Chapter 5: Discussion

"The universe is full of magical things patiently waiting for our wits to grow sharper" - Eden Phillpotts
5. DISCUSSION

A common aspect of all abiotic and biotic stresses is the enhanced production of reactive oxygen species (ROS). These ROS, namely $O_2^-$ and OH oxidize cellular constituents such as lipids, proteins and nucleic acids and can initiate chain reactions triggering cellular apoptosis. However, at low concentrations, they participate in signaling events. Plants have highly regulated and controlled enzymatic and non enzymatic mechanisms to modulate their intracellular ROS concentrations to ensure minimum damage and optimal functioning. Enzymatic mechanisms include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase to counter balance the free radical production. Non enzymatic mechanisms include antioxidant molecules like ascorbate, glutathione and tocopherols. The balance between the enzymatic and the non enzymatic mechanisms determines the steady-state level of superoxide radicals ($O_2^-$) and hydrogen peroxide ($H_2O_2$). This balance, together with sequestering of metal ions, is thought to be important for the formation of highly toxic hydroxyl radical via the metal-dependent Haber-Weiss or the Fenton reaction (Haber and Weiss, 1934; Fenton, 1894, 1899). This integrated system prevents oxidative stress in general and is therefore a common component of response of plants to various kinds of stresses.

With this rationale, the present study focused on developing the transgenics in A. thaliana for the over expression studies of antioxidant genes (Cu/Zn-SOD, Mn-SOD and APX) singly as well as the preparation of double transgenics expressing both Cu/Zn-/Mn-SOD and APX genes in A. thaliana. These transgenics have been assessed (molecularly, physiologically and biochemically) for various abiotic stresses like NaCl, PEG, low temperature and heavy metal induced oxidative stress.

The genes, Cu/Zn-SOD and APX were isolated from Potentilla atrosanguinea and Rheum australe respectively, found in Western Himalayan region. Mn-SOD was isolated from Camellia sinensis growing at Palampur. The Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) homology data showed that these genes shared a degree of similarity with the respective native gene of A. thaliana. Cu/Zn-SOD from P. atrosanguinea (EU532614.1) had 80% homology (Expect = 5e-83) with the cytosolic At Cu/Zn-SOD (NM_100757.3), Mn-SOD from C. sinensis (AY641734.2) had 74% homology (Expect = 7e-100) with the mitochondrial At Mn-SOD (NM_115493.3) and APX from R. australe (DQ078123.2) showed a homology of 75% (Expect = 4e-162) with
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the peroxisomal \textit{At APX} (NM_119666.3). These genes were cloned using the gene specific primers using the sequence submitted at the NCBI gene bank.

For the present study single copy insertions were chosen. This was done to avoid the transgene silencing known to be caused by multiple gene insertions or repeated insertions at a single locus (Hobbs \textit{et al.}, 1993; Linn \textit{et al.}, 1990) and the transgene rearrangements (Svitashov \textit{et al.}, 2002). Therefore, for more predictable transgene expression, single-copy insertions are preferred as these are less likely to be targeted by silencing mechanisms (Jones \textit{et al.}, 2005; Kohli \textit{et al.}, 2003). The expression levels or the enzyme activity has been the consideration while studying the stress response of the transgenics. In certain instances, transgenics have been selected by the reporter gene expression as well as by RT-PCR studies (Wang \textit{et al.}, 2005). Transgenics developed through \textit{Agrobacterium} infection of leaf disc were raised to maturity and next T\textsubscript{1} (Badawi \textit{et al.}, 2004; Kwon \textit{et al.}, 2002; Pitcher and Zilinskas, 1996; Sarowar \textit{et al.}, 2005) or T\textsubscript{3} (Zhao \textit{et al.}, 2006) generation were used for the stress tolerance studies. T\textsubscript{0} plants after confirmation of the transgene were also used for the analysis of stress tolerance (Behnam \textit{et al.}, 2006a; Lee \textit{et al.}, 2007a; Lim \textit{et al.}, 2007). Wang \textit{et al.}, (2004a) used both single as well double copy inserts depending upon the higher transcriptional levels. Segregation analysis has also been used to validate single copy inserts (Christou \textit{et al.}, 1989; Vanjilderj \textit{et al.}, 2005)

