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Altered Activity of Peroxidase and Oxalate Oxidase Influences Lignification in Transgenic Tobacco

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Abstract: Peroxidase and hydrogen peroxide both play important roles in the final stages of the lignification pathway. Peroxidase, in the presence of H_2O_2 catalyses the oxidation of monolignols to give lignin. In order to examine this process we looked at lignification in transgenic tobacco plants expressing a barley peroxidase gene, *HvPrx8*, either alone or in combination with a wheat germin gene, *g.f* 2.8, which encodes oxalate oxidase, thereby providing a source of H_2O_2. Elevated activity of the antioxidant ascorbate peroxidase was found in plants expressing oxalate peroxidase and was greatly increased by co-expression with the barley peroxidase, although the latter had no effect when expressed alone. An increase was observed in the oxidation of the lignin monomer, syringaldazine in cell lines over-expressing barley peroxidase, while a decrease was observed in double transformants. Plants over-expressing barley peroxidase have elevated levels of lignin deposition compared to that of wild type tobacco plants. Over-expression of the individual enzymes was also shown to enhance heat-induced programmed cell death (PCD) in cell suspension cultures, an effect which was greatly reduced in the double-expressing lines.

Keywords: Lignin, oxalate oxidase, peroxidase, syringaldazine.

INTRODUCTION

Plant peroxidases (EC 1.11.1.7) are extremely well studied proteins. They are induced by biotic [1, 2] and abiotic [3] stress and peroxidase is often the first enzyme to alter its activity after stimulation, irrespective of the stress applied [4]. Peroxidases also play a part in many physiological and biochemical plant processes, including the regulation of the level of auxin in early stages of differentiation [5], and lignification. In the latter process, peroxidase, in the presence of H_2O_2, catalyses the oxidation of monolignols to free radicals which polymerise to give lignin [6-8]. It has been demonstrated that altered peroxidase expression can have a dramatic effect on the physiology and morphology of plants. Examples include induced wilting, reduced root mass, changes in plant height, flowering time, axillary bud development and lignin content [9-11]. Peroxidases in plants are generally represented by numerous isoenzymes and can be encoded by a sizeable gene family, for example the *Arabidopsis* genome contains 73 class III peroxidase genes of which at least 53 are expressed [12]. It has however been difficult to assign specific functions to individual peroxidases due to a high redundancy factor found in peroxidase genes and the broad spectrum of substrates accepted by these enzymes. As has been observed [13] there seems to be no simple sequence similarity that allows the identification of lignin-related peroxidases across the plant kingdom.

The main function of cell wall targeted peroxidases has been shown [14] to be the oxidation of monolignols in the presence of hydrogen peroxide (H_2O_2), while it has also been demonstrated [15] that free intercellular peroxidases are associated with xylem differentiation and lignification. Poplar peroxidases with the ability to oxidise syringaldazine - a lignin monomer analogue, have been characterised [16] showing that the activity of these enzymes correlated with lignifications, and over-expressing peroxidases in tobacco [9] and tomato [8] significantly increased the lignin content in transgenic plants. A pathogen-inducible barley peroxidase gene *prx8* is closely associated with the hypersensitive response [17] and the associated ROS-induced cell death. As cell death is frequently observed feature of the differentiation of the lignified cells of the vascular tissue, this gene was a promising candidate for exploration in transgenic plants.

Another oxidative enzyme implicated in stress responses is oxalate oxidase (germin). The wheat gene for this enzyme has been transformed into tobacco [18] resulting in plants in which a range of stressing agents induced elevated oxalate oxidase activity [19] suggesting that germin plays a role in plant stress responses. Increased oxalate oxidase activity has also been found in response to salt stress [20] and fungal

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infection [21]. This increased activity can result in sustained H_2O_2 production (as opposed to an oxidative burst) [22]. In view of the key role of H_2O_2 in lignification, we hypothesised that over-expression of oxalate oxidase genes, alone or in combination with peroxidase could influence the process of lignification.

In modifying H_2O_2 levels with a view to exploring lignin metabolism, one needs to be aware of the possible consequences resulting from concomitant elevation of other ROS, including superoxide ($O_2^{\bullet-}$) and the hydroxyl radical ($^{\bullet}OH$). To counteract excessive levels of these potentially lethal ROS a battery of antioxidant defences has evolved [23-25]. We have recently demonstrated that elevated levels of some of these enzymes (superoxide dismutase, dehydroascorbate reductase, glutathione reductase, and glutathione-S-transferase) in chloroplasts influence redox cycling of antioxidants glutathione and ascorbate, leading to a modified response to abiotic stresses [26, 27]. The antioxidant ascorbate peroxidase examined in the current work (APX, EC 1.11.1.11) is found in nearly all cellular compartments, including the apoplast, cytosol, vacuole, chloroplast and mitochondrion [25].

