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Reduced Leaf Peroxidase Activity is Associated with Reduced Lignin Content in Transgenic Poplar

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Abstract

A cationic peroxidase gene (*Shpx6a*) of a forage legume species, *Stylosanthes humilis*, was transferred to poplar (*Populus tremula*) in antisense orientation under the control of 35S RNA promoter of cauliflower mosaic virus. Transformed plants were regenerated on selective media after co-cultivation of poplar stem explants with *Agrobacterium tumefaciens* and integration of transgenes was confirmed by PCR and Southern hybridization analyses. Analyses of selected transgenic plants showed reductions in total leaf peroxidase activity which was 50 % to 70 % of that measured in untransformed control plants. Transgenic poplar plants with reduced leaf peroxidase activity had 10-20 % lower lignin content than control plants. Although which isoform of poplar peroxidase(s) has been inhibited by 35*S*-*Shpx6a* antisense construct is not clearly known, our results suggested the possibility of manipulation of lignin content through inhibition of lignin-specific peroxidases.

1. Introduction

Lignin is a major component of plant cell walls. It has an important role in the adaptation of plants to terrestrial life by providing mechanical support to plant tissue. Lignin is also present in water carrying xylem elements and plays an important role in plant defense against pathogen attack (Imberty *et al.*, 1985). However, lignin may have a negative impact on the utilization of the biomass (Dean and Eriksson, 1992). During paper pulping, lignin must be removed from wood by chemical treatments that are expensive and environmentally unfriendly. Therefore, there is a considerable interest for genetic manipulation of lignin content and composition by antisense or sense suppression of genes involved in different steps of lignification (Whetten and Sederoff, 1995).

The last step in the biosynthesis of lignin is the polymerisation of cinnamyl alcohols. Two distinct classes of enzymes, peroxidases and laccases, have been proposed to perform the polymerisation of monolignols into lignin (Freudenberg, 1959; Higuchi and Ito, 1985; Takahama, 1995). However, it is still unclear whether the last step in lignin biosynthesis is catalysed by peroxidase, laccase or a combination of these enzymes (Savidge and Udagama-Randeniya, 1992; Dean and Eriksson, 1994; McDougall *et al.*, 1994; Richardson *et al.*, 1997).

The relationship between peroxidase isoenzyme groups and lignification has been investigated in many plants. Several peroxidase isoenzymes possibly participating in the synthesis of lignin were isolated from *Zinnia elegans* (Sato *et al.*, 1995), potato and tomato (Roberts *et al.*, 1988), petunia (Hendricks *et al.*, 1991) and poplar (Christensen *et al.*, 1996). Mäder and Füssl (1982) and Lagrimini *et al.* (1987) suggest that anionic peroxidases may be involved in the polymerisation of lignin monomers based on their affinity for coniferyl alcohol, their location on the cell wall, and their expression in

lignified tissue. Anionic peroxidases functioning in the peroxidase dependent oxidation of the lignin monomer analogue syringaldazine have been purified from poplar (Goldberg *et al.*, 1985; Imberty *et al.*, 1985). No definite proof, however, has been obtained for the involvement of a specific peroxidase isoenzymes in the developmentally regulated formation of lignin *in vivo*. Alteration of the expression of these enzymes by genetic engineering could specify their particular role in lignification.

The objective of this study was to examine the correlation between reduced peroxidase activity and lignification. We used a construct carrying an antisense cationic peroxidase gene (*Shpx6a*) from a forage legume, *Stylosanthes humilis* (Harrison *et al.*, 1995), to suppress peroxidase expression in transgenic poplar, and test the effect of this suppression on lignin content. Although this hypothesis could be better tested by over-expression and suppression of the Shpx6a activity in its original host (*S. humilis*), we used poplar as a model mainly for two reasons. First, in contrast to genetic transformation of *S. humilis*, which is not efficient, transformation of poplar is very efficient and thus is possible to generate the large number of transgenic plants required for antisense experiments. Secondly, *Shpx6a* gene shows DNA sequence similarity (app. 40 %) to at least one of the poplar peroxidases (D11102) (Harrison *et al.*, 1995). We report here that antisense expression of this peroxidase was effective in down-regulating total peroxidase activity in transgenic poplar plants. The effect of this down-regulation on lignin content is also discussed.

