Plant Cell Rep (2006) 25: 865–876
DOI 10.1007/s00299-006-0127-4

BIOTIC AND ABIOTIC STRESS

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Monitoring expression profiles of antioxidant genes to salinity, iron, oxidative, light and hyperosmotic stresses in the highly salt tolerant grey mangrove, *Avicennia marina* (Forsk.) Vierh. by mRNA analysis

Received: 9 September 2005 / Revised: 13 January 2006 / Accepted: 17 January 2006 / Published online: 14 March 2006

Abstract Plant photosynthesis results in the production of molecular oxygen. An inevitable consequence of this normal process is the production of reactive oxygen species (ROS) by the transfer of electrons to molecular oxygen. Plants are adequately protected by the presence of multiple antioxidative enzymes in different organelles of the plant such as chloroplasts, cytosol, mitochondria and peroxisomes. Under high light and CO_2 limiting conditions caused by environmental stress like salinity, these antioxidative enzymes play an important role in scavenging toxic radicals. To investigate the functions of antioxidative enzymes in a mangrove plant, we isolated three cDNAs encoding cytosolic Cu–Zn SOD (*Sod1*), catalase (*Cat1*) and ferritin (*Fer1*) from *Avicennia marina* cDNA library. *Sod1*, *Cat1* and *Fer1* cDNA encoded full-length proteins with 152, 492 and 261 amino acids respectively. We studied the expression of these antioxidant genes in response to salt, iron, hydrogen peroxide, mannitol and light stress by mRNA expression analysis. *Cat1*, *Fer1* showed short-term induction while *Sod1* transcript was found to be unaltered in response to NaCl stress. A decrease in mRNA levels was observed for *Sod1*, *Cat1* while *Fer1* mRNA levels remained unaltered with osmotic stress treatment. *Sod1*, *Cat1* and *Fer1* mRNA levels were induced by iron, light stress and by direct H_2O_2 stress treatment, thus confirming their role in oxidative stress response.

Keywords *Avicennia marina* · Catalase · Cu–Zn SOD · Ferritin · ROS

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Communicated by A. Altman

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Introduction

Plant growth and development are influenced by the presence of a variety of environmental factors such as availability of water, light and nutrients (Xu et al. 1996; Boyer 1982). The interactions between different environmental factors are important in determining the survival and performance as well as the productivity of plants (Boyer 1982). However, any disturbance in these environmental factors may result in an imbalance in the system, thereby affecting the plant growth and survival. There are several stress conditions that result in this imbalance such as increase in salt concentration in soil (salinity), oxidative stress and extremes of daylight, collectively termed abiotic stress factors (Holmberg and Bulow 1998; Tsang et al. 1991).

Mangroves are a group of plant communities that grow in tropical and subtropical estuarine regions of the world (Tomlinson 1986). Mangroves constitute the most well-adapted halophytes surviving in harsh environmental conditions like high external salt concentration and high light intensity that are normally unsuitable for the survival of other plants (Cheeseman et al. 1997). *Avicennia marina* (Forsk.) Vierh. is a highly salt tolerant mangrove plant species that dominates the Pichavaram mangrove forests (Chidambaram, South India). These plants survive wide fluctuations in salinity levels and are well adapted to the mangrove conditions (Tuffers et al. 2001; Selvam 2003). *A. marina* possess physical and structural attributes such as pneumatophores and salt glands. The salt resistance of *A. marina* is due to three different mechanisms: (1) salt avoidance through the roots; (2) capacity to maintain normal metabolic activity in the presence of high intracellular salt levels; (3) secretion of penetrating ions using salt glands (Waisel et al. 1986).

Abiotic stress factors, including salinity, high light intensity, high temperature, and heavy metals, lead to oxidative stress with the formation of reactive oxygen species (ROS) causing extensive cellular damage and inhibition of photosynthesis (Allen 1995; Polle 1997). ROS result from the excitation of O_2 to form a singlet oxygen (O_2^1) or from

the transfer of one, two or three electrons to O_2 to form superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) or a hydroxyl radical (OH^-) respectively (Mittler 2002). The role of ROS during abiotic stress conditions is an important area of research because ROS has been implicated in processes leading to plant stress acclimation (Dat et al. 1998, 2000).

Plants have developed non-enzymatic and enzymatic antioxidant mechanisms to counter the deleterious effects of ROS. The non-enzymatic components are low-molecular weight antioxidant molecules such as ascorbic acid, carotenoids and glutathione (Alscher and Hess 1993). The antioxidant enzymatic pathway consists of different enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), which effectively remove free radicals. The antioxidative enzymes are the principal components in the antioxidative system in plants as they are largely responsible for scavenging the ROS (Allen 1995). Superoxide dismutases constitute the first line of defence against ROS and catalyses the dismutation of the superoxide radical into hydrogen peroxide (Salin 1987). Catalases are tetrameric heme-containing enzymes that convert hydrogen peroxide to water and oxygen, protecting the cell from the damaging effects of hydrogen peroxide accumulation (Sanchez-Casas and Klessig 1994). The balance between SOD and CAT or APX activities in cells is crucial for determining the steady-state level of superoxide radicals and hydrogen peroxide and counteracting the deleterious effects of ROS (Bowler et al. 1991; Apel and Hirt 2004). More importantly, the different affinities of APX (micromolar level) and CAT (millimolar level) for H_2O_2 suggest that these two enzymes fall into two different classes of H_2O_2-scavenging enzymes—APX being involved in the fine modulation of ROS for signalling, whereas CAT is involved in the removal of excess ROS during environmental stress (Mittler 2002).

In addition to enzymatic detoxification of ROS, controlling the concentration of free transition metals is an important complementary way to prevent oxidative damage (Lobreaux et al. 1995). Among the transition metals, iron is the most abundant in biological systems, and through metal-dependent Fenton reaction, it promotes hydroxyl radical formation (Halliwell and Gutteridge 1989). To overcome the problem of biological insolubility and potential toxicity of iron in the presence of oxygen, plants have developed biological molecules, which accommodate this element in a safe, non-toxic bioavailable form. Among these molecules is ferritin, a class of multimeric protein that participates in the protection of plastids by sequestering several thousand iron atoms in their central cavity (Theil 1987; Harrison and Arosio 1996). Therefore, the balance of ROS through enzymatic detoxification and sequestration of metal ions are two important mechanisms in preventing the formation of highly toxic hydroxyl radicals and protecting the cells against oxidative damage (Lobrauex et al. 1995).

Most of the detailed studies conducted in mangroves relate to biochemical pathways for instance, catabolism of sugars, accumulation of osmolytes like betaine and adenosine metabolism (Fukushima et al. 1997; Suzuki et al. 2003; Ashihara et al. 2003). Previous studies on the effect of salt stress on antioxidant status in *A. marina* were done by monitoring the enzyme activities (Ashihara et al. 1997; Cherian et al. 1999). However, studying gene expression in response to environmental stress at the level of mRNA abundance gives a more reliable estimate of antioxidant gene activation than enzyme activity (Willekens et al. 1994b).