The site of integration of a transgene directly influences its expression level. It may be integrated in a highly transcribed region or non transcribed/least transcribed region. In the first case transgene would have high rate of transcription and so the high expression level. Also, the relative tolerance of a particular region for invasion by foreign DNA also directly affects transgene expression. In the plant genomes, heterochromatin regions, intercalary heterochromatin and repetitive DNA stretches have been shown to be associated with transgene silencing (Matzke and Matzke, 1998). In plants, the integration of DNA occurs mainly by illegitimate recombination at non-specific sites in the genome (De Buck \textit{et al.}, 1999; Hohn and Puchta, 2003). Also, the transgene expression varies in the transgenic plants generated using the same construct, even in the same environment. It may be because of position effect, transgene copy number or silencing (Bhat and Srinivasan, 2002; Fischer \textit{et al.}, 2008). Similarly, for the development of double transgenics, independent gene insertions having high levels of gene expression can be crossed to avoid the transgene silencing due to multiple copy insertions and to obtain

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transgenic plants with two different transgenes with high level of expression for both the genes (Streatfield, 2007).

A promoter is essential for driving the transcription of a gene. In most of the studies, the transgenes were transcribed by a strong constitutive promoter such as the Cauliflower mosaic virus 35S (CaMV35S) promoter (Odell et al., 1985). The promoter used for the present study was also CaMV35S. Other studies have also used constitutive promoter for the over expression studies (Badawi et al., 2004; Pitcher and Zilinskas, 1996; Sarowar et al., 2005; Wang et al., 2004b; Zhao et al., 2006). Furthermore, the constitutive promoter was also used for the over expression of Cu/Zn-SOD, Mn-SOD and APX in transgenic tobacco (Kwon et al., 2002) for imparting tolerance to MV mediated oxidative stress. Another most commonly used constitutive promoter is the Ubiquitin promoter. It has been isolated from various plants including maize (Christensen et al., 1992), Arabidopsis (Callis et al., 1990; Norris et al., 1993), potato (Garbarino and Belknap, 1994), sunflower (Binet et al., 1991), tobacco (Genschik et al., 1994) and rice (Wang and Oard, 2003). However, Kasuga et al., (1999) reported that the over expression of DREB1A under the CaMV35S promoter resulted in the stunted growth of the transgenic plants. Later Kasuga et al., (2004) used stress inducible rd29A promoter (Yamaguchi-Shizonaki and Shizonaki, 1993) with the DREB1A gene and observed drought and salt stress tolerance in transgenic tobacco without retardation in growth. Also, Behnam et al., (2006a), reported increase in cold tolerance in transgenic potato expressing DREB1A under the Arabidopsis rd29A promoter and also increases salt tolerance with proportional increase in its copy number (Behnam et al., 2006b).

In the present study, the over expression of the genes under the constitutive CaMV35S promoter did not result in retardation in growth and development of the transgenic plants. However, stress inducible promoters can provide a flexible system for gene expression (Aoyama and Chua, 1997) by providing precise regulation of gene expression resulting in the development of stress tolerant plants (Yoshida and Shinmyo, 2000). In this light, many other workers have used stress induced promoters for the over expression studies like the oxidative stress inducible SWPA2 promoter (Kim et al., 2003). Wang et al., (2005a) used this promoter for drought tolerance studies in rice. This promoter was also used in the simultaneous over expression of Cu/Zn-SOD and APX in tall fescue plants which conferred tolerance to MV mediated oxidative stress, H₂O₂ and heavy metals (Lee et al., 2007a), for chilling and MV induced stress tolerance in sweet potato.
(Lim et al., 2007) and in transgenic potato where both the genes (Cu/Zn-SOD and APX) were expressed independently under the control of SWPA2 promoter for abiotic stress tolerance (Tang et al., 2006).

