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A lignin-specific peroxidase in tobacco whose antisense suppression leads to vascular tissue modification☆

Kristopher A. Blee[a,1], Joon W. Choi[b], Ann P. O'Connell[a], Wolfgang Schuch[c,2], Norman G. Lewis[b], G. Paul Bolwell[a,*]

[a]*School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK*
[b]*Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA*
[c]*Syngenta Wheat Improvement Centre, John Innes Centre, Colney, Norwich NR4 7UH, UK*

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

A tobacco peroxidase isoenzyme (*TP60*) was down-regulated in tobacco using an antisense strategy, this affording transformants with lignin reductions of up to 40–50% of wild type (control) plants. Significantly, both guaiacyl and syringyl levels decreased in essentially a linear manner with the reductions in lignin amounts, as determined by both thioacidolysis and nitrobenzene oxidative analyses. These data provisionally suggest that a feedback mechanism is operative in lignifying cells, which prevents build-up of monolignols should oxidative capacity for their subsequent metabolism be reduced. Prior to this study, the only known rate-limiting processes in the monolignol/lignin pathways involved that of Phe supply and the relative activities of cinnamate-4-hydroxylase/*p*-coumarate-3-hydroxylase, respectively. These transformants thus provide an additional experimental means in which to further dissect and delineate the factors involved in monolignol targeting to precise regions in the cell wall, and of subsequent lignin assembly. Interestingly, the lignin down-regulated tobacco phenotypes displayed no readily observable differences in overall growth and development profiles, although the vascular apparatus was modified.

Keywords: *Nicotiana tabacum*; Tobacco; Solanaceae; Cationic peroxidase; Lignin

1. Introduction

There is considerable interest in the biotechnological modification of (woody) plant lignin content and composition, for reasons which are essentially threefold. One is to delineate the mechanism(s) of lignin assembly in different cell types (Lewis et al., 1999), whereas the other two include manipulating vascular plant biosynthetic pathways for improving the efficiency of pulp and paper making for quality, economic and environmental reasons and/or obtaining more readily digestible animal feed (Boudet, 1998; O'Connell et al., 1999; Baucher et al., 2000; Anterola and Lewis, 2002a,b). In this regard, next to cellulose, lignin is nature's second most abundant biopolymer in vascular plants.

Interestingly, tobacco (*Nicotiana tabacum*) has thus far been the primary model plant system of choice for such biotechnological manipulations, and antisense and sense suppression studies carried out have targeted a considerable number of individual steps in phenylpropanoid/monolignol biosynthesis (**1**–**3**); however, the effects obtained on the lignification process were quite variable (reviewed in Anterola and Lewis, 2002b). Nevertheless, such manipulations are yielding new insights into identification of factors, at the biochemical and cellular level, which control lignin assembly and

Abbreviations: C4H, cinnamate 4-hydroxylase; 4CL, 4-cinnamoyl-CoA ligase; CAD, coniferyl alcohol dehydrogenase; CCOMT, caffeoyl-CoA *O*-methyl transferase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyl transferase; F5H, ferulate 5-hydroxylase; IEF, isoelectric focusing; PAL, phenylalanine ammonia-lyase; SSC, standard saline citrate; S, syringyl; G, guaiacyl; H, *p*-hydroxyphenyl

☆ The nucleotide sequence reported in this paper has been submitted to the GenBank™ and EBI DataBank with accession number AF149251.

* Corresponding author. Tel.: +44-1784-443553; fax: +44-1784-443239.
E-mail address: uhbc006@vms.rhbnc.ac.uk (G.P. Bolwell).
[1] Present address: Department of Biological Sciences, California State University, Chico, CA 95929, USA.
[2] Present address: Cellfor Inc, 255 Burrard Street, Vancouver, BC, V6C 2GB, Canada.

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which ultimately afford distinct cell types, such as vessels and fibres. By contrast to these studies, there have been very few substantiated reports of the biochemical consequences on lignin and cell wall assembly, as regards manipulation of processes controlling either monomer transport to lignin initiation sites or in the subsequent oxidative polymerization stages. These are necessary to define further the strategies for global optimization of plant fibre production, and for further dissection of the lignin assembly process.

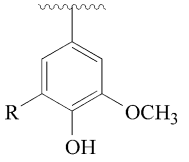
Previous manipulations of the monolignol pathway in tobacco have yielded many interesting findings: for instance, downregulation of the class I form of cinnamate-4-hydroxylase, C4H (CYP73A3) from *Medicago sativa* in tobacco, resulted in significant reduction in lignin contents with apparent decreases in their syringyl:guaiacyl, S:G ratios (Sewalt et al., 1997). In the same study, the preceding enzyme, phenylalanine ammonia lyase, PAL, was also subjected to down-regulation with an accompanying reduction in lignin levels, but in this case with an apparently increased S/G ratio. More recently a class II C4H (CYP73A15) was used to down-regulate lignin content in tobacco by sense and antisense expression with somewhat similar trends to those found for the class I enzyme (Blee et al., 2001a).

Other steps in the pathway modified in tobacco include 4-coumarate CoA ligase (4CL), cinnamoyl CoA *O*-methyl transferase (CCOMT), caffeic acid *O*-methyl transferase (COMT), ferulate-5-hydroxylase (F5H), cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) (reviewed in Anterola and Lewis, 2002). Of these, 4-CL levels have been altered in tobacco (Kajita et al., 1996) [and compared with arabidopsis (Lee et al., 1997) and aspen (Hu et al., 1999)], this resulting in reduced lignin contents and higher recoverable amounts of G compared with S residues, whereas down-regulation of CCOMT gave reductions in both S/G recoveries and lignin amounts (Zhong et al., 1998; Pinçon et al., 2001). On the other hand, a striking effect was observed for COMT down-regulation (Atanassova et al., 1995), which led to a drastic reduction of S-units with concomitant formation of the iso-electronic 5-hydroxyconiferyl alcohol **4** species; however, there was no apparent overall reduction in gross lignin content.

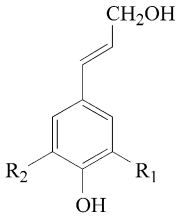
Additionally, F5H, initially considered to act on ferulic acid **5**, is now thought to act at the level of the aldehyde and/or alcohol (Humphreys et al., 1999; Osakabe et al., 1999; Li et al., 2000). CCR levels have also been altered in tobacco (Piquemal et al., 1998), and, finally, manipulation of CAD [the last enzymatic step in monolignol biosynthesis, and one of the first to be targeted (Halpin et al., 1994)], leads to the reddish-brown midrib phenotype familiar through mutational studies (Anterola and Lewis, 2002b). Nevertheless, such studies are beginning to shed light on how various aspects of the lignification process are differentially controlled in various cell wall layers and in different cell (wall) types (Dixon et al., 2001; Anterola and Lewis, 2002a,b). It must be emphasized, however, that the most severely down-regulated plants described thus far (e.g. estimated as up to 50–60% lignin reduction) show deleterious effects on the vasculature and/or growth and development (reviewed in Anterola and Lewis, 2002b).

By comparison to studies on monolignol **1**–**3** forming pathways, and the downstream effects of down-regulating their corresponding enzymes on lignin formation, there has been far less work reported on manipulation of enzymes of lignin assembly (i.e., those involved in monolignol transport to the cell wall, lignin initiation and polymerization). It would thus be instructive to assess and compare the biochemical consequences on plants down-regulated in various aspects of the lignin assembly (polymerization) process using the same overall strategy. In this context, understanding fully how assembly of the lignin biopolymer occurs, and how it can be controlled and manipulated, represents an important question in determining the overall processes involved in forming a functional vascular apparatus.