2. Materials and methods

2.1. Construction of chimeric genes and transformation of poplar

The coding region of *Shpx6a* peroxidase cDNA (Harrison *et al.*, 1995) was amplified from the plasmid pBluescript SK^+ by PCR using primers designed to introduce restriction enzyme sites to facilitate cloning. Amplification products were digested with *Xba* I and cloned into *Xba* I digested binary vector, pGA643, under the control of 35S RNA promoter of cauliflower mosaic virus and 3' end of *tmr* (cytokinin biosynthetic protein) gene of *A. tumefaciens* as terminator (An *et al.*, 1985). A clone carrying *Shpx6a* cDNA in antisense orientation was selected by sequencing the whole insert and cloning junctions and introduced into *A. tumefaciens* strain LBA 4404 by triparental mating according to Ditta *et al.* (1980).

Transformation of poplar (*Populus tremula*) was achieved by co-cultivation of stem explants with *A. tumefaciens* carrying the binary vector according to Tzfira *et al.* (1997). Following co-cultivation, stem explants were transferred to Woody Plant Medium (WPM) (Lloyd and Mc Cown, 1981) supplemented with 1 mg l^{-1} zeatin, 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. Regenerated shoots were rooted on WPM supplemented with 0.5 mg l^{-1} indole-3-butyric acid. Shoots were subcultured with fifteen day intervals and incubated in a growth chamber at 25 ℃ with a 16 hour photoperiod. Untransformed control plants were regenerated similarly under nonselective conditions, without kanamycin, and maintained under the same growth conditions as the transformants. Three-to-four weeks after transformation, putatively transformed plants were recovered and further analysed.

2.2. Molecular analysis of transgenic plants

PCR and Southern blot analyses to detect the presence of *Shpx6a* gene were performed on two-month old plants (10 cm high) according to Feuillet *et al.* (1995). Total DNA for PCR was extracted from leaf tissues of each plant by Walbot's method (1988). PCR was performed according to Dwivedi *et al.* (1994) using a forward primer (5'-TGAGCAAAGAAGCTCGCATGGGAG-3') internal to the 35S promoter and a reverse primer (5'-GTCGAACATCTGTTACTCTG-3') internal to the *tmr* terminator. For PCR, samples were kept at 94 ℃ for 10 min., followed by 30 cycles at 94 ℃ for 1 min., 50 ℃ for 1 min., 72 ℃ for 3 min. with a final extension step at 72 ℃ for 7 min. Amplified DNA was electrophoresed on a 1.2 % agarose gel and visualized after staining with ethidium bromide.

For Southern blot analysis, a DNA fragment corresponding to full length *Shpx6a* (1.2 kb) was amplified by PCR from pGA643 carrying antisense 35S-*Shpx6a* gene, labelled with DIG DNA labelling kit and used as hybridization probe. Five μg of genomic DNA from transgenic and control plants were digested with *Kpn* I, separated on a 1.0 % agarose gel and transferred to a nylon membrane (Boehringer Mannheim GmbH, Germany). Immunological detection of hybridised DNA on the membrane was performed using polyclonal sheep anti-digoxigenin fragments conjugated to alkaline phosphatase according to the manufacturer's recommendation.

2.3. Measurement of total peroxidase activity

Crude peroxidase extracts were prepared by grinding 1 g of fresh leaf tissue in 1 m*l* 0.1 M phosphate buffer (pH 7.0). The homogenate was centrifuged at 18000 g for 30 minutes at 4 ℃ . Total peroxidase activity of the supernatant was deter-

mined spectrophotometrically at 470 nm in 0.2 M phosphate buffer containing 5 mM guaiacol and 5 mM H_2O_2 (pH 5.8) (Krsnik-Rasol, 1991). Total protein content of the leaf extracts used in peroxidase assays was determined using bovine serum albumin (BSA) as standard according to Bradford (1976). Total peroxidase activity of each sample was then expressed as unit per mg protein. In addition, peroxidase isoenzymes were resolved on 10 % non-denaturing polyacrylamide gel according to Ornstein and Davis (1964). Five μg of total protein was loaded into each well and peroxidase activity was detected by incubating the gel in 0.2 M phosphate buffer containing 5 mM guaiacol and 5 mM H_2O_2 for 15 to 20 min., or until enzyme bands appeared to the desired intensity.