### 5.1 Copper Zinc Superoxide Dismutase (Cu/Zn-SOD)

Environmental stress conditions, such as drought, salinity, high light, or heavy metals, cause a rapid and excessive accumulation of ROS in plant cells (Apel and Hirt, 2004; Bartels and Sunkar, 2005; Hasegawa et al., 2000; Zhu, 2002). SODs (EC 1.15.1.1) represent the first line of defense against superoxide accumulation by rapidly converting superoxide to \( \text{H}_2\text{O}_2 \) and \( \text{O}_2 \) (Fridovich, 1995). Earlier studies have also showed that over expression of Cu/Zn-SOD conferred tolerance to MV induced oxidative stress (Aono et al., 1995; Perl et al., 1993), salt (Badawi et al., 2004, Zhao et al., 2006), cold (Sen Gupta et al., 1993a), and osmotic (Badawi et al., 2004) stress. However, Allen et al., (1997) and Tepperman and Dunsmuir, (1990) on expression of Cu/Zn-SOD in tobacco did not observe any tolerance to MV induced oxidative stress. Similarly, Aono et al., 1995 and Pitcher et al., 1991, did not observe tolerance to ozone. Importantly, Tepperman and Dunsmuir, (1990) and Pitcher et al., (1991) did not get enhanced stress tolerance even with 50 fold increase in the enzyme activity. They hypothesized that very high level of Cu/Zn-SOD expression increases the \( \text{H}_2\text{O}_2 \) titer which at high concentrations inhibits the activity of the Cu/Zn-SOD by end product/feedback inhibition. In contrast, Badawi et al., (2004) reported 4.6 fold increase in the level of SOD induced tolerance to NaCl, drought and osmotic stress since the level of the \( \text{H}_2\text{O}_2 \) was not high enough for the end product/feedback inhibition. Also Perl et al., (1993) reported that moderate increase in Cu/Zn-SOD enzyme activity has enhanced the MV mediated oxidative stress. The transgenics in the present study showed 2-3 fold increase in Cu/Zn-SOD activity resulting in the enhanced stress tolerance to NaCl, PEG, cold and copper stress.

The present study also focused on the heterologous over expression of the gene with unique features from Potentilla atrosanguinea plants growing in Western Himalaya at 3900msl (US Patent 7037697). The unique properties of the presently used Cu/Zn-SOD include the retention of scavenging activity before and after autoclaving with an activity range of sub zero temperature to >60°C. The transgenic lines over expressing Cu/Zn-SOD in the present study retained the novel autoclavable and thermostable properties of the original enzyme in the heterologous over expression model system of Arabidopsis thaliana. This result, besides providing the proof of concept implies the use of these
transgenics as bioreactors (Sharma and Sharma, 2009) for the production of autoclavable and thermostable SOD in *A. thaliana*.

Over expression of *Cu/Zn-SOD* showed enhanced salt tolerance as is discussed above, however, the present study clearly advances our insight to its relationship with lignin biosynthesis which is shown by the upregulation of phenylalanine ammonia-lyase (*PAL1*, EC4.3.1.5) and peroxidase (*AtPRX9GE*, EC 1.11.1.7) genes in the pathway. This is a novel result in the present work as no previous study has shown transgenics for *Cu/Zn-SOD* having increased enzyme activity providing NaCl stress tolerance with a concomitant increase of lignification as shown in the interfascicular cambium. While one of the advantages of enhanced SOD activity under NaCl stress would be scavenging of superoxide radical as has been discussed by previous workers, yet another advantage could be by the production of H$_2$O$_2$, particularly if the *APX* level is unaltered as is the case in the present work (Fig. 4.16). H$_2$O$_2$ has a number of regulatory roles in the plant cells. It is required in the successful cell division due to its function in cross linking of the cell walls (De Marco and Roubalakis-Angelakis, 1996). H$_2$O$_2$ is also involved in lignin biosynthesis where different subunits of lignin (4-hydroxy cinnamyl aldehyde, coniferyl alcohol and sinapoyl alcohol) are polymerized by peroxidase (*AtPRX9GE*) and hence more lignification in transgenics is expected as compared to the WT. Indeed, histological sections of inflorescence stem showed that transgenic lines had greater lignification of inter-fascicular arcs, which was not evident in the WT. Increased lignification suggested that lignin biosynthesis pathway might be modulated in transgenic lines under stress and hence critical genes of lignin biosynthesis were targeted. *PAL1* catalyzes the committed step in phenylpropanoid metabolism and plays a regulatory role in controlling biosynthesis of all phenylpropanoid compounds; including lignin (Whetten and Sederoff, 1995), whereas *AtPRX9GE* peroxidase is a key protein in lignin polymerization in *A. thaliana* (Mele et al., 2003). Genes encoding for PAL1 are expressed in a complex pattern during plant development and in response to light, pathogen challenge, mechanical damage and abiotic stresses (Liang et al., 1989; Lois et al., 1989; Lodgemann et al., 1995). Data showed an evident up-regulation of *PAL1* and *AtPRX9GE* under NaCl stress as compared to WT. Enhanced lignification of inter fascicular arcs and xylem vessels of vascular bundles is expected to provide physical support to the conducting tissue allowing better water/nutrient movement by preventing collapse, as was observed in WT. It is proposed that *Cu/Zn-SOD* mediated lignification of the conducting tissue might be yet another
mechanism of tolerance to abiotic stress in plants and this parameter may be used as a quick and simple screen to filter stress tolerant species.