We had earlier reported the existence of stress-responsive genes using Expressed Sequence Tags (ESTs) (Mehta et al. 2005). As a first step towards studying the role of individual genes in *A. marina*, we analysed the antioxidant response to salt, iron, oxidative and light and hyperosmotic stress by monitoring the mRNA levels, and focused on three genes: Cu–Zn SOD, catalase and ferritin.

Materials and methods

Seeds of *A.marina* collected from the Pichavaram mangrove forest, Tamil Nadu, India, were grown in sand-filled trays in a greenhouse at $35 \pm 2^{\circ}C$ under a 12 h/12 h (light/dark) photoperiod (illuminated from 06:00 to 18:00 h) for 1 month with daily watering (Mehta et al. 2005).

cDNA library construction

Total RNA was extracted from leaves of 1-month-old *A. marina* seedlings treated with 500 mM NaCl for 48 h, following the GITC method (Chomzynski and Sacchi 1987). cDNA was prepared from poly(A^+)-rich RNA using the SuperScript Lambda system (Invitrogen, USA) and directionally cloned in pSPORT1 plasmid vector at *Not*I–*Sal*I sites (Invitrogen). A library consisting of 1×10^5 clones was obtained (Parani et al. 2002; Mehta et al. 2005).

Isolation of cDNAs and sequencing

Sod1 was isolated from *A. marina* cDNA library using ^{32}P labelled *Eco*RI–*Xho*I fragment of *Mesembryanthemum crystallinum* Cu–Zn *Sod1* cDNA. A partial *Cat1* cDNA insert that showed identity with EST clone (BM173046) was isolated from *Avicennia marina* cDNA library using ^{32}P labelled *Cat1* cDNA fragment of *Nicotiana plumbaginifolia* (Willekens et al. 1994a). The full-length *Cat1* cDNA insert was subsequently isolated from *A. marina* cDNA library using ^{32}P labelled partial *Cat1* cDNA by restriction digestion at *Not*I–*Sal*I sites. A partial ferritin EST clone (CD777449) was used to obtain the full-length gene *Fer1* by TAIL–PCR methodology (Liu et al. 1995). A single clone was isolated to homogeneity for all cDNAs and pure plasmid DNA extracted (Sambrook et al. 1989). All the full-length putative cDNAs were sequenced using Big-Dye Chain termination method with ABI Prism 310 DNA sequencer (Applied Biosystems, Foster City, CA).

Stress treatments of *A. marina* seedlings

Salt stress treatment

One-month-old *A. marina* seedlings (four-leaf stage) were acclimatized for 3 days under 16 h light/8 h dark cycle in a growth chamber in half- strength MS medium (Murashige and Skoog 1962). The pre-adapted plants were then transferred to half-strength MS medium supplemented with 0.5 M NaCl (nearly sea-water salt concentration; Takemura et al. 2000; Mehta et al. 2005). Leaf tissues were harvested from time intervals of 0, 12, 24 and 48 h of salt stress treatment. A final sample collection was made 24 h after removal from NaCl medium.

Iron stress treatment

A. *marina* seedlings were kept in iron-deficient half-strength MS nutrient solution for a period of 1 week in 16 h light/8 h dark cycle. The nutrient solution was changed every 3 days. Iron loading in leaves of plants has been shown to be limited by the root barrier (Lobreaux et al. 1995). Therefore, to remove the root-barrier obstacle, plantlets were cut at the level of collar. Subsequently, the stem was washed with nutrient solution after 7 days in iron-free MS solution (Lobreaux et al. 1995). Iron citrate, which is the natural form of iron present in the xylem sap of plants was used for performing stress experiments (Cataldo et al. 1988). The de-rooted plants were therefore placed in freshly prepared half-strength MS nutrient solution supplemented with 1,000 μM of Fe(III)-citrate (Sigma, USA). The leaves were collected from seedlings at time intervals of 0, 0.5, 1, 3 and 6 h. At the 6 h treatment, partially bronzed and non-bronzed leaves were collected. Additionally, iron stress was withdrawn by replacing the iron stress medium with normal half-strength MS nutrient solution, and leaves were collected 3 h after withdrawal of iron stress.

Hydrogen peroxide stress treatment

For H_2O_2 stress treatment, the seedlings were transferred to half-strength MS medium supplemented with 90 mM of H_2O_2 (Merck, India), and the leaves were collected at time intervals of 0, 6, 12, 24 and 36 h after stress treatment. Additionally, leaves were harvested 12 h after withdrawal from H_2O_2 stress.

Mannitol stress treatment

Mannitol stress experiments were carried out by supplementing half-strength MS medium with 1,000 mM mannitol (Sigma, USA). The concentration of mannitol used (1,000 mM) was iso-osmotic to 500 mM NaCl (Kreps et al. 2002).

Light stress treatment

One-month-old seedlings were maintained for 3 days in half-strength MS medium under 16 h light/8 h dark cycle in a growth chamber. For evaluating the effect of white light mediated oxidative stress, plants adapted for 3 days (3D), 7 days (7D) in the dark were exposed to white-incandescent light of approximately 5,000 lux (Tsang et al. 1991). Leaf samples were harvested from treated plants at time intervals of 0.5, 1, 6, 12 and 24 h after light exposure. Samples from dark adapted 3 days (3D) and 7 days (7D) plants were harvested using a dim safety light (Tsang et al. 1991). Leaf samples were harvested from plants maintained in 16 h/8 h light/dark photoperiod conditions as a control.

Harvest of plant material after stress treatments

Leaf samples for stress experiments were taken in the light period at least 1.5 h after the beginning and before the end of the illumination period. In this study, eight leaves that are indistinguishable were harvested from different plants and pooled for RNA extractions for both control and stress treatments (Willekens et al. 1994b). All treatments were done in duplicate, and the harvested leaves were immediately frozen in liquid nitrogen and stored at –70°C for further analysis.

RNA extraction and Northern analysis

Total RNA was isolated using the GITC method of Chomzynski and Sacchi (1987). Total RNA (15 μg) from leaf samples were resolved on a 1.4% agarose gel containing 6% (v/v) formaldehyde and transferred to nylon Hybond-N^+ (Amersham Inc., USA) by capillary transfer (Sambrook et al. 1989). UV cross-linking was performed according to the manufacturer's instructions (Hoefer, Germany). Hybridizations were performed using Sigma Perfect Hyb PLUS hybridization buffer as per the instructions in the manual (Sigma, USA). Blots were probed with highly specific cDNA fragments amplified using 3′-untranslated region (UTR) primers for *Sod1*, *Cat1*, *Fer1* and *Ubc*. The amplified products were gel eluted, ^{32}P labelled using the random primer method (Rediprime, Amersham Biosciences) and column purified (Amersham Biosciences). The primers used for each of these genes are listed in Table 1. mRNA hybridizing to *Sod1*, *Cat1* and *Fer1* specific 3′ cDNA fragments in the total RNA blot would therefore be referred to simply as *Sod1*, *Cat1* and *Fer1* mRNA, respectively. A partial rbcs cDNA insert in T-vector (BD Biosciences, USA) from *Sesuvium portulacastrum* was digested with *Eco*RI. A 377 bp *rbcs* fragment was ^{32}P labelled and used for light stress experiments. Stringent washes were carried out using 0.5 × and 0.1 × SSC containing 0.5% (w/v) SDS at 65°C, sequentially. For each individual experiment, the same blot was stripped and re-probed with the other genes.

