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Salt-induced changes in cell wall peroxidase (CWPRX) and phenolic content of *Aeluropus littoralis* (Willd) Parl

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Abstract

Aeluropus littoralis as a genetic source for salinity resistance is one of the promising species for genetic improvement and performance of crop plants. We studied the cell wall bound peroxidase enzyme and some wall phenolics to find the possible role of them in wall extensibility and further effect on leaf growth. The effect of different salt concentrations on cell wall-bound peroxidase (CWPRX) activity, CWPRX gene expression and the related cell wall phenolic acid content were investigated along different ages of *Aeluropus littoralis* leaves. Growth Parameters and enzyme activity were measured in nine replications. For Study of Gene expression levels and Phenolic content changes, We used Real Time PCR and high performance liquid chromatography Systems, respectively. Salt treatment progressively inhibited elongation and expansion of the leaves. The activities of ionically and covalently bound peroxidases increased in leaves due to salt treatment. Analysis of variance of covalently bound enzyme activity measurements revealed significant differences between leaves in all treatments, whereas cell wall peroxidase gene expression increased only in leaf 4 at 200 that possibly caused induction in ferulate network production at 200 mM. Ferulic acid, p-coumaric acid and sinapic acid contents increased significantly in leaves 7 and 10 by 200 and 400 mM. These results suggested a correlation between cell wall-bound peroxidase activity and CWPRX gene expression with leaf developmental stages and salt treatment. It is concluded that cell wall bound enzyme activity and cell wall phenolic acid profile may contribute to the stiffening of cell wall during salt stress and at different leaf developmental stages.

Keywords: *Aeluropus litoralis,* Gene expression, Leaf, growth.
Abbreviations: CWPRX_Cell Wall Peroxidase, GABIT_Genetic and Agricultural Biotechnology Institute of Tabarestan, HPLC_ High Performance Liquid Chromatography.

Introduction

Salt stress is one of the major factors affecting crop plant growth and productivity (Zhu, 2001). Salinity and water deficit, caused by high solute concentration in soil, and ion related stress are inimical to plant growth and development (Blumwald, 2000). Plants apply various mechanisms to resist salinity, such as osmotic regulation, ion homeostasis and the antioxidant capacity. These strategies could be the cause of the alterations observed in different tissues, which change the shape and function of the organ, and anatomically cell division and expansion processes (Zidan et al., 1990).

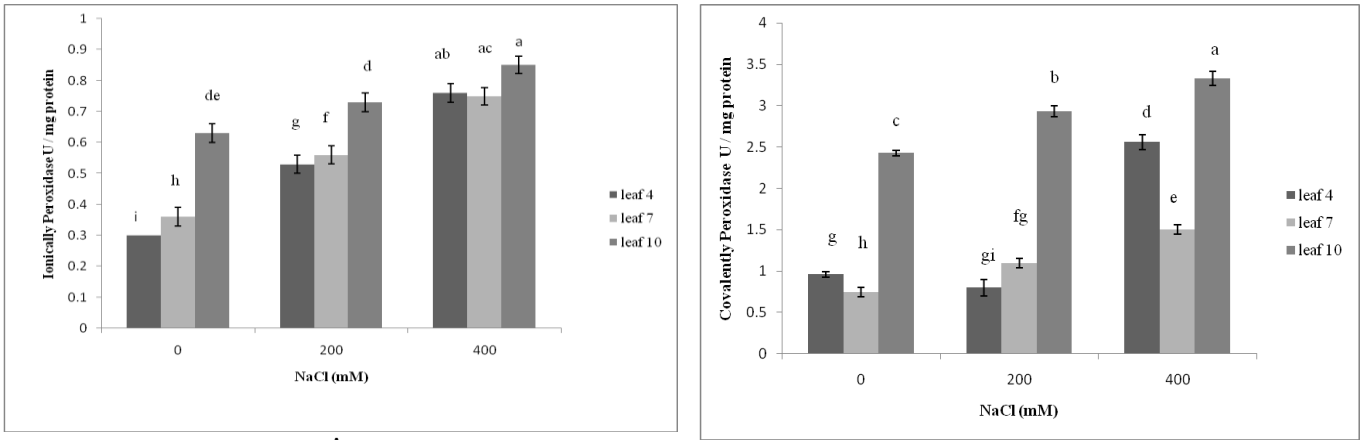
The most common response to salt stress is related to cell wall. Cell walls at different developmental stages consist of a network of polysaccharides, cellulose, proteins and phenolic compounds. The cell wall of grasses (Type II wall) is distinguished from that of dicotyledons and other monocotyledons (most common type of cell wall, Type I wall) (Carpita and Gibeaut, 1993; Carpita, 1996). Cell walls in grasses consist of a high phenolic acid content such as ferulic acid and p-coumaric acid which are linked to wall polysaccharide networks. The cross linkage among phenolic polymers matrix is related to the stiffening processes which cause cell wall rigidity, growth reduction and even growth cessation **(**Fry, 1986; Bacon et al., 1997).

