-A and B). The DPPH radical scavenging activity (in terms of %RSA) decreased under NaCl treatment beyond 1 d as compared to control, whereas it remained significantly higher than control on all time points in NaCl+TU treatment (Fig. 4.5-C). The level of malondialdehyde (MDA) also showed a significant increase in NaCl treatment until 2 d, whereas no significant effect was noticed in NaCl+TU treatment (Fig. 4.5-D).

4.6.2. The activities of pro-oxidant enzymes

At 1 d, glycolate oxidase (GO) activity increased significantly in all the treatments in comparison to control with the maximum being in NaCl+TU. After 1 d, a time dependent decrease in GO activity was seen, and at 3 d, no significant change in GO activity was observed in any treatment except the significant decline in NaCl+TU (Fig. 4.7-A). The activity of ascorbate oxidase (AO) demonstrated a significant increase in comparison to control only at 3 d under NaCl treatment, whereas on all durations in NaCl+TU treatment (Fig. 4.7-B).

4.6.3. Thiourea mediated modulation of NADP/NADPH, NAD/NADH and ATP/ADP ratios

The ratio of NADP/NADPH demonstrated significant decline under NaCl treatment at 1 d and 3 d, whereas significant increase was noted in NaCl+TU treatment beyond 1 d, as compared to that of control (Fig. 4.8-A). The ratio of NAD/NADH showed no significant change at 1 d in any treatment. After 1 d, NAD/NADH ratio declined significantly in NaCl treatment as compared to control, whereas it increased significantly in NaCl+TU treatment (Fig. 4.8-B). The ratio of ATP/ADP showed a

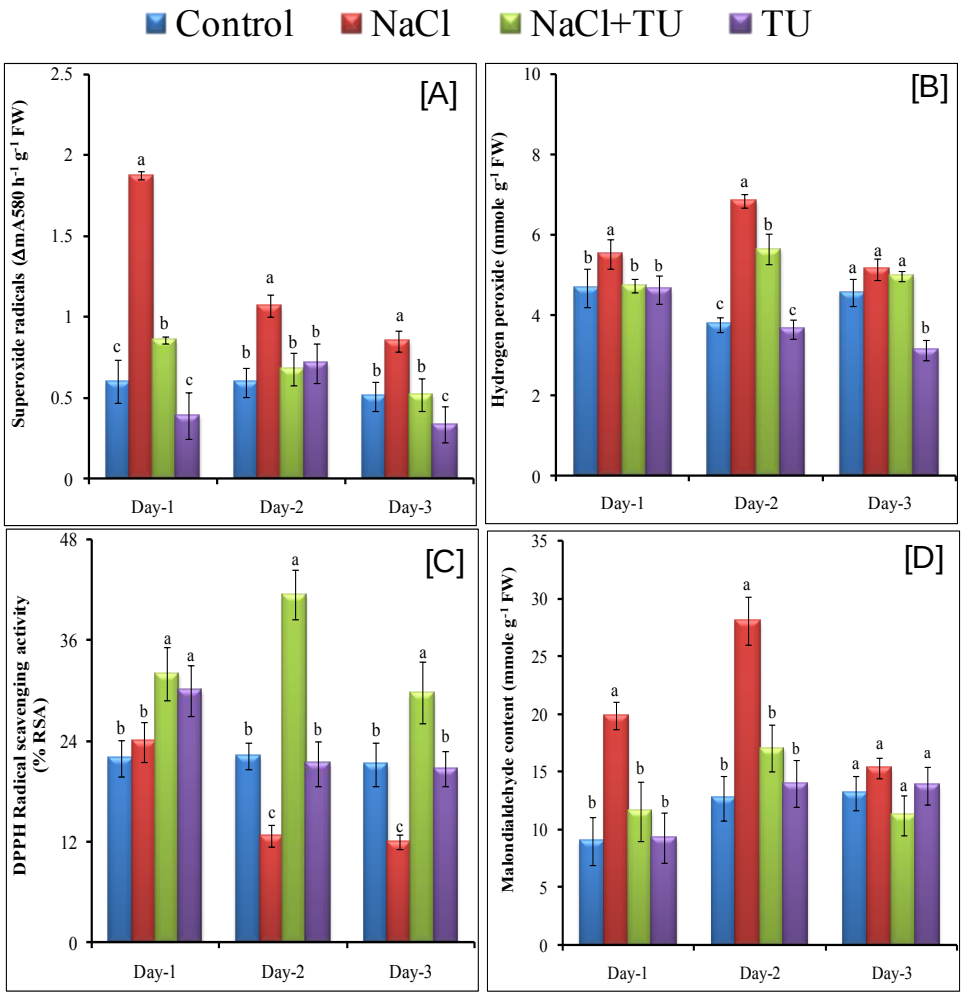


Fig. 4.5: Biochemical analysis of oxidative damage. Time dependent measurement of different ROS concentration [superoxide radical (A) and H_2O_2 (B)] and MDA content (C) was performed in 10 d old seedlings given different treatments such as NaCl; NaCl+TU; and TU alone. Control seedlings were not given any stress. All the values represent the mean of three technical and three biological replicates. Different letters indicate significantly different values at a particular duration (DMRT, P <0.05). The detailed information about the stress treatment condition is mentioned in chapter-2\section 2.3.