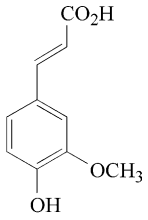
Peroxidases (Lewis et al., 1999) and laccases (Kiefer-Meyer et al., 1996) are both candidates for the one electron oxidation of monolignols prior to lignin assembly, with the former being preferred (Nose et al., 1995). Additionally, the recent discovery of dirigent proteins, which specify the outcome of stereoselective coupling of the monolignol, coniferyl alcohol **2**, in the presence of one-electron oxidative enzymes, such as peroxidase, established the presence of monolignol (radical) binding proteins (Gang et al., 1999; Burlat et



G, R=H, guaiacyl
S, R=OCH_3, syringyl



1. R_1=R_2=H, *p*-coumaryl alcohol
2. R_1=OCH_3, R_2=H, coniferyl alcohol
3. R_1=R_2=OCH_3, sinapyl alcohol
4. R_1=OCH_3, R_2=OH, 5-hydroxy coniferyl alcohol



5. ferulic acid

al., 2001; Anterola and Lewis, 2002b; Kim et al., 2002a, 2002b). It was thus proposed that *other* related proteins, harboring arrays of dirigent (monolignol radical-binding) sites, are involved in lignin assembly in order to control where lignin initiation occurs; lignin assembly is then envisaged to continue via a template polymerization process (Guan et al., 1997).

Of these different protein families, the peroxidase superfamily contains three main structural groups described as I, II, and III. In terms of catalysis, all three groups can undergo a catalytic cycle involving oxidation of the haem group by hydrogen peroxide followed by reduction by various phenolic compounds [including lignin monomers thereby generating the corresponding free-radical species (Dunford, 1991; Anterola and Lewis, 2002)]. However, only the class I (ascorbate peroxidases) and class III forms are found in plants, with most plant species containing a large number of isoforms of the latter. Although the Class III peroxidases have variable domains, all are characterised by coordinate bonding of the haem group to distal and proximal conserved histidine residues. Furthermore, since crystal structure has been determined (Gajhede et al., 1997), the individual isoforms can now be modeled using the coordinates (e.g. Blee et al., 2001b). However, members of the family still show large variations in pI and extent of glycosylation, and substrate specificity thus appears to be dependent upon residues at the active site and substrate access channel. For example, even the substrate preference for *p*-coumaryl **1** and coniferyl **2** alcohols over sinapyl alcohol **3** has been proposed as the basis for a peroxidase involved in lignification (Ostergaard et al., 2000).

Attempts have been made in tobacco and poplar to identify peroxidase isoenzymes specifically involved in lignification, which proved to be anionic (Lagrimini et al., 1987; Christensen et al., 1998, 2001; Baucher et al., 2000; summarized in Anterola and Lewis, 2002). In other species, some identification has been carried out at the biochemical level. For instance, in French bean, the immunolocalization of a cationic peroxidase isoenzyme to the middle lamella and growing secondary thickenings in secondary walls was accomplished (Smith et al., 1994) with antibodies raised against a peroxidase first purified from tissue cultures (Zimmerlin et al., 1994). At the molecular level, recent work has further resulted in the probable identification of a lignification specific form (ATP2) in arabidopsis (Christensen et al., 1998) among the 73 present in the genome. Interestingly, the French bean cationic peroxidase FBP1 Gene (GenBank™ accession number AF149277) (Blee et al., 2001b) shows ~60% sequence similarity with the arabidopsis ATP2 peroxidase, although the latter is anionic. Additionally, the molecular identification of an anionic peroxidase likely to be involved in lignification has recently been achieved from poplar (Christensen et al., 2001).

In this study, FBP1 was used to screen a cDNA library derived from tobacco stem to identify possible (cationic) peroxidases involved in lignification. In addition, the clones obtained were tested for enhanced mRNA expression during xylogenesis using a tissue culture system (Blee et al., 2001c). This work showed that only one of these was co-ordinately expressed with enzymes of lignification, and thus it was selected for down-regulation using an antisense expression approach. The overall effect of this manipulation was a very significant reduction in lignification as described below, suggesting a dominant role for peroxidase catalyzed one-electron oxidations of monolignols **1**–**3**.

2. Results

2.1. Cloning and identification of tobacco cDNAs encoding peroxidases homologous to FBP1 of French bean

Since there was evidence that the cognate protein encoded by the French bean cationic peroxidase gene *FBP1* was associated with vascular tissue development (Smith et al., 1994), full length single strand antisense RNA probes were transcribed from the cDNA *FBP1* and used to screen 500,000 pfu of a tobacco stem cDNA library (Knight et al., 1992). (The latter was obtained from 5-week-old stem tissue at a stage whereby both secondary growth and lignin biosynthesis were underway.) Three full length cDNA clones were thus obtained, of which one, *TP02*, was identical to the tobacco anionic peroxidase (GenBank™ accession number J02979, see Table 1 and Fig. 1, bottom entry) that had already been subjected to manipulation (Lagrimini et al., 1987). The other two were identical in sequence and coded for a new tobacco peroxidase cDNA designated *TP60* (GenBank™ accession number AF149251, Fig. 1 and Table 1).

TP60 had an ER signal peptide and application of von Heijne rules (Von Heijne, 1983, 1986; Nakai and Kanehisa, 1992) predicted the signal peptide cleavage site to lie between amino acid residues 19 and 20 (Fig. 1). By comparison to FBP1, *TP60* lacks a C-terminal propeptide, and additional sequence analysis revealed a single asparagine residue as a potential site for N-linked glycosylation, (N-X-S/T) (Nakai and Horton, 1999). In comparison, the number of potential glycosylation sites in the other peroxidases were 1 for the cotton peroxidase, 1 for the cationic soybean peroxidase, 4 for the cationic tobacco peroxidase, 9 for the cationic French bean peroxidase, and 4 for the anionic tobacco peroxidase (Fig. 1).

At both the amino acid and nucleic acid levels, *TP60* shared the highest sequence identity with a peroxidase of cotton and a soybean cationic peroxidase (Table 1 and Fig. 1). By contrast, the French bean cationic peroxidase *FBP1* used to acquire *TP60* had lower

Table 1
Sequence identity of tobacco peroxidase *TP60* (GenBank™ accession number AF149251) to other selected plant peroxidases

Plant species	Peroxidase type	Accession number	At the amino acid level		At the nucleotide level	
			%	Amino acid overlap	%	Nucleotide overlap
Cotton	Cationic	LO8199	86.56	305	80.33	986
Soybean	Cationic	AF039027	86.32	307	79.09	990
Tobacco	Cationic	D42064	36.21	301	57.21	458
French bean	Cationic	AF149277	35.88	301	53.32	724
Tobacco	Anionic	J02979	32.80	311	55.61	437

