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RESEARCH ARTICLE

Abiotic stress induces change in Cinnamoyl CoA Reductase (CCR) protein abundance and lignin deposition in developing seedlings of *Leucaena leucocephala*

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Abstract Aboitic stress such as drought and salinity are class of major threats, which plants undergo through their lifetime. Lignin deposition is one of the responses to such abiotic stresses. The gene encoding Cinnamoyl CoA Reductase (CCR) is a key gene for lignin biosynthesis, which has been shown to be over-expressed under stress conditions. In the present study, developing seedlings of *Leucaena leucocephala* (Vernacular name: Subabul, White popinac) were treated with 1 % mannitol and 200 mM NaCl to mimic drought and salinity stress conditions, respectively. Enzyme linked immunosorbant assay (ELISA) based expression pattern of CCR protein was monitored coupled with Phloroglucinol/HCl activity staining of lignin in transverse sections of developing *L. leucocephala* seedlings under stress. Our result suggests a differential lignification pattern in developing root and stem under stress conditions. Increase in lignification was observed in mannitol treated stems and corresponding CCR protein accumulation was also higher than control and salt stress treated samples. On the contrary CCR protein was lower in NaCl treated stems and corresponding lignin deposition was also low. Developing root tissue showed a high level of CCR content and lignin deposition than stem samples under all conditions tested. Overall result suggested that lignin accumulation was not affected much in case of developing root however developing stems were significantly affected under drought and salinity stress condition.

Keyword Abiotic stress · Cinnamoyl CoA reductase · Developing seedlings · *Leucaena leucocephala*

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Abbreviations

BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium
C3H	Cinnamate 3 hydroxylase
C4H	Cinnamate 4 hydroxylase
COMT	Caffeate *O*-methyl transferase
CCoAOMT	Caffeoyl CoA O-methyl transferase
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F5H	Ferulate 5-hydroxylase
FTIR	Fourier transform infrared spectroscopy
HIS	6 Histidine-tag
HCT	Hydroxy cinnamoyl transferase
KBr	Potassium bromide
Ni-NTA	Ni-nitrilotriacetic acid
PEG	Poly ethylene glycol
PMSF	Phenylmethylsulfonyl fluoride
PVPP	Polyvinyl polypyrrolidone
RT	Room temperature

Introduction

Drought and salinity are two major abiotic stress factors, which are becoming common to terrestrial plants. Abiotic stress leads to a series of physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang et al. 2003). Drought and salinity are often interconnected, and may induce similar cellular changes, which are manifested largely as osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Chaves et al. 2009; Langridge et al. 2006; Apse et al. 1999; Bohnert and Sheveleva 1998). High concentrations of NaCl may cause both hyperionic and hyperosmotic stress effects, which lead to a decline of turgor, disordered metabolism, and the inhibition of uptake of essential ions, as well as other problems in plant cells (Kim et al. 2007). Similarly a water deficient condition also disturbs the inherent balance of plant metabolite and renders changes in its metabolic network.

Fair amount of research has been done on the stress response induced with abiotic conditions like drought and salinity. However molecular mechanism behind the abiotic stress response is still difficult to understand. Recently, many salt-stress responsive genes and protein in plants have been identified by comprehensive transcriptomics and proteomics techniques (Kawasaki et al. 2001; Seki et al. 2002). However, the transcriptomics and proteomics techniques do not always give a clear insight into metabolite networks involved in these type of stress. Plant stress responses are also regulated by multiple signaling pathways that activate numerous transcription factors. Members of the dehydration-responsive transcription factors (DREB) and C-repeat binding factors (CBF) family are stress-inducible in themselves. Other proteins such as heat-shock proteins (Hsps) and late embryogenesis abundant (LEA)-type proteins are two major types of stress-induced proteins that accumulate upon water, salinity, and extreme temperature stress. They have been shown to play a role in cellular protection during the stress (Ingram and Bartels 1996; Liu et al. 1998; Stockinger et al. 1997; Hamilton and Heckathorn 2001).

Increase in lignification is a common stress response attributed to biotic and abiotic stress (Moura et al. 2010; Neves et al. 2010; Lee et al. 2007). The formation of stress lignin has originally been reported under pathogen attack (Vance et al. 1980). Since then, the activation of the phenylpropanoid metabolism has been observed under various stress conditions (Dixon and Paiva 1995). Lignification is a process which the plant undergoes in vascular development and normal growth. Cinnamoyl CoA Reductase is considered to be a key gene regulating carbon flux towards lignin (Piquemal et al. 1998). Role of CCR in lignification and vascular development has been well studied. CCR is known to be induced under conditions such as wound or pathogen infection (Lauvergeat et al. 2001; Kawasaki et al. 2006), However, the molecular role of lignin biosynthesis genes in abiotic stress is still unclear.