Table 1 List of primers used in this study for amplifying 3′-UTR region of *Sod1*, *Cat1*, *Fer1* and *Ubc*

Gene	3′-UTR primer sequences	GenBank accession number	PCR conditions
Sod1	Forward 5′-ATCATTACCCAGTCGCTTGT-3′ Reverse 5′- AGCAAAGATGATGTGGGAAC-3′	AF328859	94°C/2 min; 94°C/30 s, 60°C/1 min, 72°C/1 min-30 cycles; 72°C-5 min
Cat1	Forward 5′-GAGAATGGAGGCAACGTTTA-3′ Reverse 5′- TGTGTGCATCAAGAAGTTCG-3′	AF328861	94°C/2 min; 94°C/30 s, 60°C/30 s, 72°C/1 min-30 cycles; 72°C-5 min
Fer1	Forward 5′-ATCTCTATCCGTGGTTTGCC-3′ Reverse 5′-GGATTCACAGCTCCATCAAAT-3′	DQ114787	94°C/2 min; 94°C/30 s, 65°C/30 s 72 °C/30 s-30 cycles; 72°C-5 min
Ubc	Forward 5′-TCCCTTACTAGACGGTTGG-3′ Reverse 5′-AGTGACGCGTTCCTTACA-3′	AF262934	94°C/2 min; 94°C/30 s, 60°C/30 s, 72°C/30 s-30 cycles; 72°C-5 min

The GenBank accession numbers and PCR conditions are also given

Results

Isolation of cDNAs from *A. marina*

We had previously reported that cDNA inserts encoding Cu–Zn superoxide dismutase, catalase and iron homeostasis protein ferritin were represented in the genes regulated by stress, cell-defence and rescue in *A. marina* (Mehta et al. 2005). Out of the total genes involved in stress/cell-defence and rescue, about 12.5% represented isoforms encoding putative Cu–Zn superoxide dismutases, catalase and ferritin. While full-length catalase and ferritin cDNAs were isolated using partial/EST cDNAs, Cu–Zn superoxide dismutase cDNA was isolated from cDNA library using Cu–Zn *Sod* cDNA fragment of *Mesembryanthemum crystallinum*. Previous reports suggest increased tolerance to oxidative stress factors like salt and ozone stress with expression of a cytosolic Cu–Zn SOD in plants (Badawi et al. 2004; Pitcher and Zilinskas 1996) and therefore, in this study, a cytosolic Cu–Zn SOD was isolated from *A. marina*.

The insert size of cytosolic Cu–Zn superoxide dismutase cDNA clone (768 bp) included a single largest Open Reading Frame (ORF) of 459 bp (47–505 bp), 263 bp 3′-UTR and encoded a full-length putative protein of 152 amino acids (Fig. 1a). The deduced amino acid sequence of Cu–Zn superoxide dismutase cDNA insert showed 88% identity with *Paulownia kawakamii* Cu–Zn SOD cDNA. Catalase cDNA had an insert size of 1,754 bp, an initiation codon ATG at 21 bp and a stop-codon TGA at positions between 1,497 and 1,499 bp. Thus, this cDNA insert included a single largest ORF of 1,479, 20 bp 5′-UTR and a 255 bp 3′-UTR (Fig. 1b). The deduced amino acid sequence of catalase cDNA encoded a protein of 492 amino acids and was full length. Amino acid sequence of catalase showed 82% identity with *Manihot esculenta*, 81% with *Prunus persica* and 76% with *Lycopersicon esculentum Cat1*. A consensus tri-peptide S-R-L, which functions as a peroxisomal targeting signal (PTS) for import and localization to peroxisomes, is present in the carboxy-terminus of deduced catalase amino acid sequence (Willekens et al. 1994a).

Ferritin cDNA had an insert size of 1,025 bp consisting of a 10 bp 5′-UTR, 229 bp 3′-UTR with a single largest ORF of 786 bp (Fig. 1c). The translated amino acid sequence of ferritin cDNA showed 77% identity to ferritin chloroplast precursor 2 of *Nicotiana tabacum*, and encoded a full-length chloroplastic ferritin protein of 261 amino acids. Because all these cDNAs were the first isolated genes from *Avicennia marina* cDNA library, they were designated *Sod1*, *Cat1* and *Fer1*. Each of these genes encoded full-length putative protein with a predicted M_r of 16 kDa (*Sod1*), 57 kDa (*Cat1*) and 28 kDa (*Fer1*).

Effect of NaCl stress

Total RNA isolated from leaves of control and salt-stressed plants were used for RNA gel-blot analysis to study the effect of 500 mM NaCl stress on the accumulation of *Sod1*, *Cat1* and *Fer1* transcripts. The response of genes falls into two categories of transcript accumulation pattern: (1) *Cat1*

