Biochemical and phenotypical characterization of transgenic tomato plants overexpressing a basic peroxidase

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Tomato plants (Lycopersicon esculentum Mill. cv. Pera) were transformed via Agrobacterium tumefaciens with the binary vector pKYLX71 containing a tomato basic peroxidase (EC 1.11.1.7) gene, tpx1, under the control of the cauliflower mosaic virus (CaMV35S) promoter. Transgenic plants showed a 2–5-fold increase in the activity of the peroxidase ionically bound to the cell wall, whereas soluble peroxidase activity remained similar or even lower than wild-type plants. Isoelectric focusing showed the presence of a new isoperoxidase of pI ca 9 in the ionically bound extract. Western blot also showed the presence of a new band at 41 kDa that was absent in the wild-type extract. A 40–220% increment of lignin content of the leaf was found in transgenic plants. Shoot phenotype of the transgenic plants was similar to wild type, although under stress, the plants appeared wilted and the new leaves had a reduced area and were thicker than wild-type or older transgenic leaves. The root system was underdeveloped in transgenic plants, but the rooting ability of the stem was not affected by the overexpression of peroxidase. Finally, the morphogenetic response of cotyledon and hypocotyl explants from transgenic plants was evaluated. In the case of cotyledons, the percentage of explants with shoot was not different from wild-type plants. For hypocotyl, one of the transgenic lines showed a 30% reduction in the percentage of shoot organogenesis. The results are discussed in relation to the role of tpx1 in lignin synthesis.

Introduction

Peroxidases are ubiquitous plant enzymes that catalyze the oxidation of a variety of organic and inorganic substrates using hydrogen peroxide as oxidant. These enzymes have been involved in several physiological and biochemical processes, such as cell growth and expansion (Wallace and Fry 1994), differentiation and development (Gaspar et al. 1991), auxin catabolism (Lagrimini et al. 1997a), lignification (Bruce and West 1989), as well as abiotic and biotic stress responses (Mohan et al. 1993, Medina et al. 1997). However, the physiological role of a peroxidase isoenzyme has only been proven in a few cases. One approach to determine the role of a gene has been either to overexpress or to suppress its expression in transgenic plants. Thus, transgenic tobacco (Lagrimini et al. 1990, McIntyre et al. 1996, Kristensen et al. 1997) and tomato (Lagrimini et al. 1992, Sherf et al. 1993) peroxidase plants have been obtained. The most noticeable characteristics of the transgenic peroxidase plants was the wilting phenotype observed in tobacco (Lagrimini et al. 1990) and tomato (Lagrimini et al. 1992) when overexpressing an anionic tobacco peroxidase gene. This phenotype appeared when the plant reached maturity and apparently was due to the reduced development of the radicular system, as a consequence of indoleacetic acid (IAA) metabolism by the peroxidase (Lagrimini et al. 1997a). However, this phenotype was not observed in tobacco transformed with a cationic peroxidase (Kristensen et al. 1997).

In tomato, more than 12 peroxidase isoenzymes have been described and 7 of the encoding genes have been mapped (Tankersley 1985). tpx1 encodes a putative cell wall-targeted peroxidase with a pI of 8–8.5. This gene is constitutively expressed in the root and it is transcriptionally activated in this tissue when the plants are exposed to moderate NaCl concentration (Botella et al. 1994a); in the stem, its expression is induced after wounding (Botella et al. 1994b).
tpx1 is also expressed at high levels during the exponential growth phase of the tomato cell suspension growth cycle (Medina et al. 1999). The expression pattern of tpx1 has led us to propose a role for this gene in the stress adaptation process, being likely involved in the synthesis of lignin. To support the in vivo function of this gene, we have obtained transgenic tomato plants overexpressing tpx1. In this paper, we describe the transformation of plants and the effects of the overexpression of the gene on the leaf lignin content, the rooting ability of shoots, as well as on the in vitro morphogenesis of different tomato tissues.