Cell wall peroxidases are involved in growth modifications by affecting cell wall via biochemical processes. Cross-linkages between cell wall compounds are mediated by these enzymes. Several studies have investigated the roles of cell wall peroxidases and their reverse relation to cell growth by stiffening processes. It is known that cell wall peroxidases also influence plant growth by catalyzing certain limiting growth reactions through interaction with phenolic compounds that reduce wall extensibility (Goldberg et al., 1987; MacAdam et al., 1992a; Fry, 1995; DeSilva et al., 1994; Djaković and Jovanović, 2003). Leaf growth is one of the main indications of plant productivity and growth in grasses (Hu et al., 2005). Unidirectional growth in grass leaf is limited to the meristematic basal region, which is surrounded by sheaths of subtending leaves, producing cell files (MacAdam et al., 1989). The sequence of divisions and expansions that result in cell production and differentiation are influenced by age, developmental stage and environmental signals (Schnyder and Nelson, 1987; Gandar

Table1. Effect of salt on the growth of *Aeluropus littoralis*. FW: fresh weight; DW: dry weight.

NaCl(mM)	FW(mg)			DW(mg)			Leaf area$(mm)^2$		
	Leaf 4	Leaf 7	Leaf 10	Leaf 4	Leaf 7	Leaf 10	Leaf 4	Leaf 7	Leaf 10
0	10.55 ± 0.83^{ab}	11.02 ± 0.6^{a}	9.48 ± 0.57^{ac}	1.92 ± 0.13^{ac}	1.88 ± 0.1^{cb}	1.86 ± 0.13^{cd}	71.11 ± 4.3^{a}	48.9 ± 5.6^{ab}	41.66 ± 4.08^{c}
200	8.88 ± 0.7^{ae}	9.22 ± 0.75^{ade}	9.83 ± 0.9^{cd}	1.94 ± 0.13^{a}	1.91 ± 0.13^{ab}	1.93 ± 0.13^{c}	60.55 ± 5.6^{d}	54.4 ± 5.2^{de}	36.4 ± 3.2^{def}
400	6.5 ± 1.7^{fg}	7.1 ± 0.74^{f}	5.4 ± 0.3^{g}	1.56 ± 0.13^{d}	1.33 ± 0.1^{e}	1.36 ± 0.13^{ef}	49.22 ± 4.1^{g}	48.33 ± 2.9^{gh}	33.61 ± 1.7^{ghi}

Data are mean values SE of at least nine measurements.



A B

Fig 1. Peroxidase activities in two fractions of Aeluropus leaves. Growth conditions are as shown in table 1. Activity is expressed as the increase in the absorbance at 530 nm for ionically (A) and covalently (B) bound cell wall peroxidases. Data are means ± SE.

and Hall, 1988). Salt stress directly reduces leaf growth by inhibiting the leaf elongation zone and affecting growth intensity in the basal and distal domains (Bernstein et al., 1993a, 1993b). Halophytes, as naturally occurring salt-tolerant plants, have been shown to be potentially useful in biotechnological applications such as improving in salt and drought tolerance in economically important crops. *Aeluropus littoralis* is a halophyte C4 grass with small genome (324Mb), which is native to heavy saline marshes and coastal zones, has been recently used in biotechnological sterategies (Zouari et al., 2007). To our knowledge, there is no report on salt-induced leaf growth changes via antioxidant and other biochemical pathways, and its effects on plant ontogeny. In the present study, we selected three different developing leaves of *Aeluropus littoralis*, differing in age and the duration of exposure to salt stress, and analyzed peroxidase activity and the levels of cell wall-bound phenolics. In addition, the expression of a candidate cell wall peroxidase gene was monitored for its possible role in the regulation of the enzyme activities, cell wall modification under developmental stages and tolerance under salt stress.

