CHAPTER 4

Expression analysis of foxtail millet genotypes differing in salt tolerance

4.1 INTRODUCTION

Environmental conditions have been shown to affect both plant growth and development and their productivity. Plants face threat both from biotic, which includes pathogen, pests and insects, and abiotic stresses, which includes factors like salinity, osmotic imbalance, temperature extremes etc. In agricultural systems, the abiotic stresses, salinity, low temperature and drought in particular are responsible for most of the reduction that differentiates yield potential from harvestable yield (Boyer, 1982). Though enormous amount of money is being spent world wide to tackle these problems, these are still challenges to those who are involved in crop production. The cultivable land available is constantly shrinking because of human encroachment and the available lands are also being spoiled to the greater extent by accumulation of salts in high concentration especially in arid and semi-arid regions. As soil salinity is one of the important constraints, better understanding of the mechanisms that enable plants to adapt to salt stress is necessary for exploiting saline soils/water.

Salt stress can lead to changes in development, growth and productivity and severe stress may threaten survival. Salt stress results in alterations in plant metabolism including reduced water potential, ion imbalance and toxicity (Cramer *et al.,* 1994; Bohnert and Jensen, 1996). High salinity causes both hyper osmotic and hyper ionic stress effects and the consequence of these can be lethal (Hasegawa *et al.,* 2000). Most commonly, the stress is caused by high Na^+ and Cl^- concentrations in the soil solution and an altered water status most likely brings about initial growth reduction, membrane disorganization, generation of reactive oxygen species, metabolic toxicity, inhibition of photosynthesis and altered nutrient acquisition. Under these circumstances, the need to develop plants, which could withstand stress, retaining an

acceptable level of productivity, is of utmost importance. During the course of evolution different plants have developed adaptive characters against these stress factors. There are various mechanisms reported in the literature by which plants protect themselves from these stresses, including accumulation of osmoprotectants, presence of ion carriers, ion compartmentation, transporters and symporters, water channels, chaperones, superoxide radical scavenging systems and signaling molecules. All these phenomenon's have been well reviewed by several authors in different species of halophytes and glycophytes (Bohnert *et al.,* 2001; Cushman and Bohnert, 2000).

Salt tolerance is a complex trait involving responses to cellular osmotic and ionic stresses, as well as secondary stress effects. Many studies have examined the multitude effects of salt stress, and importance of protecting the plant from reactive oxygen species (ROS) seems to be one of the important components of the complex tolerance trait. The high salt concentrations normally impair the cellular electron transport within the different sub cellular compartments and lead to the generation of reactive oxygen species such as singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals. Excess of ROS triggers phytotoxic reactions such as lipid peroxidation, protein degradation and DNA mutation. The primary source of ROS is superoxide radicals, which are generated in the sub cellular compartments such as mitochondria, chloroplast and cytoplasm via a number of metabolic pathways during oxidative stress conditions (Noctor and Foyer, 1998). The degree of peroxidative damage of cells is controlled by the potency of the antioxidative peroxidase enzyme system. Though the responses are stress specific, it is believed that some of them overlap. As reported in a number of studies, environmental stresses such as salt, drought and cold stress lead to increased free radical formation (Singha and Choudhuri, 1990; Smirnoff, 1993) and lipid peroxidation (Del Río *et al.,* 1991; Leprince *et al.,* 2000). At the physiological level, the multitude of effects of salt stress such as ion toxicity and water deficit impair photosynthesis, which results in production of reactive oxygen species.

The degree of oxidative cellular damage in plants exposed to salt stress is controlled by the antioxidative systems. A correlation between the antioxidant capacity and NaCl tolerance has been demonstrated in a number of crops such as pea (Hernández *et al.,* 2000), cotton (Gossett *et al.,* 1994) and foxtail millet (Sreenivasulu *et al.,* 2000). To overcome salt-mediated oxidative stress, plants detoxify ROS by up-regulating antioxidative enzymes like superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione

peroxidase (PHGPX; E.C. 1.11.1.9), glutathione cycle enzymes and produce low molecular mass antioxidants like flavonones, anthocyanines, α-tocopherol, ascorbate, glutathione and polyphenolic compounds. Bueno *et al.* (1998), showed the up regulation of antioxidants, superoxide dismutase and ascorbate peroxidase in response to salt stress at the transcriptional and translational level. Also implicated are the protective roles played by the accumulation of specific metabolites that seem to act in more than one function; for instance preventing radical formation, acting as low molecular weight chaperones contributing to redox control and functioning as compatible solutes by decreasing the osmotic potential. A main protective role is attributed to SOD in catalyzing the dismutation of superoxide anions to dioxygen and hydrogen peroxide (H_2O_2). In order to quench the generated H_2O_2, plants evolved H_2O_2 scavenging antioxidative enzymes such as peroxidases and catalases (Halliwell and Gutteridge, 1989; Sen-Gupta *et al.,* 1993). Plant peroxidases utilize different substrates such as guaicol, ascorbate and glutathione to scavenge intracellular H_2O_2. Based on substrate specificity, peroxidases are classified into guaicol peroxidase (POX; EC 1.11.1.7), ascorbate peroxidase and glutathione peroxidase. The H_2O_2 generated in glyoxysomes and peroxisomes by the process of β-oxidation is detoxified to H_2O mainly by catalase (CAT; E.C 1.11.16), while in other sub cellular compartments H_2O_2 is converted to H_2O by ascorbate peroxidase and glutathione peroxidase.

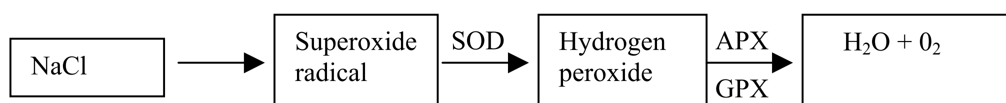

Fig. 24
A proposed model for pathways leading to the induction of reactive oxygen species (superoxide radical, hydrogen peroxide and hydroperoxides) during NaCl treatment and the role of the protective antioxidative enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and phospho glutathione peroxidase (PHGPX) in scavenging superoxide, hydrogen peroxide and hydroperoxide radicals respectively.

Glutathione peroxidases are a family of multiple isozymes, which catalyze the reduction of H_2O_2, organic hydroperoxides and lipid hydroperoxides using GSH as a reducing agent (Ursini *et al*., 1995) and thus help to protect the cells against oxidative damage (Flohé and Günzler, 1984). In animals, glutathione peroxidases have been studied extensively and various forms of GPX have been identified, which includes cytosolic GPX, plasma membrane GPX, gastrointestinal GPX and phospholipid hydroperoxide glutathione peroxidase (PHGPX). Even though all four groups reveal similarity in their primary structure, PHGPX differs from the other three by being a monomer and having ability to interact with peroxidised lipids and complex lipids, which are integrated in bio-membranes. Therefore the PHGPX reaction has been considered the main line of enzymatic defense against oxidative bio-membrane destruction in animals (Ursini *et al.,* 1995). Some reports showed the presence of PHGPX cDNAs for instance in *Citrus sinesis* (Holland *et al.,* 1993), *Nicotiana sylvestris* (Criqui *et al.,* 1992), *Spinacia oleracea* (Sugimoto *et al.,* 1997a), *Arabidopsis thaliana* (Sugimoto *et al.,* 1997b), *Lycopersicon esculentum* (Depége *et al.,* 1998), *Hordeum vulgare* (Churin *et al.,* 1999) and *Oryza sativa* (Li *et al.,* 2000). Further, PHGPX mRNA levels have been shown to increase in tissues of several plant species undergoing stress such as salinity (Gueta-Dahan *et al.,* 1997), heavy metals (Sugimoto *et al.,* 1997), herbicide resistance (Cummins *et al.,* 1999), mechanical stimulation (Depège *et al.* 1998; Depège *et al.,* 2000) and infection by viral or bacterial pathogens (Levine *et al.,* 1994).