In this present study we performed experiments to look into quantitative protein abundance pattern of a key lignin biosynthesis gene CCR paralleled with lignin deposition in developing stem and root tissue of *Leucaena leucocephala* (Vernacular name: Subabul, White popinac), a multipurpose, nitrogen fixing leguminous tropical tree species. Drought and saline conditions were created by growing *Leucaena* seedlings in 1 % Mannitol and 200 mM NaCl in the medium, respectively. Our result suggests that CCR might be playing a role in drought and salinity stress and this will open a new area of research in lignin biosynthesis.

Materials and methods

Plant material

Seeds were treated as described by Shaik et al. (2009). After 2 days in moist condition on shaker the seeds were transferred to tissue culture bottles containing ½ MS medium (Murashige and Skoog 1962) added with 1 % mannitol (*w*/*v*) and 200 mM NaCl for drought (Lipavska and Vreugdenhil 1996) and salt stress conditions, respectively. A set of seedlings was grown without any additions as a control. All seedlings were grown at 25±2 °C under 16 h photoperiod, 70 % relative humidity, and a light intensity of 24.4 μmol m^{-2} s^{-1} in a growth chamber. The growing seedlings were harvested at a definite point of time (5, 12 and 15 days). Roots and stem were separated and processed according to experimental need.

Protein extraction and ELISA

Prior to ELISA experiment, antibodies against purified recombinant CCR protein were raised in rabbit as described by Srivastava et al. (2011). Antibodies were tested for its specificity against purified soluble recombinant CCR protein (Titre; 1:100000). Cross reactivity was also tested by performing a Western blot on extracted total protein from *Leucaena* plant. Antibodies against purified CCR protein was used to prepare a standard graph with varying amount of purified CCR protein (1–32 ng) detectable by ELISA. For ELISA, root and stem were separated and crushed in buffer (100 mM Tris–HCl, pH.7.5, 2 % PVPP, 2 % PEG 4000, DTT 5 mM and PMSF 1 mM) to extract the total protein. Later the extract was kept on shaker for 1 h at 4 °C. The lysed stem and root samples were centrifuged at 15000 g and supernatant was considered to be the total soluble protein. The total protein was quantified and 25 μg was coated in 96-well titre plates (medium binding ELISA plates) in triplicates and repeated twice. ELISA was performed as described by Srivastava et al. (2011).

Histology and lignin staining

Root and stem from growing seedlings of *Leucaena* were harvested for 8 days (For lignin staining only), 12 and 15 days. Free hand transverse sections from root and stem were cut of ~200 μm thickness and were treated with 2 % phluroglucinol (in 95 % ethanol) for 10 mins. Color was developed by addition of concentrated HCl. The sections were viewed with 40× magnification and captured using AxioPlan 2 Zeiss microscope.

Immuno-cytolocalization of CCR protein

Stem and root of developing seedlings grown in light in ½ strength MS were used to represent normal (control) growing condition. Immuno-cytolocalization was performed as described by Srivastava et al. (2011). Image was captured using Axioplan 2, Zeiss microscope.

FTIR analysis

Growing seedlings in normal condition were considered in this experiment to observe any change in quality of lignin produced at different point of time. Developing seedlings (Root and stem) were harvested at 10 and 15 days time point and crushed to a fine powder in a mortar-pestle using liquid nitrogen. The powder was transferred to a glass crucible and kept at 80 °C for 2 days to remove moisture. Dried powder was used to extract lignin using modified Klason method (Yeh et al. 2005). The samples were first extracted with hexane to remove other secondary metabolites and then reacted with 72 % H_2SO_4 at room temperature with occasional stirring for 2 h. The solution was then diluted with distilled water to a 3 % H_2SO_4 concentration and autoclaved for 1 h. The reaction was filtered and acid insoluble lignin (Klason lignin) was washed 2–3 times with distilled water and dried. Klason lignin obtained was again dried to remove traces of moisture, homogenize and passed through a filter to get uniform size particle. Sample (0.85 mg) was mixed with equal amount of dried potassium bromide. Mixture was made to a fine powder and pellet was made using 10 mm dye and KBr press (Technosearch Instruments, India). The pellet was used to take readings on Perkin-Elmer Spectrum One FTIR Spectrometer. A pellet of KBr and KBr+commercial lignin (Alkali lignin, Indulin-AT, Sigma-Aldrich) was also made for baseline correction and standard lignin, respectively. FTIR scanning was done ten times for each sample and values were recorded in percent transmission. All values were corrected for baseline KBr scan.