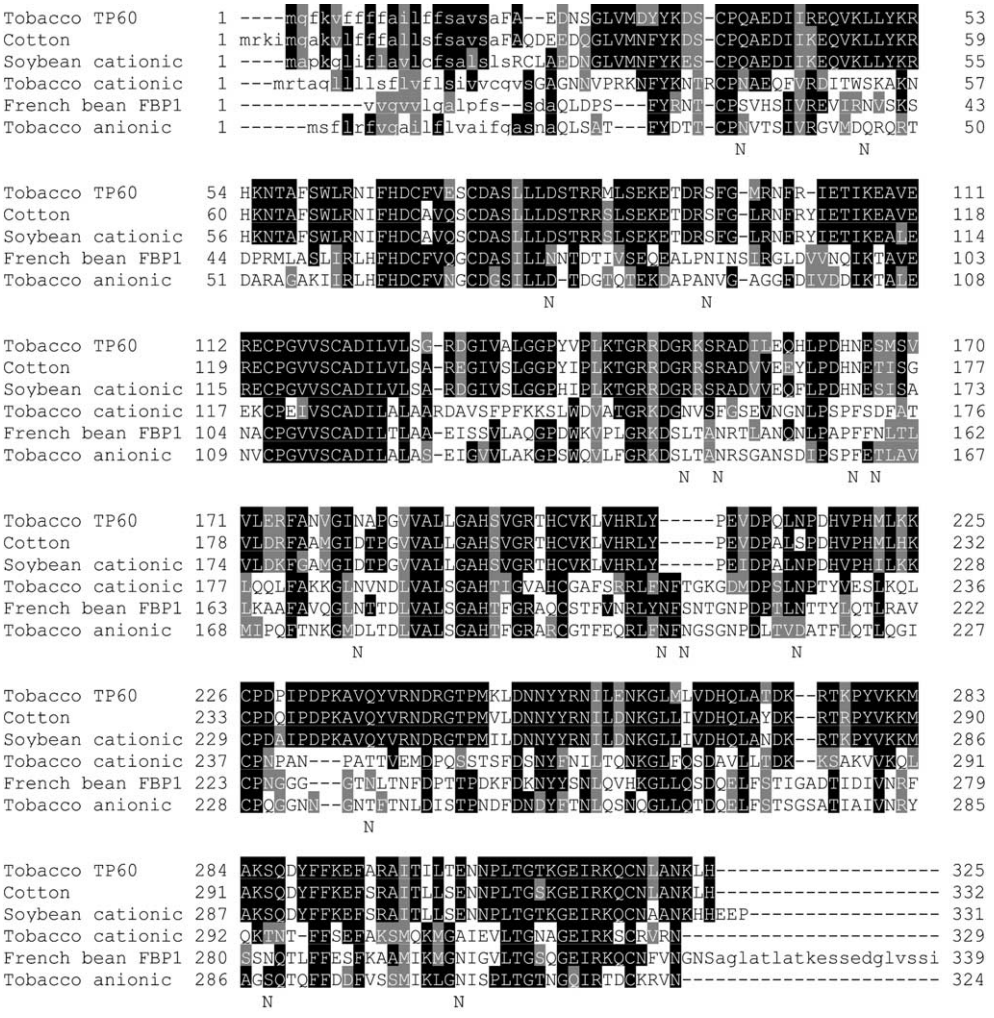


Fig. 1. Deduced amino acid sequence for *TP60* and comparison to other selected plant peroxidases. Black shaded areas represent amino acid identity, gray areas conservative amino acid changes, and non-shaded areas dissimilar amino acid residues. Uppercase letters represent sequence of the mature native enzymes, after peptide sequencing identified native N- and C-terminals for AF149277 (Blee et al., 2001b), or after application of rules for signal sequence cleavage (Nakai and Kanehisa, 1992). Lowercase letters were used to represent both N-terminal signal peptides and C-terminal propeptides. N identifies potential N-linked glycosylation sites of the target sequence N-X-S/T in one or more of the sequences above. Accession numbers; tobacco peroxidase *TP60* AF149251, cotton peroxidase L08199, soybean cationic peroxidase *PRX2* AF039027, tobacco cationic peroxidase D42064, French bean cationic peroxidase *FBP1* AF149277, tobacco anionic peroxidase J02979.

sequence identity, as did both a cationic tobacco peroxidase and the well characterized anionic form *TP02* also found in the cDNA library. Structural predictions from the full-length amino acid sequence for *TP60* were also used in searches against experimentally determined structures of plant peroxidases in the Protein Data Bank, and the deduced protein sequence modelled using coordinates for the known structure of peanut peroxidase (PDB: 1SCH). From this comparative analysis, the single putative glycosylation site for *TP60* is close to the centrally located pore leading to the haem (Fig. 2, upper image), and is in an area of positive charges (represented

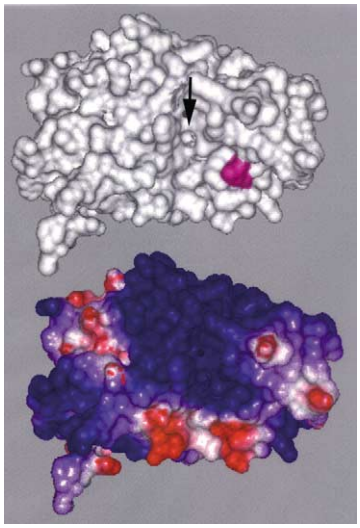


Fig. 2. Hypothetical contour mapping of the surface of *TP60*. The upper panel displays the single asparagine residue of the potential glycosylation target sequence N-X-S/T (in red), whereas the lower panel displays surface electrostatic charges, with positive charge represented by blue-purple, negative by red, white areas as neutral. The pore leading to the centrally located haem group of tobacco peroxidase 60 can be observed near the center on the molecular model as indicated by an arrow in the upper panel.

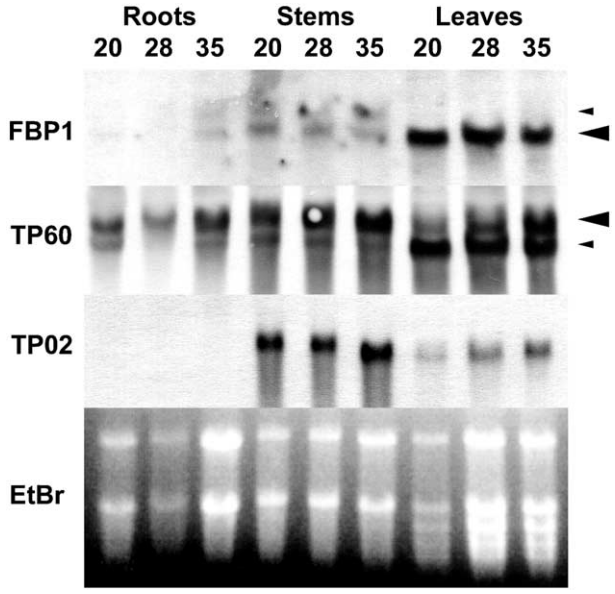


Fig. 3. Differential mRNA accumulation patterns detected in tobacco using *FBP1*, *TP60*, and *TP02* as probes. Antisense RNA probes were used in hybridizations to RNA gel blots containing total RNA (15 μg) per lane from samples of 20, 28, and 35 day roots, stems, and leaves, respectively. Multiple transcript bands were detected with *FBP1* and *TP60* probes at high stringency. Large arrowheads indicate position of the most abundant gene probe specific mRNA bands. Small arrowheads mark less abundant bands, a result of probe cross-hybridization to reciprocal primary mRNA bands, probes *FBP1* and *TP60*, respectively. *TP02* (Lagrimini et al., 1987) detected a single mRNA species mainly in stems. In the lower panel, rRNA bands were made visible with ethidium bromide staining and photographed before blotting the gel to verify loading of lanes.

by blue-purple) as assigned by the Delphi program (MSI) (Fig. 2, lower image). Other areas are negative (red) and neutral (white), with the net electrostatic charge of *TP60* being calculated as 0.0 Coulombs.