Although a wide range of significant physiological mechanisms and genetic adaptations to salinity stress has been observed, the underlying mechanisms of salt-tolerance in plants are still poorly understood. The best possible approach to explore tolerance mechanisms is to compare the components involved in stress response in tolerant as compared to sensitive plants. In foxtail millet (*Setaria italica L.*) salt sensitive and tolerant lines have been identified (Sreenivasulu *et al.,* 1999; 2000). Foxtail millet is an important food crop in India, China and Japan. Increasing salinisation of agricultural land causes toxic effects, primarily at the seedling level in plant development. Hence, improved understanding of acute adaptive and general protective mechanisms conferring enhanced salt tolerance in seedlings becomes an important issue in stress physiology to ensure further growth and yield of crop plants. An attempt has been made to study the biochemical difference between these lines in terms of the ROS scavenging system. The differential response of the system might pave the way for the better understanding of the phenomenon of salinity resistance and might in turn lead to the development of elite lines which could withstand increased salinity levels.

Present day technologies have opened ways to understand the biological principles also at the molecular/cellular level. For example newer tools are now available which allows to address the complexity of stress responses at a larger scale through genome wide expression profiling. Kawasaki *et al.* (2001), used DNA microarrays to monitor transcript abundance and expression patterns in two lines of rice differing in their response to salinity. The results indicate a progression of regulated functions such that different categories of transcripts show regulation at different developmental time scales. A difference between the two lines existed with respect to the onset of the initial response. Desikan *et al.* (2001), have undertaken a large-scale analysis of *Arabidopsis* transcriptome during oxidative stress. Using cDNA microarray technology, they identified 175 non-redundant ESTs that are regulated by hydrogen peroxide. Further, they could demonstrate that other stresses such as wilting, UV radiation and elicitor challenge also induce the expression of many of these genes. Progress is now anticipated through comparative genomic studies of an evolutionarily diverse set of model organisms. As described before, foxtail millet has the advantage to have salt tolerant and sensitive lines, and comparison of these lines for gene expression studies during high salinity stress by cDNA array analysis should be a very useful experimental tool. The discovery of novel genes, determination of their expression patterns in response to stress, and an improved understanding of their roles in stress adaptation will provide the basis of effective engineering strategies leading to greater stress tolerance.

The objectives of the following work have been

i) Using barley macroarrays as a tool to analyze related heterologous probes (foxtail millet) by gene expression studies,
ii) to study the performance of salt sensitive and tolerant lines of foxtail millet under salinity stress,
iii) to look into gene expression patterns of these two lines under high saline conditions by cDNA array analysis and
iv) to study the ROS scavenging system during salinity stress in seedlings of foxtail millet.

4.2 RESULTS

4.2.1 Growth attributes

The impact of salinity on the growth and development of crop plants has been demonstrated (Boyer, 1982). To gain knowledge about the impact of salinity on the growth and development of foxtail millet seedlings, seeds of both the tolerant (Prasad) and the sensitive (Lepakshi) variety were germinated on Hoagland medium supplemented with 0, 150, 200, 250 and 300 mM NaCl. After 5 days, seedlings were harvested and growth rates were determined by measuring the shoot and root lengths. As shown in Fig. 25, increasing NaCl concentration inhibited gradually the growth rate of both cultivars. The growth of the salt-sensitive cultivar was extensively inhibited at salt concentrations lower than 200 mM NaCl. In contrast, the salt-tolerant cultivar still developed shoots up to 250 mM NaCl, but growth was almost completely inhibited at 300 mM NaCl. The percentage of relative growth inhibition of the shoots upon increasing NaCl concentrations is indicated in the insert of Fig. 25.

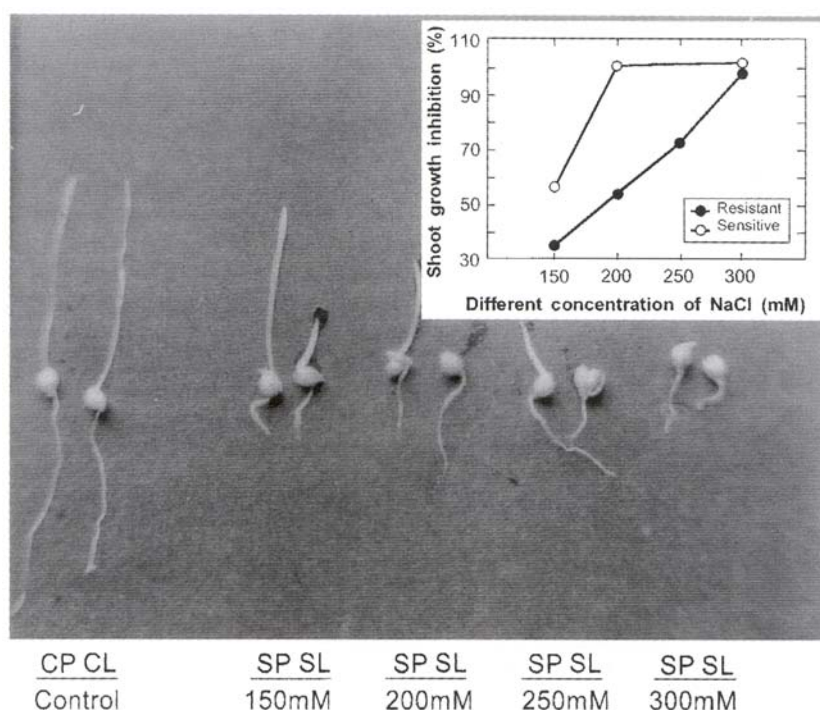

Fig. 25 Differences in root and shoot length of 5-day old seedlings of a salt-tolerant (P – Prasad) and a salt-sensitive (L – Lepakshi) foxtail millet cultivar grown under control conditions (CP – control Prasad; CL – control Lepakshi) and at different NaCl concentrations (SP – salt-treated Prasad; SL – salt-treated Lepakshi). Percentages of relative shoot growth inhibition are shown in the insert. Seedlings were grown at 25°C in Hoagland medium without NaCl (control) and with different NaCl concentrations (150, 200, 250 and 300 mM)

4.2.2 Sodium content measurements

To understand the accumulation of endogenous Na^+ in the cultivars studied, the Na^+ concentration was determined in tolerant and sensitive seedlings after five days of treatment with different concentration of NaCl. As shown in Fig. 26, seedlings of the two foxtail millet cultivars exhibited

a remarkable difference in the accumulation of endogenous Na^+ concentration upon increasing NaCl concentration in the medium, which is indicative of the differences of both cultivars in the adaptive response to salinity. In the salt-sensitive cultivar, Na^+ accumulated progressively with increasing amounts of exogenous salt. In the tolerant cultivar, the endogenous Na^+ content remains pretty low up to 150 mM NaCl treatment and increased only at 200 mM NaCl.