Another novel finding of the work has been identifying the proteins of transgenic line for Cu/Zn-SOD and WT under copper stress. Such an approach in assessing the performance of similar transgenics has not been reported earlier. Our results showed that the WT under 1 mM Cu treatment showed an upregulation of stress related proteins such as cysteine synthase and O-acetyl serine (thiol) lyase. In contrast, the transgenic line for Cu/Zn-SOD under 1 mM Cu treatment did not show upregulation of these proteins, but in certain cases, these were downregulated. Previous studies have showed that over expression of cysteine synthase lead to enhanced tolerance to heavy metal stress in A. thaliana (Domínguez-Solís et al., 2001) and in tobacco plants (Kawashima et al., 2005). Another stress related protein which was down regulated was phenazine biosynthesis like protein (putative). It is an essential component of the respiratory chain which produces ATP and also shows strong antioxidant activity against lipid peroxides. The results indicated that 2-DE protein profile for transgenics lines of Cu/Zn-SOD under stress was similar as that of the WT without stress. This implied that the transgenic plants with over expression of the additional Cu/Zn-SOD in their system increases the level of stress tolerance, therefore the germinating seeds did not show induction of stress related proteins as these seeds maintain their cellular homeostasis during copper stress which was otherwise seen as stress in WT subjected to copper stress. Accumulation of these proteins under stress in case of WT and down regulation in transgenics reveals that these are involved in the overall stress tolerance mechanism. The result also established that the native Cu/Zn-SOD is not sufficient to impart abiotic stress tolerance under stress. However, with the addition of Pa Cu/Zn-SOD in the transgenics there was an expected decrease in the ROS resulting in high germination percentage at 1mM of copper stress.

Cu/Zn-SOD over expression also leads to increased cold (4°C) stress tolerance in the transgenic plants as compared to the WT under cold stress. Since Pa Cu/Zn-SOD is known to have optimal activity at 0-5°C which could be advantageous to A. thaliana plants growing at 4°C. The enzyme activity of the transgenics showed an increase at 4°C in contrast to the WT which showed decrease in the enzyme activity at 4°C. The expression analysis of the plants under control conditions (20°C) and at cold temperature (4°C) showed that under 4°C, the native At APX and At Mn-SOD showed upregulation whereas the At Cu/Zn-SOD remained same in the transgenic lines. The gene expression of native
enzyme At Cu/Zn-SOD declines in the WT. Sen Gupta et al., (1993b) also showed upregulation in the native transcripts (At APX) under cold stress in transgenic tobacco over expressing P. sativum Cu/Zn-SOD.