Lignification is associated with tracheary element (TE) programmed cell death (PCD), but is a distinct process that can occur even after TE differentiation and death. PCD is a controlled process, under genetic regulation, leading to the organised breakdown of the cell. It occurs in normal development and in response to environmental cues. TE differentiation does not have all the hallmarks of plant PCD, it does exhibit vacuole collapse [28] and detectable DNA fragmentation [29]. We wished to investigate if our findings on lignification correlate with cell death rates. A cell morphology assay devised to measure cell death in response to heat treatment [30] has been applied to determine the influence of introduced peroxidase genes on rates of cell death [31]. The same approach has been used to explore the combined effects of peroxidase and oxalate oxidase gene expression in the current study.

MATERIALS AND METHODS

Plant Material and Culture Conditions

Tobacco (*Nicotiana tabacum* L. cv. Xanthi) shoot cultures were maintained on MS medium [32] at 25°C under a 16/8-hour light/dark photoperiod and sub-cultured as nodal cuttings every 4-6 weeks. *Nicotiana tabacum* L. cv. Xanthi (Xan nc5) seeds transformed with the oxalate oxidase gene, *gf-2.8* [18] were surface sterilised for 10 min in 10% commercial bleach ("Domestos"), washed three times in sterile distilled water, and germinated on half-strength MS medium, supplemented with 100 mg l^{-1} kanamycin sulphate.

In order to establish cell suspension cultures, leaf strips cut from axenic leaves were transferred to MS medium containing 2 mg l^{-1} NAA and 0.25 mg l^{-1} kinetin to induce the production of callus. Cell suspension cultures were established by inoculating friable callus into 50 ml liquid culture medium (MS medium supplemented with 0.1 mg l^{-1}, 2,4-D and 0.1 mg l^{-1} Kinetin) in 250 ml conical flasks with ,and incubating on a rotary shaker, at 25°C. Cultures were subcultured by transferring 10 ml to fresh medium every 10 days.

Vector Construction and Transformation

All plasmid DNA for restriction digests, PCR and sequencing was isolated using Wizard® *Plus* Minipreps or Midipreps DNA purification systems (Promega), according to the manufacturers instructions. The barley peroxidase coding sequence was cloned into a BAR-containing Ti-plasmid, as described previously [31]. A 1.2 kb *Pst*I/*Sph*I fragment of the cDNA clone, pBH-301 [17] was cloned into the *Pst*I and *Sph*I sites on a pPS48 vector derived from pBI121 (Clontech), which provided the enhanced cauliflower mosaic virus (CaMV) 35S promoter and terminator. A resulting 2.2 kb *Hind*III fragment was then isolated and cloned into the *Hind*III site of the Ti-plasmid CAMBIA 3301, containing the Basta selection gene and the β-glucuronidase (GUS) reporter gene, under the control of the CaMV-35S promoter. A positive clone was transformed into *Escherichia coli* (DH5α) using kanamycin as a selection pressure and positive clones were identified by PCR using the following primers: TDT15 [AACAGTCGTGGAAGTGCAGC] and 35S reverse [ACTGACGTAAGGGATGACGC]. Clones were also checked by sequencing using a DNA sequencer model ABI 373, Perkin-Elmer. The construct was transformed into the supervirulent *Agrobacterium tumefaciens* strain C58C1using a freeze-thaw method, as described previously [31].

That report also describes in detail the production and characterisation of transgenic plants of *N. tabacum* L. cv. Xanthi, containing the barley peroxidase gene, *HvPrx8*. The same vector and transformation procedure were used to transform *N. tabacum* L. cv. Xanthi (Xan nc5), the transgenic line expressing the oxalate oxidase gene [18] to obtain doubly transformed shoots. After rooting, PCR was used to screen for the presence of the *HvPrx8* using the primers described above. Positive plants exhibited amplification of a 356 bp product.