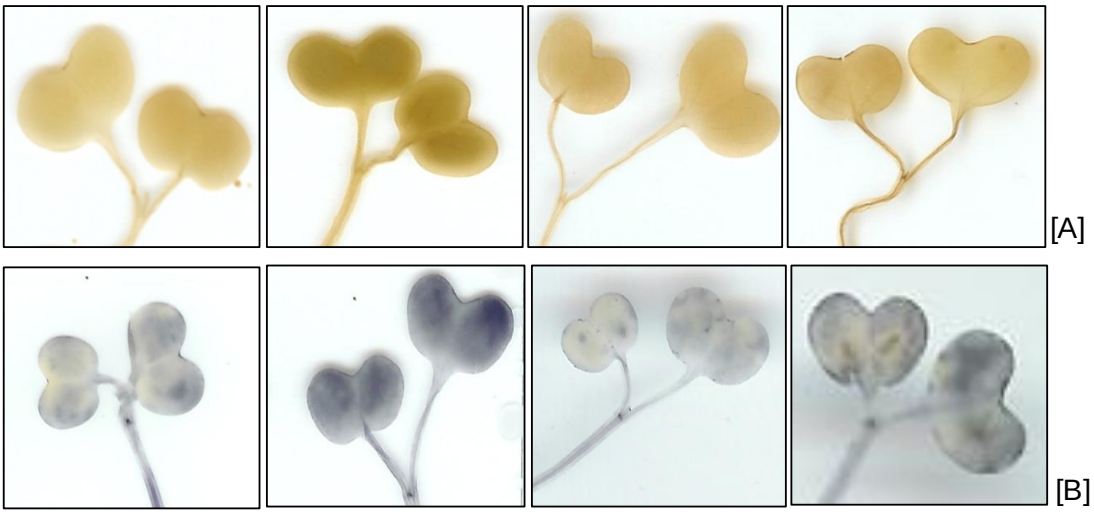


Fig. 4.6: Histochemical detection of H_2O_2 and $O_2^{\bullet-}$ radical. The staining of DAB (A) and NBT (B) was performed for H_2O_2 and $O_2^{\bullet-}$ respectively in plants which were given different treatments for 2 days. Experiments were repeated at least six times with similar results. The detailed information about the stress treatment condition is mentioned in chapter-2\section 2.3.

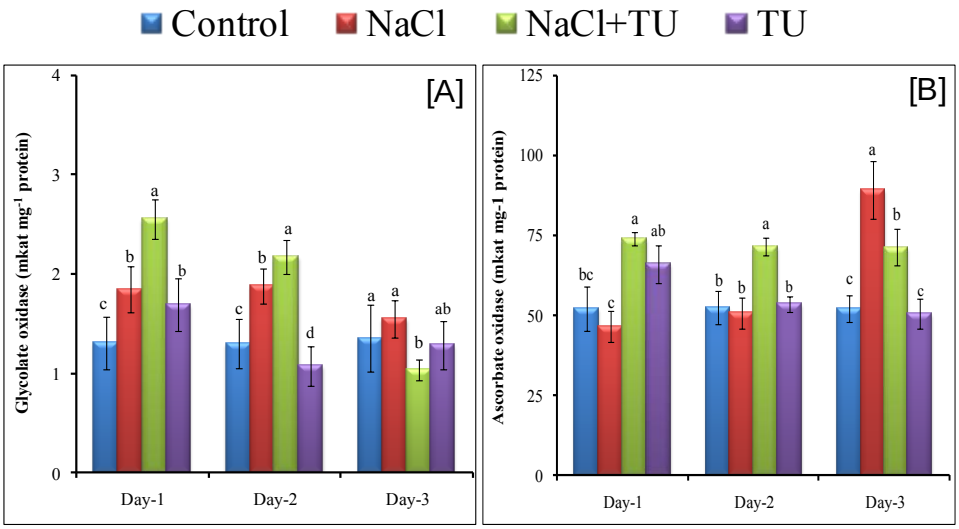


Fig. 4.7: Measurement of activities of different oxidases. Time dependent measurement of glycolate oxidase [GO (A)] and ascorbate oxidase [AO (B)] activity was performed in 10 d old seedlings given different treatments such as NaCl; NaCl+TU and TU alone. Control seedlings were not given any stress. All the values represent the mean of three technical and three biological replicates. Different letters indicate significantly different values at a particular duration (DMRT, P <0.05). The detailed information about the stress treatment condition is mentioned in chapter-2\section 2.3.

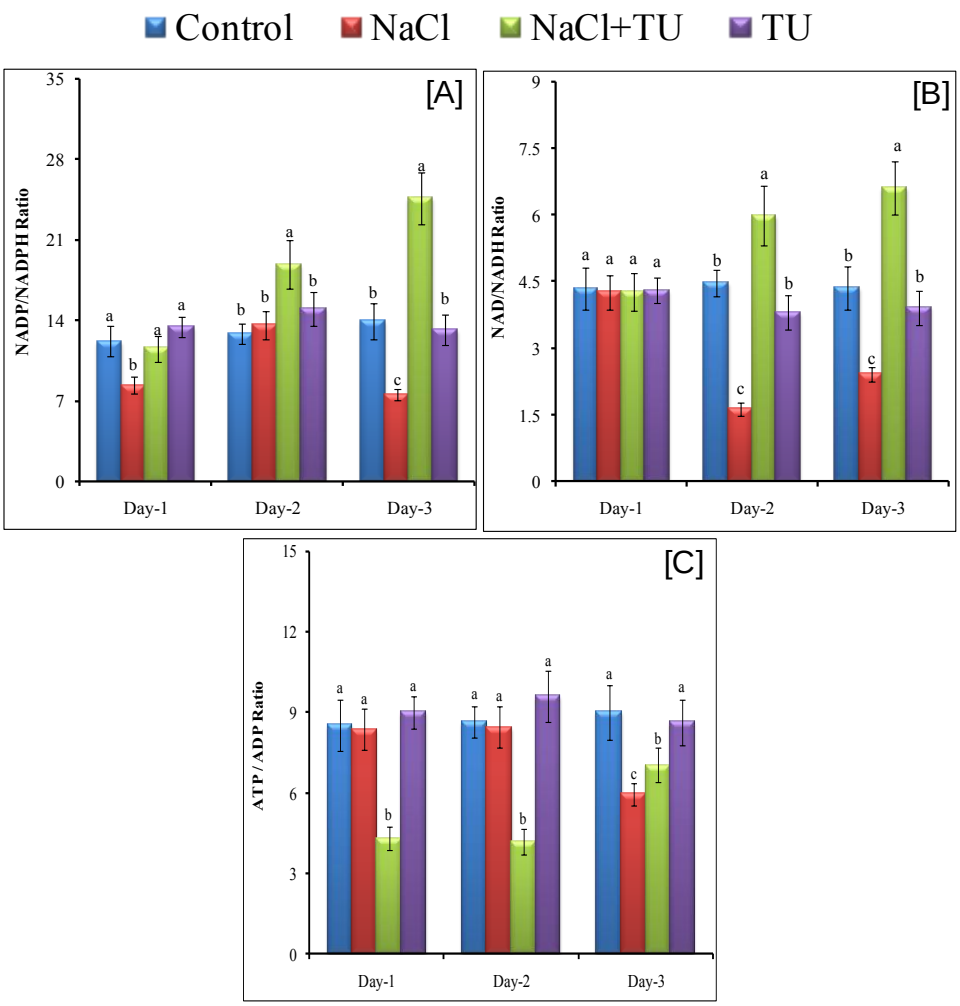


Fig. 4.8: HPLC based determination of pyridine and adenine nucleotides. The ratio of NADP/NADPH (A); NAD/NADH (B) and ATP/ADP (C) was determined by HPLC in 10 d old seedlings given different treatments such as NaCl; NaCl+TU and TU alone. Control seedlings were not given any stress. All the values represent the mean of three technical and three biological replicates. Different letters indicate significantly different values at a particular duration (DMRT, P <0.05). The detailed information about the stress treatment condition is mentioned in chapter-2\section 2.3.

significant decline only at 3 d in NaCl treatment, whereas on all durations in NaCl+TU treatment (Fig. 4.8-C). The ratios of all three couples remained near to control in TU alone treatment.