In these studies, it was found that although driven by the constitutive CaMV35S promoter, the Cu/Zn-SOD transcript levels are relatively higher in the transgenic lines under stress than under normal growth conditions. Stress inducibility of genes, in spite of being under the control of constitutive CaMV35S promoter, has been reported earlier as well (Shi et al., 2003). These results suggest that Cu/Zn-SOD transcripts may be unstable in the absence of salt stress as suggested by Shi et al., (2003) and the stress may cause post-transcriptional stabilization of the enzyme. Post-transcriptional control of transcript accumulation is an important mechanism for gene regulation under stress, and has been reported for some salt-stress regulated genes (Cushman et al., 1990; Hua et al., 2001; Shi et al., 2003) and abscisic acid and water stress regulated genes (Bartels et al., 1992; Cohen et al., 1999). Sunkar et al., (2006) showed that micro RNA (miR398) expression is downregulated transcriptionally by oxidative stresses, and this downregulation was important for posttranscriptional CSD1 and CSD2 mRNA accumulation and oxidative stress tolerance in A. thaliana.

5.2 Manganese Superoxide Dismutase (Mn-SOD)

Over expression of Mn-SOD also resulted in enhanced tolerance to MV induced oxidative stress (Bowler et al., 1991; Slooten et al., 1995), salt (Wang et al., 2004b), ozone (Bowler et al., 1991; Van Camp et al., 1994), cold (Kingston-Smith and Foyer, 2000; Mc Kersie et al., 1993), drought (Mc Kersie et al., 1996; Wang et al., 2005a), winter survival (Mc Kersie et al., 1999) and tolerance to aluminium toxicity (Basu et al., 2001) and manganese deficiency (Slooten et al., 1995; Yu et al., 1999). Our study also showed that over expression of Mn-SOD showed enhanced tolerance to NaCl stress concomitant with the moderate increase in the level of enzyme. The transgenic plants showed an increase in the root length, rosette area over the WT. The enzyme activity increased with the increase in the concentration of NaCl whereas the WT showed decreased enzyme activity at higher concentration of NaCl. These results confirmed the results obtained by Wang et al., (2004b) who got similar results by developing A. thaliana transgenics under NaCl stress. Also, the Mn-SOD transgenic showed higher germination percentage at various levels of NaCl, PEG and copper stress. The Mn-SOD showed higher percentage of germination at all concentrations of NaCl treatment in comparison with the Cu/Zn-SOD.
5.3 Ascorbate Peroxidase (APX)

APX is an essential enzyme for the conversion of H$_2$O$_2$ to water and molecular oxygen in the cells (Mittler, 2002). Over expression of APX provided tolerance against a wide range of environmental cues like chilling (Kornyeyev et al., 2003; Wang et al., 2005c), drought (Yan et al., 2003), MV induced oxidative stress (Murgia et al., 2004), salt (Wang et al., 2005c) and high light (Rossel et al., 2006). Our results with APX transgenics further confirm the tolerance imparted against NaCl stress. Also the APX transgenics had higher germination percentage than the WT at various levels of NaCl, PEG and copper stress. The APX transgenic plants also showed increased enzyme activity than the WT plants when grown on different levels of NaCl. The transgenics had moderately high enzyme activity (1.9-2.2 folds) in comparison with the enzyme activity of the WT plants and also showed higher root length and rosette area in comparison with the WT plants.

5.4 Double Transgenics (SOD and APX)

The simultaneous scavenging of O$_2^-$ and H$_2$O$_2$, by Cu/Zn-SOD and APX, is important for the maintenance of plant productivity under harsh conditions where single gene does not suffice for providing stress tolerance (Lee et al., 2007a, 2007b). Therefore, advances have been made in developing transgenic plants having two or more transgenes for alleviation of abiotic stress in various systems. Kwon et al., (2002) developed transgenic tobacco plants over expressing Cu/Zn-SOD, Mn-SOD and APX in their chloroplasts and showed enhanced tolerance to MV. Similar results were obtained by Tang et al., (2006) in potato and Lee et al., (2007a) in tall fescue by over expressing Cu/Zn-SOD and APX and obtained tolerance to MV and to high temperature in potato and also to heavy metal stress in tall fescue. Furthermore, Lee et al., (2007b) obtained tolerance against abiotic stress by over expressing Cu/Zn-SOD, APX and DHAR in the chloroplasts of the tobacco transgenics. Our results also indicate that the transgenics having both Cu/Zn-SOD and APX are more tolerant to cold stress (after 20 d at 4°C) in comparison to the WT as was evident by increased enzyme activity and decreased in situ ROS production in the double transgenics as compared to the WT plants. The transgenics for Cu/Zn-SOD and APX as well as for Mn-SOD and APX also showed enhanced tolerance to various concentrations of NaCl in comparison to that of the WT plants. The double transgenics evinced a higher enzyme activity, and shoot biomass under stress and also showed higher seed yield under high concentrations of NaCl.
5.5 Detailed Physiological and Biochemical Analysis of the Select Transgenic Lines

