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Structural and physiological changes in the roots of tomato plants over-expressing a basic peroxidase

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Previous studies on the tomato (*Lycopersicon esculentum* Mill.) peroxidase TPX1, including the development of transgenic tomato over-expressing this gene, supported an involvement of this peroxidase in the synthesis of lignin and suberin. The transgenic plants showed a wilty phenotype at flowering, but the relationship between this role in ligno-suberization and this phenotype was not clear. In the present study a histological approach and the measurement of water-related parameters have been performed in order to obtain an insight into the origin of this phenotype. Clear differences between transgenic and non-transgenic roots were observed in the cross-sections of the basal root zones where secondary growth was evident. The diameter of the xylem vessel was diminished in the transgenic plants. Total area corresponding to xylem in the basal cross-sections decreased 3.9 fold in the transgenic roots. In addition, the radial and outer tangential walls of the exodermis cells were more ligno-suberized in transgenic than in non-transgenic plants. After fruit set, predawn and midday water potentials were lower in transgenic than in-non-transgenic plants. At midday, the stomatal conductance was also lower in the transgenic plants, 494 ± 69 versus 594 ± 60 mmol $m^{-2} s^{-1}$. Root hydraulic conductances of the transgenic and non-transgenic plants were 1.4 ± 0.38 and 3.47 ± 0.19 g water min^{-1} MPa^{-1}, respectively. The results obtained support that the phenotype is caused by the anatomical differences found in the transgenic roots. These differences would be the cause of a increased resistance to water flow in the roots that would negatively affect the water supply to the shoot and, as a consequence, resulted in a decreased water potential in the leaves.

Introduction

Plant peroxidases (EC 1.11.1.7) are oxidoreductases that catalyse the oxidation of a wide range of substrates using hydrogen peroxide as co-substrate (Dawson 1988, Wallace and Fry 1999), albeit at somewhat different rates. These enzymes have been implicated in several metabolic processes such as auxin metabolism (Gazarian et al. 1996), phenol oxidation (Lagrimini 1991), cross-linking of structural proteins and polysaccharides (Epstein and Lamport 1984, Fry 1986, Schnabelrauch et al. 1996) and, lignin synthesis (Bruce and West 1989, Sancho et al. 1996). As a result, they have been involved in several physiological processes, such as cell growth and expansion (Wallace and Fry 1994), cellular differentiation and plant development (Gaspar et al. 1991, Chen et al. 2002), or responses to abiotic and biotic stress (Mohan et al. 1993, Botella et al. 1994a, b, Medina et al. 1997, van Jansen et al. 2001). However, these assignments of function are supported by correlative expression studies. The information on the amino acid sequences of many peroxidases has not improved the assignment of functions because a clear structure–function relationship has not been established (Lagrimini et al. 1997a, b, Christensen et al. 1998). An alternative approach has been the development of transgenic plants. Transgenic tobacco (Lagrimini et al. 1990, McIntyre et al. 1996, Kristensen et al. 1997, Talas-Ogras et al. 2001) and tomato (Lagrimini

Abbreviations – IAA, indoleacetic acid.

et al. 1992, Sherf et al. 1993, El Mansouri et al. 1999) plants with modified peroxidase expression have been obtained. Down-regulation of specific peroxidases did not always change the phenotype of the plant, probably because other isoenzymes compensate the reduced expression level (Sherf et al. 1993). However, peroxidase over-expression has been more fruitful, being noticeable for the wilty phenotype observed in tobacco and tomato plants over-expressing an acidic tobacco isoenzyme of pI 3.5 (Lagrimini et al. 1990, 1993). The transgenic plants showed severe wilting caused by a reduced development of the root system, that has been explained by the altered metabolism of IAA (Lagrimini et al. 1997a, b). This phenotype has not been observed in tobacco transformed with a basic barley peroxidase (Kristensen et al. 1997). However, tomato plants transformed with the *TPX1* tomato gene partially resembled some aspects of this wilty phenotype, including the development of leaves with reduced area and thicker than in wild plants (El Mansouri et al. 1999). The development of this phenotype occurred mainly after plant flowering. *TPX1* encodes a highly basic cell wall-targeted isoperoxidase with a pI value of approximately 9 (El Mansouri et al. 1999, Quiroga et al. 2000). The gene is constitutively expressed in the root and it is transcriptionally activated in this tissue when the plants are exposed to moderate NaCl concentrations (Botella et al. 1994a). Its expression is restricted to the endodermis, exodermis and protoxylem (Quiroga et al. 2000); in the stem, its expression is induced after wounding (Botella et al. 1994b). Transgenic tomato plants over-expressing the gene displayed a statistically significant increment in the amount of lignin of the leaves, whereas no hormonal imbalance was observed (El Mansouri et al. 1999). All together, the results support an involvement of *TPX1* in the synthesis of lignin rather than in IAA metabolism; but the relationship between this biochemical role and the wilted phenotype observed in the transgenic plants is not straightforward. In the present study, histochemical studies and the measurement of water-related parameters have been performed in the transgenic plants in order to get insight into the physiological determinants of the phenotype. Our aim was to propose a link between the biochemical function of TPX1 gene on lignosuberization, strongly supported by previous reports, and the development in transgenic plants of leaves that resembled those developed in wild plants under water stress.