2.2. Peroxidase transcript accumulation in tobacco

In order to establish a possible role for *TP60* in lignification, its level of expression in stem tissue was first compared with that in roots and leaves using anti-sense RNA probes from the corresponding cDNAs. For control purposes, transcript levels were also probed using *FBP1* and *TP02*, respectively, with experiments designed whereby the corresponding RNA gel blots were stripped and reprobed to allow use of all probes on a single blot. Careful examination of the data obtained revealed that each probe detected a distinct pattern of expression on the RNA blots, suggesting differential gene transcript recognition. In this way, the *TP60* probe always detected two bands in total RNA from roots, stems and leaves with stronger labeling of the upper band in stem tissues (Fig. 3, *TP60* large arrowhead) in comparison with a weaker labelling of the lower band (Fig. 3, *TP60* small arrowhead). In contrast, the heterologous use of the French bean probe for *FBP1* in tobacco identified both a strong band (Fig. 3, *FBP1* large arrowhead) in the leaves and two weak bands in the total RNA from 20, 28 and 35 day-old stems and 35 day-old roots. On the other hand, the *TP02* probe hybridized to a mRNA of different size that was primarily expressed in stems as shown previously (Lagrimini et al., 1987). Thus, the cDNA coding for *TP60,* and originating from a stem-specific library, apparently recognized relatively specifically a highly expressed transcript in lignifying stems and roots. However, it also cross-hybridized with a shorter message which is abundant in leaves, this being additionally recognized by the cDNA coding for *FBP1*; this second signal is of much reduced abundance in 35-day-old stems when the major signal is associated with lignification.

2.3. Antisense gene silencing of TP60 peroxidase in tobacco and effect on lignification

Because of the temporal and spatial expression of *TP60*, functional studies using an antisense strategy were made to test whether the cognate protein was a likely candidate for monolignol one electron oxidation to give the corresponding free radical entities required for lignin assembly. Accordingly, a full length cDNA coding for *TP60* was cloned in the antisense orientation into a pBin19 derived binary vector system with antisense transcription regulated by the CaMV/35S promoter and nopaline synthase terminator. Kanamycin resistance, similarly regulated, was also cloned into the transfer DNA of the pBin19 constructs. Following replacement of the

vectors containing antisense constructs back into *A. tumefaciens,* the transformed bacterium was then applied to tobacco leaf fragments. Successive rounds of selection with kanamycin containing media, and PCR detection of the antisense transgene in regenerated plants, identified 88 plants containing the antisense *TP60* construct (*TP60*, plants with numerical labels between 600 and 1200, data not shown). Since the *TP60* was derived from the library screened with *FBP1*, and antisense strategies using heterogeneous constructs have proven successful in manipulating lignin (Sewalt et al., 1997; Blee et al., 2001a), 81 plants containing the antisense *FBP1* construct (*FBP1*, plants with numerical labels of less than 150, data not shown) were similarly produced for comparative purposes. However, from any biotechnological context, manipulation with homologous sequences is clearly preferential.

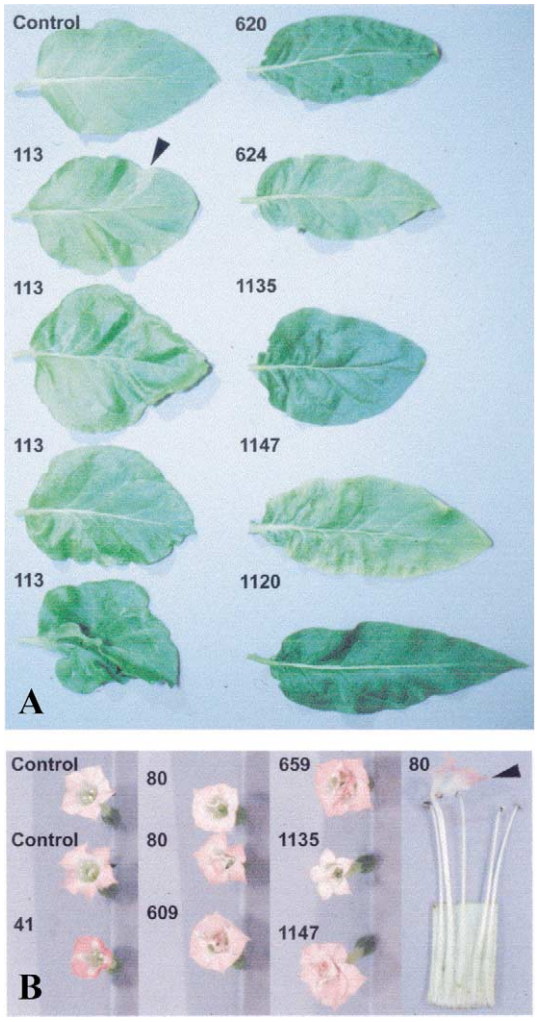


Fig. 4. Tobacco leaf and flower morphology of wild type control plants and plants transformed with antisense peroxidase constructs. **A**: Leaf tissue: Plant 113 was transformed with the antisense *–FBP1* construct, while plants 620–1147 were transformed with an antisense *–TP60* construct. Arrowhead indicates an area within a leaf from plant 113 with lighter green colour. **B**: Flower tissue: Plants 41 and 80 were transformed with the antisense *–FBP1* construct, while plants 609–1147 used the antisense *–TP60* construct. Transformed plants showed variations in petal number and corolla structure, e.g., the arrowhead indicates addition of two petals to a filament just below the anther from plant 80. Both antisense constructs for transformation were under the control of the cauliflower mosaic virus/35S promoter and the nopaline synthase transcriptional terminator.

In terms of phenotypes obtained, transformants selected for growing to maturity showed little compromising of growth with respect to plant height and stem width at all stages of development. However, there were some morphological distinctions common to a small number of both *−TP60* and *−FBP1* containing transformants involving alterations to leaves and flowers compared with wild type. Examples of changes in leaf morphology were more common with the heterologous strategy, and include that shown by plant 113 in which leaves had a rounded appearance without a sharp apex, had wavy instead of smooth margins often deeply indented where veins meet the edge, had vein branching abnormalities including the midrib sometimes forming a split leaf, and had patches of leaf much lighter in color (Fig. 4A, plant 113, see arrowhead). Alterations of leaves to plants carrying *−TP60* included an elongation and narrowing of the leaf blade (Fig. 4A, plants 620, 624, 1147, 1120). A small subset of antisense construct carrying plants contained similar flower alterations, examples of which are the reduction of petal number from the corolla from five petals to four (Fig. 4B, plants 41, 80, 659) and the addition of a petal to the filament just below attachment of the anther (Fig. 4B, plants 80, 609, 659, 1135, 1147).

It was instructive to next examine the effect of the antisense strategy on the overall peroxidase complement in both *−TP60* and *−FBP1* transformants, relative to the corresponding wild type plants, using estimations of gross peroxidase activity (i.e. of soluble protein extracts and by IEF analysis of same, respectively). The anionic peroxidase *TP02* however, was not targeted for its possible involvement in lignification, since very limited success had previously been achieved in functionally associating it with this role (Lagrimini et al., 1987; Christensen et al., 1998, 2001; Baucher et al., 2000).