The above mentioned experiments were performed in petri plates with multiple transgenic lines for each gene. From these experiments the lines which showed highest level of stress tolerance respectively, were used for comparative analysis of the single as well as the double transgenic lines under NaCl stress in pots. The results showed that total cellular SOD and APX activity in WT and in single as well as double transgenic lines (Cu/ZnSOD, Mn-SOD, APX, Cu/Zn-SOD and APX and Mn-SOD and APX), at various concentrations of NaCl and at 0, 10 and 20 d of the stress as shown Fig. 4.51 and Fig. 4.52 respectively, was significantly higher (p≤0.01) in the transgenic lines than the WT under both stressed as well as non stressed conditions. Although the activity of SOD and APX also increased in WT plants under stress, it was significantly lower as compared to that of the transgenic lines. Increased antioxidant enzyme activity with the increase in the level of stress is reported with expression of SOD or APX, individually or synchronously, in a number of studies in different plants species (Badawi et al., 2004; Bowler et al., 1991; Sen Gupta et al., 1993a, 1993b; Kwon et al., 2002; Lim et al., 2007; Murgia et al., 2004; Sarowar et al., 2005; Shi et al., 2001; Wang et al., 2004b; Wang et al., 2005a; Zhao et al., 2006). Studies with the double transgenics show that the total SOD activity increased at 50 mM of NaCl stress as compared to that of the WT than without stress. The WT showed increase in activity till 100 mM after which it declined whereas in the transgenics the enzyme activity did not decrease even at 150 mM. Similar trends were also observed in the APX activity.

Yield enhancement due to over expression of antioxidant genes has been predicted earlier (Allen, 1995). The total seed yield was higher in the transgenics than the WT. Zhang et al., (2001) reported that the transgenic Brassica napus plants over expressing AtNHX1, a vacuolar Na1yH1 antiport from A. thaliana, were able to grow, flower, and produce seeds in the presence of 200 mM NaCl. Reports showed that the transgenics for genes encoding for activity of enzymes involved in carbohydrate metabolism have greater reproductive yield in wheat and rice (Smidansky et al., 2002; 2003). Excessive production of ROS has been shown to be a major cause of reducing crop yield under stress by disrupting various physiological processes (Yan et al., 2003). In the present study, transgenic lines for Cu/Zn-SOD and Mn-SOD alone at lower levels of NaCl stress (50 mM) showed a better yield in comparison to those grown without stress. However, APX alone and in combination with either Cu/Zn-SOD or Mn-SOD, the response to stress at 50 mM
concentration is lowered. At this low concentration ROS is being produced, but is not converted to H$_2$O$_2$ which is the substrate for APX. It also goes on to show that optimal amount of H$_2$O$_2$ is essential for plant growth and developmental processes as it also serves as a signal molecule. In the double transgenics lines, (Cu/Zn-SOD and APX and Mn-SOD and APX), H$_2$O$_2$ does not accumulate at the optimum levels and hence the reduction in yield at lower levels. At higher concentrations, when the ROS production is higher more H$_2$O$_2$ is produced which is efficiently scavenged resulting in higher seed yield at 100 and 150 mM NaCl. Our work supports the earlier hypothesis that a critical minimum amount of H$_2$O$_2$ is necessary for normal development of the plant and also for enhanced tolerance to be imparted by transgenic APX. Yan et al., (2003) reported that the over expression of APX lead to the greater number of fruits leading to enhanced seed mass production. In our study, the seed yield increase could be attributed to increase in the number of seed per siliqua in transgenics as compared to WT at all concentrations of NaCl. All the transgenics lines over expressing of either a single (Cu/Zn-SOD, Mn-SOD and APX) or double transgenes (Cu/Zn-SOD and APX and Mn-SOD and APX) in their cells had greater ROS scavenging capacity as compared with the WT type plants at all the developmental stages as well as various concentrations of NaCl stress (0, 50, 100 and 150 mM). This can be inferred from the in situ ROS staining which showed that the WT had greater levels of blue stain indicative of the ROS at higher concentrations of NaCl stress as compared to the transgenics lines which had little or no blue colouration even at high concentration of NaCl stress.