**Materials and methods**

**Plant material and Agrobacterium-mediated transformation**

Tomato (*Lycopersicon esculentum* Mill. cv. Pera) explants were employed for transformation experiments. Seeds were surface sterilized in a 15% diluted commercial bleach solution (0.75% [w/v] sodium hypochlorite) and after several washes with sterile water, germinated in vitro in liquid medium containing the Murashige and Skoog (MS) basal salts (Murashige and Skoog 1962) on sterile filter paper. Ten-day-old seedlings were used as explant source. In order to develop a transformation protocol for this tomato cultivar, three transformation experiments were performed. In experiments 1 and 2, cotyledons were used as explants, whereas in experiment 3, hypocotyl sections 1 cm long were employed. Explants were excised and cultivated for 1 day on Petri dishes containing 20 ml of the shoot regeneration media. These media consist of MS salts supplemented with Gamborg’s B5 vitamins (Gamborg et al. 1968), 3% sucrose, 0.7% agar (A1296 agar; Sigma, St Louis, MO, USA) and 2 mg l⁻¹ of benzyladenine (BA) for cotyledon regeneration, and 2 mg l⁻¹ BA and 1 mg l⁻¹ IAA for hypocotyl explants (M. A. Sancho. 1994. Thesis, University of Málaga, Spain). After the induction period, explants were placed in 50-ml tubes, inoculated with an overnight-grown *Agrobacterium* culture diluted 1/10 and gently shaken for 20 min. The infected explants were blotted dry on sterile filter paper and cocultivated on the shoot regeneration medium for 2 days. In experiments 1 and 3, explants were then transferred to a selection medium, e.g., the shoot regeneration media supplemented with 25 mg l⁻¹ kanamycin and 500 mg l⁻¹ carbencillin. In experiment 2, cotyledons were transferred to a shoot regeneration medium supplemented with 500 mg l⁻¹ carbencillin without kanamycin. After 10 days of culture in this medium, the explants were transferred to the selection medium. Shoot regeneration occurred after 6–8 weeks of culture. Regenerated shoots were isolated, elongated and rooted in MS basal medium supplemented with 50 mg l⁻¹ of kanamycin and 200 mg l⁻¹ of carbencillin. Standard in vitro culture conditions were 25 ± 1°C temperature and a 16-h photoperiod under 20 μmol m⁻² s⁻¹ irradiance for shoot regeneration and 40 μmol m⁻² s⁻¹ for shoot elongation and rooting, provided by Sylvania Gro-lux lamps. The selected plants were acclimatized, transferred to the glasshouse and grown until fruiting under natural photoperiod and a 28/18°C (day/night) temperature regime.

In the presence of kanamycin was confirmed by polymerase chain reaction (PCR) analysis. DNA was isolated from leaf tissue by the hexadecyl trimethyl ammonium bromide (CTAB) method (Rogers and Bendich 1994) and 200–500 ng of DNA was used to amplify the *nptII* gene according to Lipp-Joao and Brown (1993). The possibility of *Agrobacterium* contamination was checked by the amplification of the *VirD1* bacterial gene (Lipp-Joao and Brown 1993). The stable integration and inheritance of the T-DNA was tested by segregation analysis of the seeds obtained from T0 and T1 plants.

**Bacterial strain**

tpx1 cDNA was transferred from the pBSII cloning vector to the binary plasmid pKYLX71 (Schardl et al. 1987) by PCR amplification of the tpx1 insert of the cDNA clone and insertion in the expression cassette downstream of the cauliflower mosaic virus (CaMV35S) promoter. The pKYLX71-tpx1 vector was transferred to *Agrobacterium tumefaciens* LBA4404 strain by electroporation.

**Peroxidase assay and isolectric focusing**

Total peroxidase was extracted in 50 mM phosphate buffer, pH 6, containing 1 M KCl and 8% polyvinylpolypyrrolidone (PVPP). A different protocol was used to separate the soluble peroxidase fraction from the peroxidase fraction ionically bound to the cell wall. One gram of leaf tissue was homogenized in an Ultra-Turrax (Janke&Kunkel, IKA-Labortechnik, Staufen, Germany) with 5 ml of 50 mM phosphate buffer, pH 6. The extract was centrifuged at 30 000 g and 4°C for 20 min and the supernatant was the soluble fraction. The pellet was washed three times with the phosphate buffer, resuspended in 3 ml of 50 mM phosphate buffer, pH 6, supplemented with 1 M KCl and maintained under continuous agitation for 3 h. The extract was centrifuged at 30 000 g and the supernatant, containing the ionically bound to the cell wall fraction, was dialyzed overnight. Peroxidase activity was measured as previously described (Quesada et al. 1992) using o-dianisidine as substrate. One arbitrary unit corresponds to an increase in absorbance at 460 nm of 1.0 min⁻¹ under the assay conditions.

Isoelectric focusing (IEF) was performed in ready-to-use agarose plates (FMC Bioproduct Europe, Vallensbæk Strand, Denmark) in the 3–10 pH range, following the manufacturer’s instructions. Peroxidase activity in the gel was developed with 0.26 mM o-dianisidine and 8.8 mM H₂O₂.