Materials and methods

Plant material and greenhouse experiments

Tomato plants, *Lycopersicon esculentum* Mill., cv. Pera were transformed via *Agrobacterioum tumefaciens* as described (El Mansouri et al. 1999). Among the three transgenic lines previously characterized (El Mansouri et al. 1999), we selected the transgenic line TP3 with a single copy of the *TPX1* cDNA insert and displaying the highest value of peroxidase activity. The experiments were conducted in a greenhouse at the Estación Experimental La Mayora, CSIC, in the south-east of Spain. Water relationships and growth parameters were assessed in transgenic and non-transgenic plants grown under natural climatic conditions during the spring–summer season. Individual plants were grown in 16-l plastic pots filled with gravel (particle size between 2.5 and 5 mm) or hydroponically, in plastic pots with 12 l aerated nutrient solution that was changed every 7 days. In both growth conditions nutrient solution contained: 12.0 m*M* NO_3^-, 0.5 m*M* NH_4^+, 1.5 m*M* PO_4^{3-}, 7.0 m*M* K^+, 4.0 m*M* Ca^{2+}, 2.5 m*M* Mg^{2+}, 2.0 m*M* SO_4^- and 0.5 m*M* HCO_3^-; pH 5.5 (Cánovas 1995).

Plant water related parameters

Leaf water potential (Ψ_w) was measured with a pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA; Scholander et al. 1965). Measurements were developed on fully expanded leaves, early in the morning, between 0800 and 0900 h, and at midday, between 1200 and 1300 h Measurements were performed before and after fruit set occurred in the plants. The measurements dates were 30 March and 7 May. Midday measurements were only performed at the second date.

Instantaneous measurements of the exchange of water between leaves and surrounding air were made by infrared gas analysis in an open system at ambient partial pressures of carbon dioxide and water vapour using a portable photosynthesis system (LI-6400; Li-Cor Inc., Lincoln NE, USA). At the time of measurements, the leaf temperature was 27–28°C and the light photon flux density was 800 μmol m^{-2} s^{-1}. Stomatal conductance was calculated by the on-board software. Five to 10 young fully expanded leaves, of similar ages to those used for the measurements of leaf Ψ_w, were selected for gas exchange analysis.

Root hydraulic conductance was measured as described by Joly (1989). For this purpose healthy plants of both genotypes with 5–6 leaves were grown hydroponically for a period of 1 month. These plants were moved to a 1-l container designed to be enclosed in the pressure chamber and filled with nutrient solution. Plants were cut just below the cotyledonary node. A 30-cm-long rubber sleeve was attached to the stump and the assembly placed in the pressure chamber with the rubber sleeve protuding through a rubber gasket. The pressure in the chamber was increased to 0.1, 0.2, 0.3, 0.4 and 0.5 MPa, thereby generating a range of sap flows that included whole-plant transpiration rates. Each applied pressure was held constant until sap flow rate became stable. Then sap was collected three times, for 5 min-periods each, in glass vials and weighted in an electronic balance. The flow rate of sap exudate was determined for each pressure as the average exudate weight per minute, and hydraulic conductivity was estimated as the slope of exudate flow rate versus applied pressure. These

measurements were carried out twice, with at least three plants each time.

At the end of the experimental period, fresh and dry weight of separated roots and shoots were recorded on six randomly selected plants per genotype. Total leaf area was also determined with a Delta-T Image Analysis System (Delta-T, LTD, Cambridge, UK).