Results

Differential CCR protein accumulation under drought and salinity stress conditions

The expression pattern of CCR protein in developing seedling under control condition was in agreement to what we have recorded earlier for 5, 10 and 15 days time points. It shows around 3–4 fold increase in CCR protein expression from 5 to 15 days (Srivastava et al. 2011). However in this experiment 12 days time points were taken into consideration instead of 10 days time point. The overall expression of CCR protein was 2–3 fold higher in root than in corresponding stem sample at most of the time points. In case of stem, the decrease of CCR expression from 5 to 15 days time point was more prominent in 200 mM NaCl treated developing stem samples however in case of 1 % mannitol treated samples the expression peaked at 12 days (Fig. 1a). In case of root a decrease was seen in 200 mM salt and 1 % mannitol treated root samples at 12 days (Fig. 1b). However there was a increase in CCR protein expression at 15 days time point in treated developing root samples (Fig. 1b). It was also noted that developing root under normal condition showed a gradual increase in CCR protein expression from 5 to 15 days.

CCR protein was immuno-cytolocalized near developing vascular tissue

ELISA based expression studies of CCR protein showed that it was differentially expressed under different abiotic stresses. To confirm this we performed immuno-cytolocalization of CCR protein on free hand transverse sections (~200 μm thickness) of 12 days old root and stem samples. The dark brown to grey colour precipitate was observed around the lignified tissues indicated by arrows in developing stem and root tissues (Fig. 2). There was a notable decrease in immuno-cytolocalized CCR protein in mannitol and salt treated developing seedling and the protein was localized near the vascular tissue where ligninfication and vascular development is in process.

Lignin deposition and CCR accumulation under salt stress condition in developing *Leucaena*

CCR protein expression profiling was also paralleled with lignin staining at the same time points to understand the relation between lignin deposition in vascular tissue and CCR protein expression under normal and stress condition. Five days old developing seedlings were not used for lignin staining. The seedlings growing under 200 mM NaCl stress were characterized with stunted growth and smaller roots when compared to the normally growing seedlings. Interestingly, in developing stem under salt stress, level of lignin deposition

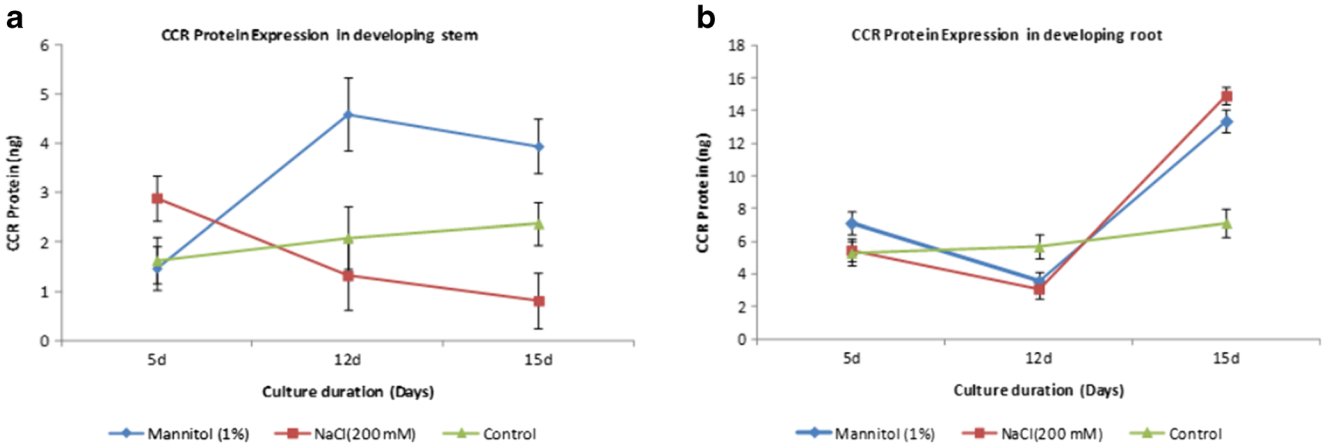

Fig. 1 ELISA profile of CCR protein Expression: **a** Stem **b** Root. The CCR protein was quantified in normal as well as stress treated samples using recombinant CCR protein standard graph. Two factor ANOVA was performed. (*p value for Fig.* 1*a*: *0.380158*; *Fig.* 1*b*: *0. 0.672243*)

was lower at all time points when compared to the developing stem under normal condition. This was in accordance with the CCR protein accumulation at that time point, which shows a gradual decrease from 5 to 15 days (Fig. 3g, h and i). The amount of lignin deposition maintained a low level at 12 days time point which was in harmony again with the CCR protein expression and immuno-cytolocalization at 12 days old developing stem. Salt stress condition in developing stem showed that the lignin deposition was toward a lower level at all time points when compared to seedlings under normal and mannitol stress condition (Fig. 3). However in case of developing root, the lignin deposition followed an increasing pattern, with highest at 15 days time point (Fig. 4g, h and i) and almost equivalent or higher to the developing root under