Before describing the data obtained, it is noteworthy that possession of the antisense constructs in each case was confirmed by DNA gel blot hybridization analysis using both *FBP1* and *TP60* cDNA's as probes. Additionally, the DNA gel blot analysis indicated multiple insertions of *−FBP1* in plant 89, and multiple insertions of *−TP60* in plant 624 (data not shown). Furthermore, for control purposes, wild type plants were harvested after 4 weeks growth, with stems being sectioned into 12 segments, these being separated according to internode (where 1 represents the internode closest to the apex). As expected, the gross soluble peroxidase activity using the non-physiological substrate guaiacol (in the presence of H_2O_2) increased with increased internode age

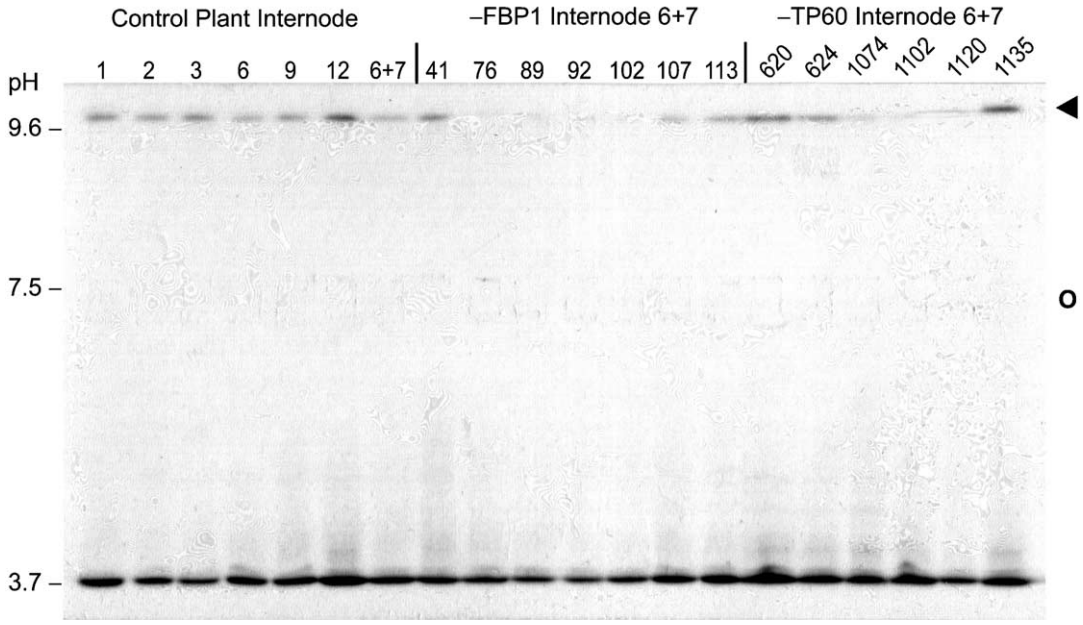


Fig. 5. Isoelectric focusing of soluble protein extracts from tobacco stem internodes, stained for gross peroxidase activity and taken from wild type (control) and antisense peroxidase transformants, respectively. Internode extracts were subjected to isoelectric focusing and gels were incubated in an assay to detect peroxidase isoenzymes. Profiles are first shown for a developmental series of internodes in the wild type plants. In the case of transformed plants, internodes 6 and 7 were selected for comparison with wild type. **O** indicates the sample origin before electrophoresis. Arrowhead marks the position of the cationic peroxidase isoenzyme with reduced abundance in various antisense construct carrying plants. Twenty microgrms of total protein were run in each lane.

(data not shown). IEF analysis, on the other hand, containing equivalent amounts of soluble protein from each internode segment revealed the presence of two differentially migrating peroxidases at pI ~9.5 and ~3.7, respectively, with peroxidase visualization using guaiacol/H_2O_2 as before (Fig. 5).

For comparison between the transformants *−TP60* and *−FBP1* and the wild type plants, internodes 6 and 7 were selected and combined for each plant specimen, with each individually analysed to ascertain the effects of antisense peroxidase expression. Fig. 5 thus shows the results obtained with various lines taken at random: with both *−TP60* and *−FBP1* transformants, the most obvious effect on readily soluble peroxidases was the near disappearance of the cationic peroxidase (pI ~9.6) in several (but not all) of the transformants, whereas the effect on the peroxidase(s) at pI ~3.7 was apparently less dramatic. Thus, because *FBP1* was used to isolate *TP60* and the two nucleic acid probes cross-hybridize under stringent conditions, it can be provisionally considered that antisense gene silencing attempts from both constructs are effective in preferentially reducing expression of the gene encoding the "cationic" peroxidase, *TP60*.

2.4. Chemical analysis of tobacco xylem tissue for lignin content and compositions

A number of transformed plant lines were next selected from 3 and 4 month old transformants, with internodes 2–5 being subjected to detailed lignin determinations, i.e. using both Klason (Dence, 1992) and acetyl bromide (Iiyama and Wallis, 1990) estimations of gross lignin contents, as well as by thioacidolytic (Lapierre et al., 1986; Rolando et al., 1992) and nitrobenzene (Iiyama and Lam, 1990) degradative analyses to estimate the relative amounts of recoverable guaiacyl (G) and syringyl (S) components.

Thus, Fig. 6A–D summarizes the results obtained by plotting the %G and %S contents recoverable from the cell wall residue (see Section 4) relative to the corresponding measured acetyl bromide lignin contents, respectively. As expected, due to differences in efficacy of overall *TP60* down-regulation in the various transformants, there was considerable variability in the levels of down-regulation obtained. Nevertheless, in essentially every case relative to the controls, a progressive reduction in overall measured lignin content was accompanied by a concomitant decrease in recoverable G and S amounts. This trend was noted regardless of the type of degradative analysis undertaken (i.e. via either nitrobenzene oxidation (Fig. 6A,C) or thioacidolysis (Fig. 6B,D) or by the method of lignin estimation, i.e., acetyl bromide or Klason lignin (data not shown). There was as expected, however, more variability in the Klason lignin data, since the latter is not a particularly useful method for herbaceous plants due to the presence of non-lignin contaminants (Anterola and Lewis, 2002b). As for such analyses, the recoveries of %G and %S in both cases ranged from 13.5 to 45.1% of the