2.4. Determination of total lignin content

Lignin content was determined using thioglycollic acid (TGA) method (Campbell and Ellis, 1992). Stem explants of two-month-old plants (10 cm) were homogenized and dried for four hours at 105 ℃. One hundred μl of thioglycollic acid, 0.75 ml of water, 0.25 ml of 37 % HCl was added to the 50 mg dried sample. After three hours of incubation at 80 ℃, homogenate was centrifuged at 12000 g for 10 min. Pellets were washed with water and resuspended in 1 ml of 1 M NaOH and mixed for 16 hours at 4 ℃. The homogenate was centrifuged at 12000 g for 10 min. and retained supernatant was taken to a new tube. Two hundred μl of 37 % HCl was then added and incubated at 4 ℃ for four hours to reprecipitate the pellet. Centrifugation step was repeated and the pellet was dissolved in 1 ml of 1 M NaOH. The absorbance was measured spectrophotometrically at 280 nm.

3. Results

3.1. Antisense expression of Shpx6a peroxidase in poplar

After transformation of poplar with a construct carrying antisense *Shpx6a* cDNA under the control of 35S promoter of cauliflower mosaic virus, forty-five putatively transformed kanamycin-resistant shoots were obtained. Six randomly selected poplar transformants were further analysed by PCR and Southern blot to confirm the transgenic status of the plants. PCR amplifications using primers designed to amplify the *Shpx6a* gene located on the T-DNA region of the pGA643 showed the existence of amplification products with sizes equal to the expected size of *Shpx6a* gene in all tested transformants **(Fig. 1-A)**. Genomic DNA from all these six plants was also subjected to Southern blot analysis to determine the number of T-DNA insertions. Using a DNA fragment corresponding to *Shpx6a* gene as probe, we showed that all tested transgenic plants have at least one T-DNA insertion **(Fig. 1-B)**. This provided further evidence that the antisense *Shpx6a* gene was stably integrated into the genome of the transgenic poplar plants.

3.2. Analysis of peroxidase activity and lignin content in transgenic poplar plants

To determine whether the expression of antisense *Shpx6a* construct was effective in reducing peroxidase activity of transgenic poplar, crude extracts from the leaves of six transgenic plants and five control plants were analysed for peroxidase activity. This analysis showed that the reduction in total peroxidase activity in transgenic plants was between 50-70 % of that found in untransformed control plants **(Fig. 2-A)**. Native gel electrophoresis analysis of the leaf extracts from transgenic and untransformed control plants also supported the results obtained from peroxidase enzyme assays. In antisense transformed plants the intensity of the perox-

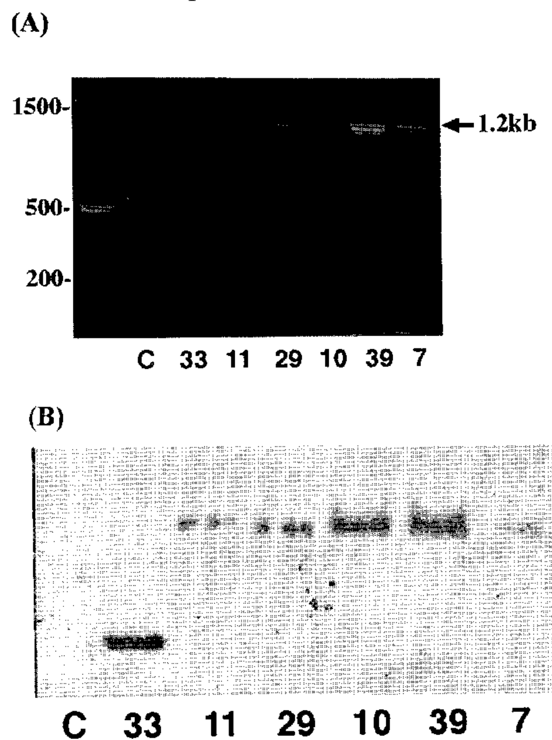
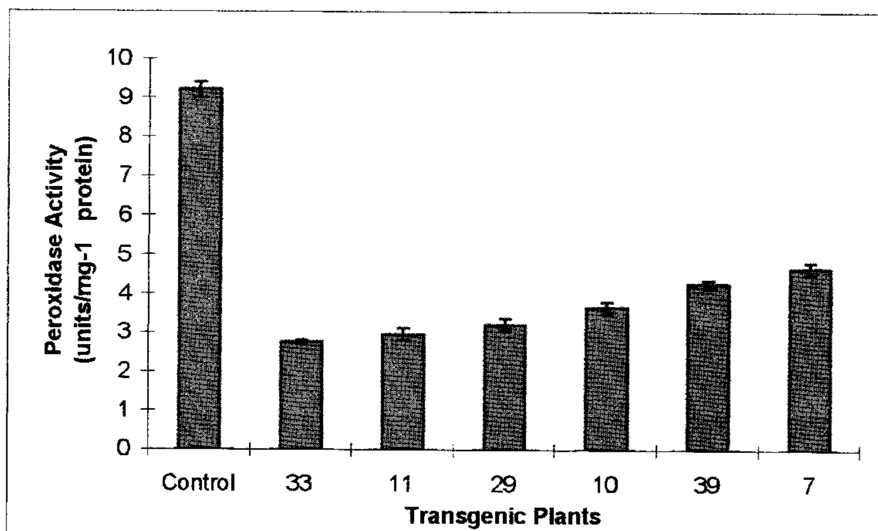
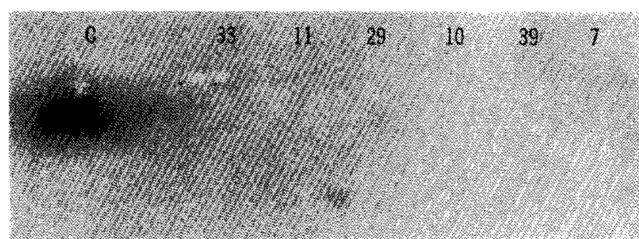