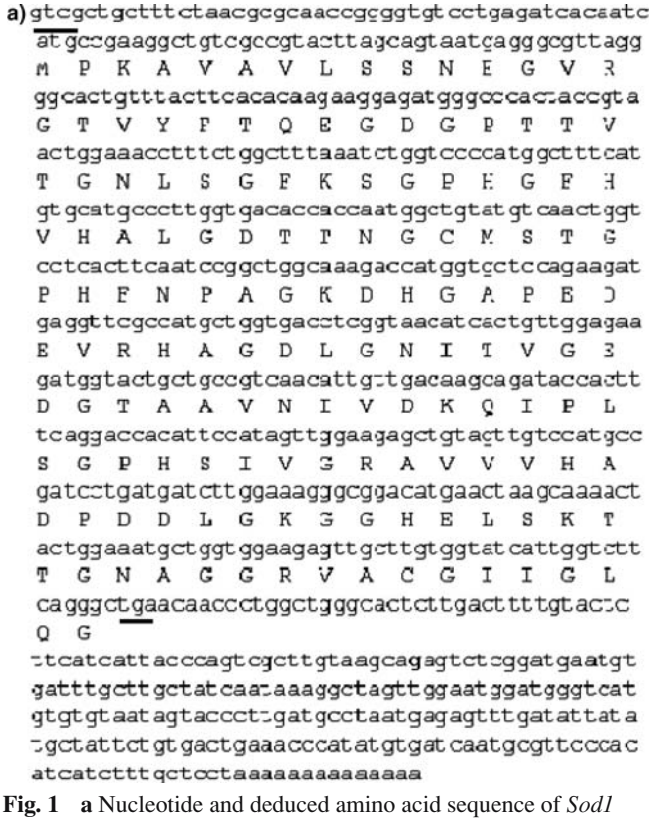

Fig. 1 **a** Nucleotide and deduced amino acid sequence of *Sod1*

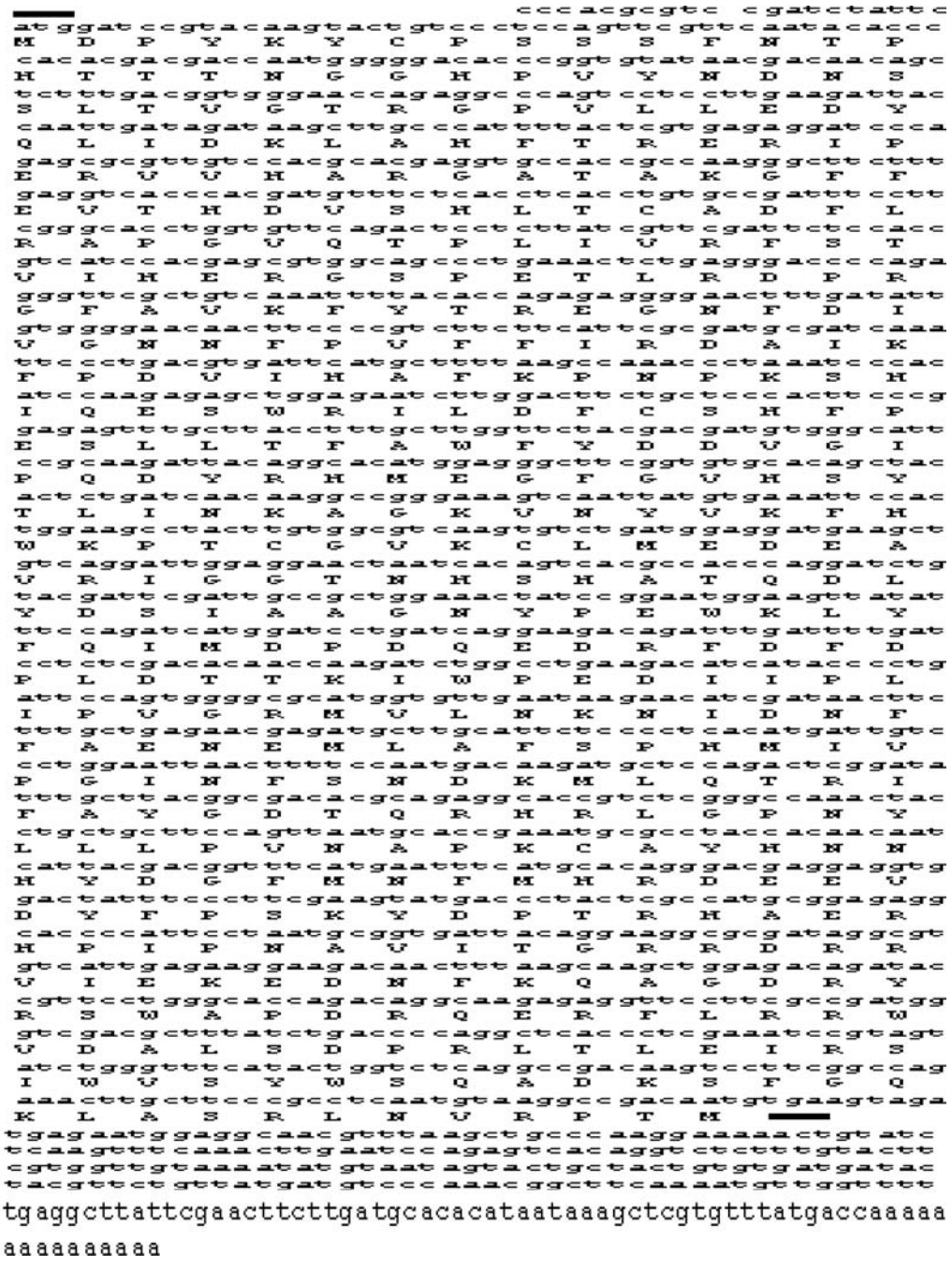

Fig. 1 **b** Nucleotide and deduced amino acid sequence of *Cat1*

and *Fer1* mRNA levels showed resemblance in expression profiles. An increase in *Cat1* and *Fer1* mRNA levels was observed with 12 h of NaCl stress. However, it was also observed that mRNA levels subsequently decreased after 24 h and at 48 h of salt stress. When the plants were transferred to half-strength MS-unstressed conditions, normal mRNA levels were restored for both *Cat1* and *Fer1* comparable to 0 h. (2) *Sod1* mRNA levels, on the other hand, were unaltered and maintained constant levels throughout the time intervals studied in NaCl stress treatment (Fig. 2).

Effect of iron stress

The effect of iron overload on the transcript levels of *Sod1*, *Cat1* and *Fer1* was studied with de-rooted plants (Lobreaux et al. 1995). Iron stress treatment resulted in plants showing leaf bronzing at 6 h of treatment indicating iron stress (Fig. 3). It was found that with iron overload, transcripts for *Cat1* increased and peaked by 6 h. *Fer1* mRNA level showed a rapid increase, and differed from the response of *Cat1* by the induction at 3 h. Moreover, *Fer1* mRNA levels showed a continuous increase starting from 0.5 to 6 h. This

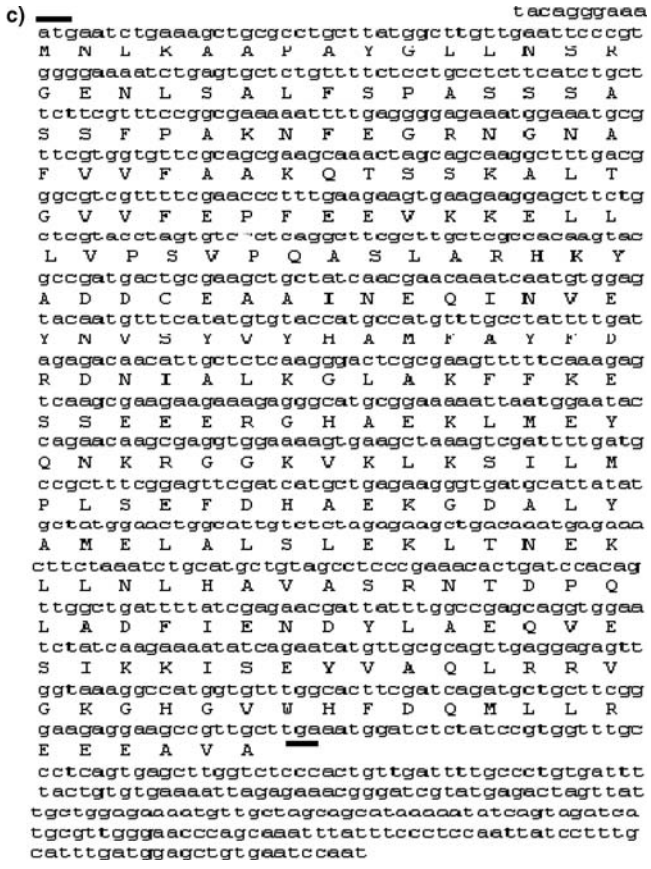

Fig. 1 **c** Nucleotide and deduced amino acid sequence of *Fer1*. The nucleotide sequence is indicated by lower case letter while the amino acid sequence is indicated by single code upper case letter. The start and stop codons are indicated by a line