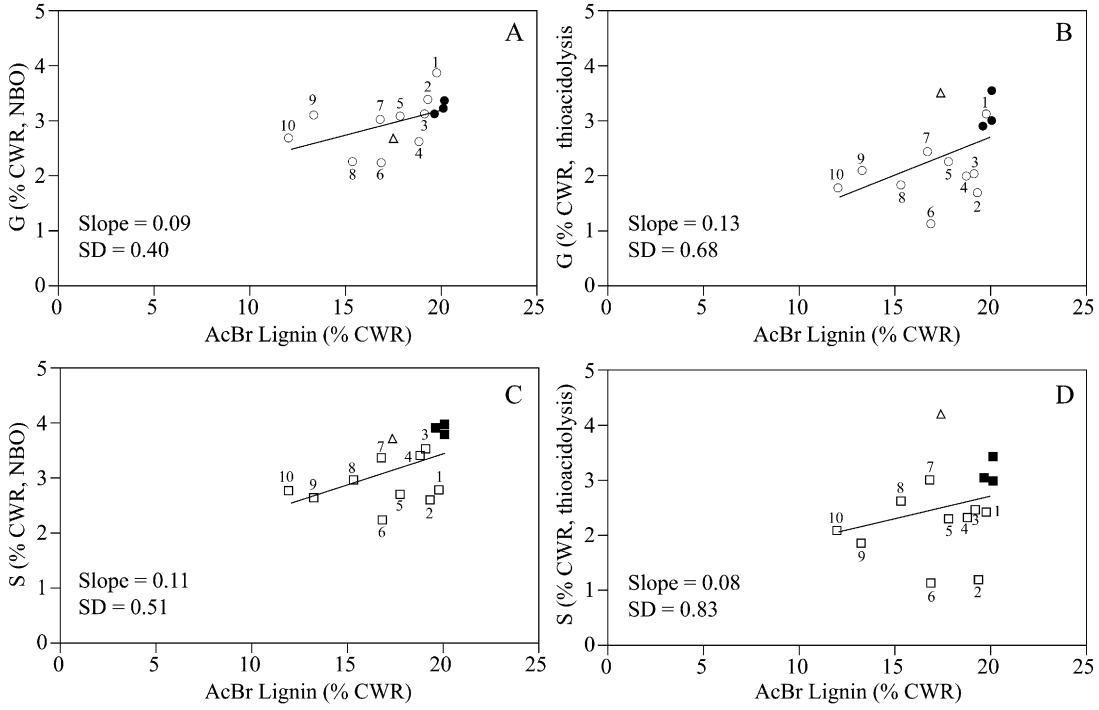


Fig. 6. Estimated acetyl bromide lignin contents versus guaiacyl (G, ○, ●, and △) and syringyl (S, □, ■, and △) recoveries of cell wall residues (CWR) of control (solid circles) and TP60 downregulated transgenic lines (open circles) in tobacco. [Δ refers to a −FBP1 downregulated transformant]. A and C, data from nitrobenzene oxidation (NBO); B and D, data for thioacidolysis. Numbers 1–10 on graphs refer to different transgenic lines whose AcBr lignin levels are: (1) Line 1102, 19.81±0.62%; (2) Line 636, 19.41±0.70%; (3) Line 629, 19.21±0.15%; (4) Line 1135, 18.85±0.94%; (5) Line 624, 17.84±0.61%; (6) Line 651, 16.89±0.88%; (7) Line 1077, 16.81±0.16%; (8) Line 1074, 15.42±1.18%; (9) Line 659, 13.31±0.70%; (10) Line 1147, 12.05±0.33%, of CWR, whereas for the controls and the *−FBP1* downregulated transformant the AcBr levels are 20.11±0.22, 19.64±0.10 and 17.42±0.34%, respectively.

estimated acetyl bromide lignin, this recovery level being a limitation of the degradative analyses currently employed. Interestingly, unlike many other previous studies on monolignol pathway down-regulation (Anterola and Lewis, 2002b), there appeared to be no obvious deleterious effects on plant growth and development, even when overall reductions in lignin content were approximately ~40–50%.

2.5. Histochemical lignin analysis of selected transformants

The so-called Wiesner reaction (phloroglucinol–HCl staining) is widely used to detect guaiacyl-rich lignins in various tissue sections and gives a characteristic red color in its presence (Johansen, 1940). This coloration is widely considered (but not unambiguously proven) to be due to presence of low levels of coniferaldehyde end-groups in the lignin polymer, whereas with syringyl-rich lignins the staining may be either faint or undetectable (Sarkanen and Ludwig, 1971).

Fig. 7A shows a cross-section of wild type tobacco xylem containing secondary growth (from internode four), whose lignified xylem elements (~20% lignin, S/G ratio ~0.84) are clearly detectable using the phloroglucinol–HCl test, i.e., as revealed by the intense uniform pink staining across the secondary xylem tissue inward from the cambium to the pith. In contrast to the wild type specimen (Fig. 7A), however, phloroglucinol–HCl staining of the comparable antisense sections from transgenic lines 1074 (Fig. 7B) and 1102 (Fig. 7C) were considerably reduced. Of these, the staining intensity of line 1074 (acetyl bromide lignin content ~15.4%, S/G ratio ~1.21) was strongest nearest the stem interior (oldest tissue) but decreased dramatically towards the cambium (newly formed cells). Moreover, the lignified cells in line 1074 qualitatively appeared to be larger in size, this presumably reflecting the decrease in vascular integrity. Unexpectedly, line 1102 (lignin content ~20%, S/G ratio ~0.67) also gave very faint staining with files of relatively unlignified cells with respect to intensity of staining even though the actual estimated content was not significantly reduced. As for 1074, the staining was most evident in the older xylem cells closest to the pith tissue.

3. Discussion

It is an important goal to accomplish the molecular dissection of the individual roles of the various peroxidase isoenzymes, and to identify those specifically

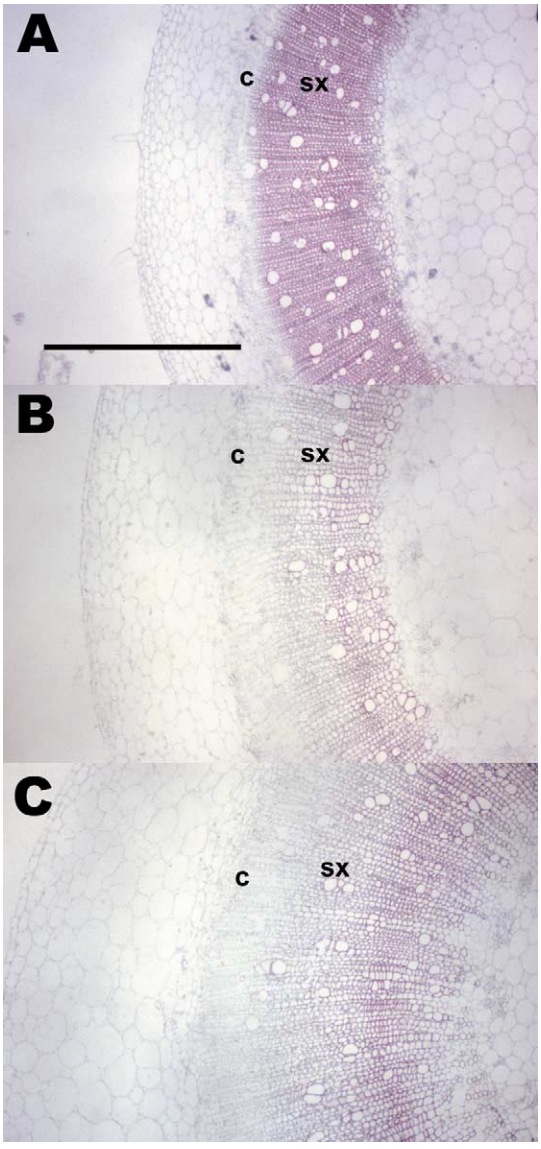


Fig. 7. Histochemical lignin (phloroglucinol–HCl) staining of secondary growth in tobacco stems of wild type and peroxidase down-regulated plant lines. Sections (20 μm) of tobacco stems (fourth internode) displaying secondary growth were subjected to phloroglucinol–HCl staining to detect lignin (visualized as red pink colour). A, wild type parental tobacco; and transgenic TOB60 antisense plants B, 1074 and C, 1102. All sections received identical treatment before photography, and all are represented at identical magnification. Abbreviations; c, cambium; sx, secondary xylem. Bar = 1.0 mm.

involved in monolignol oxidation leading eventually to lignification. In this regard, functional identification of various suspected lignin-specific peroxidase isoenzymes had been previously attempted by modification of the expression of particular anionic peroxidases in tobacco (Lagrimini et al., 1987, 1997a,b; Lagrimini, 1996) and poplar (Christensen et al., 1998; Baucher et al., 2000); these manipulations, however, had no substantial effect on lignin deposition of biotechnological significance. For instance, the effect on manipulation of a tobacco anionic peroxidase, identical to the one isolated as *TP02* in this study, has been extremely well studied through both overexpression and antisense strategies. While no notable effects on lignin biosynthesis were noted, *TP02* does possibly have a role in the wounding response (Lagrimini et al., 1997a). Accordingly, it remained to be established whether alteration of peroxidase levels is a valid strategy for manipulating lignin contents and/or compositions in the vascular apparatus.

More recent evidence has been reported for the molecular identification of an arabidopsis isoform ATP2 that is specifically involved in lignification (Ostergaard et al., 2000). Furthermore, attempts have also been made to identify lignin-specific peroxidase isoenzymes by purification from species such as French bean (Zimmerlin et al., 1994). In that case, the enzyme was immunolocalised to the middle lamella and the inner-face of the secondary wall in differentiating xylem (Smith et al., 1994). It was thus quite unexpected to learn of the view in a recent report (claiming to provide a new model for lignin biosynthesis), that peroxidases (as well as other proteins) could not be targeted to the cell wall because they were too large (Önnerud et al., 2002)! There are already more than 200 proteins considered to be targeted to the cell wall (Chivasa et al., 2002).