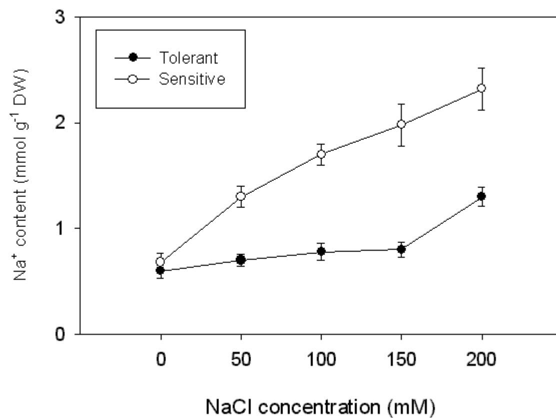

Fig. 26 Na^+ accumulation in 5-day-old seedlings of the tolerant and sensitive foxtail millet cultivar grown at different NaCl concentrations. The presented data are the average of 3 independent sets of experiments. DW - dry weight.

4.2.3 Effect of salinity on electrolyte leakage

The extent of membrane damage by salinity stress was assessed by an indirect measurement of solute leakage in tolerant and sensitive seedlings. The electrolyte leakage was subsequently measured after growing the seedlings in Hoagland medium with (different concentrations) and without (control) NaCl. The estimated ion leakage of seedlings correlates with increasing NaCl concentrations. While the sensitive cultivar showed the highest electrolyte leakage after incubation with 150 mM NaCl, the salt-tolerant cultivar displayed a 1/3 lower leakage at this NaCl concentration (Fig. 27). Results show a lower impairment of membrane permeability in the cells of the tolerant seedlings in comparison to the sensitive one.

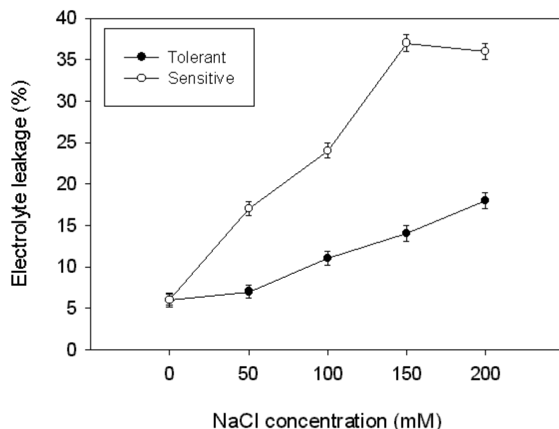

Fig. 27 Electrolyte leakage rate measured in cells of 5-day-old seedlings of the tolerant and sensitive foxtail millet cultivars grown at different salt concentrations. The presented data are the average of 3 independent sets of experiments.

4.2.4 Effect of salinity on malonaldehyde content (MDA)

The extent of membrane damage was assessed by measuring the MDA content of five-day old seedlings of the tolerant and sensitive cultivar grown on different NaCl concentrations and without NaCl (control). In both cultivars, increased salt concentration caused a significant increase in the MDA level. However, MDA accumulation was higher in the salt-susceptible one (Fig. 28). Up to 100 mM NaCl, no significant difference was found in the MDA level of the two cultivars. At higher NaCl concentrations, the amount of MDA remains nearly constant in the tolerant cultivar (around 20 μmol at 100 mM and 28 μmol at 250 mM NaCl), but increases to more than double the amount (around 55 μmol) in seedlings of the sensitive cultivar exposed to 250 mM NaCl.

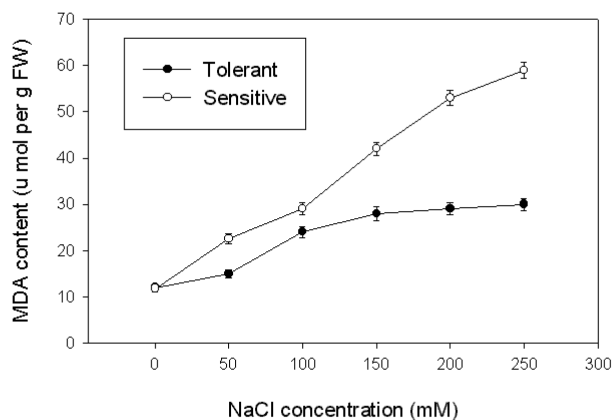

Fig. 28 Variation in the MDA content of salt-tolerant and salt-sensitive seedlings of foxtail millet grown under different concentrations of NaCl. The presented data are the average of 3 independent sets of experiments.

4.2.5 High-throughput expression analysis of salt stress responsive genes

In the present study macroarray containing 711 barley cDNA fragments described in Chapter 1 was used to study salt stress responses. It includes 58 stress responsive genes. To monitor NaCl-stress induced gene expression, mRNA samples from five-day-old tolerant and sensitive foxtail millet seedlings grown upon 250mM NaCl and without salt treatment (control) were isolated and reverse transcribed into first-strand cDNA probes. Four different ^{33}P-labelled second-strand cDNAs, two generated from tolerant (salt-treated, control) and two from sensitive seedlings (salt-treated, control) were hybridized to the barley cDNA array. The complete hybridization procedure was repeated using independently grown seedlings as material for probe preparation. The global expression changes among all the expressed sequence tags (ESTs) on the macroarray were compared between tolerant and sensitive cultivars during high NaCl treatment and control conditions. From these expression analyses, 16 non-redundant ESTs were found to be up regulated 2-fold and higher in the salt treated tolerant cultivar with respect to control, whereas in the salt-

sensitive cultivar these transcripts were not up regulated to a significant extent (see Table 13). In order to determine whether the transcripts up regulated in the salt-tolerant cultivar belong to a particular class of genes, we associated expression data with the functional classification. The functional classification of ESTs was described in annotation section of Chapter 1. The ESTs *up* regulated in the salt-tolerant cultivar represent mainly hydrogen peroxide scavenging enzymes and additionally, hypothetical and unknown genes. Some transcripts such as heat shock proteins are generally down regulated in both lines.

