Phenylalanine Ammonialyase Gene Expression and Activity in Relation to Lignin Deposition in Salt Stressed Aeluropus Littoralis

Sedighe Kelij 1,2*, Ahmad Majd 1, Ghorbanali Nematzade 3,
Parisa Jonoubi 1 and Leila Haghighi 1
1 Department of Plant Biology, Faculty of Life Science
Kharazmi University, Tehran, Iran
2 Department of Biology, Faculty of Basic Sciences
University of Mazandaran, Babolsar, Iran
3 Genetic & Agricultural Biotechnology Institute of Tabarestan (GABIT)
University of Agriculture Science and Natural Resources, Sari, Iran
* corresponding author: S.Kelij@umz.ac.ir

Copyright ©2013 Sedighe Kelij et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Phenylalanine ammonia-lyase is the first enzyme of phenylpropanoid pathway and has an important role on plant cell wall lignification. The influence of different concentrations (0, 200 and 400 mM) of NaCl on growth, lignin content and PAL gene expression and activity were investigated at different internode positions (first, fourth and seventh internodes) in a halophyte grass, Aeluropus littoralis (Gouan) parl. Salt stress inhibited the growth, increased the PAL mRNA level and induced PAL activity as well as lignin deposition. Level of gene expression and activity of PAL changed during internode maturity and decreased basipetally along the stem. Growth inhibition under NaCl stress can be the consequence of incorporation of lignin in the cell wall. The result suggests that A. littoralis with growth retardation, higher PAL gene expression and activity and increment of lignin content could have better tolerance at saline conditions.
Keywords: Phenylalanine ammonia lyase, Lignin, Growth, salinity

1 Introduction

Excessive soil and water salinity is a serious threat to agricultural lands. Salt stress has a major impact on plant growth and it can reduce yield and quality and therefore, agricultural productivity (Yaycili and Alikamanoglu, 2012). One way to avoid this problem is improving salinity tolerance of crop plants by identifying resistance mechanisms in tolerant plants. Lignin, one of the phenolic compounds in cell wall, provides mechanical support and durability for plant tissues and plays an important role in growth and development. It is not only gives structural rigidity, but also forms a mechanical barrier against various biotic and abiotic stresses (Hu et al., 2009). Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5.) is the first enzyme of phenylpropanoid pathway that catalyzes the conversion of L-phenylalanine to trans-cinnamic acid. Positive effect of PAL on plant cell wall lignification has been established (Bidlack et al., 1995) and Down regulation of PAL resulted in lignin modification in transgenic plants (Ogras et al., 2000). Studies have shown that PAL activity respond to various stresses, such as wounding, drought, salinity, heavy metals and infection by viruses, bacteria or fungi, therefore, PAL is involved in the defense response of plant cells (MacDonald and D'Cunha, 2007, Gao et al., 2008). Since, PAL has been shown to play important role in lignification and in view of the fact that lignin is a resistance factor to environmental stresses and is essential for plant growth (Moura et al., 2010), the study of lignification in concern to changes in related enzymes such as PAL under environmental stresses in tolerant plant species, may provides main information for manipulation of plant mechanical strength. Aeluropus littoralis (Gouan) Parl is a graminaceous halophyte and very important plant because of high tolerance salt, drought and heat stress levels and small diploid genome (342 Mb) that has recently been discovered as a genetic resource for biotechnological researches (Zouarie et al., 2007). Although effects of salinity on growth and some physiological and biochemical responses in A. littoralis have been studied, no reports on the effect of salt stress on lignification of A. littoralis tissues are available. So the aims of this study were determine the effects of salt stress on growth and lignin content in relation to PAL gene expression and activity during internode maturation of A. littoralis.

2 Materials and Methodes

2.1 Plant growth and salt treatment

Seeds of A. littoralis were prepared from Pakan Seed Research Center, Isfahan, Iran. After sterilization, seeds were planted in acid-washed sand in a greenhouse (50-60% relative humidity, an average minimum temperature of 23°C and an average maximum temperature of 28°C, 14 h of light). The pots were irrigated as
needed with Hoagland nutrient solution. After 45 days, plants were treated with Hoagland nutrient solution containing 0 (control), 200 and 400 mM NaCl. 15 days after the beginning of salt treatment, different internodes, 1\textsuperscript{st} (first, upper and youngest internode), 4\textsuperscript{th} (fourth internode from the top) and 7\textsuperscript{th} (seventh internode from the top) were taken. The samples were immediately frozen in liquid nitrogen and kept at -80°C for future analyses.