With the subsequent cloning of the cDNA for the French bean enzyme family containing putative lignification-specific peroxidases (Blee et al., 2001b), it was possible to screen a cDNA library derived from lignifying tobacco stems for possible peroxidases involved in lignification. Among the clones obtained, *TP60* showed a regulated high expression when analysed in northern blots of mRNA expressed during xylogenesis using a differentiating tissue culture system (Blee et al., 2001c). Importantly, the expression of the peroxidase *TP60* was coordinated with F5H, CCR and CAD, these being presumed specific to the lignin pathway in these cells; however, a different pattern of expression was observed for PAL, C4H and COMT, which are also involved in the phenylpropanoid pathway.

The *TP60* gene was compared for expression with that of the anionic peroxidase *TP02* (Lagrimini et al., 1987), and both displayed different expression patterns with *TP60* being relatively more dominant in lignifying stem and root tissues. Accordingly, the functional analysis of *TP60* was next investigated by antisense expression, the transformants of which showed some limited phenotypic changes without any apparent significant effects on growth.

The major physiological significance of down-regulating *TP60*, under control of the CaMV35S promoter, was on overall lignin deposition, with the same trends being noted regardless of the lignin analysis methodology employed. For example, in Fig. 6A–D, there is a progressive reduction in both recoverable G and S levels as lignin amounts decrease down to ca. 40–50% of wild type levels, and this trend was noted for G and S recoveries

not only from nitrobenzene oxidation but also from thioacidolysis analyses. In contrast, the corresponding three wild type controls displayed little variability in either lignin composition and/or amount. It was also noteworthy that Klason lignin determinations showed greater variability (data not shown); however, Klason lignin analyses are known to suffer from the presence of non-lignin contaminants in non-woody tissues, and these can skew overall estimations (Anterola and Lewis, 2002b).

The reductions in lignin contents obtained by down-regulating TP60 in this way compare very favorably with the best examples obtained thus far in monolignol pathway manipulation (Anterola and Lewis, 2002). However, in the present case, the reductions in both G and S recoveries follow a more or less linear trend as lignin contents decreased. These data thus appear to suggest that some form of feedback mechanism is operative at the plasma membrane/cell wall interior which prevents accumulation/transport of monolignols should oxidative capacity be diminished. If this assumption is correct, this would provide an additional dimension as to how metabolic flux is controlled in lignifying cell walls. Previously, only the steps involving Phe supply and the relative activities of C4H/C3H have been demonstrated to have rate-limiting roles (Anterola et al., 2002a,b) in the pathway, and not the other enzymes downstream involved in metabolism leading to the monolignols (Anterola and Lewis, 2002b). This question will be the topic of further study.

The phloroglucinol–HCl histochemical staining of cross-sections of *TP60* down-regulated tobacco stems was also generally consistent with a progressive decrease in lignification, but interestingly the main effect appeared to involve only the stages of secondary thickening/lignification, i.e. as indicated by the differences in staining at the lignified regions closest to the pith and cambium, respectively. It will thus be important in the future to examine the transformants in greater detail, in order to identify what effects on vascular integrity and individual cell wall development have actually occurred.

Finally, from an applied point of view, the identification of lignification-specific peroxidases and their downregulation using anti-sense manipulation thus appears to be an additional valid strategy for modifying the lignin content of woody species.

4. Experimental

4.1. Materials

The tobacco strain used throughout was *Nicotiana tabacum* cv. Samsun, and was grown in equaml amounts of Minster Economy No. 3 Compost (Heatherwood Nurseries, Ashington, Wimborne, United Kingdom) and Sinclair Growers Potting and Bedding Compost (William Sinclair Horticulture Ltd, Firth Road, Lincoln, United Kingdom) with 10 ml of Osmocote Plus controlled release fertilizer (Scotts Europe B.V., The Netherlands) in 6-inch pots. The plants were grown in the glass house and received natural lighting.

4.2. cDNA library screening

Plaques, representing a cDNA library of poly (A) RNA from stems of 5-week-old tobacco (Knight et al., 1992), were generated according to (Sambrook et al., 1989). Plaque lifts representing 500,000 pfu were hybridized with digoxigenin-UTP-labeled antisense RNA probes generated from in vitro transcriptions off the *FBP1* cDNA. Positive hybridizing plaques were detected using the Genius System (Boehringer Mannheim Corporation, Indianapolis, IN, USA) and purified after two additional rounds of plating at low titer and screening, allowing single plaque isolation. Plasmid clones were obtained by excision of the pBluescript phagmid from the purified plaques using the Exassist/Solar System for Lambda ZAP vectors (Stratagene, La Jolla, CA). Plasmids containing the cDNA clones were isolated (Del Sal et al., 1988) and the inserts were sequenced commercially (Oswell Laboratory, University of Southampton, Southampton, UK). The cloned sequence *TP60* was submitted to GenBank™ and assigned accession number AF149251.

4.3. Molecular modelling of peroxidases

Homology modelling of peroxidases employed the molecular graphics modelling packages Modeller and Insight II 97 (MSI) with electrostatic charges assigned by the Delphi programme (MSI). The protein sequence for the tobacco peroxidase 60 was derived from the novel clone accession AF149251 and was modelled using homology to the peanut peroxidase model (PDB:1SCH).

4.4. RNA gel blot analysis

Total RNA was isolated individually from pooled samples of liquid nitrogen ground root, stem, and leaf tissues of 20, 28, and 35-day-old tobacco plants, respectively, according to Logeman et al. (1987), and was loaded (15 μg) onto formaldehyde gels containing 1% (w/v) agarose, then separated, and blotted onto nitrocellulose (Ausubel et al., 1999). Digoxigenin-UTP-labeled antisense RNA probes were generated from full length *in vitro* transcriptions off the peroxidase cDNAs (*FBP1* and *TP02*) according to Stratagene methods (Stratagene, La Jolla, CA, USA). Following the Genius System methods (Boehringer Mannheim Corporation, Indianapolis, IN, USA), prehybridizations and hybridizations were carried out in 5×SSC, 50% (v/v) formamide,

0.1% (w/v) *N*-lauroyl-sarcosine, 0.02% (w/v) SDS, and 5% (w/v) blocking reagent at temperatures ranging from 50 to 65 °C. Hybridizations were carried out overnight using probes at 50 ng ml^{-1}, with two washes at room temperature for 5 min each in 2 SSC, 0.1% (w/v) SDS, and finally with two high stringency washes at 68 °C for 1 h each in 0.5×SSC, 0.1% (w/v) SDS. Blots were stripped between hybridization experiments with 2 consecutive 10 min incubations in freshly boiled 0.1% (w/v) SDS. Replicate blots were used to independently confirm differential expression patterns for the probes.