Results and Discussion

Leaf growth

Leaf growth analysis was performed by measuring fresh and dry weights and the leaf area for leaf 4, leaf 7 and leaf 10 of *Aeluropus littoralis*. Table 1 shows the effects of NaCl on leaf growth parameters. Salt stress was found to have adverse effects on growth. A salt concentration of 400 mM decreased all leaf growth parameters, examined here, more significantly than a 200 mM salt concentration. In addition, the change in leaf area under the 400 mM salt treatment appeared to decrease in an age-dependent manner, with older leaves showing the greatest reduction.

Cell wall bound peroxidase activity

The activity of cell wall peroxidase during leaf developmental stages are shown in Fig 1. Both ionically and covalently bound cell wall peroxidases were present in the cell walls of Aeluropus leaves (Fig 1A and Fig 1B). The activity of wall bound peroxidases increased significantly in all three leaves under salt stress. Covalently bound peroxidase increased significantly only in leaves 4 and 7 under a 400 mM NaCl treatment (Fig 1B). Therefore, the covalently bound peroxidase appeared to be affected by both salt stress and leaf type, whereas the ionically bound peroxidase was only affected by salt stress.

Cell wall phenolic content

Phenolic content esterified to wall polysaccharides of leaves under salt stress and the control is shown in Fig 2. Ferulic acid, p-coumaric acid and sinapic acid were analyzed with high performance liquid chromatography. Extracts from cell walls indicated a correlation between salt treatment and leaf age with changes in the levels of cell wall bound phenolic acids. The amount of all wall bound phenolic acids increased due to salt treatment in leaves 7 and 10. The comparison between different treatments indicated that the amount of FA monomer was dependent on leaf type at 0 and 200 mM. The pattern of p-coumaric acid and sinapic acid changes were the same at 400 mM for the three leaves in all treatments. Comparison between leaves in all treatments showed a significant difference for these phenolic acids. These results

Table 2. The expression level of cell wall peroxidase gene in Aeluropus leaves. Gene expression was analyzed by Real time RT-PCR. Values were normalized with the levels of actin rRNA as an internal standard. Data are means ± SE from three replicated samples.

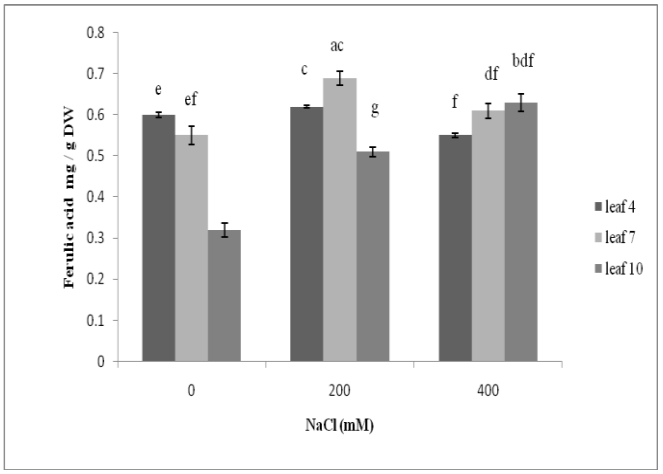
[NaCl] (mM)	Relative gene expression		
	Leaf 4	Leaf 7	Leaf 10
0	0.9±0.06	0.93±0.06	0.76±0.03
200	1.5±0.06	0. 86±0.03	0.84±0.07
400	0.5±0.03	0.6±0.05	0.32±0.04

suggested that p-coumaric acid does not contribute greatly to the phenolic acid content in the cell walls of Aeluropus leaves, however, its content is increased following salt treatment, and changes during leaf developmental stages.