Table 13 cDNA clones that are preferentially expressed in salt-treated tolerant seedlings

EST-id	Putative function	Exp1 ct	Exp1 tt	Exp1 ratio	Exp1 cs	Exp1 ts	Exp1 ratio	Exp2 ct	Exp2 tt	Exp2 ratio	Exp2 cs	Exp2 ts	Exp2 ratio
	hydrogen peroxide scavenging												
HY09D05	glutathione peroxidase homolog	20,00	44,23	**2,21**	30,36	22,68	**1,31**	18,9	39,0	**2,1**	19,0	29,0	**1,5**
HY10B17	l-ascorbate peroxidase, cytosolic	75,98	164,28	**2,18**	61,21	57,06	**1,07**	54,0	111,9	**2,1**	110,0	80,9	**1,4**
HY03C01	catalase	18,00	37,66	**2,09**	17,52	20,54	**1,17**	21,0	45,0	**2,1**	24,9	33,7	**1,4**
	fuctose metabolism												
HY06N11	fructose-bisphosphate aldolase, cytosolic	8,26	17,54	**2,12**	5,29	12,81	**2,42**	9,2	18,0	**2,0**	8,0	14,0	**1,8**
	purine metabolism												
HY03K17	phosphoribosylformylglycinamidine	21,00	42,75	**2,03**	12,98	28,11	**2,16**	18,0	36,4	**2,0**	25,2	36,2	**1,4**
	lysine biosynthesis												
HY03O15	dihydrodipicolinate synthase 2	26,04	*52,76*	**2,02**	19,61	21,59	**1,10**	32,1	66,5	**2,1**	25,2	33,1	**1,3**
	energy production												
HY01A07	peptidyl-prolyl cis-trans isomerase	9,00	23,21	**2,58**	3,59	16,79	**4,68**	14,0	32,1	**2,3**	12,0	25,4	**2,1**
HY05K13	peptidyl-prolyl cis-trans isomerase	7,77	18,00	**2,30**	4,28	10,39	**2,43**	6,6	17,0	**2,6**	8,1	15,3	**1,9**
	protease inhibitor												
HY05L03	alpha-amylase/trypsin inhibitor cmd	110,00	229,81	**2,09**	118,00	149,48	**1,27**	108,7	218,3	**2,0**	119,3	133,0	**1,1**
	non-classified												
HY10M15	open rectifier potassium channel protein	291,98	1136,2	**3,89**	404,49	703,07	**1,74**	321,0	816,9	**2,5**	341,8	680,3	**2,0**
HK04B02	dihydrolipoamide dehydrogenase	27,00	55,84	**2,06**	24,51	22,68	**1,08**	15,2	29,8	**2,0**	7,2	9,3	**1,3**
HY06G06	open rectifier potassium channel protein	30,18	61,25	**2,03**	28,96	39,74	**1,37**	29,9	65,5	**2,2**	48,0	49,6	**1,0**
HY02G05	carboxyvinyl-carboxyphosphonate	26,00	52,69	**2,02**	22,47	29,03	**1,29**	23,6	49,0	**2,1**	32,0	34,5	**1,1**
HW01M06	amphiregulin precursor, human	13,28	26,04	**2,00**	8,75	16,55	**1,89**	14,9	29,2	**2,0**	12,6	18,6	**1,5**
HY09N04	argonaute protein	36,00	72,00	**2,00**	19,59	45,17	**2,31**	36,8	74,7	**2,0**	43,5	61,0	**1,4**
HY09O22	npc derived proline rich protein 1, mouse	71,00	142,00	**2,00**	64,19	72,42	**1,13**	63,3	124,5	**2,0**	50,9	62,2	**1,2**

Clones that are preferentially expressed in salt-treated tolerant cultivar are defined as those that give ratios of control: NaCl-treatment signal intensity larger than 2 with higher signal intensity values and absolute signal intensities greater than 5 au in the two independent array experiments. Putative function of secured annotated clones is given. ct – control tolerant; tt - treated tolerant (250 mM NaCl); cs –control sensitive; ts – treated sensitive (250 mM NaCl).

4.2.6 Hydrogen peroxide scavenging enzymes

Concerted attempts have been made to understand the expression of salt-mediated oxidative stress-induced hydrogen peroxide scavenging genes such as phopholipid hydroperoxide glutathione peroxidase (PHGPX), ascorbate peroxidase (APX) and catalase I (CATI). It was shown before that APX transcript and protein levels are up regulated under salt-treatment (200 mM) only in the tolerant cultivar (Sreenivasulu *et al.,* 2000). Using the cDNA array analysis we confirmed the observed changes in transcript levels of APX. As shown in Table 13, the PHGPX transcript level increased 2-fold in the tolerant cultivar under high concentrations of NaCl (250 mM), whereas no remarkable difference was found in the PHGPX expression level of salt-treated sensitive seedlings. We have evaluated the validity of the PHGPX-specific array results by using a fragment of the millet PHGPX gene as probe in northern blot analysis. The membrane was hybridized with a PHGPX-specific fragment 316 bp in length amplified from millet RNA by RT-PCR (see below). As shown in Fig. 29, the level of the PHGPX transcript is induced to higher levels in the tolerant cultivar during salt treatment, whereas in salt-sensitive cultivar PHGPX mRNA was expressed to the lower levels. Comparing the results of the two methods, i.e. cDNA array analysis and Northern blotting, nearly the same profile of PHGPX-mRNA expression was found.

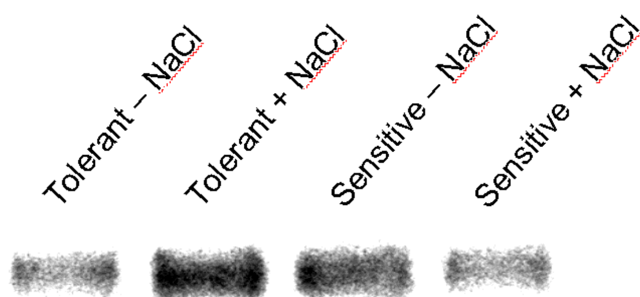

Fig. 29 Northern blot analysis of PHGPX mRNA accumulation following salt treatment (250 mM NaCl) in the tolerant foxtail millet cultivar as compared to the salt-sensitive cultivar. Total RNA was prepared from control and salt-treated seedlings, approximately 15 µg total RNA was loaded per lane, electrophorased, blotted and hybridized with ^{32}P-labelled SiGPX (*Setaria italica* PHGPX) probe.

4.2.7 Isolation and identification of a cDNA coding for a PHGPX from millet

To isolate PHGPX cDNA from millet, conserved domains of known PHGPX sequences from different species including barley sequences (Churin *et al.,* 1999) together with the barley PHGPX EST/cDNA spotted on our cDNA macroarray were used to design degenerated primers for RT-PCR. Total RNA was isolated from seedlings of the salt-tolerant and the salt-sensitive cultivar grown upon 250 mM NaCl. The RNA probes were used as template for RT-PCR. Bands were amplified only from that RNA sample extracted from tolerant seedlings (data not shown). After