4.5. Tobacco transformation with Agrobacterium tumefaciens

Disarmed *A. tumefaciens* LB404 harboring binary vector pBin19 was used for all the experiments. Within the T-DNA region, the pBin19 constructs contained kanamycin resistance and, in the reverse orientation with respect to the promoter, either the entire full length cDNA for *FBP1* (antisense *FBP1* construct, *−FBP1*) or the entire full length cDNA for *TP60* (antisense *TP60* construct, *−TP60*). CaMV/35S promoter and nopaline synthase terminator were then used to regulate expression from the antisense constructs and kanamycin resistance, respectively, within the T-DNA. After transformation of *A. tumefaciens* with pBin19 antisense constructs, the cultures were initiated in Luria–Bertani medium (100 ml) containing kanamycin (50 μg ml^{-1}) and allowed to grow for 12 h at room temperature with shaking. *A. tumefaciens* cells were collected by centrifugation and resuspended in liquid Murashige and Skoog medium. Leaf strips were cut from genetically identical tobacco plants (*N. tabacum* cv. Samsun), generated by vegetative propagation, then incubated in the resuspended *A. tumefaciens* cells for 20 min before being placed onto solid Murashige and Skoog medium containing 6-benzylaminopurine (1 mg ml^{-1}) and α-naphthalene acetic acid (0.1 mg ml^{-1}) for 2 days. The leaf strips were transferred to solid Murashige and Skoog medium containing 6-benzylaminopurine (1 mg ml^{-1}), α-naphthalene acetic acid (0.1 mg ml^{-1}), carbenicillin (500 mg ml^{-1}), and kanamycin (100 mg ml^{-1}) and left for 5 weeks to form shoots. Shoots were excised from the leaf strips and inserted into solid Murashige and Skoog medium containing carbenicillin (500 mg ml^{-1}) and kanamycin (100 mg ml^{-1}) and allowed 4 weeks for rooting. Regenerated plants were respectively screened for the presence of either transgene (*−FBP1* or *−TP60*) using PCR. DNA was isolated from leaf tissue using the Fast DNA Kit for plants (Bio 101, Carlsbad, CA, USA) and added to Ready to Go PCR beads (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) with CaMV/35S promoter and nopaline synthase terminator primers 5′-CAATCCCACTATCCTTCGC-3′ and 5′-CATCGCAAGACCGGCAACAG-3′, respectively. Cycling proceeded after 7 min at 95 °C with 40 cycles of 1 min at 95 °C, 1 min at 58 °C, and 2 min at 72 °C. Rooting on selective medium (described earlier) of transgenic plants was repeated before transferring rooted plants to solid growth matrix and glasshouse grown exactly as described (see Section 4.1).

4.6. DNA gel blot analysis

Genomic DNA was isolated from liquid nitrogen ground leaf tissue (1 g) with the resulting frozen powder extracted with 50 mM Tris–HCl buffer (6 ml, pH 8) containing 20 mM Na_2EDTA, 0.32 M NaCl, 0.4% (w/v) *N*-lauryl sarcosine, and 42% (w/v) urea, and phenol (6 ml), (phenol equilibrated with an equal volume of 1 M Tris–HCl at pH 8:chloroform:isoamyl alcohol at 25:24:1). After vortexing the mixture for 60 s, the resulting suspension was centrifuged to aid phase separation. Supernatant was collected and the DNA was precipitated by addition of 7.5 M ammonium acetate (3 ml) and isopropanol (3.6 ml). The precipitated DNA was removed by spooling, rinsed in 70% (v/v) aqueous ethanol, air dried, then re-dissolved in sterile water (200 μl). Five microlitres of 10 mg ml^{-1} RNAase A was added, and the mixture was incubated at 37 °C for 30 min. After RNAase A incubation, the samples were again subjected to phenol:chloroform:isoamyl alcohol extraction, ethanol precipitation, and resuspended in sterile water. After quantification of DNA by the absorbance at 260 nm, 20 μg of restriction enzyme digested DNA was loaded and separated in each lane of 0.7% agarose gels containing ethidium bromide before blotting onto nylon membranes (Ausubel et al., 1999). Gel blots were generated that contained DNA samples from each plant digested with only *Eco*RI to release the entire antisense cDNA (either *−FBP1* or *−TP60*) and that contained samples from each plant digested with only *Eco*RV. *Eco*RV cuts once within the cDNAs of *−FBP1* and *−TP60*, with any additional cuts being dependent upon the site of transgene insertion into the genome thereby allowing identification of possible multiple insertions of the transgene. Probing followed that of the RNA gel blot analysis earlier.

4.7. Protein extraction, peroxidase assays, and isoenzyme analysis

Internodes from 4-week-old plant stems were harvested and immediately frozen in liquid nitrogen. After grinding in liquid nitrogen, to the ground tissue (0.3 g) was added 2% (w/v) PVPP and 50 mM MOPS (900 μl, pH 7.0) containing 1 mM Na_2EDTA, 1 mM PMSF, and 1 mM DTT. After brief vortexing and 30 min incubation on ice, the samples were centrifuged at 10,000*g* with the supernatant collected for protein analysis using the BCA reagent of Pierce (Pierce Biochemical, UK) for

quantification. Peroxidase activity was determined using guaiacol and H_2O_2 as substrates with monitoring at 436 nm. Assays contained 1 ml of 0.1 M sodium acetate at pH 4.4, 10 mM guaiacol, and 0.1 mM H_2O_2 to which 20 μl of prepared extract was added to start the reaction.

Isoenzyme analysis of the extracts was performed after isoelectric focusing in Ampholine PAGplate precast gels pH 3.5–9.5 (Pharmacia Biotech, UK) by assaying the gel for peroxidase activity. Immediately after electrophoresis the gel was washed three times for 10 min each with 50 mM sodium acetate pH 4.4 containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Next the gel received a final incubation in the same solution containing methanol–H_2O (15:85) to which 4-chloro-1-naphthol and H_2O_2 were added to final concentrations of 0.5 mg ml^{-1} and 0.5 mM, respectively. The gel was allowed to incubate in this assay mixture as long as required (30–60 min) at room temperature before photographing the results. For best results in detecting the cationic isoform, extraction, protein assays, guaiacol assays, and IEF with staining were all performed on the same day without freezing the samples.

4.8. Lignin staining

Paraffin embedded stem sections (20 μm thick) from selected down-regulated plants were obtained using a sliding microtome. The sections were taken from the fourth internode of 3-month-old stems and mounted on glass slides coated with a 1% gelatin solution using 4% formaldehyde in H_2O. Mounted sections were deparaffinized with xylene, rinsed in 100% ethanol and allowed to dry before staining. A lignin staining solution of 0.1% phloroglucinol in 16% (v/v) HCl in 95% ethanol, was applied to the slides before immediate addition of coverslips and sealing of the coverslips with rubber cement. The sections were then allowed to stain at room temperature for 25 min before photographic analysis.

4.9. Sample preparation for lignin analysis

The xylem tissues of transgenic tobacco plants from internodes 2–5 were carefully isolated from both phloem and cortex, respectively, using a scalpel with each tissue ball-milled to a fine powder as described previously (Blee et al., 2001a). Extractives were removed through sequential extraction with water, ethanol and ethanol/toluene (1/1, v/v), using a soxhlet extraction apparatus for each solvent for at least 8 h with the resulting extractive-free samples dried in vacuo and stored in a desiccator until needed.

4.10. Estimations of lignin content

Two estimations of lignin contents were used, namely Klason (Dence, 1992) and acetyl bromide methods (Iiyama and Wallis, 1990); the latter was carried out exactly as described in Blee et al. (2001a). Acetyl bromide lignin contents were estimated by using an absorptivity value for lignin of 20.09 $g^{-1}\,l\,cm^{-1}$ for the absorbance at 280 nm. All data were mean values of three determinations.

4.11. Estimation of lignin monomeric compositions by thioacidolysis and nitrobenzene oxidation

Thioacidolysis was carried out as described exactly in Blee et al. (2001a), following the general procedures of Lapierre and Monties (1986) and Rolando et al. (1992), respectively. Nitrobenzene oxidation was carried out as described in Iiyama and Lam (Iiyama and Lam, 1990).

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