Protein Extraction and Peroxidase Activity Assay

Leaf material was homogenised in extraction buffer, (50 mM sodium phosphate, pH 6.2; 100 mg/ml tissue/buffer-volume ratio), and the crude extract centrifuged at 16,000 g for 10 min. Total soluble protein in the supernatant was quantified according to Bradford [33] using BSA as standard protein. Peroxidase activity was determined by adding 1-20 µl supernatant to 1 ml reaction mixture (0.28% guaiacol and 0.30% H_2O_2 in 0.05 M Na_2HPO_4, pH 6.2) and measuring the increase in absorbance for 3 min at 30 second intervals at 420 nm.

Cellular Fractionation

A modified form of the method of McDougall [34] was used. For leaf material, 10 x 1 cm diameter discs were cut with a cork borer, weighed and washed in sterile water. For cell suspension cultures, cells were filtered and a known weight of cells used. Samples were immersed in 5 ml sterile water and vacuum infiltrated at 15 mm Hg for 5 minutes, before being dabbed dry on sterile filter paper. They were then transferred to 5 ml syringe barrels, inserted into 30 ml universals, and centrifuged at 1,500 g for 15 min. The infiltrate was used to determine the soluble enzyme activity in the apoplast. To determine the combined soluble and ionically bound enzyme activity in the apoplast, the procedure

was repeated with additional leaf discs immersed in 10 mM $CaCl_2$. The residual leaf material from this extraction was ground in liquid nitrogen containing 5 ml buffer (10 mM sodium phosphate, pH 7.2). The homogenate was centrifuged at 16,000 g and the supernatant was used to determine cytosolic and vacuolar activity. The pellet was transferred to 1 g Cellulase and 600 mg Macerozyme (Sigma) in 100 ml of the same buffer, incubated overnight at 25°C before centrifugation at 16,000 g. The supernatant was used to determine cell wall bound activity.

Ascorbate Peroxidase Assay

A method from Asada [35] was modified as follows. Leaf tissue (0.3 g) was ground in liquid nitrogen and 10 ml of extraction buffer (50 mM sodium phosphate, pH 7.0, 1 mM ascorbic acid, 1 mM EDTA, 1% Triton X-100 and 1% PVP) was added. Once thawed the extract was transferred to a 15 ml tube, vortexed and spun at 800 g for 15 min at 4°C. Ascorbate peroxidase activity of the supernatant was determined by adding it to the reaction mixture (50 mM sodium phosphate buffer, pH 7.0, 100 mM H_2O_2) to a final concentration 10 µl of 50 mM of 1%. The increase in absorbance at 290 nm was read over a period of 3 minutes after the addition of 10 µl of 50 mM ascorbate.

Oxidation of Syringaldazine

This assay was carried out on apoplastic fluid. 10 x 1 cm diameter leaf discs were cut with a cork borer, immersed in 5 ml sterile water and vacuum infiltrated for 5 min at 15 mm Hg. They were then left to stand for 10 min and dabbed dry on sterile filter paper. They were then centrifuged in a syringe barrel for 15 min at 1,500 g. The liquid collected was apoplastic fluid. Oxidation of syringaldazine was detected in reaction mixture containing 5 mM Tris-Cl, pH 7.5, 20 µM syringaldazine, 0.03% H_2O_2 and 0.5% of apoplastic fluid. The increase in absorbance was measured at 430 nm for 30 seconds.

Lignin Determination

According to the method from Weiting *et al.* [36] stem material was ground in liquid nitrogen subjected to extraction with methanol five times over a two day period, before drying the insoluble residue at 60°C overnight. 100 mg samples of this were incubated in 5 ml 10% thioglycolic acid in 2 N HCl at 95°C for 4 h. The pellet was washed in 5 ml water, resuspended in 5 ml 0.5 M NaOH and incubated overnight at 4°C, before centrifugation and washing with 2 ml. water. The NaOH extract and pellet washings were combined and 1 ml concentrated HCl was added to re-precipitate the ligninthioglycolic acid (LTGA) complex. The solution was kept at 4°C for 2 days to aid precipitation of the LTGA complex. The precipitate was collected by centrifugation, resuspended in 2 ml 0.1M HCl and left at 4°C overnight. The precipitate was collected by centrifugation at 1,500 g for 10 min and then dissolved in 1 ml of 0.5 M NaOH at 25°C for 24 hours. A further spin at 1,500 g for 3 min removed the insoluble material and the absorbance was then measured at 280 nm. The concentration of lignin was calculated from a standard curve (0-100 µg) prepared using commercial alkali lignin.

Xylem Staining

Hand cut stem sections were stained with phloroglucinol (5% phloroglucinol in 78% ethanol). A drop of phloroglucinol solution was added to the section, left for 4 min and then followed by a drop of concentrated HCl. Lignin stained pink and sections were examined under a Leitz microscope equipped with a 16x lens on.