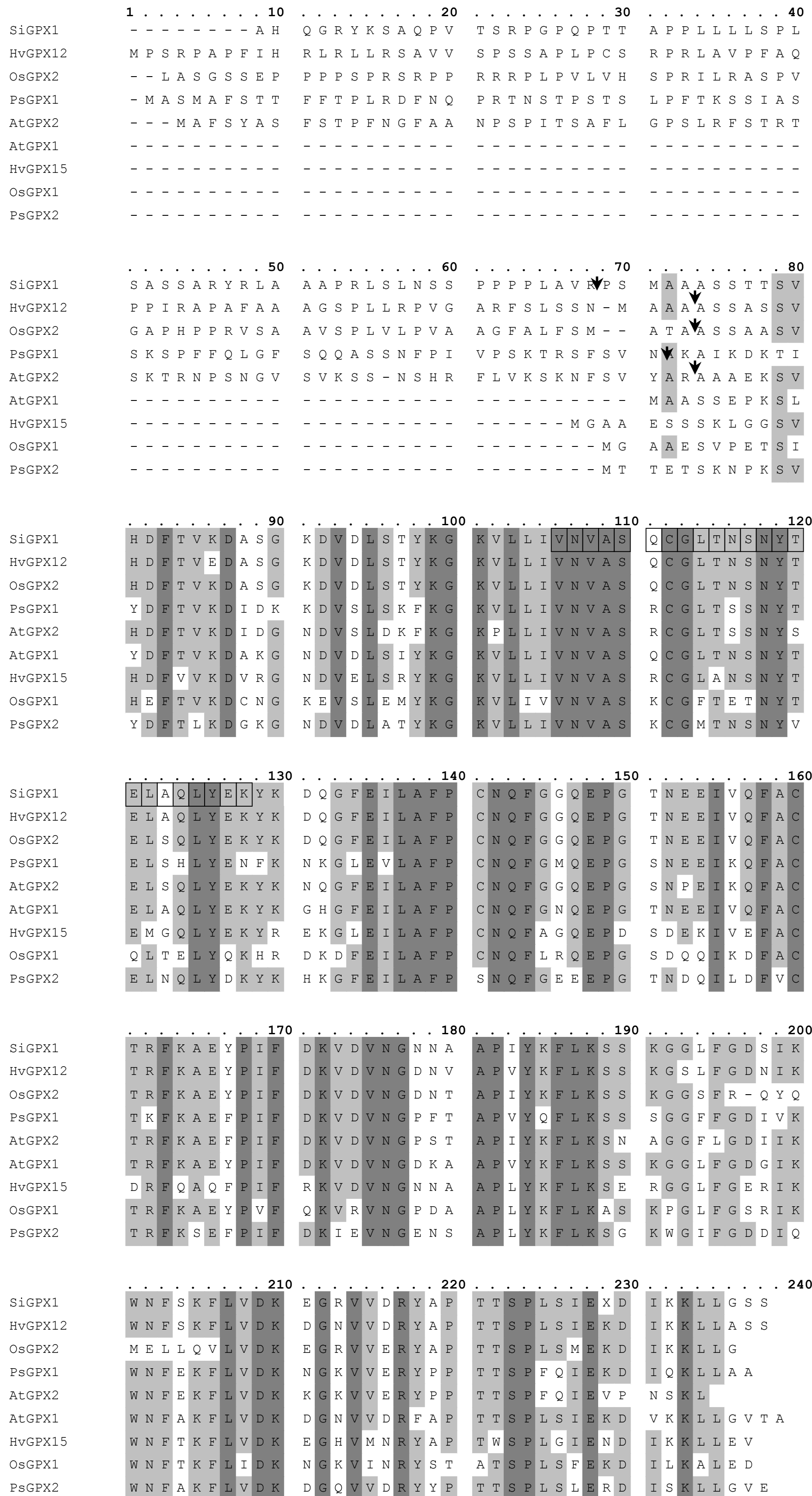

Fig. 30 Amino acid sequence alignment of cDNA clone isolated from *Setaria italica* PHGPX (SiGPX) with PHGPX sequences from other species. Database searches were carried out using the BLAST program and sequene alignment was performed with OMEGA 2.1 program. Amino acid sequences of PHGPX are aligned as follows: *Setaria italica* (SiGPX1); *Horedium vulgare* (HvGPX12-Acc Nr CAB 59893: HvGPX15-ACC Nr CAB 59894); *Oryza sativa* (OsGPX2-Acc Nr

BF430853: OsGPX1- Acc Nr CAC 17628); *Pisum sativum* (PsGPX1- Acc Nr CAA04142: BF430853; OsGPX1-Acc Mr CAC 17628); *Pisum sativum* (PsGPX1- Acc Nr CAA04142: PsGPX2); *Arabidopsis thaliana* (AtGPX2- Acc Nr CAB 40757: AtGPX1- Acc Nr AAC 09173). Residues common to all 9 proteins are shown by dark gray shading, whereas those shared by 6 to 9 proteins are shadowed by light gray colour. The putative transmembrane domains are predicted by the TMHMM program. Arrow head indicates the predicted chloroplast cleavage site. The boxed region represents the peptide sequence identified from purified protein.

cloning of the RT-PCR fragments, sequence analyses showed that the fragments obtained with two different combinations of primers (see Material and Methods) represent the same PHGPX gene. The RT-PCR amplified millet cDNA fragment (316 bp in length) shared 85% and 95% homology with the PHGPX EST from barley at the nucleotide and amino acid level, respectively. This cDNA fragment was used to screen a cDNA library prepared by Dr. Winfriede Weschke from salt-treated (200 mM) tolerant foxtail millet seedlings at high stringency (65°C). Sequence analysis of seven independent positive clones of different length revealed that all represent the same PHGPX gene. The longest cDNA was chosen and designated as millet PHGPX1. The alignment of the amino acid sequence of millet PHGPX1 to recently published putative plant and mammalian PHGPX proteins is shown in Fig. 30. Although a very high degree of homology was found to the plastid targeting sequence of barley PHGPX12, an additional in-frame ATG located in the 5′ region of PHGPX12 could not be identified in the 5′ region of the millet gene (compare Fig. 30). On the other hand, a second glutathione peroxidase found in barley HvGPX15 has not been detected in millet. Up to now, it is unclear whether the millet PHGPX1 cDNA contains the complete 5′ transcribed region of the corresponding gene. Starting with the ATG identified up to now (see Fig. 30), the predicted millet PHGPX1 protein has 237 amino acids and a molecular mass of 25.7 kD.

4.2.8 Identification of a PHGPX gene family in millet

To analyze the genomic organization of millet PGHPX1, genomic DNA from seedlings of the tolerant and sensitive cultivar was isolated, digested by different restriction enzymes and subjected to Southern blot analysis using the ^{32}P-labelled 316 bp RT-PCR fragment as a probe. Because none of the enzymes used to cut the genomic DNA has a restriction site within this fragment, different fragments visible to nearly the same high intensity within one lane of the Southern blot should represent different genomic loci of highly homologous genes. Therefore, at least two nearly identical PHGPX genes can be expected within the millet genome (see Fig. 31). Furthermore, Southern blotting revealed no differences between the two varieties in the major bands whereas a polymorphism is visible in minor bands (identified by Fig. 31).