With the increase in the level of stress, the difference in the root biomass between the different transgenics reduces. Overall, the root biomass is more than that of the WT at all levels of NaCl at day 10. In shoot, the differences in shoot biomass of the transgenics are relatively more amongst the transgenics. The differences in biomass between the transgenics and WT were marginally increased with the increased level of stress. Sami et al., (2002) reported an increase in plant biomass after pyramiding Mn-SOD genes in chloroplast and mitochondria of alfalfa.

The transgenics had significantly higher rate of photosynthesis ($P_N$) ($p \leq 0.01$) than that of the WT without NaCl stress. At various levels of NaCl stress (0, 50, 100 and 150 mM), the $P_N$ values of all the lines progressively declined. The rate of photosynthesis also declined in the plants without stress over the time because of bolting observed at around 35-37 d of growth followed by the onset of senescence in transgenics and WT. These
results were in consonance with those observed by Stessman et al., (2002), who observed that photosynthetic rates attained peak levels early in the expansion phase of development, and thereafter upon bolting decline. The increase in the $P_N$ value could be attributed to the change in the levels of $\text{H}_2\text{O}_2$ in the transgenics lines and the WT, since $\text{H}_2\text{O}_2$ also acts as a signaling molecule for the opening of stomata enhancing the gaseous exchange and the receptivity of $\text{CO}_2$ (Yan et al., 2003). A decrease in the $P_N$ after 20 days of stress in the double transgenic line of $\text{Mn-SOD}$ and $\text{APX}$ may be due to the greater scavenging of the $\text{H}_2\text{O}_2$, decreasing its titer and thereby decreasing the $P_N$ (Pei et al., 2000).

The present study brings out some novel observations that are likely to unfold avenues for research particularly on the aspect of lignification during salinity stress and the usage of plants as bioreactors for the production of SOD. There are several reports of transgenic expression in plants under stress (Badawi et al., 2004; Shi et al., 2001; Tang et al., 2006; Wang et al., 2004b); however, none of the previous reports project enhanced grain productivity and details of number of fruits and seed characters. Although the present study was done in pots under controlled and in contained conditions, the results provide an important insight into the number of seeds per silique being an important parameter for the increase of total seed yield per plant in all the transgenic lines. Perhaps, the lignification of the inflorescence stem provides structural strength and facilitates the translocation of water/nutrients to the sink. This enhanced sink capacity could be responsible for the production of higher number of seeds per silique thereby increasing the total seed yield. A long-standing hypothesis in plant development has been that sink strength can influence photosynthetic output and the excess photosynthetic capacity can be tapped in case of altered demand (Paul and Foyer, 2001). It has been proposed that this increase in the photosynthetic efficiency induced by increased sink strength, may lead to greater biomass production (Toenniessen, 1991), which is reflecting in the form of seed yield in our study. A proposed mechanism for sink-induced increase in photosynthetic output involves release of feedback inhibition on the process of photosynthesis resulting from more rapid use of photosynthate (Paul and Foyer, 2001). Plants typically bring to maturity far fewer reproductive structures than are genetically possible (Yoshida, 1981), opportunities exist to manipulate plant development in ways that permit utilization of some of this unfulfilled reproductive potential (Smidansky et al., 2002, 2003). Our results, through the development of transgenics seem to corroborate with the above mentioned hypothesis. As a corollary, the present study also reflects on the relevance of $\text{SOD}$ and $\text{APX}$ combination.
in transgenic development. Moderate increase in the SOD combined with over expression of APX can also lead to the change in the H₂O₂ homeostasis of the cell. Therefore, double gene i.e. SOD and APX should be over expressed together only if the level of expression and enzyme activity of SOD are high leading to substantial generation of H₂O₂ requiring the over expression of APX. On the basis of these results, relevant crop improvement strategies can be evolved.