Induction and Measurement of Cell Death

Suspension cultures, seven days after subculture were spun at 250 g for 5 min to pellet the cells. The spent medium was replaced with hormone-free fresh medium before subjecting the cells to 20 min heat shock at temperatures between 25°C and 85°C. After returning to 25°C and shaking for 24 hours, equal volumes of cells and fluorescein diacetate (FDA; a 0.1% w/v stock diluted 1:50 with culture medium), were placed on a microscope slide for examination of epifluorescence. Single cells and cells in clusters of <6 were scored for fluorescence. Cells that did not stain with FDA were scored as dead and those in which cytoplasm had condensed and shrunken were counted as having undergone PCD. This cell death morphology was previously described for *Arabidopsis* and carrot cells killed by a 55°C heat shock [37] and has since been demonstrated in tobacco cell lines [31]. Cells that swelled and lysed were scored as necrotic.

RESULTS

Generation of Transgenic Plants

Cloning of the *HvPrx8* vector construct was carried out as described in the methods [31]. The construct was transformed into *Agrobacterium tumefaciens*, which was then used to transform both wild type and Xan nc5 tobacco plants. Transformation was confirmed by the expression of the reporter gene β-glucuronidase (data not shown) and PCR (Fig. **1**).

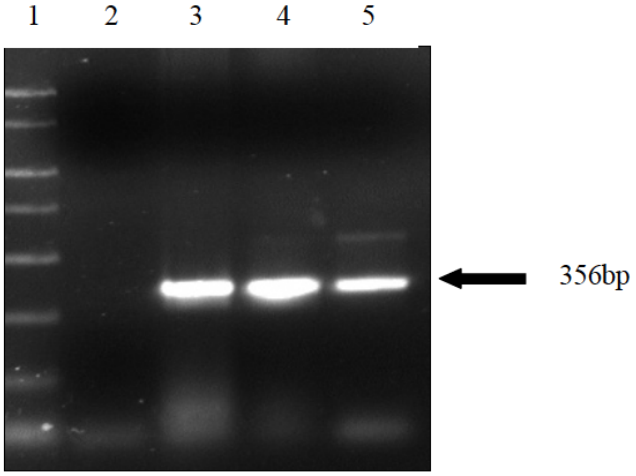

Fig. (1). PCR on tobacco plants transformed with the barley peroxidase gene, *HvPrx8*. PCR was carried out on DNA from regenerated shoots from wild type tobacco leaf discs transformed with HvPrx83201, to identify positive transformants. The primers *HvPrx8* forward and *HvPrx8* reverse were used, resulting in a 356 bp product. Lane 1: 100 bp ladder, lane 2: wild type tobacco, lane 3: HvPrx83201 plasmid, lanes 3 and 4: positive transformants.

Assignment of Peroxidase Activity to Cellular Compartments

Two separate lines of wild type tobacco transformed with the *HvPrx8* gene were studied and the results presented in (Fig. **2**). Fig. (**2A**) displays the data from the $CaCl_2$ extract and shows the specific activity of peroxidase, which is either freely soluble in the apoplast or ionically bound to the cell wall. Fig. (**2B**) shows the activity tightly bound to the cell walls, and only released by enzymatic digestion, while (Fig. **2C**) represents the activity of those compartments retained by the plasmalemma (cytosolic and vacuolar). For all three fractions, there is a large increase in specific activity of peroxidase (ranging from about 5 to 8-fold) in the two representative tobacco lines transformed with the barley peroxidase gene *HvPrx8* compared to the wild type line. The bulk of the activity in both wild-type and transformed lines is apoplastic.

