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Activation of Lignin Biosynthetic Enzymes During Internodal Development of *Aeluropus littoralis* Exposed to NaCl

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Abstract

Lignin is one of the major characteristics of plant secondary cell wall that provides structural rigidity for the cells and tissues and hydrophobicity to tracheary elements. Internode tissues of *Aeluropus littoralis* as a halophyte grass were sampled at different developmental stages (from the first to the fifth internodes) and under different NaCl concentrations. The influences of NaCl and internode maturity on lignin content and activities of phenylalanine ammonia-lyase (PAL) and cinnamyl alcohol dehydrogenase (CAD) were investigated. Salt stress induced the activities of PAL and CAD and increased the lignin content. Data indicated that the highest level of PAL activity was found at the first internode and CAD activity in the apical and young parts of stem was higher than the old and basal parts of it. Lignin accumulation correlated positively with PAL and CAD activities under salt stress, but during internodal maturation lignification correlated negatively with PAL and CAD activity. The results suggest that induction of PAL and CAD activities and consequently increasing of lignin deposition at internode tissues can be a strategy for high salinity tolerance in this halophyte.

Key words: *Aeluropus littoralis*, Cinnamyl alcohol dehydrogenase, Phenylalanine ammonia lyase, Salt stress

Introduction

Lignin is a biopolymer composed of three different monomers that are derived from phenylalanine, through the phenylpropanoid pathway. Lignin deposition in the plant cell walls provides structural support and stability to the cells and plays an important role in water transport (Boerjan *et al*., 2003). Presence of lignin and other phenolic compounds in the plant cell walls cause plant cell resistance against digestion and attacking birds and insects. Cell walls lignified act as a mechanical barrier against physical injuries and penetration of the pathogens (Santiago *et al*., 2013). Different stresses such as low temperatures, water deficit, light, UV-B and mechanical injuries can cause changes in the plant lignin content and composition (Moura *et al*., 2010). Lignin content in tobacco cells treated with excess boron is higher than that cells under the control conditions (Ghanati *et al*., 2002). The changes in lignin content and composition are due to differences in spatial and temporal activity of certain enzymes in lignification. Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) and cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) are important enzymes in the biosynthesis of lignin precursors. The initial step of phenylpropanoid pathway, conversion of L-phenylalanine to trans-cinnamic acid is catalyzed by PAL (Qurraan *et al*., 2012). Induction of PAL activity and increment of lignin content are considered as usual plant responses to stressful conditions (Pawlak-Sprada *et al*., 2011). The final step of the monolignol biosynthesis, reversible conversion of the cinnamylaldehydes to the corresponding alcohols or monolignols is catalyzed by a multifunctional enzyme, CAD (Zhang *et al*., 2006). Under various stresses, as pathogen infection, water loss and injured leaves, *Arabidopsis taliana* plants showed increased expression of genes involved in lignification, coding for the enzymes such as CAD (Moura *et al*., 2010). CAD is one of the targets for manipulating of lignin content and composition (Hirano *et al*., 2012). PAL and CAD not only are

major rate limiting enzyme in lignin biosynthesis, but also play a role in the resistance mechanism to environmental stresses (Nakashima *et al.,* 1997). *Aeluropus littoralis* (Gouan) Parl is a perennial halophyte (*Poaceae*) growing in saline and arid areas or marshes and as a precious genetic resource for improving tolerance to environmental stresses in economically important crops (Zouarie *et al.*, 2007). Understanding the lignification process and activity of related enzymes in *A. littoralis* as a model system provides baseline information for the metabolic control of lignification in order to manipulate and improve plant responses to environmental stresses.

Materials and methods

Plant material and growth conditions

Seeds of *A. littoralis* were planted in pots containing acid-washed sand in a green-house (50- 60% relative humidity, average temperature 23-28°C, 14 h of light). After 45 days, plants were treated with Hoagland nutrient solution containing 0 (control), 200 and 400 mM NaCl. From the first (I1, upper) to the fifth (I5, lower) internodes were sampled separately after 15 days of salt treatment.

PAL activity assay

Protein concentration was determined by the Bradford method. The reaction mixture contained 1ml of the extraction buffer, 0.5 ml of 10 mM L-phenylalanine, 0.35 ml of double distilled water and 0.15 ml of enzyme extract were incubated for 1 h at 37°C and after this time the reaction was finished by the addition of 0.5 ml of 6 M HCl. The product was extracted with 15 ml of ethyl acetate, after evaporation to remove the extracting solvent the solid residue was suspended in 3 ml of 0.05 M NaOH. For PAL activity assay amount of cinnamic acid was quantified spectrophotometrically at 290 nm (Ziaei et al., 2012).