The scope of the present work was limited to include transgenics having a single copy of the gene. However, it would be interesting to study the dose effects of the gene on its enzyme activity and stress tolerance. The transgenics with different copy number should be compared to provide an insight into the relation between the copy number and expression level along with the enzyme activity. Although there are no reports of antioxidant enzyme imparting biotic stress tolerance, however, further enquiry in this direction could be fruitful.

In the background of the above mentioned successes and limitations of the present work, the thesis opens up new avenues for future researchers to pursue upon the following. The results obtained showed, that the transgenics developed by over expressing genes for antioxidant enzymes impart tolerance against abiotic stresses. Also, a 10% increase in the seed yield has been observed. Since all these studies have been done in transgenic A. thaliana, the present results can lead to the deployment of these genes at least in the relevant crops of the Brassicaceae family like mustard (Brassica campestris) and rape seed (Brassica napus). This needs to be done with marker free gene insertions. This is best achieved by developing marker free constructs that randomly get inserted and expressed when placed alongside a promoter region either independently or by employing the Cre-Lox system (Wang et al., 2005b) to remove the gene encoding for the selection marker. Also, stress inducible promoters (rd29A, SWPA2) can be used to overcome the problem of stunted growth of the transgenics under control conditions. Stress inducible promoters also are less taxing on the cellular machinery as they are upregulated only during stress and the transcripts are produced only when and where these are required (Kasuga et al., 2004).

The transgenics obtained should be tested against multiple stresses as in the field environment, single stress seldom occurs (Mittler, 2006). Majority of experiments on abiotic stresses performed under controlled conditions evaluate single stress, whereas in the field multiple stresses occur. For instance, high temperature stress would invariably be accompanied by dehydration in the plant. High temperature would cause the opening of the
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Leaf stomata for cooling by transpiration, but then dehydration of plant would not permit the opening of its stomata as it would cause further loss in moisture due to transpiration. Since plants have different mechanisms for coping with these stresses and the responses of the plant system (molecular and metabolic) to drought and heat stress are unique, it cannot be extrapolated from the present results to scenarios with multitude of stresses in a growing season of the plant (Rizhsky et al., 2002).

Since the copy number of the transgene does not govern the level of expression of that gene (Allen et al., 1993, 2000; Henikoff, 1998) therefore regardless of the copy number in transgenics developed, the lines which have the maximum enzyme activity can be used for developing these plants as bioreactors for production of these enzymes in comparison to the cell line usage (Sharma and Sharma, 2009).

In conclusion, the present study effectively achieved most of the objectives laid down for the development and assessment of the single (Cu/Zn-SOD, Mn-SOD and APX isolated at IHBT) as well as the double gene transgenics (Cu/Zn-SOD and APX as well as Mn-SOD and APX) in plants of *A. thaliana*. The transgenics were characterized and single copy inserts were selected and raised to T3 generation. The three single gene transgenics showed high degree of tolerance during seed germination and plant development *in vitro* on different levels of NaCl, PEG, cold and Cu stress. Furthermore, transgenics containing two transgenes (Cu/Zn-SOD and APX, Mn-SOD and APX) along with the above single gene transgenics, in general, performed better than the WT under NaCl stress assessed in pots. This was evident from a higher rate of photosynthesis, better shoot biomass, and efficient ROS scavenging capacity as seen from the *in situ* NBT assays. Importantly, these results lead to higher grain productivity giving insights into single gene deployment and cases where both SOD and APX need to be deployed. The present work would pave way for the judicious use of these genes effectively into the relevant crop plants leading to optimum growth and enhanced yield under environmentally stressed conditions.