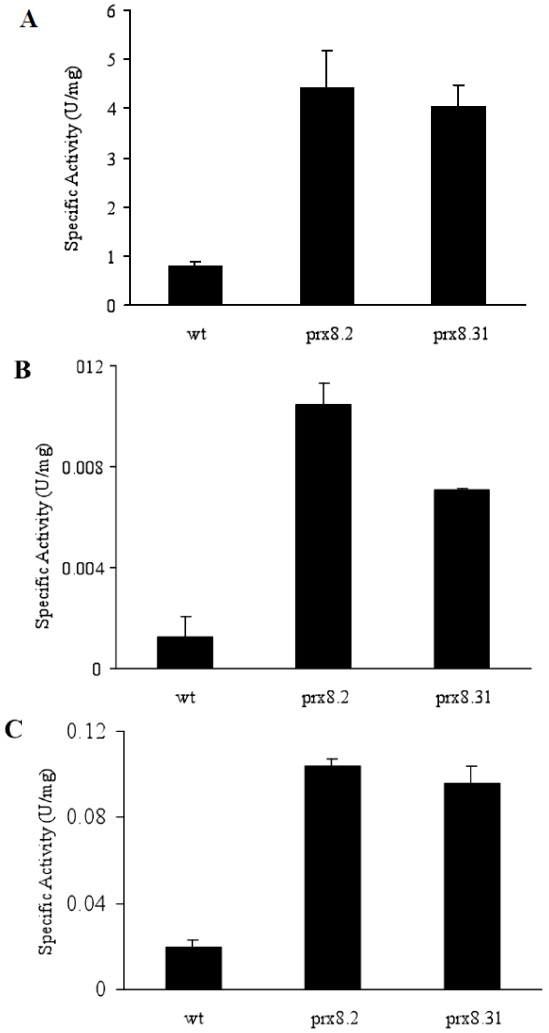

Fig. (2). Assignment of peroxidase activity to cellular compartments. The compartments were identified, and their peroxidase activities determined as described in the Materials and Methods. (**A**) Soluble and ionically bound apoplastic activity. Values are the means from three experiments. (**B**) Peroxidase activity covalently bound to the cell wall. Values are the means of four replicates. (**C**) The activity of those compartments retained by the plasmalemma (cytosol and vacuole). Values are the means of four replicates and the bars represent the standard errors of the mean (SEM).

Induction of Ascorbate Peroxidase (APX)

A number of tobacco transformants expressing barley peroxidase were found to have similar APX activity levels to wild type tobacco (Fig. **3**). However, Xanthi nc5, the oxalate oxidase producing tobacco plants, showed elevated levels of ascorbate peroxidase activity, an effect that was greatly enhanced by the simultaneous expression of the barley peroxidase gene.

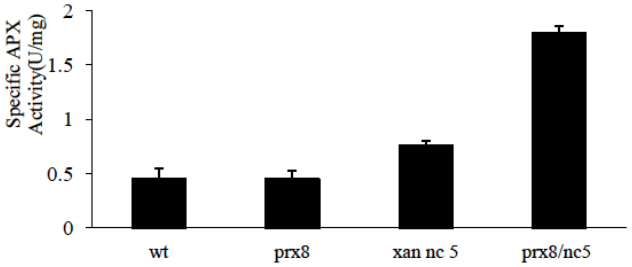

Fig. (3). Ascorbate peroxidase activity in leaf tissue of tobacco plants transformed with the barley peroxidase gene. Protein was isolated from leaf material using a buffer containing ascorbic acid as detailed in methods. The scavenging of H_2O_2 was measured and expressed as μ moles H_2O_2 consumed/min/mg protein (Units/mg). An extinction coefficient of 2.8 M/cm was used. Bars represent the SEM.

Oxidation of Syringaldazine

The capacity of the peroxidase present in the apoplastic fluid from two-month old tobacco plants (wild type, wt; peroxidase expressing, prx8; oxalate oxidase expressing, Xan nc5; and double transformants, prx8/nc5) to oxidise syringaldazine is illustrated in (Fig. **4**). All lines exhibit a capacity to oxidise syringaldazine, but this oxidation activity is significantly enhanced in those lines transformed with *HvPrx8* and shown to have elevated apoplastic peroxidase activity.

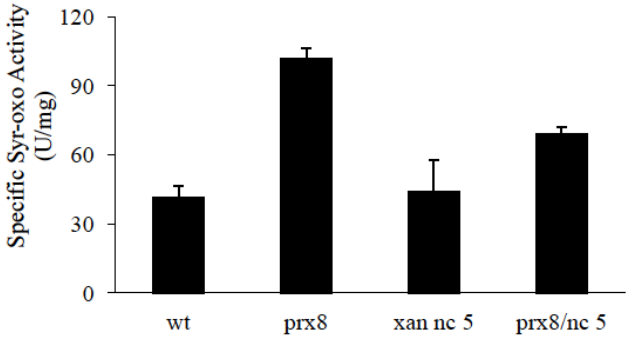

Fig. (4). Oxidation of syringaldazine by peroxidase located in apoplastic fluid. Apoplastic fluid was extracted from leaf material as described in methods and the ability of peroxidase to oxidise the lignin precursor analogue, syringaldazine was measured. Bars represent the SEM.