CAD activity assay

The enzyme was extracted in 0.1 M potassium-phosphate buffer (pH 8) containing 2 mM dithiothreitol and 2% polyvinylpoly- pyrrolidone at 4°C for 20 min. The supernatant was collected for enzyme assay. The reaction mixture consisted of 800 µl 0.1 M Tris HCl (pH8.8), 50 µl enzyme extract**,** 100 µl 2 mM $NADP^+$ and 50 µl 2mM coniferyl alcohol. CAD activity was determined following the oxidation of coniferyl alcohol at 30°C and the formation of coniferaldehyde at 390 nm (Esmaeilzadeh Bahabadi *et al.*, 2012).

Total lignin content

To measure the total lignin, cell walls were isolated and then Cell wall material was treated with a mixture (total of 2.5 ml) of 25% (w/w) acetyl bromide (ME-8.21926) in glacial acetic acid and 0.1 ml of 70% perchloric acid at 70°C for 30 min with shaking at 10 min intervals. After cooling with ice, the digestion mixture was transferred to a 25 ml volumetric flask containing 5 ml of 2M sodium hydroxide and 6 ml acetic acid and fill up to 25 ml with acetic acid. Acetyl bromide soluble lignin (%ABSL) was determined with absorbance at 280 nm using a specific absorption coefficient of 20.0 g^{-1} L cm^{-1} (Ghanati *et al.*, 2002).

Statistical analysis

Data were analyzed in ANOVA program using the software package of SPSS, version 19 and Duncan's method were performed to compare the mean values at $p \leq 0.05$.

Results and Discussion

Exposer to increasing of NaCl concentrations (200 and 400 mM) induced the PAL activity in all internode positions (Fig. 1). There were no significant differences between 200 and 400 mM NaCl at third and fourth internodes. At the fifth internode PAL activity decreased in 400 relative to the 200 mM NaCl. In general, PAL activity decreased from apex to base (Fig. 1) indicating that maximum activity of PAL occurs in drastically elongating internodes. This reduction was associated with greater fluctuation at 200 mM NaCl.

Our findings showed that CAD activity increased strongly with salinity increment (Fig. 2) which agrees with other evidences suggesting the involvement of CAD in the resistance mechanism of plant against stressful conditions (Cheng *et al*., 2013). In the second internode difference between 200 and 400 mM NaCl was not statistically significant. The highest level of CAD activity was

found at the sub-apical internodes in control and 200 mM NaCl, but in 400 mM NaCl the highest rate of CAD activity was at the apical internode (Fig. 2). In general, CAD activity in the apical and young parts of stem was higher than the old and basal parts of it and it appears that CAD becomes active later than PAL (Morrison *et al*., 1994).

Total lignin content was significantly increased associated with increasing of NaCl concentrations from 0 to 400 mM in all internode positions, except for the fourth internode that there no was a significant change between 0 and 200 mM NaCl (Fig. 3). Deposition of lignin in the cell wall confer stability and high static properties to the cell walls (Degenhardt and Gimmler, 2000). The greater lignin content in cell walls provides hydrophobicity for the cells and restricts water vapor from cells to the surrounding medium, further reduces cell wall extension and plasticity and therefore decreases cell expansion and growth (Li *et al*., 2013).

Lignin deposition showed an evident trend of increasing from the apical internode, I1 to the basal internode, I5 in all three treatments (Fig. 3), indicating active lignin synthesis during internodal development that causes a greater stem mechanical strength. Biosynthesis of lignin in plants is regulated both developmentally and environmentally (Zhong *et al*., 2000).

Correlation analysis showed that Lignin content has a positive correlation with PAL and CAD activities, as the increasing of salinity level.

In contrast, lignification correlated negatively with PAL and CAD activities during internodal maturity (Table 1). Results suggest that PAL and CAD activities are essential for lignin biosynthesis and the induction of PAL and CAD activities is resulted in lignin increment under salt stress. In a research, PAL activity was assayed in some crops and it was found the possetive effect of it in lignification, but was no seen a direct relation between PAL activity and lignification. It seems that maximum activities of these enzymes is not synchronous with maximum lignin accumulation during internodal development (Bidlack *et al*., 1995). Similar results were also reported by Luo (2008).