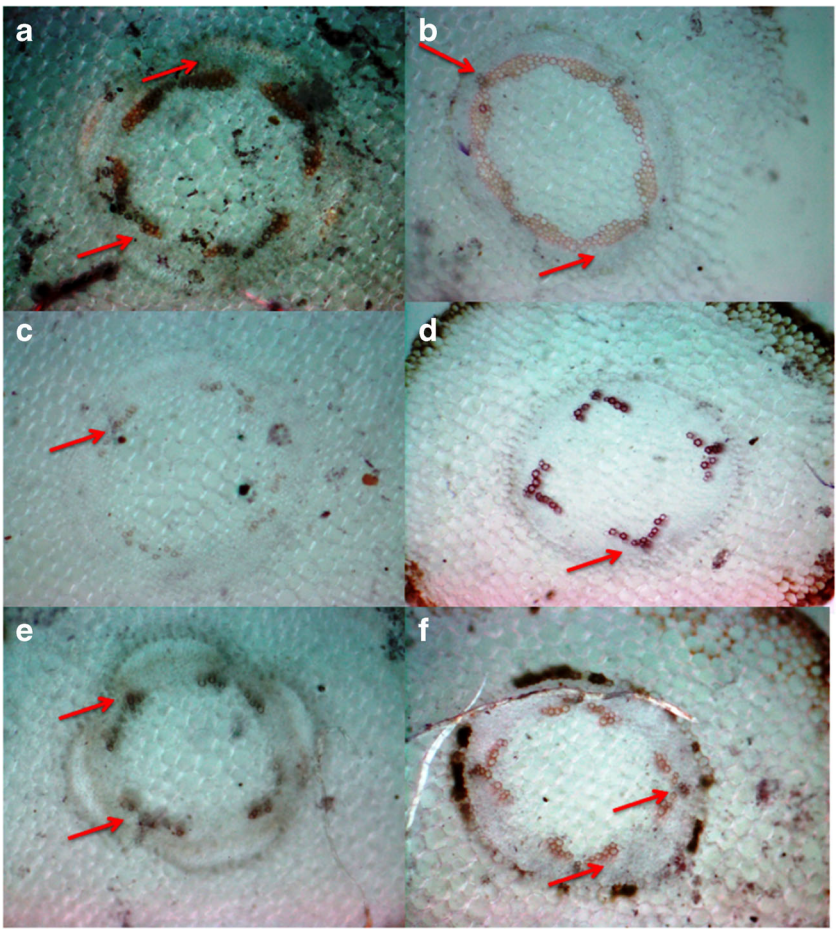

Fig. 2 Immuno-cytolocalization of CCR protein in 12d stem and root tissues under normal as well as stressed conditions. **a** Control 12 day stem samples, **b** Control 12 day root samples, **c** Manitol (1 %) treated 12 days stem sample, **d** Manitol (1 %) treated 12 days root sample. **e** NaCl (200 mM) treated 12 days stem sample, **f**: NaCl (200 mM) treated 12 days root sample. All pictures were taken at 40× magnification