**Lignin determination**

For lignin determination, leaf tissue was homogenized in an Ultra-Turrax (Janke&Kunkel, IKA-Labortechnik) with absolute methanol, filtered through Whatman GF/C and dried at 60°C for 24 h. Lignin from 15–20 mg of the alcohol-insoluble residue (AIR) was solubilized with thioglycolic acid following the procedure of Bruce and West (1989), and the concentration was estimated by the absorbance at 280 nm.

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weeks of culture. The percentage of shoot regenerating explants was recorded after 10 days of culture, explants were excised and germinated in MS medium with 0.7% agar (Sigma A1296). Don and hypocotyl sections were employed. Seeds were cultured in the shoot regeneration media optimal for cotyledon growth (2% agarose) and after 14 days of culture, explants were excised and germinated in MS medium with 0.7% agar (Sigma A1296). In the rooting experiment, axillary shoots 10–20 cm long with 2–3 leaves from wild-type and transgenic T0 plants were employed. Immediately after being detached from the plant, shoots were placed individually in tubes filled with water and maintained in a growth chamber at 22°C under a 16-h photoperiod of 80 μmol m⁻² s⁻¹ photon flux. After 2 weeks, the number and length of the roots were recorded.

For the in vitro shoot organogenesis experiment, cotyledon and hypocotyl sections were employed. Seeds were germinated in MS medium with 0.7% agar (Sigma A1296) agar) and after 14 days of culture, explants were excised and cultured in the shoot regeneration media optimal for cotyledon and hypocotyl explants previously described. The percentage of shoot regenerating explants was recorded after 10 weeks of culture.

Results
Transformation of tomato cv. Pera
The results of the three transformation experiments performed are shown in Table 1. The highest percentage (10.7%) of regenerated shoots after 14 weeks on selection medium with 25 mg l⁻¹ kanamycin was obtained in experiment 1, where cotyledons were used as explants and kanamycin was applied just after cocultivation. The other two experiments only yielded 2% of regenerating explants. Transforms from this first selection were isolated and transferred to a medium containing 50 mg l⁻¹ kanamycin. Only 6 out of 40 shoots of experiment 1 survived in the presence of this kanamycin concentration. None of the shoots obtained in experiments 2 and 3 were able to root in this medium and died after 4 additional weeks of culture. The final transformation frequency obtained in experiment 1 was 1.7% based on kanamycin resistance. The presence of the T-DNA was confirmed by amplification of the nptII gene by PCR (Fig. 1A). In the same DNA samples, no amplification of the VirD1 gene was observed when using specific primers, indicating the absence of Agrobacterium contamination (Fig. 1B). One of the transgenic lines showed a reduced growth in vitro and died during the acclimation process. The other five lines were successfully acclimated and transferred to the greenhouse. Three transgenic lines were self-pollinated and the seeds were collected. The kanamycin-resistant trait segregated as an integrated gene in T1 generation, e.g., a Mendelian segregation was observed in all the cases (Table 2). The clone TP39 showed a 15:1 kanamycin segregation and, therefore, it contained...
two loci of the T-DNA, whereas the clones TP3 and TP15 showed a 3:1 ratio, indicating the stable integration of the gene construct in one locus.

### Biochemical characterization of transgenic plants

Total activity, including soluble and cell wall-bound peroxidases, was assayed in leaf tissue using an extraction buffer at high ionic strength. No differences in total peroxidase activity were found between transgenic and control plants (results not shown). However, when soluble peroxidase activity was distinguished from peroxidase ionically bound to the cell wall, a 2–5-fold increase in peroxidase activity was observed for the cell wall-bound fraction of the transgenic plants (Table 3). Peroxidase activity was also assayed in the three washes carried out with the low ionic concentration buffer to determine the efficiency of the extraction procedure employed. Activity found in the last wash never exceeded 10% of the total amount obtained when the high ionic strength buffer was included. In the TP39 plant, the increment of ionic peroxidase was concomitant to a decrease in soluble activity. However, the homozygous clones corresponding to one-copy transgenic plants (TP3 and TP39) showed a slight increment in the soluble peroxidase activity. Soluble and ionic root peroxidase activities were not higher in the transgenic clones (results not shown).

Cell wall-bound extracts from leaves of TP39 and TP15 were analyzed by IEF and western blot (Fig. 2). The IEF revealed the presence of an isoperoxidase with high activity at pI of ca 9 in the transgenic clones (Fig. 2A). Western blot is shown in Fig. 2B. The polyclonal antibodies recognized at least 8 bands with molecular mass ranging from 30 to 90 kDa. One band of 41 kDa was present in the transgenic extracts, but absent in the control extract. A similar IEF and western blot pattern was observed in ionic extract from the other selected line TP3 (results not shown).