4.6.4. Modulation in the level of GSH and ASC and ratios of GSH/GSSG and ASC/DHA

The level of reduced glutathione (GSH) was increased significantly in NaCl and NaCl+TU treatments on all durations with a higher level of GSH in NaCl+TU (Fig. 4.9-A). However, the ratio of reduced to oxidized glutathione (GSH/GSSG) declined significantly on all durations in NaCl treatment in comparison to control, whereas at 3 d only in NaCl+TU treatment (Fig. 4.9-B). In TU alone treatment, the level of GSH (Fig. 4.9-A) remained, in general, at par to control except for GSH at 2 d, however, the ratio of GSH/GSSG showed significant increase until 2 d (Fig. 4.9-B). The level of ASC increased significantly under NaCl and NaCl+TU treatments but remained similar to control level in TU alone treatment on all durations (Fig. 4.9-C). However, the ratio of reduced to oxidized ascorbate (ASC/DHA) did not show a significant change in any treatment (Fig. 4.9-D).

4.6.5. Differential responses of antioxidant enzymes

In NaCl treated seedlings, superoxide dismutase (SOD) activity demonstrated a significant decline beyond 1 d in comparison to control, whereas in NaCl+TU treatment, the activity was found to increase significantly on all durations except at 3 d (Fig. 4.10-A). In contrast to the response of SOD, the activity of catalase (CAT) increased

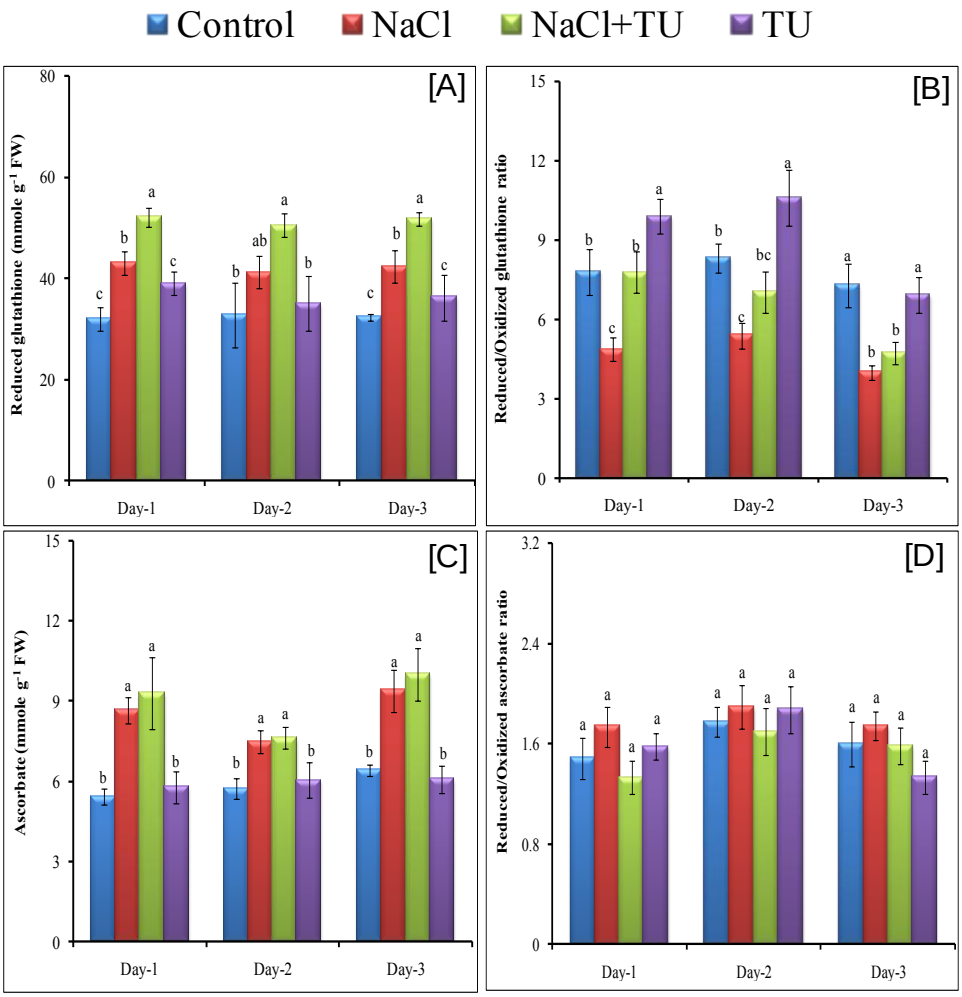


Fig. 4.9: Measurement of GSH/GSSG ratio and ascorbate content. Time dependent measurement of GSH content (A) and ratio of GSH/GSSH (B) and ascorbate content (C)] was performed in 10 d old seedlings given different treatments such as NaCl; NaCl+TU; and TU alone. Control seedlings were not given any stress. All the values represent the mean of three technical and three biological replicates. Different letters indicate significantly different values at a particular duration (DMRT, P <0.05). The detailed information about the stress treatment condition is mentioned in chapter-2\section 2.3.