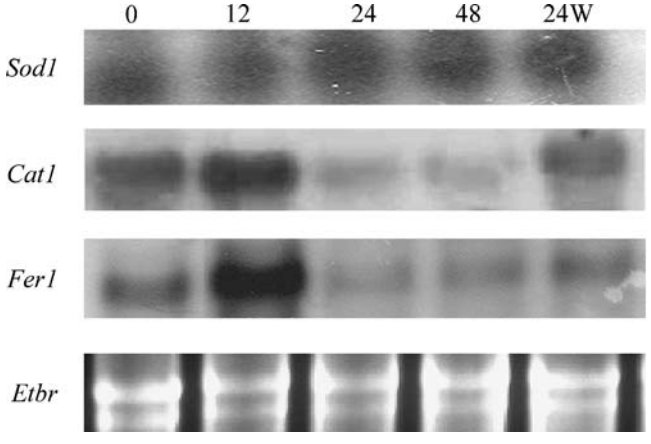

Fig. 2 Effect of salt stress on the mRNA level of *Sod1*, *Cat1* and *Fer1*. *Sod1*, *Cat1* and *Fer1* mRNA levels after 0, 12, 24, 48 h of exposure to 500 mM of NaCl (salt stress) and 24 h after withdrawal from salt stress treatment (24 W). For RNA control loading, the gel was stained with ethidium bromide (Etbr)

showed that iron overload led to a significant accumulation of *Fer1* transcripts in the first few hours of stress treatment. *Sod1*, however, showed a transient increase attaining maximal expression at 3 h, and decreased by 6 h of stress treatment (Fig. 4a). mRNA levels of these three genes were not induced by wounding at 3 and 6 h caused by cutting the

Fig. 3 Visible phenotype in *A. marina* plants after treatment with excess iron. One-month-old seedlings (roots excised) showing signs of leaf bronzing after keeping it for 6 h in the presence of 1,000 μM Fe(III)-citrate (iron stress)

roots, thereby confirming an iron-specific effect (Fig. 4b).

Effect of hydrogen peroxide stress

Studies with H_2O_2 stress showed a delayed response for *Sod1* and *Cat1*. Both the genes showed increased mRNA levels with 24 h of H_2O_2 treatment. However, the response of *Cat1* differed by a pronounced hybridization signal at 24 and 36 h of treatment. The mRNA levels for *Fer1*, however, showed an initial increase at 6 h but did not show a continuous increase in transcript accumulation. Nevertheless, increased mRNA levels were observed at 36 h in comparison to 0 h treatment (Fig. 5).

Effect of mannitol stress

To study the response of all the three genes to osmoticum, mannitol stress treatments were carried out. *Sod1* and *Cat1* mRNA levels decreased after 6 h of mannitol treatment while *Fer1* mRNA levels remained unaltered after 6 h (Fig. 6).

Effect of light stress

In this study, *Sod1* maintained constant mRNA levels after 3-day dark adaptation and with subsequent exposure to light (Fig. 7a). However, a prolonged 7-day dark adaptation resulted in decreased *Sod1* mRNA levels in comparison to 16 h/8 h light/dark photoperiod conditions (Fig. 7b). *rbcs* also showed a decrease in mRNA levels after 7 days of dark adaptation in comparison to 3 days dark and control 16 h/ 8h light/dark photoperiod conditions (Fig. 7c). Therfore, effect of light stress on *Sod1*, *Cat1* and *Fer1* was

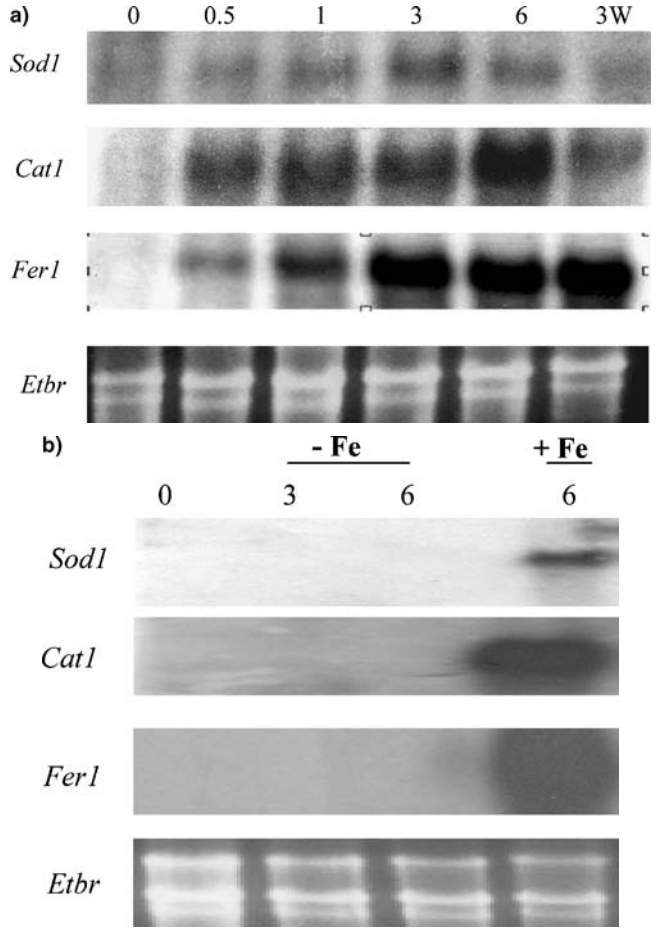

Fig. 4 Iron specific induction of *Sod1*, *Cat1* and *Fer1* in de-rooted *A. marina* plants *A. marina* seedlings were pre-adapted in the absence of iron for 7 days. The roots were excised and then seedlings were transferred to half-strength MS containing 1,000 μM Fe(III)-citrate (iron stress). For RNA control loading, the gel was stained with ethidium bromide. **a** *Sod1*, *Cat1* and *Fer1* mRNA levels after 0, 0.5, 1, 3, 6 h of exposure to iron stress and 3 h after withdrawal of iron stress (3 W). **b** First three lanes consists of total RNA from de-rooted plantlets incubated in half-strength MS for 0, 3 (− Fe) or 6 h (− Fe) and last lane has total RNA of de-rooted plantlets incubated for 6 h in a 1,000 μM Fe(III)-citrate half-strength MS solution (+ Fe)