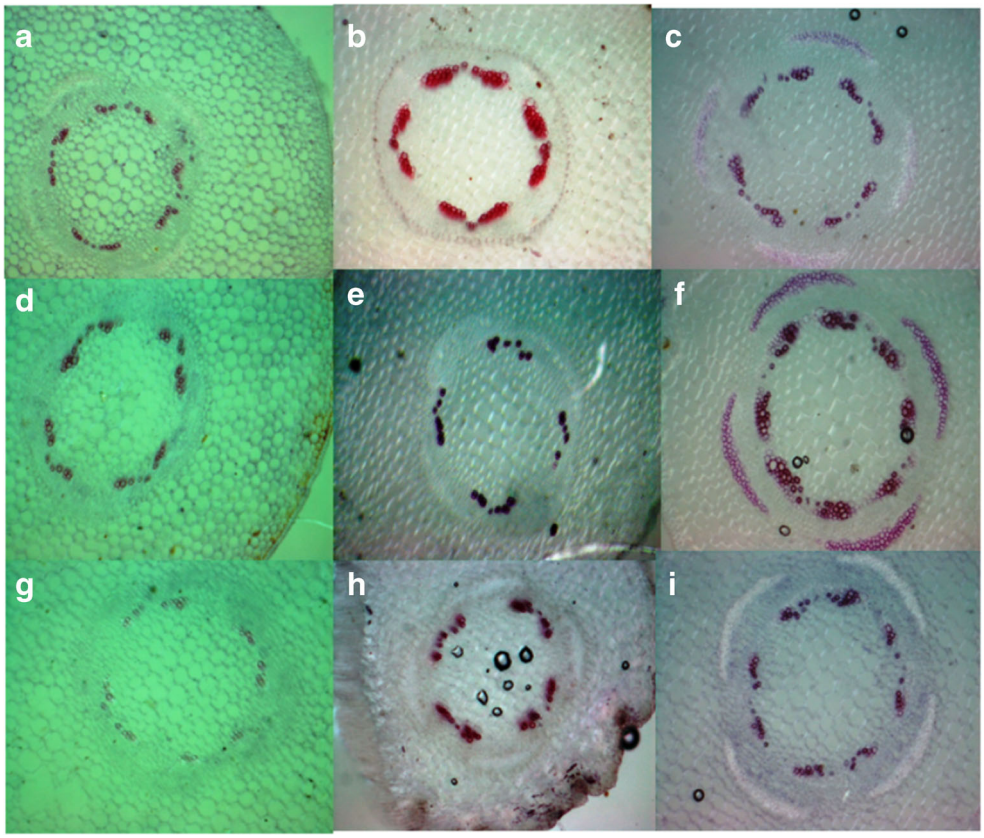

Fig. 3 Lignin staining (Phluoroglucinol) of developing stem under normal as well as stressed conditions. (**a**, **b**, **c**). Control 8, 12 and 15 days samples, respectively. (**d**, **e**, **f**). Mannitol (1 %) treated 8, 12 and 15 days samples, respectively. (**f**, **g**, **h**). NaCl (200 mM) treated 8, 12 and 15 days samples, respectively. All pictures were taken at 40×magnification

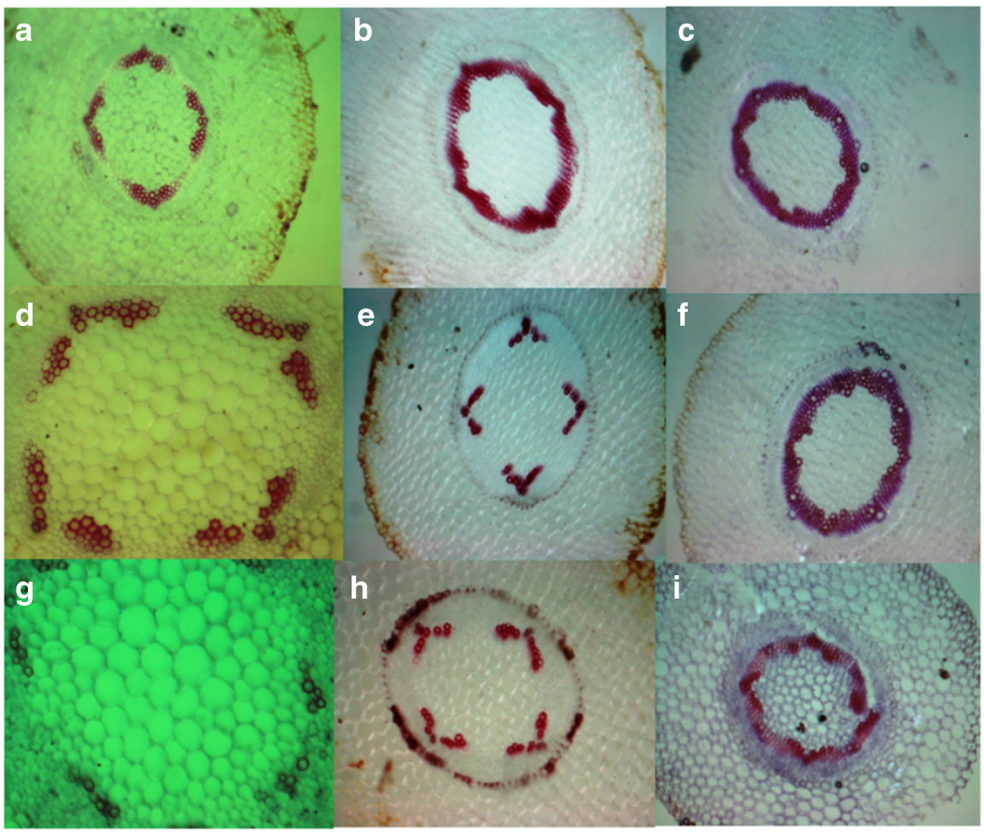

Fig. 4 Lignin staining (Phluoroglucinol) of developing root under normal as well as stressed conditions. (**a**, **b**, **c**). Control 8, 12 and 15 days samples, respectively. (**d**, **e**, **f**). Mannitol (1 %) treated 8, 12 and 15 days samples, respectively. (**f**, **g**, **h**). NaCl (200 mM) treated 8, 12 and 15 days samples, respectively. All pictures were taken at 40× magnification except Fig. 4d and g which were taken at 100× magnification

normal condition. This suggest that stem was affected more due to salt stress condition than the water deficient condition.