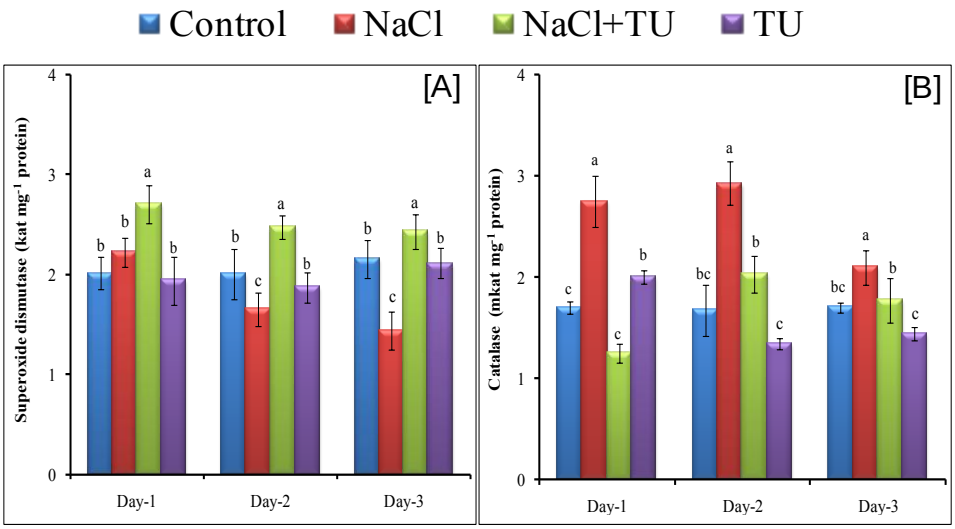


Fig. 4.10: Measurement of different antioxidant enzyme activities. Time dependent measurement of superoxide dismutase [SOD (A)] and catalase [CAT (B)] activity was performed in 10 d old seedlings given different treatments such as NaCl; NaCl+TU and TU alone. Control seedlings were not given any stress. All the values represent the mean of three technical and three biological replicates. Different letters indicate significantly different values at a particular duration (DMRT, P <0.05). The detailed information about the stress treatment condition is mentioned in chapter-2\section 2.3.

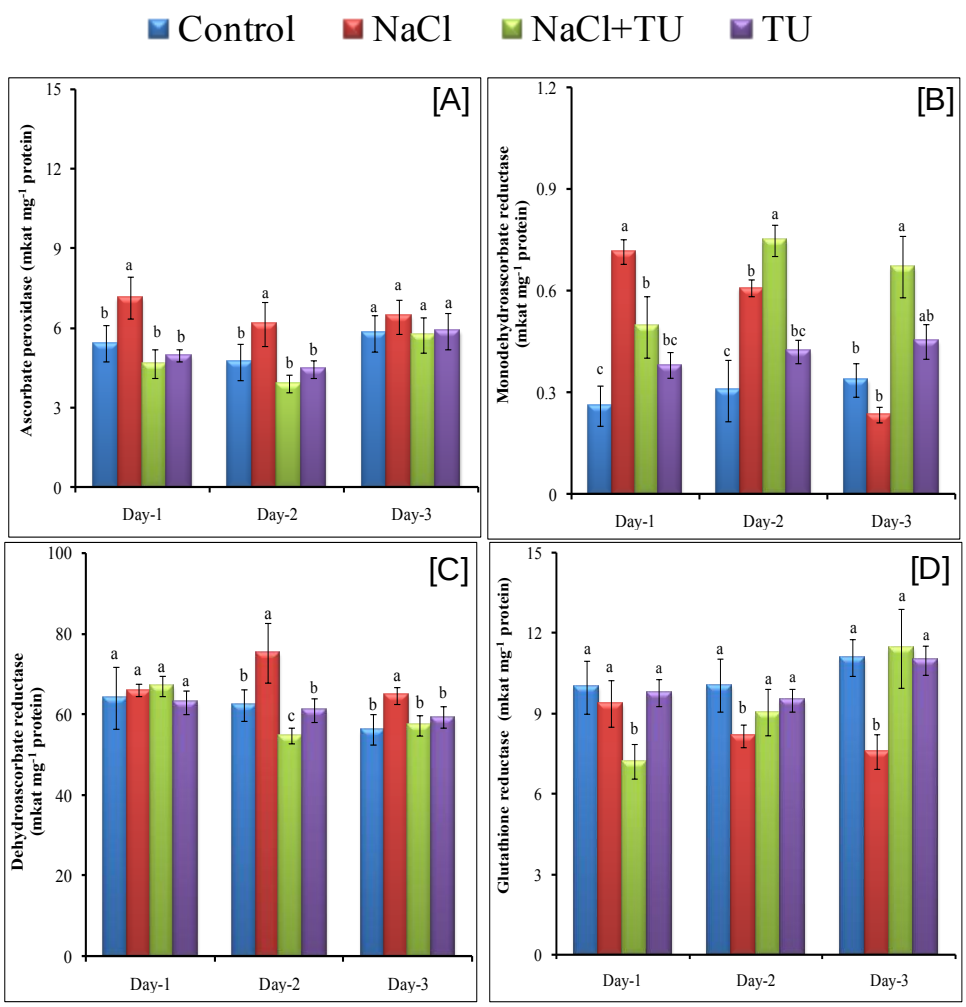


Fig. 4.11: Measurement of enzymes activities involved in ascorbate-glutathione cycle. Time dependent measurement of ascorbate peroxidase [APX (A)]; monodehydroascorbate reductase [MDHAR (B)]; dehydroascorbate reductase [DHAR (C)] and glutathione reductase [GR (D)] activity was performed in 10 d old seedlings given different treatments such as NaCl; NaCl+TU and TU alone. Control seedlings were not given any stress. All the values represent the mean of three technical and three biological replicates. Different letters indicate significantly different values at a particular duration (DMRT, P <0.05). The detailed information about the stress treatment condition is mentioned in chapter-2\section 2.3.

significantly higher than control on all durations in NaCl treatment, whereas it did not show significant changes in NaCl+TU and TU alone treatments except at 1 d where TU alone treatment showed a significant increase in CAT activity (Fig. 4.10-B).

Under NaCl stress, the activities of ascorbate peroxidase (APX) (Fig. 4.11-A) and monodehydroascorbate reductase (MDHAR) (Fig. 4.11-B) demonstrated significant increase as compared to control until 2 d but did not change significantly at 3 d. In contrast, in NaCl+TU treatment, APX activity did not show any significant change, whereas MDHAR activity increased significantly in comparison to control on all durations. Both APX and MDHAR did not show any significant change in TU alone treatment. The activity of dehydroascorbate reductase (DHAR) showed significant increase in NaCl treatment beyond 1 d, whereas it did not exhibit any significant alteration in NaCl+TU and TU alone treatments except at 2 d in NaCl+TU (Fig. 4.11-C). The activity profile of glutathione reductase (GR) showed a contrasting time dependent pattern in NaCl and NaCl+TU treatments. At 1 d, NaCl treatment caused no significant change in GR activity but produced significant decline beyond 1 d. In contrast, in NaCl+TU treatment, GR activity declined significantly at 1 d but thereafter remained at par to control. No significant change in GR activity was observed in TU alone treatment (Fig. 4.11-D).