Lignin Content and Number of Lignified Cells

Results of lignin analysis in the different lines are illustrated in (Fig. **5**). The increased apoplastic peroxidase activity, associated with expression of *HvPrx8*, was correlated with elevated extractable lignin levels in the stem, while no effect of oxalate oxidase expression could be detected. How-

ever, examination of transverse sections from the eighth internodal stem region did reveal a concerted effect of elevated peroxidase and oxalate oxidase on formation of lignified cells in the xylem. Representative sections are illustrated in (Fig. **6**). When wild type (Fig. **6A**), and transgenic stems expressing barley peroxidase (Fig. **6B**) are compared, the width of the xylem bands is similar but the number of lignified cells is higher in the transgenic line, as the individual cells are of smaller mean diameter. In wild type plants there was a mean of 23.25 (SEM = 0.68) lignified cells across the width of the xylem band in the eighth internodal section of a four month old plant, while the equivalent value for a transgenic line was 30 (SEM = 0.81). In both cases these values are the means of single counts from ten sections. Fig. (**6C**) illustrates a transverse stem section from tobacco expressing barley peroxidase and oxalate oxidase. The width of the band is larger and the size and density of the lignified cells resemble that of wild type plants.

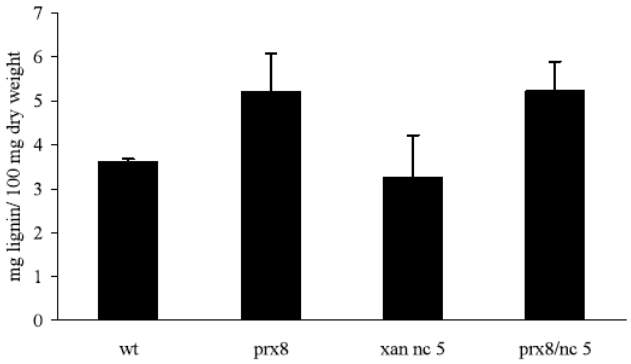

Fig. (5). Analysis of extractable lignin content in stem material of wild type and oxalate oxidase-expressing tobacco plants which were transformed with the barley peroxidase gene, *HvPrx8*. Lignin was extracted by acidic thioglycolsis and expressed in mg/100 mg of dried insoluble stem material. The results were the means from at least three separate experiments using different plant material, and the bars represent the SEM.

Induction of Cell Death in Cell Cultures

Wild type, Xan nc5, *HvPrx8*, and doubly transformed *HvPrx8*/nc5 cells were harvested 7 days after subculture and subjected to heat shock treatments, as described in Materials and Methods. Cells were scored for viability, PCD or necrosis.

The results obtained at a range of temperatures are shown in Fig. (**7A**). At 25°C, a background level of cell death can be slightly increased due to centrifugation and handling of the cells. The level of PCD never rises substantially at 35°C. At 45°C the cells must either commit to die or survive the treatment. Heat treatments below 55°C, gave consistently higher PCD in the *HvPrx8* transgenic cell line, the PCD rate after 45°C being almost twofold higher in the peroxidase transgenic line than it is in the wild type cells and doubly transformed cell lines. From 25°C to 55°C the oxalate oxidase expressing culture was comparable to wild type tobacco cultures. At 55°C, all cells are dead with usually at least 90% of the cells having died by PCD. At higher temperatures all the cells are dead, but the proportion that have died through PCD, as opposed to necrosis, declines with temperature. We highlighted the 45°C heat treatment since at this temperature there is room for fluctuation of cell death levels. The percentage of cell death at 45°C remaining after the removal of background cell death levels was plotted (Fig. **7B**). This figure emphasises the large increase in programmed cell death in *HvPrx8* transgenic cell lines compared to wild type and double transformants (*HvPrx8*/nc5).