Gene expression analysis

Expression of a cell wall peroxidase candidate gene, analyzed in Aeluropus leaves, during salt stress is given in Table 2. A decrease in gene expression was observed in all leaves at 400 mM after 14 days of treatment. Changes in the levels of expression were leaf type-dependent under both treatments and the control. The three leaf types examined here showed a significant difference in gene expression under salt stress. The fourth leaf showed the greatest alterations in the expression under salt stress, increasing at 200 mM and decreasing at 400 mM NaCl concentration. Such mixed alterations in gene expression are indicative of a developmental and stress-related regulation of cell wall peroxidase gene expression. The results in table 2 suggest that the upregulation of the candidate peroxidase gene may be partly responsible for the increased activity in covalently bound cell wall peroxidase in leaf 4 at 200 mM (Fig 1C), however, the decreased expression level at 400 mM did not correspond to the higher peroxidase activity observed at this NaCl concentration. Kukavica et al., (2012) suggested that both developmental signals and the environmental condition can cause alterations in the amount of cell wall bound peroxidase activity. After 14 days, enzyme activity increased while gene expression decreased in basal leaf at 400 mM. Whereas, both gene expression and enzyme activity increased in basal leaf at 200 mM (Bacon et al., 1997; Awad et al., 2004). An increase in wall bound peroxidase activity coincided with a reduction in leaf growth parameters (Fig 1 and Table 1), suggesting that the induction of wall bound peroxidase may be associated with a reduction in leaf elongation. Thus, the greater reduction in leaf area at 400 mM may be due to a reduction in cell division as a result of a limited enlargement of cell walls with respect to related peroxidase activity enhancement. On the other hand, the reduction in leaf growth caused by salinity, which was exacerbated in the older leaves, may have resulted from the longer duration of exposure to salt stress (Hu et al., 2005). These results indicated that the activation of both wall-bound peroxidases in our experimental condition coincides with a progressive deposition of related cell wall phenolic acids in *Aeluropus littoralis* mature leaves. In addition, the activity of cell wall peroxidases may have a potential role in catalyzing covalent linkages between polysaccharides and phenolic polymers, and in the polymerization of phenolic monomers. These processes appear to be involve an H_2O_2-dependent oxidative coupling (Fry, 1986; Pandolfoni et al., 1992). In addition, phenolic compounds such as phenolic acids play an important role in scavenging free radicals produced during salt stress in plants (Ksouri et al., 2007; Hichem et al., 2009).

With increased gene expression at 200 mM, phenolic content also increased in the fourth leaf, while a reduction in gene expression did correlate with the increase in phenolic



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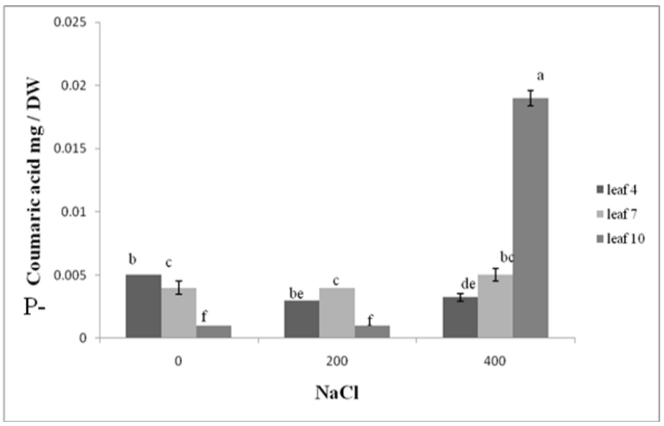

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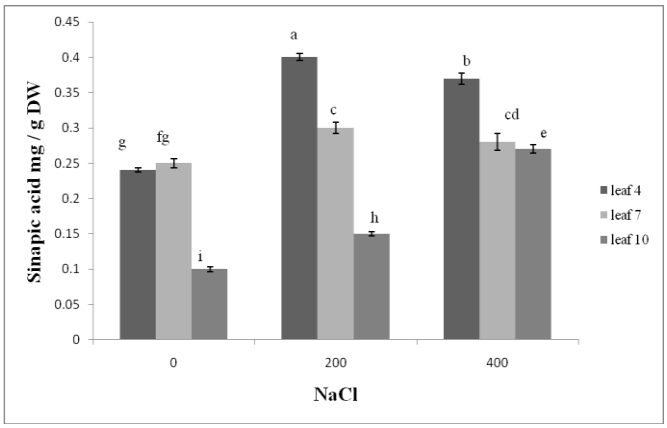