4.6.6. Accumulation of Na^+ and K^+ ions

The data on ion analysis showed that the Na^+ ion content (Fig. 4.12-A) as well as the Na^+/K^+ ratio (Fig. 4.12-B) were significantly higher in NaCl and NaCl+TU treated seedlings as compared to that of control. However, the extent of ion accumulation was lower in NaCl+TU than that of NaCl treatment. At 3 d, in NaCl+TU treated seedlings, the

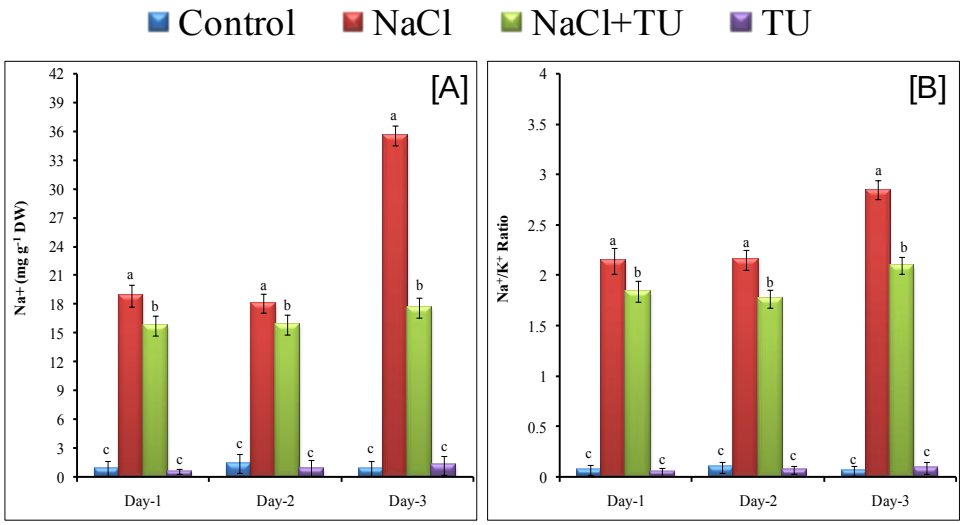
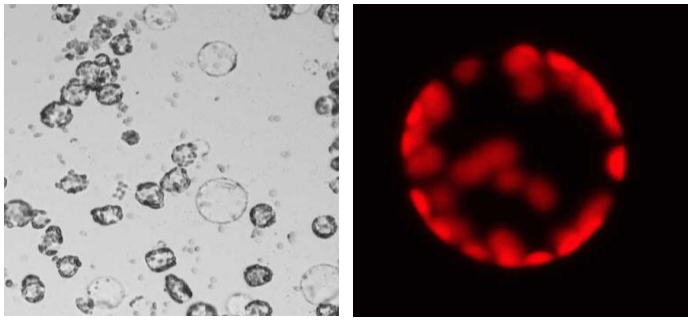


Fig. 4.12: Measurement of Na^+ content and Na^+/K^+ ratio. Time dependent measurement of Na+ content [A] and Na+/K+ ratio [B] was performed in 10 d old seedlings given different treatments such as NaCl; NaCl+TU and TU alone. Control seedlings were not given any stress. All the values represent the mean of three technical and three biological replicates. Different letters indicate significantly different values at a particular duration (DMRT, P <0.05). The detailed information about the stress treatment condition is mentioned in chapter-2\section 2.3.



A B

Fig. 4.13: Binding study of FITC-TU on isolated root protoplast. The intact protoplast was isolated from the leaves of 10 d old Brassica seedlings and incubated with FITC-TU for 1 h. The panel-A and -B shows the representative image of protoplast under bright field (10X) and fluorescence (100 X) microscope, respectively.

level of Na^+ ion was 50% less than that of NaCl treatment. The status of ions in TU alone treatment was similar to that of control.

4.6.7. Binding study of FITC-TU on isolated protoplast

The binding of the TU on the plant cell was studied using the FITC-TU. The FITC-TU was incubated with isolated plant protoplast for 1 h and then fluorescence microscopic data was recorded. The result indicated that TU binds at the level of plasmamembrane (Fig. 4.13). However, further studies are underway with ^{35}S-TU to further validate the findings.

4.7. Discussion

The present experiment was performed to understand the mechanism of TU-imparted salinity stress tolerance, which was earlier demonstrated at the field level (Sahu et al., 2005; Sahu et al., 2006). A preliminary analysis of differential phenotype of the seedlings re-confirmed that TU alone treatment per se did not impose any negative impact on the seedling growth and also that its supplementation to NaCl allowed the seedlings to maintain growth similar to that of control plants (refer to Chapter-4\Section-I; Srivastava et al., 2011). Apart from this, the comparative evaluation also showed that the biochemical responses in TU alone treatment were almost at par to that of control.

The series of events leading to the perturbation of cellular metabolism under NaCl stress are suggested to be as follows: less water availability, stomata closure, altered gaseous exchange, inhibition of photosynthesis, effect on electron flow in ETC in chloroplast and mitochondria, increase in the production of ROS and disturbed status of adenine (ATP) and pyridine nucleotides (NADH, NADPH) [Munns and Tester, 2008; Miller et al., 2009]. Hence, the imposition of oxidative stress and altered energy reactions