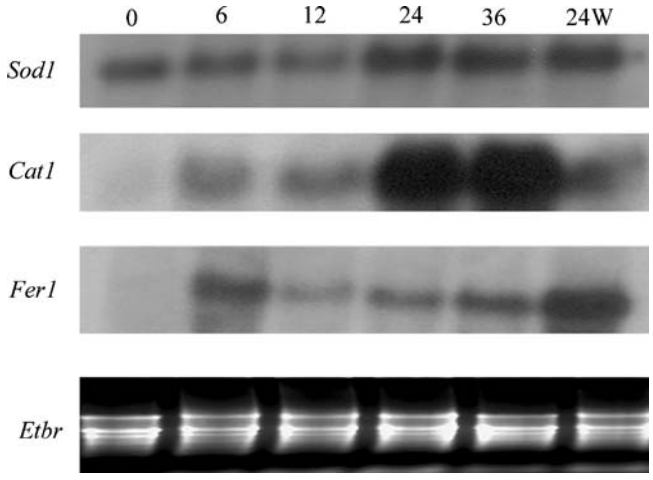

Fig. 5 Effect of oxidative stress (H_2O_2) on the accumulation of *Sod1*, *Cat1* and *Fer1* mRNA. *Sod1*, *Cat1* and *Fer1* mRNA levels after 0, 6, 12, 24, 36 h of exposure to 90 mM of H_2O_2 stress and after 24 h withdrawal of H_2O_2 stress (24 W). For RNA control loading, the gel was stained with ethidium bromide

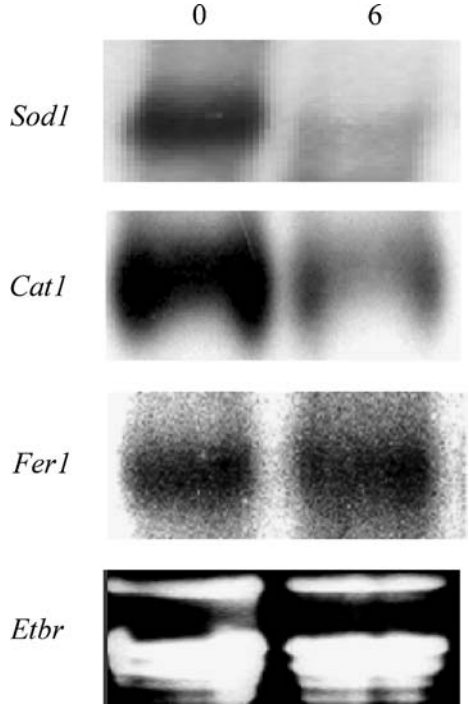

Fig. 6 Effect of mannitol stress on the accumulation of *Sod1*, *Cat1* and *Fer1* mRNA. *Sod1*, *Cat1* and *Fer1* mRNA levels after 0, 6 h of 1,000 mM of mannitol stress. For RNA control loading, the gel was stained with ethidium bromide

studied after 7 day dark adaptation. mRNA levels of *Sod1*, *Cat1* and *Fer1* showed induction and peaked by 24 h of light exposure following a 7 day dark adaptation. In contrast, *Ubc* mRNA levels remained unaltered throughout the time intervals studied (Fig. 7b).

Discussion

Mangrove plants like *A. marina* grow in anoxic soils in coastal areas with high salinity, often under conditions of high temperature and light that could potentially lead to ROS production (Mehta et al. 2005). Plants respond to these toxic radicals by increasing the transcription of genes involved in antioxidant pathway, like superoxide dismutase, catalase and ferritin (Willekens et al. 1994b; Briat et al. 1999). *A. marina* may serve as a good model to study antioxidant response in mangroves, because mangroves have developed effective protection from photosynthetic damage by the active involvement of ROS scavengers (Cheeseman et al. 1997). Hence, in this study, full-length cDNA inserts encoding *Sod1*, *Cat1* and *Fer1* were isolated for studying the response to abiotic stress treatments.