Fig. 1. PCR and Southern blot analyses of poplar plants transformed with 35S-*Shpx6a* antisense construct (A) PCR amplification of *Shpx6a* gene from transgenic (33, 11, 29, 10, 39, 7) and untransformed control (C) plants. M: 100 bp DNA ladder used as DNA size marker. (B) Southern blot analysis of DNA from transgenic (33, 11, 29, 10, 39, 7) and untransformed control (C) plants cut with Kpn I and probed with labelled *Shpx6a* cDNA.

(A)



(B)



C 33 11 29 10 39 7

Fig. 2. (A) Total leaf peroxidase activity of transgenic (33, 11, 29, 10, 39, 7) and control (C) plants. Results are mean of three independent measurements. Total peroxidase activity was expressed as unit per mg protein used in the assay. Error bars are also shown for each plant. (B) Non-denaturating PAGE of leaf proteins from transgenic (33, 11, 29, 10, 39, 7) and untransformed control (C) plants stained for peroxidase activity. Peroxidase bands were also visible in the leaf extracts of transgenic plants (data not shown) after extended incubation (more than 20 min).

idase bands was strongly reduced as compared with the extract from control plants **(Fig 2-B)**. After prolonged incubation (longer than 20 min), however, peroxidase bands were also visible in the extracts from the leaves of transgenic plants (data not shown).

Next, we analysed total lignin content in transgenic plants with reduced peroxidase activity. These analyses showed that the amount of total lignin in stem was also reduced in antisense plants. The highest reduction in total lignin content, which was 20 % lower than that measured in untransformed control lines was observed in the transgenic line, T33. Consistent with this observation, line T33 had the lowest peroxidase activity of all transgenic lines studied **(Fig. 3)**. Although reductions in lignin content were relatively small in other transgenic lines, it was reproducibly consistent across all transgenic lines. As a result, the overall correlation between reduced peroxidase activity and reduced lignin content was highly significant ($r= 0.96$, $p> 0.05$).

4. Discussion

Peroxidases represent one of the most ubiquitous enzymes in plants. Previous studies examining the role(s) of peroxidases in physiological processes have been hampered mainly due to very high redundancy of peroxidase genes distributed throughout plant kingdom. It has been demostrated that even the genome of *Arabidopsis* could potentially encode more than forty different peroxidases (Welinder *et al.*, 1996). Additionally, these peroxidases use a very broad range of substrates with similar immunological properties (Christensen *et al.*, 1998). This makes functional differentiation of these isoenzymes even more difficult. Nevertheless, one of the functions putatively assigned to peroxidases is lignification. Existence of lignification specific peroxidases has been shown in a number of plants such as lupin (Ros Barcelo *et al.*, 1988; Ferrer *et al.*, 1990),