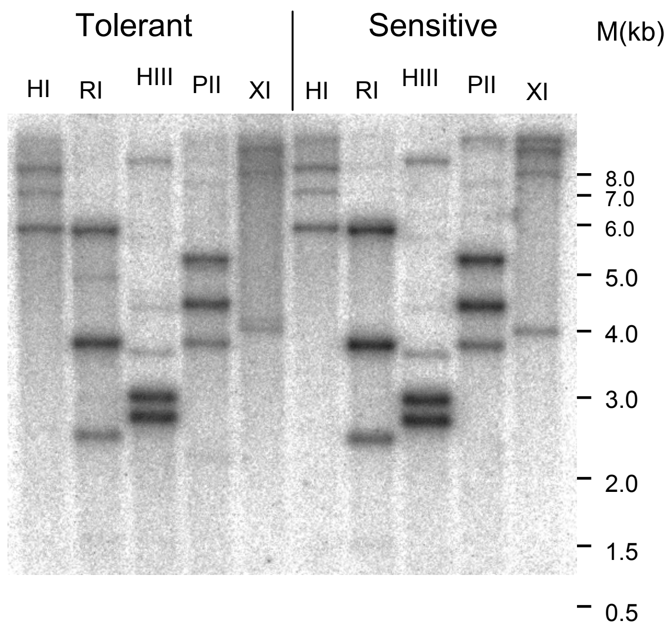

Fig. 31 Southern blot analysis of *Setaria italica* PHGPX gene. Genomic DNA (10 µg) from foxtail millet seedlings of the tolerant and sensitive genotypes were digested with restriction endonucleases (HI-Bam HI ; RI-Eco RI ; HIII-HindIII ; PII-PVUII ; XI-XhoI) Digested DNA was subjected to electrphoresis and hybridized with the ^{32}P-labelled SiGPX cDNA probe. Fragment length of the standard DNA marker (M; 1 Kb ladder, BRL) are indicated in kilo bases (kb) on the right hand side.

4.2.9 Salt-specific induction of the PHGPX protein (25 kD)

Proteins were extracted from tolerant seedlings exposed to NaCl (24 hours, 250 mM NaCl), drought (24 hours, 20% polyethylene glycol 8000), higher temperature (45^{0}C, 5 hours) and cold (4^{0}C, 5 hours) and separated on a 12-15% SDS-PAGE gradient gel. The protein patterns obtained after silver staining of the gradient gel indicate that a 25 kD protein is specifically induced by NaCl stress. Only a slight increase in concentration of this protein was registered under temperature stress (Fig. 32).

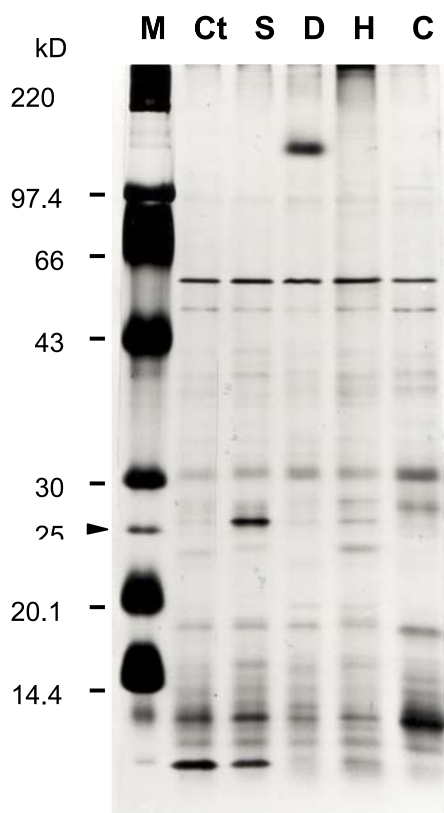

Fig. 32 Protein patterns of 5-day-old tolerant seedling samples grown under control (Ct) conditions and different types of stress such as 150 mM NaCl (S), drought (D), high temperature (H) and cold (C) depicted on a 12-15% gradient acrylamide gel. Positions of the marker (M) proteins (220, 97.4, 43, 30, 20.1 and 14.4 kilo Daltons, kD) were represented on the left hand side of gel. The arrowhead marks the position of the of 25 kD protein induced under salt treatment.

4.2.10 Purification of the salt-induced 25 kD PHGPX protein

The 25 kD protein specifically induced under salt treatment in seedlings of the tolerant cultivar was detected in the soluble fraction. The protein extract was subjected to DEAE ion-exchange chromatography. Fractions number 12-18 eluted at 0.6 M NaCl exhibited a clear protein peak and high peroxidase activity (Fig. 33a). The fractions were pooled, lyophilized and subjected to two-dimensional (2D) gel analysis. The DEAE-sepharose pooled fraction contains a 25 and a 27 kD protein with apparent isoelectric point of 4.8 and 3.7, respectively (Fig. 33b). The DEAE-Sepharose fraction containing 25 and 27 kD proteins were subjected to Superose FPLC. The 25 kD and the 27 kD protein were eluted at 141 and 121 min respectively (Fig. 33c). The homogeneity of the salt-induced 25 kD purified protein is shown in the insert of Fig. 33c.

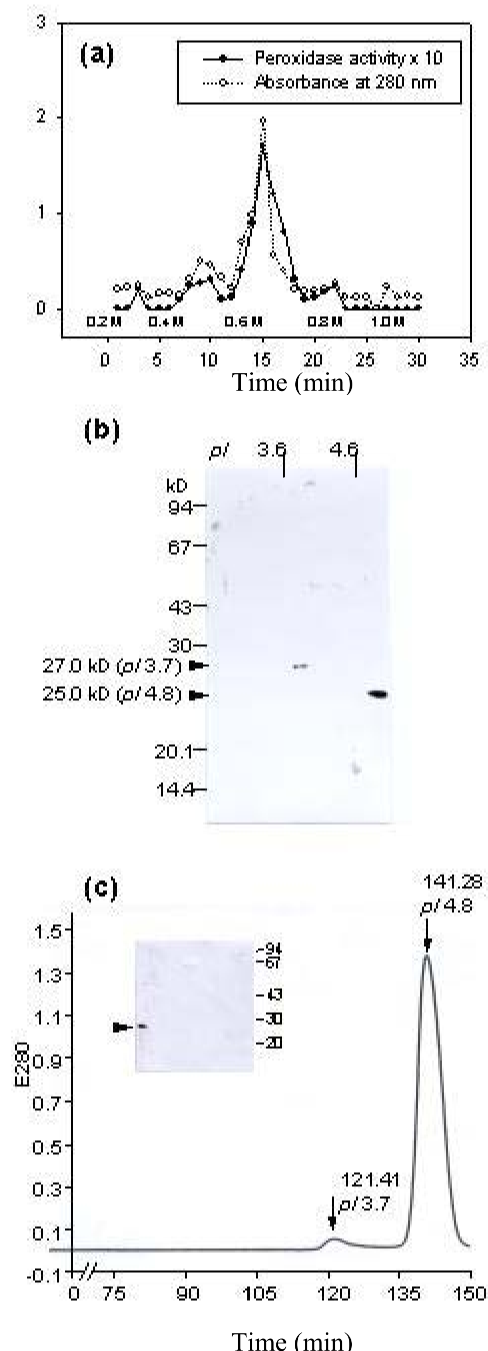

Fig. 33 Purification of the salt-induced 25 kD protein from NaCl-treated tolerant seedlings by DEAE-Sepharose and FPLC.