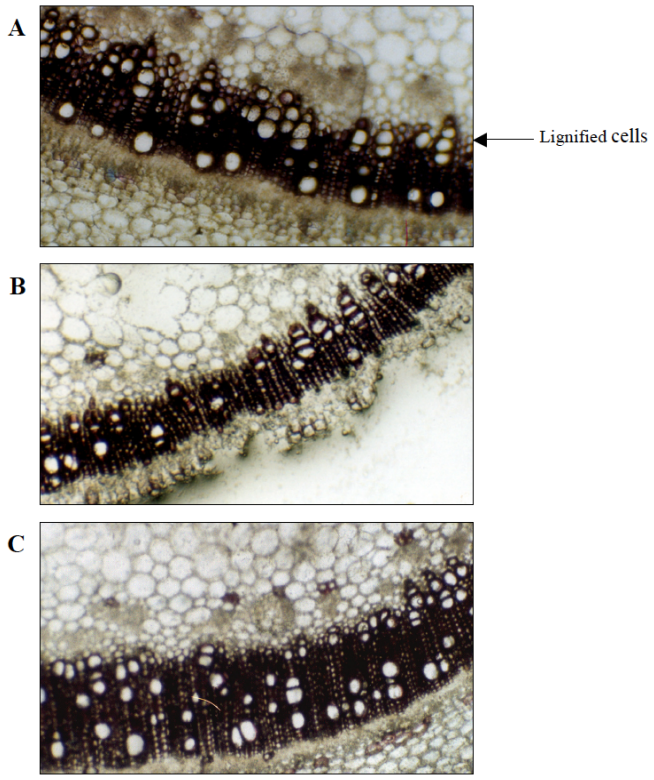

Fig. (6). Representative transverse sections from the eighth internode of wild type and transgenic plants showing the band of lignified cells. Phloroglucinol was used to stain the hand cut sections. Magnification: 160x. (**A**) = wild type tobacco, (**B**) = tobacco transferred with barley peroxidase gene and (**C**) = tobacco expressing barley peroxidase and oxalate oxidase

DISCUSSION

Subcellular localisation of peroxidase activity illustrated that the majority of activity in transgenic *Nicotiana tabacum* plants expressing barley peroxidase (*HvPrx8* plants) is located in the soluble and ionically bound fraction from the apoplast. Relatively little activity was found in the cytosolic/vacuolar fraction. This is in agreement with an earlier expression study in the diploid *Nicotiana benthamiana* which showed the barley *HvPrx8* is expressed in a form indistinguishable from the native in terms of intercellular localisation and posttranscriptional processing [38]. In view of the pivotal role of peroxidase in lignin synthesis, it might be anticipated that these elevated apoplastic levels could influence lignification. This surmise is supported by the increased capacity of the apoplastic fluid from the *HvPrx8* plants to oxidise a lignin precursor analogue, syringaldazine, for which cell wall peroxidases involved in lignification have a high affinity. The oxidation of syringaldazine by a peroxidase was suggested to be indicative of its involvement in the