The three transgenic lines analyzed displayed a statistically significant increment in the amount of lignin content of the leaves when compared to the wild type (Fig. 3). The percentage of increment over the control were 40, 60 and 225% in the transgenics TP3, TP15 and TP39, respectively.

### Phenotypical characterization of transgenic plants

Transgenic tomato plants overexpressing the *tpx1* peroxidase gene displayed a characteristic phenotype. During most of their life cycle, they resembled wild-type plants. However, during flowering or under stress conditions, as acclimation to the greenhouse or growth at low light intensity, the morphology of the new developing leaves in transgenic plants was different to the leaves of control plants growing under the same conditions, e.g., leaves of transgenic plants were thicker and showed a reduced leaf surface (Fig. 4A,B). This phenotype was maintained when axillary shoots from T0 plants were isolated and rooted in water, and also in the T1 generation of the three transgenic lines. Transgenic plants displayed a lower root biomass than control plants (Fig. 4C). In the case of the TP39 clone, a high variability in plant growth and root biomass was observed in the T1 generation resistant to kanamycin (Fig. 4D). Of these kanamycin-resistant seedlings, 26% displayed a very low root biomass and died during the acclimation process.

### Effect of peroxidase overexpression on shoot rooting ability

The rooting ability of the plants overexpressing peroxidase was analyzed to determine if this was the cause of the low development of the root system. Axillary shoots from T0 plants corresponding to the TP15 and TP39 lines were collected and grown in water in a growth chamber. After 2 weeks, more than 80% of the shoots had formed new roots; a similar performance was obtained when using shoots from control plants. A more detailed analysis of the TP15 line showed no differences compared with control shoots on percentage of rooted shoots, root number per shoot and minimum and maximum average root length (Table 4). However, when rooted shoots of similar size were transplanted to Perlite, the root biomass in the transgenic line was about 70% of the wild type after 1 month of culture (9.1 ± 1.4 g per plant in the TP15 vs 13.5 ± 1.1 g per plant in wild type, mean ± se, n = 6). Since the leaf and stem biomass was similar among genotypes, an increment in the shoot/root ratio was evident in the transgenic line TP15 (2.8 vs 2.1 in the wild type).
Effect of peroxidase overexpression on in vitro morphogenesis

The morphogenetic response of the transgenic plants was evaluated in the seedlings of the homozygous lines TP15 and TP3, both with a single locus of the transgene (Fig. 5). The percentage of cotyledon explants regenerating shoots obtained for both transgenic lines was similar to that of the control. However, hypocotyl explants from clone TP3 displayed a 30% reduction in the regeneration rates when compared with the control or the TP15 line.

Discussion

Agrobacterium-mediated transformation of several tomato cultivars has been established (Hamza and Chupeau 1993 and references cited). Although kanamycin selection in the range 50–100 mg l\(^{-1}\) is usually employed, we did not succeed using these concentrations with cv. Pera. Among the three transformation strategies used in the present work, only when cotyledon pieces were used as explants and a low kanamycin selection was applied after cocultivation, plants were recovered. This protocol yielded a 1.7% transformation rate and included a first selection with low kanamycin concentration (25 mg l\(^{-1}\) ) and a second selection with higher kanamycin (50 mg l\(^{-1}\)) once the putative transgenic shoot was isolated. Due to the low kanamycin concentration applied during explant regeneration, a high percentage of the plants recovered were chimeras, since they did not survive 50 mg l\(^{-1}\) of kanamycin.

The results obtained showed that the transformation of tomato plants with a root-specific basic peroxidase under the control of the 35S promoter resulted in an increased leaf peroxidase activity in the cell wall fraction. The IEF and western blot analysis of the ionic extracts from transgenic lines confirmed the features of the protein encoded by the \(tpx1\) gene (Botella et al. 1994a). It is a cell wall-targeted isoperoxidase, highly basic, with pI ca 9, and a molecular mass around 40 kDa. In relation to the soluble activity of transgenic plants, this was slightly higher in the homozygous line with one locus, but lower in the two loci line, where the ionic activity was the highest. The reason for the decrement of soluble peroxidase in the TP39 is unknown, and further experiments would be needed to clarify this result. As a preliminary hypothesis to be tested, overexpression of \(tpx1\) may result in a depletion of precursors for other mature active peroxidase proteins.