caused by ETC over-reduction affect general metabolism and growth. The results of ROS and MDA in the present study also suggested towards the built-up of oxidative stress under NaCl stress, whereas a significant alleviation of the stress was noticed upon TU supplementation to NaCl (Fig. 4.5 and 4.6). At 3 d, MDA level was found at par to control in NaCl treatment also. This can be attributed to altered composition of polyunsaturated fatty acids (PUFA) in the membranes due to salinity stress imposition since it has been suggested that a decline in PUFA under stress may lead to a decline in MDA level (Gupta et al., 1996). Further, it has been suggested that the level of fatty acid unsaturation is correlated to tolerance and a sensitive plant/phenotype is often related to a decline in PUFA (Upchurch, 2008). The results of ROS and MDA indicated that upon TU supplementation either ROS scavenging activity was significantly stimulated as a whole or the processes of production and dismutation of ROS were properly coordinated. The differential level of ROS was correlated with the DPPH radical scavenging activity and the data indicated that the %RSA was significantly higher in NaCl+TU than in NaCl treated seedlings (Fig. 4.5-C). Thiourea, itself, is well known as a direct scavenger of hydroxyl radicals, $O_2^{\bullet-}$ and H_2O_2 (Kelner et al., 1990; Gao et al., 2008). Further, the role of TU has also been demonstrated in competitively inhibiting the reduction of cytochrome c by the xanthine/xanthine oxidase superoxide-generating system (Kelner et al., 1990). This suggested that dismutation of ROS would have occurred at a comparatively higher rate in NaCl+TU than in NaCl treatment leading to the observed differences in oxidative stress. In our previous study, TU supplementation was found to maintain the functionality of seed mitochondria under high degree of salinity stress, which indicates that it might have also affected the rate of ROS production since

mitochondria are one of the prime sites for the ROS production (Srivastava et al., 2009). In addition, a time dependent decrease in Na^+ ion accumulation and an increased potential to retain the K^+ ion (as indicated by the Na^+/K^+ ratio) was noticed in NaCl+TU treated seedlings as compared to NaCl (Fig. 4.12), which would have also led to differential ROS production. The comparative analysis of Na^+-ion specific transporters and antiporters in NaCl and NaCl+TU treated roots is presently underway to unravel the exact mechanism of TU mediated decrease in Na^+ accumulation. The observed positive response of TU supplementation was due to combined effect at the level of ROS production (due to the decrease in the Na^+ ion accumulation) and dismutation (due to the improved antioxidative potential). To reveal this, various components of redox and antioxidant metabolism were further investigated.

In the seedlings subjected to NaCl stress, an over-reduction of ETC in chloroplasts and mitochondria apparently occurred as indicated by the significant decline in the ratios of NADP/NADPH and NAD/NADH, respectively. In contrast, no such negative impact occurred in NaCl+TU treatment as the ratio of NADP/NADPH and NAD/NADH demonstrated some increase. In our study, the role of TU supplementation in maintaining the water homeostasis has been demonstrated (refer to Chapter-4\Section-I; Srivastava et al., 2010b). Thus, TU supplementation might positively affect stomatal movement under stress through maintenance of water homeostasis and hence the gaseous exchange. However, this possibility needs to be investigated further. Under NaCl-stressed conditions, an increase in photorespiratory pathway has previously been observed as O_2 evolution decreases due to closure of stomata leading to higher oxygenase activity of Rubisco (Srivastava et al., 2008b). However, photorespiration would facilitate O_2

consumption, transport of reducing equivalents from chloroplast and allow energy dissipation (Foyer et al., 2009). An increase in GO activity might be a helpful strategy. In the present study, GO activity was stimulated significantly in both NaCl and NaCl+TU treatment, however to a higher level in NaCl+TU treatment and the activity came down with an increase in duration (Fig. 4.7-A). The alternative pathway to avoid NADPH accumulation was also increased to higher level under TU supplementation. Although there is a possibility that H_2O_2 level may also increase due to enhanced GO activity as there was no stimulation of CAT activity in NaCl+TU, but this H_2O_2 might have been taken care by antioxidants other than CAT viz., GSH and ASC (Corpus et al., 2001). Further, the ATP/ADP ratio was also lower in NaCl+TU as compared to NaCl treatment (Fig. 4.8-C) due to a greater ATP consumption to support various energy consuming tolerance mechanisms such as exclusion/sequestration (a lower ion accumulation is observed in this study) of the ions and synthesis of different osmolytes etc. All these analyses suggested that NaCl+TU treated seedlings faced a lower increase in ROS production as compared to that of NaCl treated seedlings. Further experiments were conducted to see whether seedlings also differed in the responses of ROS scavenging antioxidant processes as well.

Proper maintenance of cellular redox environment is crucial for plants and reduced redox state is favorable for growth (Mittler, 2002). The results of GSH and ASC showed that the values of GSH and GSH/GSSG were significantly higher in NaCl+TU than in NaCl treatment but the levels of ASC and ASC/DHA were similar in NaCl and NaCl+TU treatments. In plants, both GSH and ASC are present in very high concentrations and hence, they are the major regulators of the redox state and antioxidant

potential of plants (Noctor, 2006). Thus, the higher level of GSH might lead to significant difference in the antioxidant potential of plant. For example, both ASC and GSH need to be present in reduced state for the proper functioning of ASC-GSH cycle, an important pathway of H_2O_2 degradation, and thus, significant decline in GSH/GSSG ratio would negatively affect the operation of this cycle and ROS scavenging. The observed increase in the GSH content might be attributed to the stimulated biosynthetic reactions (Noctor, 2006). The inability of NaCl-treated seedlings to maintain GSH/GSSG ratio could also be linked to significant decline in GR activity beyond 1 d (Fig. 4.11-D).

Among the different antioxidant enzymes, the role of SOD as a determinant of salinity tolerance has been demonstrated (Hernandez et al., 2000). Thus, the significant increase in the SOD activity in response to NaCl+TU as compared to that of NaCl treatment appears to be another factor causing differential response of the seedlings to NaCl-induced oxidative perturbations. Further, the increased SOD activity in NaCl+TU treatment also occurred in conjunction with other positive reactions, such as increased NADP/NADPH ratio and an early activation of alternative salvage pathway (via GO) as compared to that of NaCl treatment. Thus, the TU supplementation performed a two-sided action: minimizing the extent of $O_2^{\bullet-}$ production and ensuring its efficient scavenging through the increased activity of SOD.