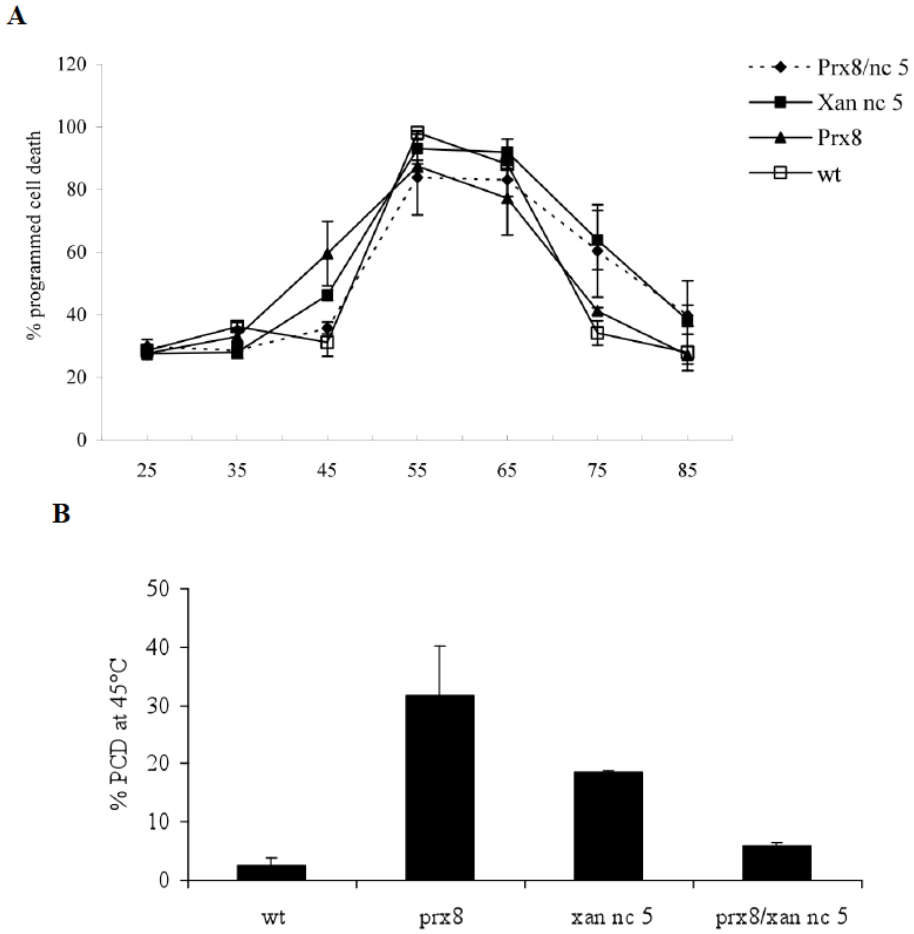

Fig. (7). The effect of peroxidase, oxalate oxidase and their combined expression on the induction of PCD. (**A**) Cells were subjected to heat treatments for 20 min, returned to 25°C and scored 24 hours later. Cells were scored under the light microscope for PCD morphology, as described in the Materials and Methods, and the percentage of cells undergoing PCD was determined. (**B**) The percentage of cell death at 45°C remaining after the removal of background cell death levels. Data shown are representative of three independent replicate experiments. Error bars represent SEM, which is derived from independent fields of at least 100 cells for each experiment.

synthesis of lignin [39, 16]. Plants transformed with both barley peroxidase and oxalate oxidase genes (*HvPrx8*/nc5 plants) showed a reduction in syringaldazine oxidase activity compared to lines transformed with the barley peroxidase gene alone. We propose that this is due to reduced peroxidase activity in doubly transformed lines based on peroxidase inactivation by H_2O_2 [40].

The activity of ascorbate peroxidase has been shown to accumulate in response to stresses which result in an increase in ROS production [35]. This lends an explanation to why transgenic plants expressing oxalate oxidase (nc5 plants), a source of H_2O_2, show an increase in ascorbate peroxidase activity. Since double transformants expressing barley peroxidase and oxalate oxidase have potentially two sources of H_2O_2 [41], it was not surprising that these plants had the highest ascorbate peroxidase activity. However in the absence of H_2O_2 measurements in the present study, this interpretation must remain speculative.

Tobacco plants expressing high levels of barley peroxidase showed an increased level of extractable lignin compared to that of wild type tobacco plants. This finding is consistent with the increase in the oxidation of the lignin precursor analogue, syringaldazine, in the same plants. As mentioned earlier other reports have associated peroxidases present in tissues undergoing lignification with *in vitro* activity towards syringaldazine [42, 16]. Therefore, we suggest that the apoplastic barley peroxidase in these transgenic tobacco plants was active at sites of lignification. The width of the xylem band in both plants was similar but it was observed that the xylem band of the *HvPrx8* plants consisted of a greater number of smaller lignified cells. Increased lignification can decrease cell wall plasticity and consequently there could be reduction in cell expansion. A difference in xylem band width was observed in the transgenic line expressing both peroxidase and oxalate oxidase. The band is much wider in *HvPrx8*/nc5 plants, compared to either wild type or barley peroxidase tobacco and an increased level of lignin was found in this double transgenic line compared to wild type or oxalate oxidase tobacco, although the level was similar to that of *HvPrx8* plants. It is proposed that the additional expression of oxalate oxidase, a possible source of H_2O_2, could also effect lignification. However, there was no direct evidence produced from lignin level measurements that expression of oxalate oxidase alone could lead to the modification of lignin content.

We investigated the effect that elevated levels of peroxidase may have on the induction of programmed cell death in plant cells. We utilised cell morphology to measure rates of cell death after heat shocks [37] and could observe the influence of the introduced peroxidase gene on these rates. At 45°C, large differences in PCD levels were observed while at 55°C the majority of cells died regardless of the death-inducing agent. In cell cultures expressing barley peroxidase only, there was a substantial increase in PCD at 45°C, a finding consistent with our earlier observations [31]. Although H_2O_2 is a known activator of PCD [43-46] direct measurement of H_2O_2 levels would be needed to confirm that changes in those levels are responsible for the altered PCD response in the transgenic plants.

To summarise, studies on tobacco plants with enhanced levels of apoplastic peroxidase suggest that modest increases in peroxidase activity can influence cell wall-related processes such as the peroxidase–dependent phenomenon of lignification. Furthermore, this effect can be influenced by the simultaneous expression of a known H_2O_2-generating enzyme, oxalate oxidase.

ABBREVIATIONS

APX	=	Ascorbate peroxidase
Class III	=	Peroxidase
ROS	=	Reactive oxygen species
PCD	=	Programmed cell death

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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