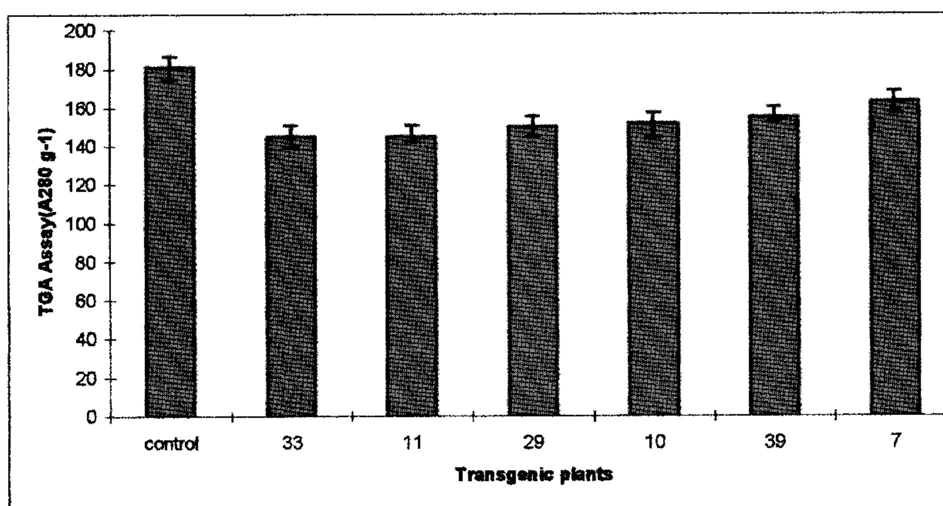


Fig. 3. Total lignin content in transgenic plants transformed with the 35S-*Shpx6a* antisense construct (33, 11, 29, 10, 39, 7) and untransformed control plants determined by TGA method. Results are averaged from three different determinations. Error bars are also shown for each plant.

poplar (Imberty *et al.*, 1995), tobacco (Goldberg *et al.*, 1985), flax (McDougall, 1992), peach (Abeles and Biles, 1991) and *Coleus* (Hepler *et al.*, 1972). However, a direct link between reduced peroxidase activity and lignification has not been conclusively shown. In this study, we down-regulated total peroxidase activity of the transgenic poplar by antisense expression of *Shpx6a* gene and examined the effect of reduced peroxidase activity on lignification.

Analysis of transgenic plants with lower peroxidase activity revealed a slight reduction in total lignin content of these plants. The correlation between reduced lignin content and reduced peroxidase activity was highly correlated suggesting that the expression of antisense *Shpx6a* gene resulted in inhibition of certain lignin specific peroxidase isoenzymes. However this reduction in lignin content was relatively small, possibly due to following reasons. First of all, the number of independent transgenic lines studied was relatively small. It is possible that transgenic plants exhibiting highly reduced lignin and peroxidase activity could be found if more plants transformed with the 35S-*Shpx6a* antisense construct or more homologous antisense peroxidase constructs were examined. Secondly, a vital process like lignification has evolved to have several redundant pathways as shown recently by Zhong *et al.* (1998). Such redundancy suggests that for higher reductions in lignin content, activities of more than one enzyme (e.g. CAD, OMT, CCOMT etc.) should be simultaneously inhibited (Ros Barcelo, 1997). This approach could increase the likelihood of more substantial reductions in lignin content. Similarly, it is well-established that there are several peroxidase isoenzymes involved in lignification. In this case, inhibition of the activity of one enzyme can be partially compensated by certain other related isoenzymes, resulting with only modest reductions in lignin content.

Although antisense *Shpx6a* gene used in this study reduced peroxidase activity and lignification in an heterologous host, exact function of the *Shpx6a* gene in its original host is unknown. Previously, this gene has been shown to be strongly induced at the early stages of infection of its natural hosts by anthracnose pathogen *Colletotrichum gleoesporioides* (Harrison *et al.*, 1995). Although it is still somewhat speculative, function of *Shpx6a* in its natural host may also involve strengthening of cell wall components or increase lignification during infection. Such mechanism(s) would provide better tolerance against pathogen challenge and tentatively explain how constitutive over expression of *Shpx6a* in tobacco and canola resulted in higher tolerance to fungal pathogens (Kazan *et al.*,1998). Further analysis of transgenic plants expressing *Shpx6a* peroxidase is now underway to determine whether lignin content of transgenic plants has also been modified.

In conclusion, our results assigned a putative but more direct link for peroxidase involvement in lignification, although the number and the types of peroxidases inhibited in transgenic poplar plants are not known. Further characterisation of transgenic plants to determine which peroxidases are specifically down-regulated may help for isolation of certain lignification specific peroxidase isoenzymes.

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