(a) DEAE-Sepharose column chromatography of bound protein eluted at different concentrations of NaCl (0.2, 0.4, 0.6, 0.8 and 1.0 M). The elution time-scale is given on X-axis. Peroxidase activity and absorbance values at 280 nm were scaled on Y-axis.

(b) Two-dimension gel analyses of 0.6 M eluted fraction to confirm the molecular weights of partially purified fraction of 27 kD (*pI* 3.7) and 25 kD (*pI* 4.8) proteins. The standard molecular weight marker were represented in kD on the left hand side and Standard pI markers are represented on of the gel.

(c) Superose FPLC chromatogram of purified 25 kD (*pI* 4.8) and 27 kD (*pI* 5.5) proteins represented as eluted peak at 121.41 min and 141.28 min, respectively.

4.2.11 Amino acid (AA) sequence analysis of the salt-induced 25 kD protein

The purified 25 kD protein (*pI* 4.8) was subjected to amino acid analysis of the N-terminal region. Since the N-terminal region of the protein was blocked, it was subjected to trypsin digestion. The generated peptides were isolated by reversed-phase HPLC and sequenced (by Prof. Prakash). The amino acid sequence of the major well resolved peptide (VNVASQCGLTNSNYTELAQLYEK) showed high homology to non-selenium glutathione phospholipid hydroperoxide peroxidase from plants and human as shown in Fig. 30.

4.3 DISCUSSION

4.3.1 Application of barley macoarrays to foxtail millet

It is well known that, in the grasses, conservation of gene orders (synteny) extends beyond species and even tribes (Chao *et al.,* 1988; Whitkus *et al.,* 1992; Ahn and Tanksley 1993; Peterson *et al.,* 1995). Establishment of the relationship between the genomes of different species provides researchers with the possibility of trans-genomic gene identification and isolation. Foxtail millet and other millet species for which little is known at the genomic level will benefit greatly from the EST information already available in other grasses, especially in cereals such as barley. Since several cereals are phylogenetically close to foxtail millet, we explored the ability of barley-based arrays to examine the expression patterns in foxtail millet genotypes differing in salt tolerance. Since we worked with a heterologous system (barley chips and millet probes), hybridizations were performed at 60^0 C. Based on expression data presented in the result section (Table 13), the conclusion can be drawn that barley macroarrays are a very useful tool to analyze foxtail millet with a minor loss in sensitivity in comparison to homologous probes (data not shown). Using foxtail probes we identified specific mRNAs induced by NaCl in the tolerant cultivar to a 2-fold levels (Table 13). In only a few studies, mRNA profiles resulting from cDNA array analyses can be compared to both, protein amount and enzyme activity of the same gene. In order to validate the data generated by array experiments we selected PHGPX and APX (Sreenivasulu *et al.,* 2000) genes to conduct independent confirmation by northern blot analysis. Further we isolated the PHGPX gene from the salt-tolerant foxtail cultivar “Prasad” to follow the line mRNA abundance → protein amount → enzyme activity and to analyze whether the increase in mRNA expression seen on the macroarray filter is reflected at the level of enzyme activity. Northern analyses using a PHGPX and APX probe from foxtail millet as probe in principle confirmed the array data. The induction of the PHGPX transcript was correlated to the increased level of the PHGPX protein in the tolerant foxtail millet cultivar treated by NaCl treatment.

4.3.2 Differential response of physiological parameters to salinity stress in salt-tolerant and salt-sensitive seedlings of foxtail millet

As salinity is one of the important limiting factors in agriculture, a better understanding of the behavior or responses of plants to salinity is necessary to develop plants, which could withstand higher salinity. Therefore, an attempt has been made to understand the mechanism behind salt tolerance taking two cultivars of foxtail millet differing in their sensitivity to salinity. The analysis of endogenous Na^+ ions content showed a higher accumulation of Na^+ in the sensitive cultivar as compared to that of the tolerant one (Fig. 26). Growth of the salt tolerant foxtail millet cultivar is certainly favored by the maintenance of a constant Na^+ ion level in the plant. In this context, it is important to mention that an antiserum raised against proteins of the salt-tolerant cultivar recognized a putative Na^+/H^+ antiporter, which is highly enriched in the tolerant foxtail millet seedlings as compared to the sensitive one (Sreenivasulu *et al.,* unpublished data). Similar observations on a relationship between endogenous Na^+ concentrations and salt tolerance have been reported for different organs of rice (Yeo and Flowers, 1983; Dionisio-Sese and Tobita, 1998). Furthermore, salinity causes stronger electrolyte leakage of sensitive seedlings in comparison to the tolerant seedlings (Fig. 27). Likewise, strong differences in electrolyte leakage between tolerant and sensitive cultivars were described for 3-week-old rice seedlings grown under salinity stress (Dionisio-Sese and Tobita, 1998). Increase in MDA content in sensitive seedlings was observed at 150 mM NaCl treatment (Sreenivasulu *et al.,* 1999) whereas tolerant seedlings maintained constant MDA levels even up to 200 mM NaCl (Fig. 28). The constant value of electrolyte leakage in combination with a low content of MDA observed in the tolerant seedlings, provide some evidence for lower lipid peroxidation and less affected membrane integrity in comparison to the sensitive seedlings.

4.3.3 The possible role of hydrogen peroxide scavenging enzymes in salt-mediated oxidative stress tolerance

Results of biochemical studies showed that better growth of tolerant foxtail millet seedlings under high salt conditions is accompanied by an efficient antioxidative system as part of tolerance mechanism to cope-up salt mediated oxidative stress (Sreenivasulu *et al.,* 1999; 2000). It was noticed earlier that salt induces the up regulation of superoxide dismutase (SOD) activity mainly due to the induction of cytosolic Cu/Zn-SOD and Mn-SOD isoforms in tolerant, but not in the salt-sensitive foxtail millet cultivar, is expected to scavenge superoxides, which in turn resulted in formation of H_2O_2 (Sreenivasulu *et al.,* 2000). SOD converts relatively less toxic $O_2^{\cdot -}$ radicals to the more toxic H_2O_2. The excess H_2O_2 produced under salt stress needs to be scavenged at the

production site itself in order to achieve salt-induced oxidative tolerance during long-term salt stress (Foyer *et al.,* 1994). Plants have evolved various protecting mechanisms to adapt themselves against such menace and one such system is the presence of antioxidant enzymes such as peroxidases and catalases, which detoxify H_2O_2. During NaCl treatment the salt-tolerant foxtail millet cultivar exhibited a higher activity of the H_2O_2-detoxifying enzyme APX (Sreenivasulu *et al.,* 2000). The increase of both SOD and APX activity shown by our previous results (Sreenivasulu *et al.,* 2000) is in agreement with the induction of both activities in a salt-treated *Arabidopsis* mutant line (PS1) with salt tolerance (Tsugane *et al.,* 1999) and in salt-tolerant peas treated with NaCl (Hernandez *et al.,* 1993).