Peroxidase extraction and assay

Soluble peroxidase activity was extracted in 50 m*M* sodium phosphate, pH 6. One gram of whole root tissue from individual plants was homogenized using an Ultra turrax (Janke & Kunkel, Staufen, Germany). The extract was centrifuged at 30 000 *g* and 4°C for 20 min and the supernatant was the soluble fraction. Peroxidase activity was evaluated using 0.6 m*M* *o*-dianisidine and 2.8 m*M* hydrogen peroxide as substrates, at pH 6 in 50 m*M* phosphate buffer. One arbitrary unit corresponded to an increase in absorbance at 460 nm min^{-1} under the assay conditions (Quesada et al. 1992).

Peroxidase activity was also evaluated in the medium where hand-made transverse sections of the roots were incubated for histological study, as described in the next section.

Root sections staining procedures and image processing

One centimetre root segments were prepared from non-transgenic and transgenic plants grown hydroponically, and immediately fixed in ethanol. Similarly to Quiroga et al. (2000), root sections corresponding to middle and basal parts of the roots were used for analysis. The middle zone corresponded to sections obtained in the range of 6–9 cm from the tip and basal sections were obtained from root segments distant 13–15 cm from the tip. Care was taken to select those roots with sections and vascular cylinders of similar diameters, as well as comparable cortex development. Cortical cell size and the number of cortex cell layers were used as developmental criteria.

After ethanol fixation, serial free-hand transverse sections were made and transferred to multichamber holders (Brundrett et al. 1988). Several sections from two replicate roots of each genotype were stained and analysed as described below.

A specific staining procedure to visualize both exodermis and endodermis was carried out as described by Brundrett et al. (1988). Sections were incubated in 0.1% (w/v) berberine hemi-sulphate (Sigma, St Louis, MO, USA) for 1 h. After washing, sections were counter-stained with 0.5% (w/v) aniline blue WS (Sigma) for 30 min, to partially quench the fluorescence of the epidermal and the cortical cells. Berberine has a high affinity for cell walls containing suberin or lignin. Root cross-sections were also incubated with 0.01% (w/v) safranin-o (Sigma) in 50% ethanol for 3 min. This dye binds to aliphatic components such as those present in the suberin and it has been previously used in tomato cotyledons to detect this polymer (Davis and Raymon 1991). Lignins were detected using the Wiersner test (specific for cinnamaldehyde groups). In this case, the sections were incubated in 1% (w/v) phloroglucinol (Sigma) in 1 : 3 (v/v) HCl : ethanol for 10 min (Ros-Barceló 1999).

Root sections were also stained for peroxidase activity. Ethanol-fixed sections were incubated for 20 min in a staining solution containing 0.1 mg ml^{-1} 3, 3′, 5, 5′-tetramethylbenzidine-HCl (Sigma) and 0.03% hydrogen peroxide, in 50 m*M* Tris-acetate buffer, pH 5.0. Controls were performed in the same conditions but including hydrogen peroxide as the only substrate (Ros-Barceló 1999). Peroxidase activity was quantified in the staining solutions, in which root sections had been incubated, by measuring the absorbance at 654 nm in 1 ml aliquots. The absorbance values were normalized to the weight of the root sections to allow proper comparison between middle and basal zones.

Stained sections were viewed either in white light or in ultraviolet light of a Laborlux 12 Leitz microscope (Oberkochen, Germany) and photographed using 100 ASA slide film with a WILD MPS 45 Photoautomat micrography system. Then, a charge-coupled device video camera (Sony, Tokyo, Japan) was used to acquire images from the slides. The video signal was used as input into the Visilog 5.2 image-processing system (Noesi, Noesis, France) and cross-sectional areas of xylem vessels from pholoroglucinol-stained sections was determined.

Results and discussion

Transgenic tomato plants that over-expressed the TPX1 isoperoxidase gene showed a wilted phenotype after flowering (El Mansouri et al. 1999). This water-stress-like phenotype was experimentally confirmed by the measurement of the leaf water potentials (Fig. 1).

Prior to plant flowering, predawn leaf water potential (Ψ_w) was lower but not significantly different in the transgenic plants in comparison with the non-transgenic ones. However after fruit set, Ψ_w was significantly lower in the transgenic leaves (Fig. 1). At this stage, the difference in leaf Ψ_w between transgenic and non-transgenic