The product of SOD activity is H_2O_2, which is still toxic and must be eliminated. In plants, CAT and APX are of prime importance in regulating H_2O_2 levels, however these enzymes have different affinities for H_2O_2 [APX in μM range and CAT in mM range], which determine their functionality in different cellular organelles and in various stress situations (Mittler, 2002). In the present study, the H_2O_2 detoxifying enzymatic

mechanisms were not stimulated significantly in NaCl+TU treatment as, except MDHAR, the activities of CAT, APX, DHAR and GR were, in general, close to control values. This could be linked to the lower level of Na^+ ion in NaCl+TU treated seedlings, which in turn led to decreased H_2O_2 level and hence the increased activity of enzymes involved in its removal was not required. Upon TU supplementation, the detoxification of low level of H_2O_2 would have been efficiently managed through non-enzymatic detoxification by GSH and ASC, both of which showed significant stimulation upon TU supplementation. In NaCl+TU treatment, the activity of MDHAR was increased at all the time points as compared to that of control. This might be required for the regeneration of ASC being consumed in non-enzymatic detoxification reactions other than in the ASC-GSH cycle. To this end, significant increase in the activities of CAT, APX, MDHAR as well as DHAR in NaCl treatment stress appears to be a compensatory mechanism to scavenge the higher H_2O_2 levels. However, the activity of GR demonstrated a treatment duration dependent decline while the GSH/GSSG ratio declined significantly to lower levels than in the control at all time points determined. Hence, the proper functioning of ASC-GSH cycle would have been hampered by a lower availability of GSH in the reduced form in NaCl treatment. The critical role of GR has been proposed for determining the character of salt tolerance (Meloni et al., 2003). The TU-mediated modulation in the activities of different enzymes could be due to their direct interaction with TU or change in the level of their corresponding genes. The exact mechanism needs to be investigated in detail.

The TU-imparted stress tolerance might also be attributable to an early perception of the stress. In this perspective, possible role of AO, which is considered to play a

crucial part in signaling mechanisms related with stress tolerance (Yamamoto et al., 2005), was analyzed. In this study, AO activity was found to be stimulated significantly at an early stage (1 d) in NaCl+TU whereas in NaCl stress the comparable level of induction was observed only at 3 d (Fig. 4.7-B). An early induction of AO activity hints that TU treatment perhaps helps in quick sensing of the stress and hence, induction of different tolerance mechanisms at an early stage. However, the present results are in contrast with the previous observation, which suggested that a suppressed activity of AO induces salt tolerance in Arabidopsis plants through maintenance of ASC reduced state and low H_2O_2 levels (Yamamoto et al., 2005). The binding study performed with FITC-TU on isolated protoplast indicates its binding at the plasmalemma. The binding of TU at the plasmalemma may initiate downstream signaling leading to the activation of salt-tolerant mechanisms.

4.8. CONCLUSION

The study highlights the importance of reduced redox state (augmented by –SH containing compound; TU) as an intrinsic defense strategy to combat the NaCl stress induced oxidative damage in *Brassica juncea*. The TU supplementation along with NaCl stress allowed plants to channel their energy (ATP) for ion exclusion/avoidance leading to reduced ion accumulation and in effect the ROS load. In addition, ROS scavenging was coordinately regulated with stimulation of only the required components of antioxidant machinery. The positive effects of TU supplementation were also attributable to an early sensing of the NaCl stress.

CHAPTER 5

SUMMARY

Conclusion

The major findings of the present thesis are summarized as below:

1. The addition of TU protects the plants from the NaCl-stress induced damage. This was demonstrated both at the seed germination as well as the seedling stage.
2. The transcripts responsible for the differential response of seeds under NaCl stress with/without TU treatment were identified. The data obtained revealed the early and co-ordinated induction of ABA, redox and calcium based signalling and effector mechanism only in NaCl+TU treatment. TU was also shown to maintain the proper functioning of mitochondria. All these changes together help the seeds to cope with the NaCl-stress induced damage and thereby maintaining their viability and germination ability.
3. The ameliorative effect of TU under NaCl stress was also demonstrated at the seedling stage. The TU treatment differentially modulated the expression of aquaporins in a time dependent manner. This was positive in maintaining comparatively better root-water status in NaCl+TU as compared to NaCl treatment. Further, the NaCl+TU treatment was found to reduce the ROS load which helps the plants to maintain the reduced redox state more efficiently than in NaCl stress. This also curtailed the requirement to stimulate different antioxidants. Owing to this, plants were able to channel their energy towards Na^+-ion exclusion/avoidance resulting into the significant alleviation of NaCl-stress induced oxidative damage.
4. The effect of TU was mediated at the level of plasmalemma.