CCR protein accumulation and lignin deposition under water deficit condition in developing *Leucaena*

CCR protein accumulation was observed to be higher in 1 % mannitol treated developing stem at 12 and 15 days time points (Fig. 1a). Developing stems under 1 % mannitol stress condition showed a very low level (at 5 days) to high level (approximately 2–4 fold high in 15 days) of lignin deposition. There was a gradual increase in lignin deposition unlike the CCR accumulation at corresponding time points. Lignin deposition was even more than the developing stem under normal condition at 15 days time point (Fig. 3d, e and f). However, in case or developing root under 1 % mannitol stress showed a zigzag expression of CCR protein from high at 15 days, lowest at 12 days was recorded. The corresponding lignin deposition also showed a zigzag pattern comparable to CCR protein accumulation at these time points except at 12 days which showed a very low CCR expression and low lignin deposition. Moreover developing roots under 1 % mannitol stress showed an equivalent amount of lignin deposition to roots under normal condition at 15 days (Fig. 4d, e and f). 8 d sections are showed for initial lignin deposition (Figs. 3 and 4; extreme left panel). Overall result suggested that water deficient conditions have physiological stress in initial stages of development. As soon as the plant stabilizes its osmotic stress it starts developing vascular tissue and other peripheral support tissue (lignified cells).

Lignin composition at 10 and 15 days normal developing seedlings

To characterize the composition of lignin deposited at 10 d and 15 d old developing stems and root under normal condition was considered for FTIR analysis. FTIR analysis revealed that there is no qualitative difference in lignin composition of 10 and 15 days old developing stem (Fig. 5) and 10 and 15 days old developing roots (Fig. 5). FTIR scan of commercial lignin (Alkali lignin, Indulin-AT, Sigma-Aldrich) was also performed to characterize signature peaks of Lignin. Peaks with wave number 1040 cm^{-1} denote dialkyl ether linkage (between cinnamyl alcohol subunits), peak 1270 cm^{-1} corresponds to C-O stretching, aromatic (methoxy), peak 1595 cm^{-1} is aromatic ring with C=O stretching and peak 1510 cm^{-1} represents aromatic ring with C-O stretching (MacKay et al. 1997). Peaks with wave number in the range of 1595 to 1599 cm^{-1}, 1040 to 1062 cm^{-1}, 1250 to 1270 cm^{-1} and 1510 to 1512 cm^{-1} are characterized to be signature peaks in FTIR of tissue samples. Signature peaks of commercial lignin were 1044, 1268, 1512 and 1598 cm^{-1}.

Discussion

It has been well documented that biotic and abiotic stresses are responsible for the increase in cell wall lignifications (Moura et al. 2010; Neves et al. 2010; Lee et al. 2007; Chazen and Neumann 1994; Katerji et al. 1997). In a research report, the analyses of primary metabolites after salt stress treatment in *Arabidopsis thaliana* cell cultures have suggested presence of phenylpropanoid metabolites (Kim et al. 2007). In another report EST analysis of salt treated and untreated cDNA libraries of *Tamarix hispida* roots showed presence of lignin biosynthesis pathway genes (Li et al. 2009). Similarly, it was also observed that there was a high activation of lignifying enzymes in water-deficit stress conditions in white clover (*Trifolium repens* L.) (Lee et al. 2007). There is no doubt about an active role of lignin biosynthesis genes in abiotic stresses such as saline and drought conditions. Lignification is a dynamic process and is tightly regulated at different levels during normal development and in response to different stresses. Cinnamoyl CoA reductase is a key enzyme in lignin biosynthesis and is often considered as a control point of monolignol biosynthesis (Piquemal et al.1998; Kawasaki et al. 2006), moreover in situ CCR activity is often linked with lignin deposition in the xylem vessels (vascular tissue). The expression of CCR under abiotic stress has not been studied to a greater extent.