Using the EST approach, we dissected the oxidative stress effects of complex-salt tolerance trait, and identified salt-mediated-oxidative stress induced hydrogen peroxide scavenging transcripts such as PHGPX, APX and CATI in tolerant seedlings under high NaCl treatment (250 mM NaCl). In order to investigate the molecular mechanism of salt-mediated oxidative stress, a detailed study was initiated including molecular cloning of the PHGPX cDNA as well as characterization and purification of the protein. In the present study, data obtained from expression analysis by using barley ESTs indicates that the transcript accumulation of PHGPX was increased significantly at 250 mM NaCl treatment in the tolerant cultivar (Table 13). Our findings are consistent with other reports, for instance about induction of PHGPX during NaCl treatment in citrus (Beeor-Tzahar *et al.,* 1995) and barley (Churin *et al.,* 1999). The present results are also in agreement with results of Gueta-Dahan *et al.* (1997) with respect to higher expression of PHGPX under NaCl treatment when compared to control conditions. On the other hand, Gueta-Dahan *et al.* (1997) showed that long-term exposure of salt resulted in similar increases in the amount of PHGPX in both salt-sensitive and salt-tolerant citrus cells. As part of their recent investigations they reported that short-time NaCl treatment increased PHGPX more in salt treated sensitive seedlings of citrus cells than in salt-tolerant ones (Avsian-Kretchmer *et al.,* 1999), which is in contrast to our present findings. Recently, Hernánndez *et al.* (2000) showed that PHGPX mRNA level was significantly induced in the NaCl-tolerant pea variety in comparison to the NaCl-sensitive variety during long-term salt stress. This data strongly correlate with our results and suggest that induction of PHGPX is at least part of the tolerance mechanism in tolerant millet as well as salt-tolerant pea genotypes under long-term salt-stress.

In plants, PHGPX was not known until recently. In animals, PHGPX is considered as one of the key enzymes involved in scavenging hydroxyl radicals and hydrogen peroxide. Both the plant and

mammalian PHGPX enzyme contain three conserved domains: NVASQ(C/X)G, ILAFPCNQF and IKWNF(S/T)DFL(V/I)DK with catalytic residues X, Q and W respectively. The presence of the amino acids selenocysteine, glutamic acid and tryptophan is known as being critical for the catalytic activity in mammalian PHGPX enzyme (Chu *et al.,* 1993). The most prominent difference between plant and mammalian PHGPX is replacement of selenocysteine (present in the mammalian enzyme) by cysteine in plant PHGPX. This replacement results in a drastic decrease of the activity of the plant enzyme in comparison to the mammalian PHGPX one (Eshdat *et al.,* 1997). Similar to other plant PHGPXs, millet PHGPX contains cysteine instead of selenocysteine, suggesting that millet PHGPX is one of the non-selenium PHGPX. However, the two other amino acids, glutamic acid and tryptophan (see Fig. 30) known to be critical for the catalytic activity, are present in the millet PHGPX sequence.

Based on amino acid alignments of many plant PHGPXs, at least two protein/enzyme classes could be discerned (see Fig. 30). The longer cDNA most likely encodes an isoform targeted to the plastid and the shorter cDNA a cytosolic isoform of PHGPX. In barley, Churin *et al.,* (1999) have isolated 3 PHGPX cDNA clones, out of them two clones are up regulated under salt stress. The longer isoform would be targeted by a transit peptide to the chloroplast, whereas the isoforms lacking such transit peptide would be localized within the cytoplasm. In the predicted protein derived from the SiGPX cDNA an in-frame methionine residue was identified (position 63), which corresponds to the start codon of the cytoplasmic isoform. On the other hand, computer analyses have predicted a transit peptide located in the region upstream of this methionine residue, which shares similarity to the probable transit peptide of the barley HvGPX12. It seems that the millet cDNA 5′-region is incomplete and possibly, also possesses a plastid transit peptide. Most likely, the SiGPX is localized in plastids as recently demonstrated for a PHGPX from barley, *Arabidopsis* and pea (Mullineux *et al.,* 1998; Churin *et al.,* 1999). Interestingly, the third PHGPX isoform found in barley is down regulated under salt stress (Churin *et al.,* 1999). As expected from that finding, expression of this cDNA could not be demonstrated in the salt-treated tolerant millet cultivar, but its existence would explain the Southern analyses result, which hints to the presence of a small PHGPX gene family in the millet genome (Fig. 31).

Until now, the salt-induced PHGPX was purified from the cytosolic fractions in citrus (Ben-Hayyim *et al.,* 1993; Beeor-Tzahar *et al.,* 1995) and another PHGPX isoform was purified to homogeneity from total membrane fractions of *Hansenula markii*, a yeast strain resistant against lipid hydroperoxide treatment (Tran *et al.,* 1993). In the present study, we purified the 25 kD millet salt-induced PHGPX protein (*pI* 4.8) from the soluble fraction of 5-day-old seedlings of a

tolerant foxtail millet cultivar (see Fig. 33b). Expression of the 25 kD protein was found under high salt conditions; it was not induced by other abiotic stresses (see Fig. 32). Thus, the induction of protein expression can be correlated to salinity tolerance. Furthermore, the peptide sequence of the purified 25 kD protein (*pI* 4.8) showed high similarity to mammalian PHGPX. Because a high similarity was found to several PHGPX sequences from other plant species, and further on, the isolated peptide could be assigned to the expected region of the PHGPX proteins (Fig. 30), PHGPX from *Setaria italica* (SiGPX) should have the physiological function of a glutathione peroxidase, i.e. to reduce directly peroxidized membrane lipids.

4.4 SUMMARY

On the basis of cDNA array analysis, hydrogen peroxide scavenging genes such as PHGPX, APX and CAT were found to be up regulated in the tolerant cultivar under long-term 250 mM NaCl exposure. Further on, the strong increase of PHGPX mRNA expression was the starting point for a detailed investigation of one of the most interesting antioxidant enzyme in plants. The transcript induction of PHGPX gene in salt-tolerant cultivar during NaCl treatment revealed by macroarray analysis can be juxtaposed to protein level. The induction of hydrogen peroxide scavenging genes in the salt tolerant cultivar in response to salt (250 mM NaCl) stress corroborates earlier data (Mullineux *et al.,* 1998; Churin *et al.,* 1999). The present study shows substantial differences in the cellular reactivity between the salt-tolerant and salt-sensitive foxtail millet cultivars in response to salinity stress. Upon high NaCl concentrations, seedlings of the salt-sensitive cultivar show a high internal Na^+ concentration, resulting in symptoms of oxidative damage, which are accompanied by a decrease in PHGPX, APX and CAT gene activity. The process leads to electrolyte leakage and ultimately cell death. Growing upon the same NaCl concentration, seedlings of the tolerant cultivar show maintenance of cellular intactness and display a significantly lower Na^+ accumulation. The increase of antioxidative enzyme activity induced in parallel at low intracellular Na^+ concentration represents a part of the complex salt tolerance mechanism. This could explain the ability of the tolerant foxtail millet cultivar to grow at higher NaCl concentrations than the sensitive one.