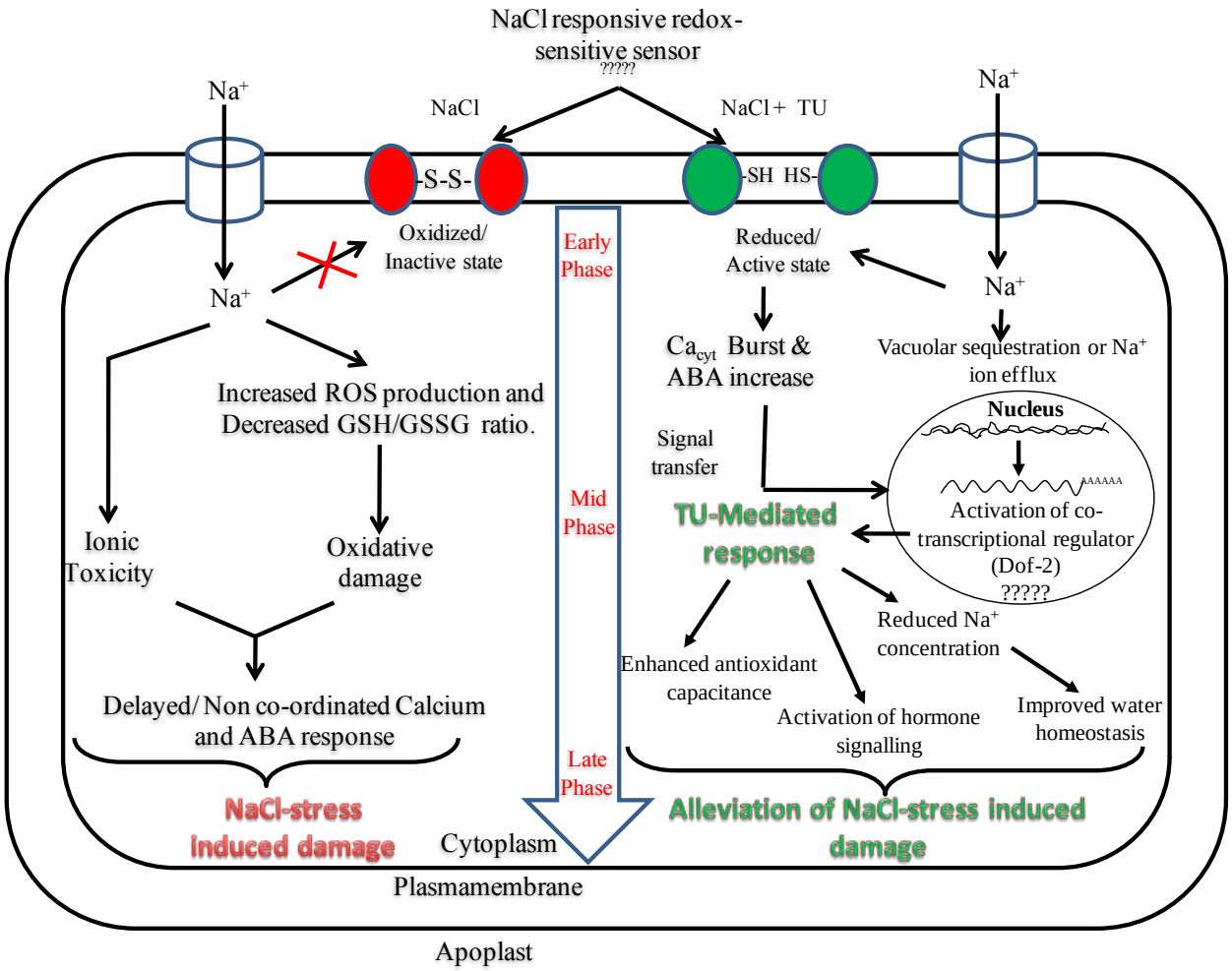


Fig. 5.1 Proposed mechanisms for thiourea mediated action.

Future directions

The proposed mechanism for the thiourea mediated action under salt-stress is depicted in Fig. 5.1. The finding proposes the central role of reduced redox state for the NaCl-stress perception and tolerance. Although there might be several sensors for the perception of NaCl-stress, the present results postulate that TU treatment maintains a specific redox-sensitive and NaCl stress-responsive sensor in the active form (-SH state) so that the NaCl-stress is perceived at an early time point and downstream signaling is activated in a coordinated manner. The signal is transduced to the nucleus through calcium/ABA signaling and results in the induction of some common regulator required for the activation of different protective mechanisms. The preliminary analysis of promoter elements in TU modulated NaCl-stress responsive transcripts indicated that they can be co-regulated by the transcription factor, Dof-2. However, this needs to be explored further to confirm the hypothesis. In the absence of TU, the redox-specific NaCl sensor may get inactivated (-S-S- state) due to the induction of oxidative stress in the apoplastic region. Consequent to delayed perception of NaCl through a different pathway, the coordinated induction of various responses is not achieved under NaCl alone stress. Further studies are required to identify the proposed redox-sensitive as well as alternate NaCl-specific sensors involved in the perception of NaCl stress. The present thesis also reveals various signaling and effector genes responsible for the regulation of salinity stress tolerance. In future, these genes could be used as candidate genes for the purpose of crop improvement. Activation of a gene or set of genes consequent to the exposure of a bioregulator (such as TU) could also leave epigenetic marks that may facilitates quicker and more potent responses under subsequent stress exposure. Further research is needed to exploit the concept of stress memory in sustaining crop productivity under challenged environments.

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LIST OF PUBLICATIONS

[A] Papers published in peer-reviewed International Journal

1. **Srivastava AK**, Srivastava S, Suprasanna P, D'Souza SF (2011) Thiourea orchestrates regulation of redox state and antioxidant responses to reduce the NaCl-induced oxidative damage in Indian mustard (*Brassica juncea* L.). Plant Physiology and Biochemistry 49, 676-686.
2. **Srivastava AK**, Ramaswamy NK, Suprasanna P, D'Souza SF (2010) Genome-wide analysis of thiourea modulated salinity-stress responsive transcripts in seeds of *Brassica juncea* L.: Identification of the signaling and effector components of stress tolerance. Annals of Botany (London) 106, 663-674.
3. **Srivastava AK**, Suprasanna P, Srivastava S, D'Souza SF (2010) Thiourea mediated regulation in the expression profile of aquaporins and its impact on water homeostasis under salinity stress in *Brassica juncea* roots. Plant Science 178, 517-522.
4. **Srivastava AK**, Ramaswamy NK, Mukopadhyaya R, Chiramal Jincy MG, D'Souza SF (2009) Thiourea modulates the expression and activity profile of mtATPase under salinity stress in *Brassica juncea* L. Annals of Botany (London) 103, 403-410.

[B] Reports

Srivastava AK, Ramaswamy NK, D'Souza SF (2008) Bioregulatory role of "thiourea" in multistress tolerance of the Indian mustard (*Brassica juncea*). Founder's Day special issue of **BARC News Letter** Issue No. 297.

[C] Abstracts presented in national and international conferences

1. **Srivastava AK**, Suprasanna P, Ramaswamy NK, D'Souza SF (2010) Molecular and biochemical investigation of thiourea mediated responses reveals various signaling and

effector components of salinity stress tolerance. [Poster presented in Cold Spring Harbour Symposium on "From Plant Biology to Crop Biotechnology" held at Suzhou, China during October 25-29, 2010].

2. **Srivastava AK,** Suprasanna P, Srivastava S, D'Souza SF (2009) Thiourea mediates regulation of water homeostasis and expression profile of different aquaporin isoforms in roots of *Brassica juncea* under salt and multistress. Accepted for Poster presentation in 9th International Plant Molecular Biology Congress held at St. Louis, USA during October 25-30, 2009.
3. **Srivastava AK,** Ramaswamy NK, D'Souza SF (2007) Rapid transcriptome changes induced by thiourea reveal the role of calcium and calmodulin related proteins in multistress tolerance of *Brassica juncea*. Poster Presented in International symposium on "Calcium based signaling systems in plants" held at Dublin, Ireland during 5th-7th December, 2007 {Received New Phytologist Trust award}.
4. **Srivastava AK,** Ramaswamy NK, D'Souza SF (2007) Regulatory role of mtATPase subunits of *Brrasica juncea* identified through differential display and quantitative Real-time PCR. Oral Presentation in International symposium on "Light and Life" held at University of Hyderabad 29th -31st August, 2007.

[D] <u>Gene Bank Submissions</u>

EU143655; EU143656; EE191543; EE111314; EE111313; FJ654732; GQ243695; GQ243696; GQ243697; GQ243698; GQ243700; GQ243701; GQ243702; GQ243703.