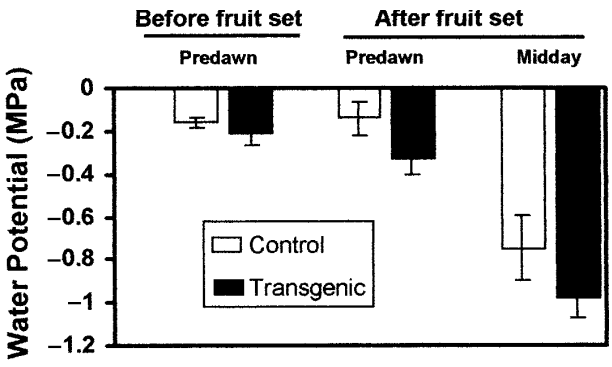

Fig. 1. Leaf water potentials of control and transgenic plants. Predawn measurements were performed prior and after fruit set. Midday measurements were also performed after fruit set, when shoot water demand is higher. Means ± standard deviations (n = 5).

plants was maintained at midday, when maximum air vapour pressure deficit is reached in the greenhouse. The results presented correspond to line TP3 but a similar behaviour was observed in the transgenic line TP 15, which was assayed in parallel (results not shown). Both lines contained a single locus of the transgene (El Mansouri et al. 1999), but the differences observed in leaf water potential were higher in line TP3, which also showed the highest values of peroxidase activity, and later studies were focused on this line.

As water availability in the soil phase was similar for control and transgenic plants, the lower water potential found in transgenic leaves may be caused by an increased loss of water from the leaves and/or a greater resistance to water flow in the roots, as has been reported for rice (Yambao et al. 1992). The stomatal conductance measurements gave the values of 594 ± 60 and $494 \pm 69\,\text{mmol}\,\text{m}^{-2}\,\text{s}^{-1}$ for the non-transgenic and transgenic plants, respectively. This lower stomatal conductance of the transgenic leaves discredits the hypothesis that the lower water potential in these plants was due to a higher loss of water from the leaves. Thus, the lower leaf Ψ_w observed in the transgenic over-expressing TPX1 plants after fruit set, when the water demand by the shoot increases, could result from a decrease of the water supply from the root. The measurements of the root hydraulic conductance from control and transgenic roots support this last hypothesis. The effect of increasing external pressure from 0.1 to 0.5 MPa on the flow of water through the whole root system is shown in Fig. 2. In both genotypes, water flow increased linearly with pressure but the slope, which corresponds to the hydraulic conductivity, was lower in the transgenic roots. This value was $3.47 \pm 0.19\,\text{g water}\,\text{min}^{-1}\,\text{MPa}^{-1}$ for non-transformed plants and $1.4 \pm 0.38\,\text{g water}\,\text{min}^{-1}\,\text{MPa}^{-1}$ for the transgenic line, and the differences were maintained when related to root biomass. Independent measurements of the water flow rate through the whole root system performed at constant external pressure (0.4 MPa) yielded similar estimates of hydraulic conductance, 2.23 ± 0.79 and $0.97 \pm 0.26\,\text{g water}\,\text{min}^{-1}\,\text{MPa}^{-1}$ ($n = 4$) for control and transgenic plants, respectively. Therefore, the lower leaf Ψ_w recorded on transgenic plants at early morning, when evaporative demand is very low, is probably related to the lower root hydraulic conductance observed in these plants. This would determine a lower capacity to recover the leaf water status during the night period. The difference in leaf Ψ_w between transgenic and non-transgenic plants becomes greater as the whole plant water demand increases, either after fruit set or at midday.

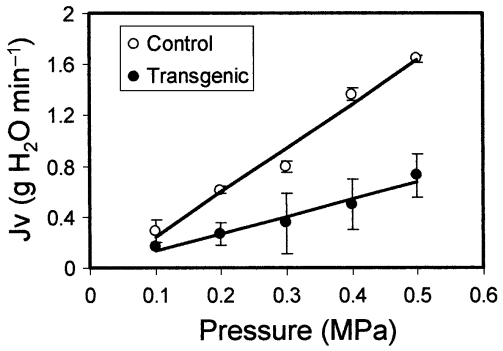

Fig. 2. Effect of increasing pressures on water flux (JV) of root systems from control and transgenic tomato plants. Hydraulic conductance was determined as the slope of the flux–pressure relationship. The results presented are means ± standard deviations of measurement performed in three independent plants.