C

Fig 2. The amounts of FA (A), p-CA (B) and SA (C) (mg/g DW) in cell walls of A.litoralis leaves. Growth conditions are as shown in table 1. Phenolic acids were analyzed by HPLC. Data are means ± SE from three replicated samples.

monomer content in leaf 10. The accumulation pattern of phenolic acids in leaves at different developmental stages may have been interpreted as an effect of the developmental stage on the content of phenolic acids (Choi et al., 2006; Barros et al., 2007). Ashraf et al., (2010) demonstrated a positive correlation between plant growth and leaf phenolic contents. Furthermore, Hichem et al., (2009) reported that a correlation between leaf age and the content of phenolic acids under salt stress may be caused by the capacity of the plant in preventing the detrimental effects of stress at different growth stages. Wakabayashi et al., (2012) suggested a possible role for the wall bound phenolic acid network in strengthening cell walls in the process of the development of an immature cell wall to a mature one, which apparently inversely affects growth, and leads to a reduction in cell expansion (MacAdam et al., 1992a, 1992b). Reduction in growth parameters under salinity further confirms the behavior of the Poaceae as a facultative halophyte (Barhoumi et al., 2007).

Materials and Methods

Cultivation of plants and the experimental design.

Seeds of *A. littoralis* were surface sterilized and then planted in plastic pots containing acid washed sand and grown in a growth chamber (60%-80% relative humidity, 14h photoperiod at 800-1000 µmol photons m^{-2} s^{-1} of photosynthetically active radiation). After 45 days, they were treated with complete Hoagland medium containing three different concentrations (0, 200 and 400 mM) of NaCl for 14 days. On the 14th day, plants were harvested and washed with distilled water. Selected leaf 4, leaf 7 and leaf 10 were frozen in liquid nitrogen and stored at -80 ºC.

Cell Wall Peroxidase activity

Peroxidases were extracted and measured in two fractions (Pandolfoni et al., 1992).. The reaction mixture consisted of 0.018 mM of syringaldazine buffer (Sigma), 0.05 mM of H_2O_2 buffer, 60 mM phosphate sodium buffer (pH 6) and the enzyme extract. The activities of ionically and covalently bound peroxidases were expressed as the increase in absorbance at 530 nm mg^{-1} protein with the extinction coefficient of 27 mM^{-1} cm^{-1} (Quiroga et al. 2000).

Quantitative real-time RT-PCR

Total RNA was prepared using the Trizol method, and later treated with DNAse (RNase-Free DNase Set, Fermentas). Single strand cDNA was synthesized using Revert Aid^{Tm} MINUS 1st strand cDNA synthesis kit (Fermentas, cat#: K1632). Real time RT-PCR reaction was studied using the Real Time PCR System with SYBR Green PCR Master Mix from Qiagen (cat #: 204052). All data were normalized with respect to actin as a reference gene. Primers were designed based on conserved regions of cell wall peroxidase genes (GenBank Accession #s: AF387866, AF014468, AF014469, AF014470, L36093). Primer sequences for cell wall peroxidase gene (CWPRX) were: Forward; 5′ ACCACATCACCGACAACAC 3′ and Reverse: 5′ AGTCGATGTATGTTTCCCA 3′. The RealTime PCR condition was 95 °C for 5 min, followed by 95 °C for 15 s, 58 °C for 45 s.

Analysis of phenolics by high performance liquid chromatography (HPLC)

Cell wall bound phenolics were measured according to the method of Franke et al., (2002). All chemical materials were of HPLC grade. Ferulic acid, p-coumaric acid and sinapic acid were purchased from Sigma. Extracts were separated on HPLC Agilent 1100 with XDB, a C-18 column using Mobile phase (A: 0.02% trifluoroacetic acid in water; B: 0.02% trifluoroacetic acid in methanol).

Statistical analysis

Data were performed by one-way analysis of variance (ANOVA) using statistical software (SPSS 19.0). Duncan's multiple range test was used to detect a significant difference between means at a significant level of $P < 0.05$. All analysis were conducted by three replication for Phenolic acid and gene expression and nine replication for enzyme activity and growth parameters.

Conclusion

In the present work, the overall pattern of wall bound peroxidase activitiy correlates with a reduction in growth parameters and alterations in the examined phenolic acids during exposure to salt, suggesting an important possible role for wall peroxidases in the regulation of cell wall elongation and modification.

Acknowledgements

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