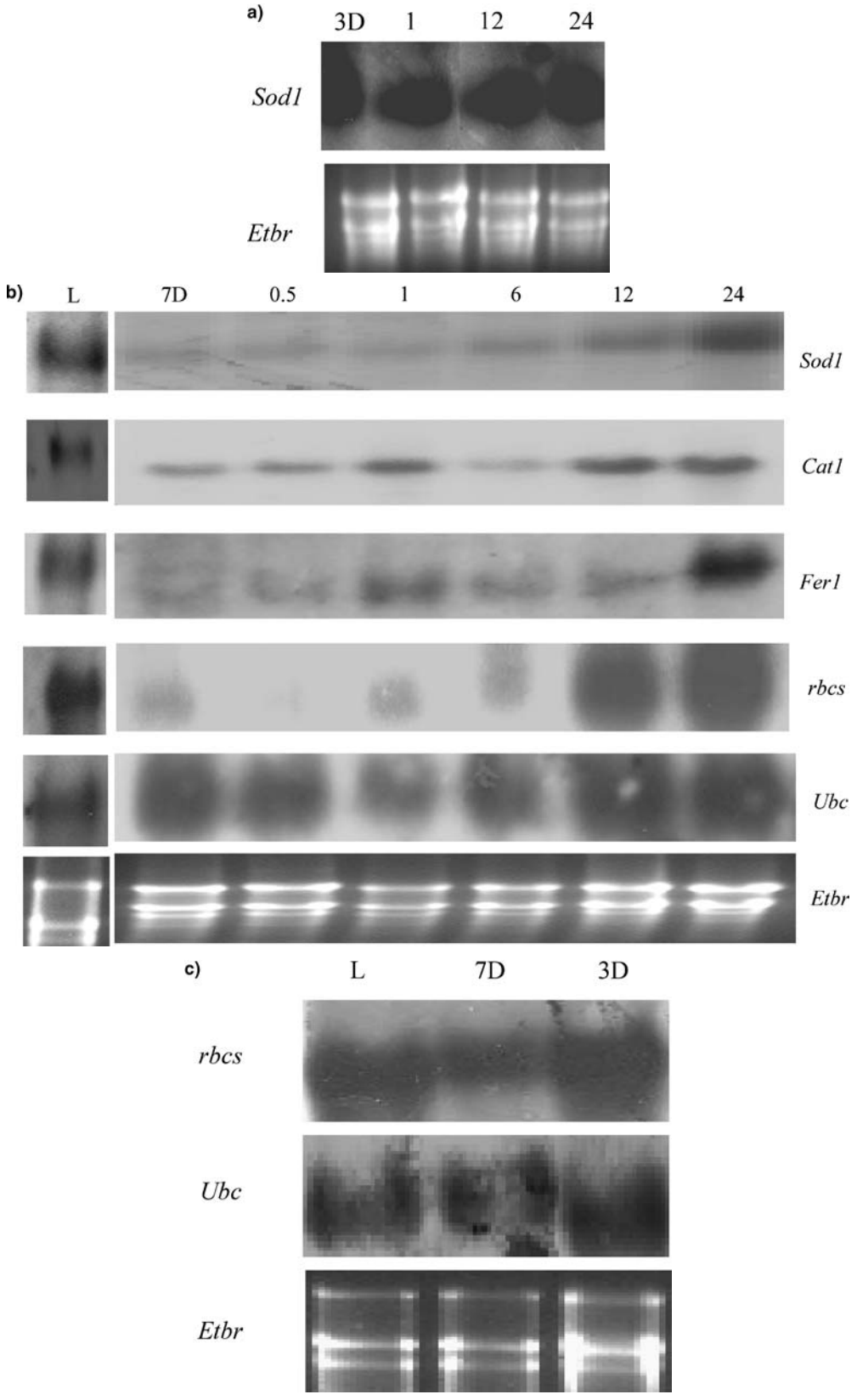

Fig. 7 Effect of light stress on the accumulation of *Sod1*, *Cat1* and *Fer1* mRNA. One-month-old seedlings were dark adapted for a period of 3 (3D), 7 days (7D) and then exposed to white light (5,000 lux). Control plants grown in 16 h/8 h light/dark photoperiod is also shown (L). For RNA control loading, the gel was stained with ethidium bromide. **a** *Sod1* mRNA levels after 3 days dark adaptation and 1, 12, 24 h of exposure to white light. **b** *Sod1*, *Cat1*, *Fer1*, *rbcs* and *Ubc* mRNA levels after 7 days dark adaptation and after 0.5, 1, 6, 12, 24 h of exposure to white light. **c** *rbcs* and *Ubc* mRNA levels after 3 and 7 days dark adaptation

Salt stress elicits short-term response of *Cat1* and *Fer1* mRNA while maintaining constant mRNA levels for *Sod1*

The accumulation of ROS during salt stress is mainly attributed to the inhibition of photosynthesis and a decline in CO_2 fixation. In agreement with this, concentrations of H_2O_2 in shoot tissues of rice have been shown to increase upon salt stress (Fadzilla et al. 1997).

Increased time of exposure to NaCl stress caused a decline in *Cat1* and *Fer1* mRNA levels. There are few reports on the effect of NaCl stress on antioxidant mRNA levels in mangroves though enzyme activity studies have been reported. Salt stress inhibition of antioxidant enzymes like SOD and CAT were observed at high NaCl concentrations in *A. marina* (Cherian et al. 1999); *Suaeda nudiflora* Moq (Cherian and Reddy, 2003) and in *Bruguiera parviflora* (Roxb.) (Parida et al. 2004). We observed increased mRNA levels of *Cat1* and *Fer1* in leaf tissues with 12 h of NaCl stress treatment. This clearly showed that in *A. marina*, *Cat1* and *Fer1* mRNA levels increase in response to short-term salt stress.

On the contrary, *Sod1* mRNA levels were unaltered during NaCl stress treatment. Cyt Cu–Zn SOD used in this particular study, showed no distinct dissimilarities at the amino acid level in comparison to the salt stress inducible (82% with *O. sativa SODCc2*) and non-inducible forms of SODs in maize and rice (81% with *O. sativa SODCc1*). Therefore, the differences between different Cu–Zn SOD isoforms might reside in functional responses (Fink and Scandalios 2002; Benavente et al. 2004).

Salt stress affects the plant in multiple ways—osmotic, ionic and even oxidative. Therfore, to study the regulation and time response of these genes, treatments with oxidative stress (iron, hydrogen peroxide, light) and osmotic stress (mannitol) were carried out.

Effect of oxidative stress on *Sod1*, *Cat1* and *Fer1* mRNA accumulation

Many recent studies in plants revealed that iron overload causes increased oxidative stress and elicit rapid antioxidant responses in plants (Lobreaux et al. 1995; Kampfenkel et al. 1995). Most of the earlier studies in glycophytes reporting the involvement of antioxidant genes have not shown time-related responses (Pekker et al. 2002). Again, theses studies were performed for changes in total SOD or CAT activity with iron stress treatment (Kampfenkel et al. 1995). Therefore, to clarify the role of antioxidative genes during iron stress, time-dependent response of *Sod1*, *Cat1* and *Fer1* mRNA level was studied.

Iron-treated *A. marina* plants showed symptoms of toxicity by uptake of iron from the nutrient solution at 6 h. Oxidative damage was evident in these plants with leaf tissue damage and bronzing (Fig. 3). We observed increased *Fer1* and *Cat1* mRNA levels while *Sod1* mRNA levels showed a transient increase. *Fer1* mRNA levels increased with 1 h of iron stress treatment. Induction of ferritin synthesis is an early event of the plant response to oxidative stress caused by iron toxicity (Briat et al. 1995; Lobreaux et al. 1995). Interestingly, the response of *Cat1* was also rapid and shown to increase with time. The induction of *Cat1* and *Sod1* by iron treatment in this study provided additional evidence for the involvement of ROS with iron-mediated oxidative stress in the mangrove plant, *A. marina*.

We also studied the direct effect of hydrogen peroxide, a constituent of oxidative plant metabolism and a product of peroxisomal and chloroplastic oxidative reactions (Del Rio et al. 1992). *A. marina* plants showed physiological signs of wilting in stress treatments, indicative of H_2O_2 stress. We observed increased *Cat1* mRNA levels in stress treatments as observed in other studies (Guan et al. 2000). Additionally, *Sod1* and *Fer1* mRNA levels were also upregulated. Hence H_2O_2, which acts as an intermediate molecule mediating antioxidant responses to different stresses, activates all the three genes directly and confirms the participation of *Sod1*, *Cat1* and *Fer1* in the antioxidative pathway.

Finally, the effect of light mediated oxidative stress was analyzed. *Arabidopsis* seedlings exposed to excess light, resulted in photoinhibition accompanied by ROS and H_2O_2 accumulation (Karpinski et al. 1997). When tobacco plants deficient in the H_2O_2 dismutating enzyme, catalase (*Cat*1AS) were exposed to excess light, it resulted in leaf bleaching caused by H_2O_2 accumulation in the peroxisomes, providing further proof that H_2O_2 is a mediator for cellular toxicity during light stress (Willekens et al. 1997).