These differences in the water economy of the plant could be related to the modified expression of the TPX1 isoperoxidase in the root tissue of the transgenic plants. However, it was difficult to detect quantitative differences because peroxidase isoenzymes are very abundant in the roots of tomato plants (Quiroga et al. 2000, 2001) and expressed in all tissues. Then, although TPX1 was clearly over-expressed two to three times in the leaf tissues of these plants (El Mansouri et al. 1999 and results not shown), total peroxidase activity was only slightly higher in the whole-root extracts of transgenic plants (129 ± 27 versus $107 \pm 34\,\text{U}\,\text{g}^{-1}$ fresh tissue). Similar results have been reported for tobacco transgenic plants over-expressing an acidic isoperoxidase (Lagrimi et al. 1997a). As an alternative approach, we focused our study in specific zones of the roots and based in previous results (Quiroga et al. 2000) we selected the middle and basal parts of the roots obtained from hydroponically grown plants (Fig. 3). The middle zone corresponds to the root part exhibiting primary growth (Fig. 3A), whereas secondary growth was evident in the sections corresponding to the basal zone (Fig. 3B). When these sections were incubated in the medium containing the substrate for peroxidase activity, the sections corresponding to middle and basal regions of the roots gave higher values for transgenic than for non-transgenic plants (Fig. 3C and D). This difference was statistically significant in the activities of the basal zones where major anatomical changes were observed.

Differences in water conductance of the whole root system must be due to anatomical differences that would limit the axial and/or radial flow of water through the root (Steudle and Frensch 1996, Nobel 1999, Steudle 2000). Therefore, putative targets of modification by TPX1 over-expression would be the vascular cylinder and the exodermis and endodermis. Although TPX1 expression is under the control of the 35S constitutive promoter, the effect of TPX1 over-expression on phenolic polymerization in lignin and the aromatic domain of suberin would occur in these tissues in which all the other enzymes and metabolic precursors involved in lignin and/or suberin synthesis are present.

The results obtained with root sections of both genotypes stained with dyes that detect lignin and suberin are shown in Fig. 4. Differences in the root anatomy between non-transformed and transformed plants were minor in the sections corresponding to the middle zone (Fig. 4A and B), but clear differences between transgenics and non-transgenics were observed in the cross-sections of