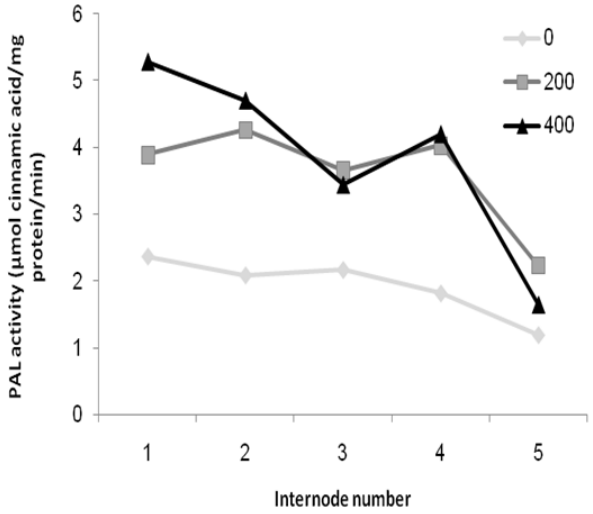

Fig. 1. Change in PAL activity under salt stress during internodal development. 1-5 Internode number from top; 1: upper and youngest internode and 5: lower internode. NaCl treatment: 0, 200 and 400 mM NaCl.

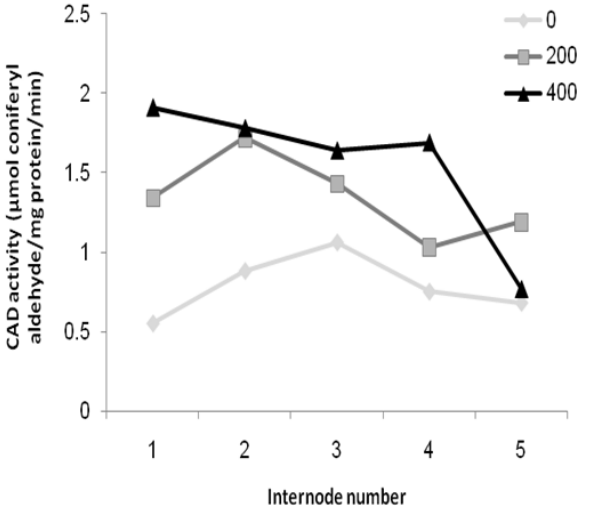

Fig. 2. Change in CAD activity under salt stress during intermodal development. 1-5 Internode number from top; 1: upper and youngest internode and 5: lower internode. NaCl treatment: 0, 200 and 400 mM NaCl.

Table 1. Correlation of lignin content with PAL and CAD activity: I1- I5: Internode number from top. T: NaCl Treatmeant; 0, 200 and 400 mM NaCl. *r* = correlation coefficient, * $p < 0.05$; ** $p < 0.01$

		PAL activity *r*	CAD activity *r*
Across treatments, within internodes	I1	0.80**	0.95**
	I2	0.84**	0.71**
	I3	0.73*	0.76**
	I4	0.65*	0.92**
	I5	0.58*	0.29
Across internodes, within treatments	T0	0.86*	0.08
	T200	0.84*	0.48*
	T400	0.85*	0.83*

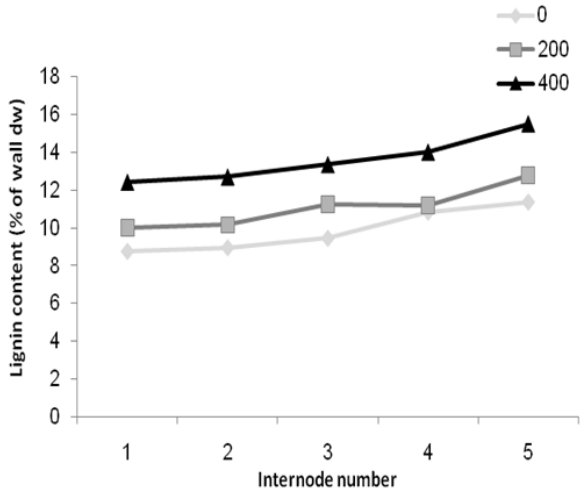

Fig. 3. Change in lignin content under salt stress during internodal development. 1-5 Internode number from top; 1: upper and youngest internode and 5: lower internode. NaCl treatment: 0, 200 and 400 mM NaCl.

In conclusion, PAL and CAD activities and lignin deposition all were significantly different during internodal development. Activities of PAL and CAD enzymes decreased basipetally along the stem, but lignification increased from apex to base. Salinity induced PAL and CAD activities and consequently increased lignifica- tion that it can be a mechanism of adaptation of *A. littoralis* to saline conditions.

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