To determine the regulation of these genes in response to a burst of higher fluence of light, *A. marina* seedlings were initially adapted to 3-day dark conditions and then exposed to white light. Superoxide dismutases have been shown to be upregulated in plants when exposed to high light intensity primarily due to oxidative stress (Tsang et al. 1991). However, *Sod1* mRNA levels remained unaltered after 3 days of dark pre-adaptation (3D) and subsequent light exposure (Fig. 7a). *rbcs* (rubisco small subunit) known to be strongly affected by light were used for comparisons. *rbcs* mRNA levels also showed no decrease at 3 days dark adaptation (Fig. 7c). Therefore, the period of dark adaptation was increased to 7 days. *Sod1* mRNA levels decreased after 7 days of dark exposure (7D) in comparison to 16 h/ 8h light/dark photoperiod as observed by Tsang et al. 1991. In addition, *Sod1* mRNA levels showed induction at 24 h of subsequent light exposure (Fig. 7b). The induction of *Sod1* is in agreement with previous results (Tsang et al. 1991; Kliebenstein et al. 1998). A similar trend was also observed for *rbcs* suggesting that the observed decrease in mRNA levels during dark period and subsequent induction on light exposure was a light specific response.

The observed increase in abundance for *Sod1*, *Cat1* and *Fer1* mRNA levels could reflect the regulation of these genes upon light-mediated oxidative stress. In order to test this, we analyzed the light responsiveness of ubquitin (*Ubc*). *Ubc* mRNA levels were reported to be unaffected by salt stress, which is a composite stress including oxidative, osmotic and ionic stress (Parani et al. 2002). Our results show that *Ubc* mRNA levels remained unaltered (Fig. 7b).

We observed increased *Fer1* mRNA level in comparison to *Sod1* and *Cat1* at 24 h of light exposure. It is possible that the signalling cascade that senses ROS concentration increases, leading to active induction of ferritin (Murgia et al. 2001). Chloroplasts are the major cellular compartment affected by light-mediated oxidative stress, and the induction of *Fer1* (localized within chloroplasts) confirms that *Fer1* is a part of the light-mediated oxidative stress network. Interestingly, our results also reveal that oxidative damage in chloroplasts could affect other cellular compartments and influence changes in mRNA levels of genes that are outside chloroplasts like *Sod1* in cytosol and *Cat1* in peroxisomes.

Response of *Sod1*, *Cat1* and *Fer1* to high Osmoticum

To study whether these genes, which are responsive to oxidative stress factors, are also activated by an osmoticum, gene expression studies were done after providing mannitol stress. Six hours of mannitol stress treatment caused a decrease in *Sod1* and *Cat1* mRNA levels while *Fer1* remained unaltered. The response with *Sod1* and *Cat1* reveals that the mRNA level inhibition is due to its osmolarity.

In conclusion, our data confirms that oxidative stress positively regulates the functioning of *Sod1*, *Cat1* and *Fer1*. These genes are upregulated upon oxidative stress to counter the production of increased amounts of accumulating H_2O_2 (Fig. 8). Osmotic stress, on the other hand, either down-regulates (*Sod1* and *Cat1*) or causes no alteration (*Fer1*) in mRNA levels at the time-intervals studied.

Mangroves have to deal with many environmental stress factors, especially, salt stress and light. Taken together, the time response of these genes with salt stress reveals an induction with 12 h of NaCl beyond which osmotic and ionic stress may take effect. NaCl stress in plants, apart from its increased osmolarity, is ionic in its toxic effects (Streb et al. 1993; Munns 2002). It is also possible that after 12 h, NaCl specific effects could be severe in hydroponically grown *A. marina* plants. A study of ionic accumulation in *A. marina* leaves revealed that Na^+ and Cl^- ions reached saturation levels by 18 h of 500 mM NaCl stress treatment (Ashihara et al. 1997). The response of *Cat1* and *Fer1* can be categorized as short-term response while *Sod1* performs a constitutive role in NaCl stress. This particular isoform, *Sod1* may not have a contributory role in salt stress response of *A. marina*. Currently, it is not known whether the short-term increases in *Cat1* and *Fer1* could play a role in recovery from salt stress conditions in *A. marina* plants apart from other salt-induced adjustment strategies (Parida et al. 2004).

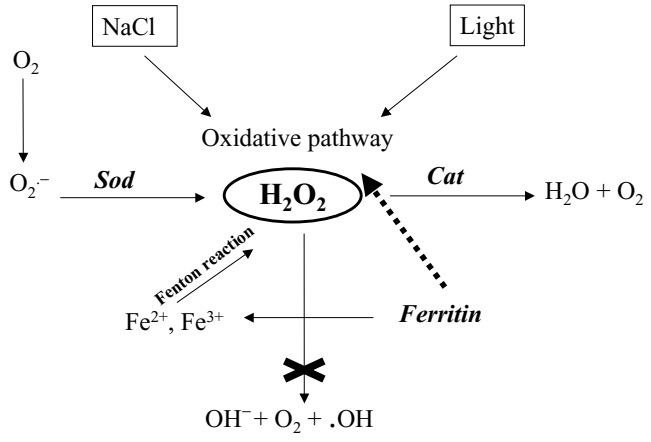

Fig. 8 Schematic representation showing ROS generation and detoxification mechanisms. Enhanced H_2O_2 levels under different oxidative stress caused by stress factors like NaCl, light are efficiently scavenged by the free radical detoxification enzymes like *Sod*, *Cat*. Iron that catalyzes Fenton reaction by reacting with H_2O_2 is scavenged by the iron scavenger *Fer* thus reducing hydroxyl radical formation. The involvement of these genes in the oxidative pathway reduces the production of ROS during environmental stress

Comparative studies on the photosynthetic performance with high light intensity reveal that *A. marina* was more sensitive to changes in light intensity, has a higher light requirement than *Bruguiera gymnorrhiza*, and shows photoinhibition (Naidoo et al. 1998). Our data suggest that oxidative stress-induced increase in transcripts like *Sod1*, *Cat1* and *Fer1* might have a role in mitigating the deleterious effects of oxidative stress with increased incident light in *A. marina*.

This is the first report of studies on antioxidant gene expression in a mangrove, *A. marina*. Observed differences in mRNA levels with either an induction or decrease or a constitutive expression in each of these stresses could be easily differentiated. Additionally, an early or delayed response was also shown with these genes in different stresses, confirming the specificity of individual stress components on differential gene regulation. This study and the earlier study (Willekens et al. 1994b) confirm that relative mRNA expression levels could be used as indicators to study the role of individual genes of multigene family in each of these stresses.

Acknowledgements We acknowledge Department of Biotechnology, Department of Atomic Energy and CSIR, India. We thank Dr. Gopalakrishnan, MSSRF, for helping in the collection of *A. marina* seeds. We thank Laboratorium voor Genetica, VIB, Universiteit, Gent, Belgium for providing *Nicotiana plumbaginifolia Cat1* plasmid, John Cushman, Oklahoma for *Mesembryanthemum crystallinum* Cu–Zn SOD plasmid and Shankar, MSSRF, for *Sesuvium portulacastrum rbcs* plasmid.

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