2.2 Growth measurements
Ten plants from each treatment were measured to determine the stem length and the length of the first, fourth and seventh internodes. Stem fresh weight was determined; dry weight of stem was measured after drying at 60°C oven for 3 days.

2.3 Cell wall isolation and lignin content
Cell walls were isolated by sequential washing of the dried internode samples (100 mg) with distilled water, 4 volumes of EtOH, 10-20 volumes of EtOH, CHCL-MeOH (2:1) and acetone, followed by air-drying. Acetyl bromide soluble lignin (ABSL) was determined. Briefly, 4 mg of fine-powdered wall preparation was treated with a mixture (total of 2.5 ml) of 25% (w/w) acetyl bromide (ME-8.21926) in acetic acid and 0.1 ml of 70% HClO\textsubscript{4} 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 NaOH and 6 ml acetic acid and made up to 25 ml with acetic acid. %ABSL was determined by measuring the absorbance at 280 nm using a specific absorption coefficient of 20.0 g\textsuperscript{-1} L cm\textsuperscript{-1} (Ghanati et al., 2002).

2.4 Total RNA isolation, cDNA synthesis and quantitative real-time PCR
Total RNA from frozen internode samples (100 mg) was extracted using the TRIzol reagent (Invitrogen, Inc., CA, USA) and then was treated with DNase (RNase-free DNase I, Fermentas) to eliminate residual genomic DNA according to the manufacturer’s instructions. The concentration and integrity of the total RNA were verified with spectrophotometer (Biochrom WPA Biowave II) and an agarose gel. First-strand cDNA was synthesized from 1 µg of purified RNA using a first-strand cDNA synthesis Kit (Fermentase) based on the manufacturer’s instructions. PAL primers were designed by Oligo7 software based on conserved regions and sequences comparison of PAL gene in Graminaceae (especially, rice). The primer sequence used as follow: F: 5\textsuperscript{ʹ} - ATAGAGCGGGAGGTCAACTC-3\textsuperscript{ʹ}, R: 5\textsuperscript{ʹ} - AAGGCAGACCGTTGGTAG-3\textsuperscript{ʹ}. A. littoralis actin gene was used as a housekeeping gene and a reference to normalize the cDNA concentration among all samples. For PAL gene, qRT-PCR was performed by CFX 96, Real-Time system, Bio-RAD. 10 µl of reaction mixture contained: the diluted cDNA 1 µl, QuantiFast SyberGreen PCR MasterMix (Qiagen) 5 µl, 1 µl of each gene specific primer, water 2 µl. Three replicas were set for each sample. The temperature cycle was: 95°C for 5 min; 40 cycles of 95°C for 10 s; 60°C for 30 s. In order to verify the specificity of the amplified product, melting curve was analyzed at the end of the reaction. Threshold values were calculated by the internal software of
system according to the method of $2^{\Delta \Delta Ct}$.

2.5 PAL activity assay
The PAL activity was assayed according to the method of Wang (2006). Internode samples (300 mg) were homogenized in 6.5 ml Tris-HCl buffer at pH 8.8 containing 15 mM of β-mercaptoethanol. The homogenate was centrifuged at 12,000 rpm for 30 min and the supernatant was used for enzyme activity assay. 1 ml of the extraction buffer, 0.5 ml of 10 mM L-phenylalanine (ME-1.07256), 0.35 ml of double distilled water and 0.15 ml of enzyme extract were incubated for 1 h at 37°C in a water bath and the reaction was stopped by adding 0.5 ml of 6 M HCl. The product was extracted with 15 ml ethyl acetate, followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 ml of 0.05 M NaOH and the amount of cinnamic acid was quantified spectrophotometrically (Biochrom WPA Biowave II) at 290 nm.