In an interesting report in maize, Fan et al. (2006) observed that there was an increase in transcripts of CCR1 and CCR2 in root elongation zone under water deficit conditions. They also suggested a reduction in root growth than the apical region. This reduction was also associated with increased deposition of lignin. Till date, limited reports are available on the effect of abiotic stress such as salinity and drought in relation to CCR protein accumulation and lignin deposition. We report here a quantitative profile of CCR protein paralleled with lignin staining to draw the link between accumulation of CCR protein under salt and draught stress conditions and lignification. As CCR gene transcript expression could be more dynamic for each type of stress and CCR protein is the end result of the its gene transcript, we performed a comprehensive protein expression profile in developing seedlings of *Leucaena* to correlate with lignin deposition as a more reliable measure of CCR protein accumulation. Our data suggested that the expression of CCR protein and lignin deposition was dependent on the salt and drought stress conditions in *L. leucocephala* developing seedlings.

Salt stress has a negative effect on lignin deposition and CCR expression in developing stem and no conclusive effect on developing root. As CCR expression is directly linked to increase in lignification (Srivastava et al. 2011), we attempted to link the CCR protein expression under salt stress and lignin deposition. Salt stressed seedlings were characterized with stunted growth and smaller roots. CCR expression in developing stem was recorded towards a lower level and the

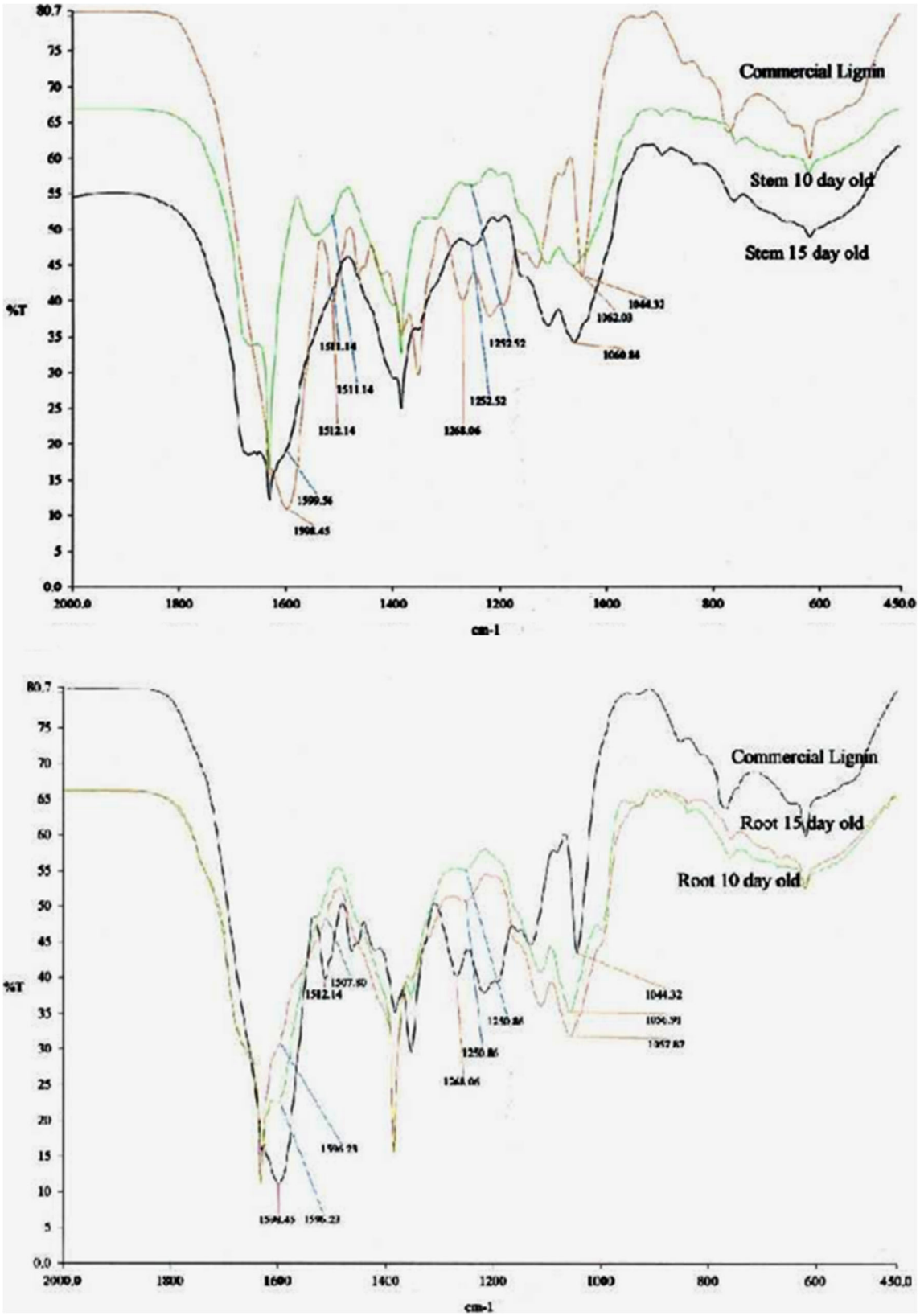

Fig. 5 *Upper panel*: FTIR analysis of normal 10d old and 15 d old stem samples. *Lower panel*: FTIR analysis of 10 d and 15 d old normal root samples