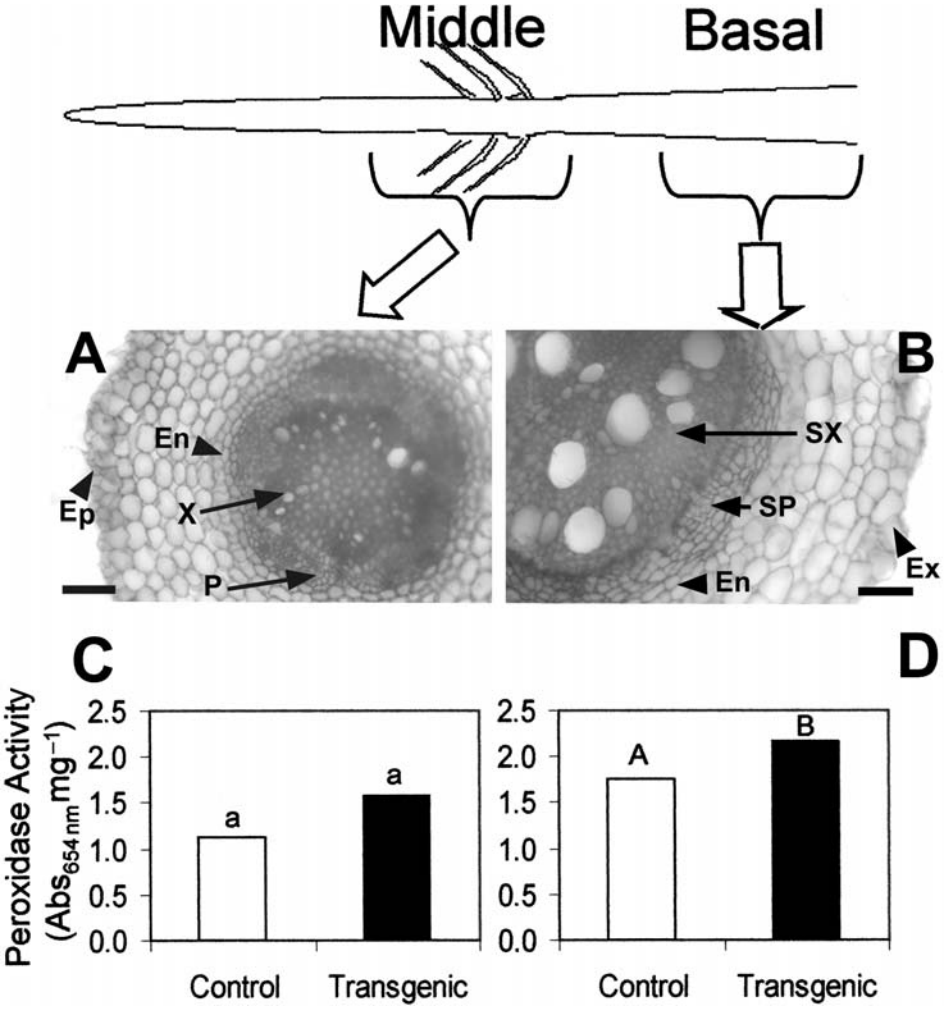

Fig. 3. Panels A and B correspond to middle (A) and basal (B) sections of tomato roots stained for peroxidase activity, these representative sections have been selected to show the anatomical differences between the middle and basal zones in wild-non-transformed plants. Bars correspond to 80 μm. Ep, Epidermis; En, endodermis; Ex, Exodermis X, xylem; P, phloem; SX, secondary xylem; SP, secondary phloem. (C) Peroxidase activity in the medium where root sections corresponding to middle sections of control and transgenic plants were incubated. (D) Peroxidase activity in basal sections. Comparison between control and transgenic roots were performed in the middle and basal sections by *t*-test (n = 4). Means with different letters are statistically significant at the 5% level.

the basal root zones, both stained with safranin and phloroglucinol (Fig. 4C–D and E–F, respectively). Using several independent sections of both genotypes we found that the differences were apparent in the size of the xylem vessel rather than in the number of vessels, which was similar for both plants. Thus, xylem vessels with area above 1500 μm^2 were absent in the transgenic roots and, conversely, the percentage of xylem vessels with areas below 500 μm^2 was higher in these plants (Fig. 5). The average areas of the xylem vessel for the non-transgenic and transgenic plants were 1843 and 458 μm^2, respectively (significant at $P < 0.05$, ANOVA analysis). An estimation of the total area corresponding to xylem in the basal cross-sections gives a 3.9-fold decrease in the transgenic roots relative to the non-transgenic. There is strong anatomical support for a decreased axial water flow through the roots of the transgenic plants because the volume of fluid moving in unit time along a cylinder is proportional to the fourth power of its radius (Nobel 1999).

In addition, although not very evident with the sections stained with safranin and phloroglucinol, a higher staining was observed in the exodermis of the transgenic plants (Fig. 6A and B) and this difference in ligno-suberization was clearly confirmed using berberine (Fig. 6C and D). The fluorescence revealed that the radial and outer tangential walls of the exodermis cells in the transgenic roots were more ligno-suberized than in-non-transgenic roots. Differences in the fluorescence of the endodermis were also observed between transgenic and non-transgenic roots, although the variability in the pattern of staining was higher in the endodermis than in the exodermis. In general, the outer tangential walls of the endodermis of the non-transgenic roots were slightly more fluorescent than those from transformed roots (Fig. 6E and F). However, the presence of ligno-suberization in radial and inner tangential walls was only observed in some basal sections of the transgenic roots (Fig. 6F). Radial ligno-suberization would correspond to a casparian band. These differences would also explain a decrement of the hydraulic conductance of the root system (Cruz et al. 1992). A greater deposition of apoplastics barriers in the tangential and, most importantly, in the radial walls of the exodermis and the endodermis would limit the radial water flow in the roots (Steudle and Frensch 1996, Steudle 2000, 2001). Suberin is a