2.6 Statistical analysis
All of data have at least three replications and were subjected to analysis of variance (ANOVA) and then means were compared by Duncan’s method in software package of SPSS, version 19. Differences at P < 0.05 were considered to be significant. The figures were plotted using Microsoft Excel 2007 software.

3 Results
Fresh and dry weights of stem decreased about 54.5 and 59.2%, respectively as the salinity level varied from 0 to 400 mM NaCl treatment. Stem length reduction was about 40.2%. There was no significant difference between 200 and 400 mM NaCl treatment in stem length, fresh and dry weights (Table 1). Reduction in the length of internodes was also observed with increment of salinity and it was 57.1% for the first, 49.61% for the fourth and 32.26% for the seventh internode as shown in Figure 1. At all three treatments the lowest internode length was obtained at the first, while the highest value was found at the fourth internode. Salinity treatment caused a significant increase (P < 0.05) in lignin content in A. littoralis internodes. In all three internode positions increasing of lignin deposition was evident as the salinity increased from 0 to 400 mM NaCl, except for the fourth internode, where lignin content in 200 mM concentration remained at the control level (Figure 2). In the same way, in all three treatments, lignin accumulation increased significantly as the internode position changed from first to the seventh internode (Figure 2).

Real-Time quantitative PCR was used for the analysis of PAL expression level. The PAL mRNA level increased in response to salinity and was different significantly (P < 0.05) among three treatments in all three internode positions (Figure 3). The amount of PAL mRNA was decreased with internode maturation (Figure 3). The lowest level of the PAL gene was at the seventh internode and the highest level of the PAL gene obtained from first internode, except for the 200
mM NaCl treatment, where PAL mRNA level was highest at the fourth internode. Salinity effects on the PAL activity were significantly (P < 0.05) different from those of controls in all three internodes (Figure 4). The PAL activity was increased strongly at the first internode as the salinity level increased. At the fourth internode no distinct changes in PAL activity were observed between 200 and 400 mM NaCl treatments. Significant different was between 0 and 200 mM NaCl treatments at the seventh internode, but no meaning different were between 400 with 0 and 200 mM NaCl treatments. Internode maturation led to a significant decrease in PAL activity (Figure 4). The PAL activity obtained from first internode was mainly higher than other internodes, whereas, the lowest level of PAL activity was found at the seventh internode.

4 Discussion

All growth parameters (stem length, fresh and dry weights and length of internodes) were negatively affected by salinity. These results are in accordance with other research reports (Barhoumi et al., 2007a and Sobhanian et al., 2010). Adverse effect of NaCl on the internode length was more obvious in the first internode than the others (Figure 1). In agree with other studies (Wang et al., 1997 and Barhoumi et al., 2007a), our findings revealed that, decreasing of internode length led to the reduction in stem length under salinity. Growth inhibition at salinity conditions is a consequence of 3 important factors: 1) osmotic shock, 2) ion toxicity, 3) ionic imbalance, which ultimately influence on physiological processes and cause a reduction in photosynthetic capacity (Bagci et al., 2003), which adversely affect cell division and expansion and therefore growth (Alizadeh et al., 2011). Decreasing of growth factors in *A. littoralis* under salt stress confirms report of Barhoumi and et al (2007b) that it is a facultative halophyte. Lignin deposition in many halophytes, be positively related with salinity, as in *Leptochloa fusca* (Abd Elbar etal. 2012) and some Chenopodiaceae (Grigore etal. 2007). In *Phaseolus vulgaris*, lignification in vascular root tissue increased under salt stress (Cachorro et al., 1993). Also, Horie and et al (2012) stated that amount of lignin in the most salt- tolerant rice cultivars is higher than those that are sensitive. These results suggest, lignification contributes to the protective mechanism against compression and torsional stress in cells under salinity (Wang et al., 1997). Lignin deposition causes an increased number of tracheary elements and followed by, high speed water transport that probably, reduces ion uptake (Sanchez-Aquayo et al., 2004). In the seventh internode, represent oldest internode, lignin content was highest in all three treatment (Figure 2), therefore lignin deposition is regulated developmentally (Bi et al. 2011) and increased gradually with stem maturity.