![Fig. 3. Effect of \(tpx1\) peroxidase overexpression on the leaf lignin content, measured as the increment in absorbance at 280 nm per mg of AIR. The results correspond to the mean value of three samples (four leaves per sample) per line. Means with different letters are significantly different at the 5% level by LSD.](image)
Among the different roles assigned to peroxidases, the cross-linking of cell wall proteins and pectin, and the involvement in the biosynthesis of lignin and suberin are the most widely accepted for cell wall-bound isoenzymes (Gaspar et al. 1991). tpx1 gene is constitutively expressed in root tissue (Botella et al. 1994a), and its expression is induced in wounded vascular tissue (Botella et al. 1994b). Since lignosuberization occurs in both cases, this gene was proposed to be involved in the oxidation of cinnamyl alcohols previous to their polymerization to form the aromatic domain of lignin. Our results with transgenic plants support the involvement of the basic tpx1 isoperoxidase in the lignification process, since the three transgenic lines displayed a significant increment in their lignin content. Moreover, the highest lignin content was found in the transgenic line with two copies of the gene, which also displayed the highest peroxidase activity in the cell wall-bound fraction. Previous studies in the same tomato cultivar showed an increment in lignin-like compounds of cell suspensions adapted to grow in the presence of 256 mM NaCl and this was linked to a 10-fold increment in peroxidase activity in the culture medium of the salt-adapted cells (Sancho et al. 1996). The major change in the isoelectric profile of peroxidases from the culture medium of these cell suspensions was observed in the range of pl 7–9 (Medina et al. 1999). All together, these results indicate a crucial role of this basic peroxidase in the polymerization of phenolics during lignin synthesis. Previous reports have proposed that the acidic isoperoxidases are involved in lignification processes (Bruce and West 1989, Gaspar et al. 1991, Lagrimini et al. 1993), and, according to Mohan et al. (1993), the synthesis of lignin and suberin would be mediated by different anionic peroxidases. However, in spite of all the lines of evidence suggesting the role of peroxidases in lignin synthesis, it is surprising that the lignin content remained unchanged in transgenic plants, where significant reductions of specific peroxidase isoenzymes have been achieved by anti-sense or ribozyme constructs (Sherf et al. 1993, McIntyre et al. 1996, Lagrimini et al. 1997b). In these three cases, an acidic isoperoxidase was used. Thus, it is possible that basic, rather than acidic isoperoxidases, are responsible for the polymerization of the phenolic moieties, as it has been proposed for maize pollen (Liu and Ger 1997).

Table 4. Effect of peroxidase overexpression on rooting. Axillary shoots from transgenic TP15 and wild-type plants were detached and rooted in water. After 14 days of culture, the root number and root length were recorded. Data represent the average of two independent experiments with a minimum of 8 shoots per genotype (mean ± SD).

<table>
<thead>
<tr>
<th>Wild type</th>
<th>TP15</th>
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<tr>
<td>Rooted shoots (%)</td>
<td>85.9 ± 5.8</td>
</tr>
<tr>
<td>Shoots with more than 10 roots (%)</td>
<td>58.3 ± 11.7</td>
</tr>
<tr>
<td>Maximum root length (cm)</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td>Minimum root length (cm)</td>
<td>0.3 ± 0.1</td>
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A reduced growth seems to be a general trend in plants overexpressing peroxidase (Lagrimini et al. 1990, 1993, Kristensen et al. 1997). In our case, we did not observe either a significant reduction in growth or a delay in flowering time in the tpx1 transgenic plants. However, as previously observed in tomato transformed with the tobacco acidic peroxidase gene (Lagrimini et al. 1993), the morphology of the plants changed after flowering and under some stress conditions. This phenotype resembled, although to a lesser extent, the chronic wilting phenotype obtained by Lagrimini et al. (1993), but a daily wilting cycle was not observed in our case in any of the transgenic lines. As previously suggested by Lagrimini et al. (1997a), the low root biomass of the tpx1 transgenic plants could account for the wilting appearance of the shoot. The low development of the root system was not due to a lack of rooting ability, since no differences in rooting capacity were detected among shoots from wild-type and transgenic plants. Similarly, overproduction of the anionic tobacco peroxidase interferes with the development of lateral roots and it was likely due to a hormonal imbalance induced by the catabolism of IAA by this isoperoxidase (Lagrimini et al. 1997a). Further evidence of alteration of hormonal levels induced by peroxidase can be deduced from the results of Lagrimini et al. (1994b) Induction of a tomato peroxidase gene in vascular tissue. FEBS Lett 347: 195–198.


References


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