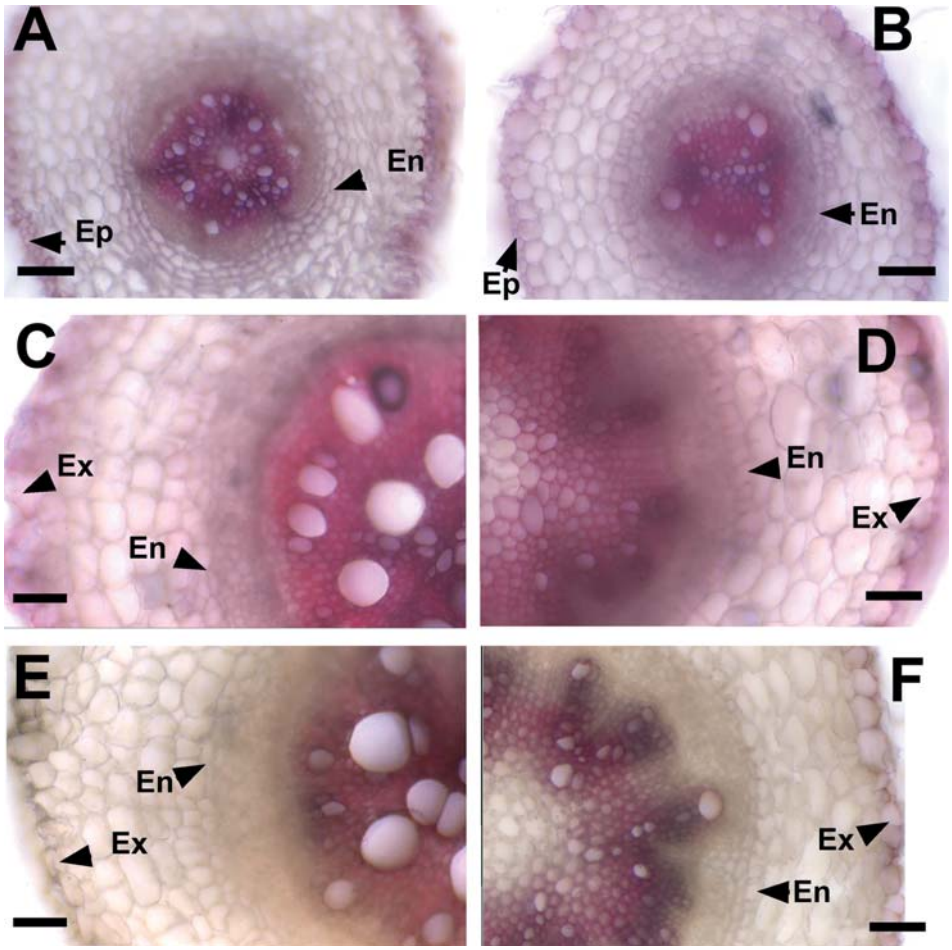

Fig. 4. (A) and (B) Middle sections of control (A) and transgenic roots (B) stained with safranin. Parts (C) and (E) correspond to basal sections of control roots stained with safranin and phloroglucinol, respectively. Parts (D) and (F) correspond to basal sections of transgenic roots stained with safranin and phloroglucinol, respectively. Bar: 80 μm. Ep, Epidermis; En, endodermis; Ex, exodermis; X, xylem; P, phloem; SX, secondary xylem; SP, secondary phloem.

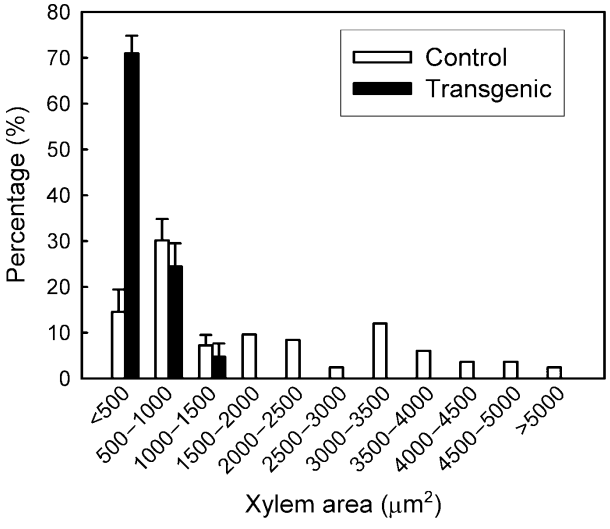

Fig. 5. Frequency distribution of xylem areas observed in the cross-sections of control and transgenic plants. Three independent cross-sections per genotype were analysed. The mean number of xylem vessel evaluated per section ranged between 25 and 30. Values are means of three independent cross-sections ± standard deviations.

better candidate than lignin for this role, although the apoplastic water transport barriers formed as a result of suberin deposition in the exodermis and endodermis of the roots seem to be less perfect than those formed in the suberized potato periderm (Schreiber et al. 1999).

In the present study, we have not estimated the specific contributions of radial and axial resistances to the decreased hydraulic conductance of the whole root system, although it is known that the radial rather than the axial component of water transport limits water uptake by the roots (Steudle and Peterson 1998, Steudle 2001). However, the changes here reported in the xylem vessels, increasing axial resistance, are significant and may explain some percentage of the total resistance. In any case, both resistances would result in a reduced water supply to the shoot and would probably cause a decreased water potential in the leaves.