corresponding lignin deposition (vascular development) also at low level. It clearly suggests an inhibitory effect of salt stress in vascular development. Developing root samples showed a different pattern of lignin deposition and CCR

protein expression. No conclusion can be made on this data. One reason to explain this phenomenon could be that till 12 d of treatment the roots are struggling to cope up with the stress and various metabolic pathways including osmolyte biosynthesis, transport, lignin synthesis and homeostasis (Li et al. 2009) are perturbed and eventually the root tissue acclimatize to such condition with compromised vascular development, stunted growth and smaller roots.

Drought stress condition has no clear effect on developing root but has a positive effect on lignin deposition and CCR expression in developing stem. Drought stress (1 % mannitol) showed an increase of lignifications in the stem of developing seedlings (Fig. 3d, e and f). The corresponding CCR protein accumulation was also higher in mannitol treated stem samples than salt stress and control condition. The extent of developed vascular tissue in mannitol treated stem sample was even more than normal 15 days old developing stem sample (Fig. 3c and f). Up-regulated CCR transcript in root was recorded within a period of 48 h in water deficit condition by Fan et al. 2006, which was in accordance with our results in which CCR protein was estimated to be more at 5 and 15 days time points.

To identify any major differences in type of lignin deposited during early vascular development, composition of deposited lignin was compared by FTIR analysis for 10 and 15 days control developing stem and root samples. Apparently no change in lignin composition was observed (Fig. 5) in these samples or we were limited by our FTIR technique. This result was also in accordance with Vincent et al. 2005, who reported no change in lignin composition in leaves subjected to water deficit. The result could be extrapolated to 200 mM NaCl and 1 % mannitol treated stem and root samples and can be suggested that there would be no major difference in lignin composition but the quantity of lignin would vary according to the type and intensity of stress which the plant is undergoing.

There are few reports about CCR expression and lignin deposition in abiotic stress like salt and mannitol. In many reports, different isoforms of CCR has been reported to perform differential functions like defense, development and stress. In *Arabidopsis*, two genes coding for CCR (*AtCCR1* and *AtCCR2*) are known to be differentially expressed (Lauvergeat et al. 2001). *Arabidopsis AtCCR2* was induced following the infection of plants with *Xanthomonas campestris* pv. campestris and was proposed to be involved in defense against plant pathogens. Studies on *Oryza* CCR and CCR-like genes showed that *OsCCR1*, which is induced by sphingolipid elicitor, is involved in defense against *Xanthomonas oryza* infection (Lauvergeat et al. 2001; Kawasaki et al. 2006). As one isoform of CCR is reported from *Leucaena*, The possibility of a tightly regulated single CCR isoform performing such functions in *Leucaena* cannot be overruled. In another report on maize, two important lignin biosynthesis genes (CAD and COMT) was also found to be over expressed in drought which again suggests the association of lignin biosynthesis under abiotic stress conditions (Hu et al. 2009). In our earlier experiments we recorded CCR expression at 5, 10 and 15 days old developing seedlings under normal condition, which showed a gradually increasing CCR expression from 5 to 15 days (Srivastava et al. 2011). It was interesting to note such an expression pattern and lignifications in early development of vascular tissue in stem and root. CCR accumulation (ELISA based) also corroborated to the immune-cytolocalization of CCR protein and lignin deposition pattern of both root and stems tissues of normal as well as treated samples, which suggests that the CCR has a major role to play in developing vascular tissue in root and stem tissue undergoing salt and drought stress in developing seedlings of *Leucaena*.

Conclusion

To conclude the findings, it would be reasonable to suggest that lignin related enzymes such as CCR, whether alone or in combination with other lignin biosynthetic genes are associated with the drought and salt stress tolerance mechanisms as shown in other studies (Chazen and Neumann 1994; Kawasaki et al. 2001; Kim et al. 2007; Li et al. 2009; Lee et al. 2007). However, more experiments need to be performed to dissect the molecular mechanism of CCR involvement in lignin deposition during salt and drought stress conditions.

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Conflicts of interest The authors declare that they have no conflicts of interest.

Authors' contributions SS and BMK conceived, designed the experiments, and wrote the manuscript. SS, RKV, SKG and YAA performed the experiments. All authors read and approved the final manuscript.

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