What is interesting to note, is that very significant increase of PAL mRNA level under salinity, especially in 400 mM NaCl treatment and in all three internode positions (Figure 3). PAL activity also increased as a result of NaCl treatment, as shown in Figure 4 (Gao et al., 2008). These findings confirm that PAL protein is a
marker of stress conditions in plant species (Pinto et al., 2003) and it can be a good candidate for the technology of genetic engineering of crops towards the salt tolerant. These two responses (PAL mRNA level and PAL activity) were not coordinated entirely under salinity. At the first internode, both PAL gene expression and activity exhibited a similar pattern, but at the fourth and seventh internodes PAL activity has a distinct pattern of PAL transcript level (Figure 3,4). This disparity happens because of the gene expression level in a cell is determined by several factors, including transcriptional and post-transcriptional events (Pawlak-Sprada et al., 2011). This study showed that maturity and internode development influence on PAL gene expression and activity (Figure 3,4). Both PAL transcript level and PAL activity decreased basipetally along the stem, indicating the maximum of both responses occur in actively-elongation internodes. Similar results were also reported by Morrison (1994) and Bidlack (1995). In this work, NaCl treatment of *A.littoralis* plants results in increased PAL protein that has been related to increment of lignin content and has been associated with reductions in stem growth. This suggests that PAL probably is involved in the regulation of plant growth (Huang et al., 2010). Simply, PAL enzyme induces lignification and lignin accumulation in cell wall led to form a three-dimensional network that gives rigidity to the cell wall and therefore, limits the cell growth (Bido et al., 2010). Due to the negative relationship between lignin deposition and growth rate, appears for genetic engineering of crops, genes must be selected that also, participate in resistance mechanism and have no significant effects on plant growth. Because of developmental regulation of lignification, it is expected that genes responsible for lignin biosynthesis will also be developmentally regulated, (Bi et al., 2011). As observed here, apical part of stem, chiefly the first internode shows convinced and conclusive changes under salinity, indicating that, it can be suitable for studying the effects of various stresses.

In conclusion, this study suggests that *A. littoralis* with growth retardation, increasing PAL gene expression and activity as well as lignin content could have better tolerance at high concentration of NaCl. Data also indicated that internode maturity has negative effects on PAL mRNA level and activity, whereas internode maturity is associated with increased deposition of lignin. Finally, lignin is responsible for increasing of mechanical strengthening and inhibition of growth in *A.littoralis* under salt stress.

**Acknowledgments**

This work was supported by Kharazmi University and GABIT (Genetic & Agricultural Biotechnology Institute of Tabarestan).

**References**


Table 1. Changes in the stem length, fresh and dry weights of the 15-day *A. littoralis* plants treated with NaCl. Similar Letters indicates no significant difference at $P < 0.05$. Values are the means ± SE from three independent experiments.

<table>
<thead>
<tr>
<th>NaCl Treatment</th>
<th>Stem length (mm)</th>
<th>Stem Fresh Weight (mg)</th>
<th>Stem Dry Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>201.29 ± 9.93$^a$</td>
<td>44 ± 2.7$^a$</td>
<td>14 ± 1$^a$</td>
</tr>
<tr>
<td>200 mM</td>
<td>149.29 ± 11.81$^b$</td>
<td>24 ± 1.4$^b$</td>
<td>5 ± 0.06$^b$</td>
</tr>
<tr>
<td>400 mM</td>
<td>120 ± 9.7$^b$</td>
<td>20 ± 2.1$^b$</td>
<td>5.7 ± 0.06$^b$</td>
</tr>
</tbody>
</table>

Fig. 1 The effect of salinity levels on length of internodes in the 15-day *A. littoralis* plants treated with NaCl. Bars, followed by the same small letter (between NaCl treatment, in each internode) and by the same capital letter (between internodes, in each NaCl treatment). Similar Letters indicates no significant difference at $P < 0.05$. Values are the means ± SE from three independent experiments.
Fig. 2 The effect of salinity levels on lignin content in the 15-day *A. littoralis* plants treated with NaCl.

Fig. 3 The effect of salinity levels on expression of PAL gene in the 15-day *A. littoralis* plants treated with NaCl.

Fig. 4 The effect of salinity levels on PAL activity in the 15-day *A. littoralis* plants treated with NaCl.

Received: August 1, 2013