The reduction of root hydraulic conductance in the transgenic plants explain the wilty phenotype previously reported (El Mansouri et al. 1999), and this also agrees with previous results on TPX1 expression in wild plants, especially in relation to salt stress. The *TPX1* gene is transcriptionally activated in tomato root tissue when the plants are exposed to moderate salt concentration (Botella et al. 1994a). With the same salt treatment, a

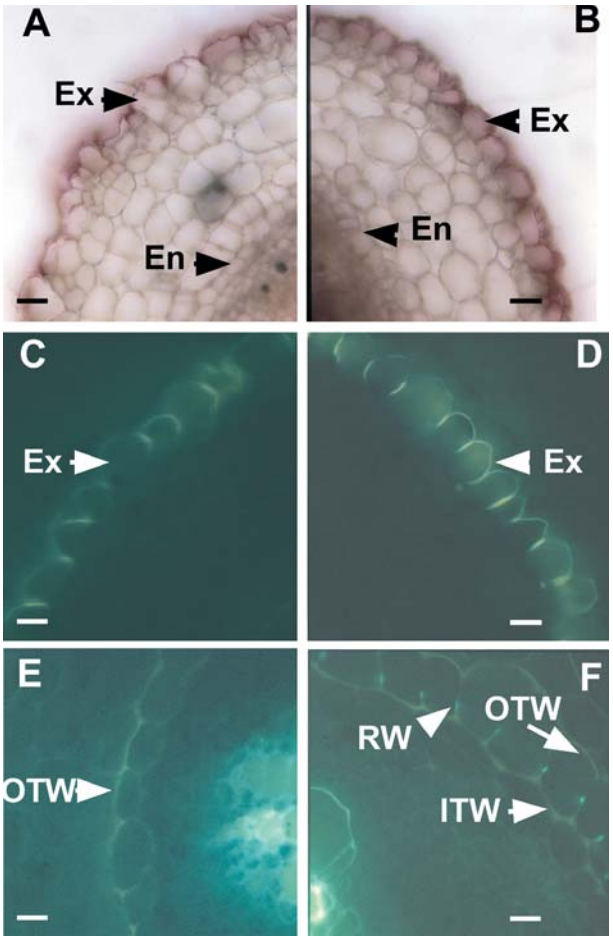

Fig. 6. Basal sections stained with phloroglucinol (A, B) and berberine (C–F). (A), (C) and (E) correspond to control roots and (B), (D) and (F) to transgenic roots. Bars: 80 μm in (A) and (B); and 32 μm in (C)–(F). OTW, outer tangential walls; ITW, inner tangential walls; RW, radial walls; Ex, exodermis; En, endodermis.

decreased hydraulic conductivity has been reported for tomato roots of the same cultivar (Peyrano et al. 1997). Similarly, decreases of the hydraulic conductivity of soybean and sorghum roots have been reported as the result of salt- and water-deficit treatments in these species (Joly 1989, Cruz et al. 1992). The control of the radial movement of water through the roots seems to be involved in the salt and water stress and would include the development of apoplastic barriers in the root exodermis and endodermis (Schreiber et al. 1999). Furthermore, *in situ* mRNA hybridization of TPX1 transcripts has also shown an enhanced expression of this gene in the endodermis, exodermis and protoxylem, which are tissues where lignin and/or suberin synthesis occurs (Quiroga et al. 2000). Moreover, the purified TPX1 protein showed the highest values of catalytic efficiency for syringaldazine, a lignin monomer analogue; and coniferyl alcohol (Quiroga et al. 2000, 2001); and TPX1 overexpression significantly increased the lignin-like polymer content of the transgenic tomato plants (El Mansouri et al. 1999). Therefore, a putative role for TPX1 expression during salt-stress response would be the development of apoplastic barriers that would allow a better control of the radial movement of water and ions through the root.

The presence of apoplastic barriers in the exodermis would increase the ability of the roots to retain water under water shortage, as found by Taleisnik et al. (1999). Its involvement in ion movement would be at the endodermis level because this layer represents a barrier to the back diffusion to the root cortex of ions released into the apoplast of the stele (Steudle and Peterson 1998, Steudle 2000). Further experimental evidence including isolation of the exodermis and endodermis (Schreiber et al. 1999) and the use of apoplastic tracers is required to provide additional support for this proposal.

In conclusion, transgenic tomato plants overexpressing TPX1 peroxidase gene have a modified root anatomy, resulting in a lower root hydraulic conductance and, in consequence, a lower availability of water in the leaves. The results presented in this work, in conjunction with those previously obtained with wild tomato plants, suggest an important role for this gene during water and salt stress response.

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