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Improved germination under osmotic stress of tobacco plants overexpressing a cell wall peroxidase

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Abstract The cell wall is a fundamental component in the response of plants to environmental changes. To directly assess the role of the cell wall we have increased the expression and activity of a cell wall associated peroxidase (TPX2), an enzyme involved in modifying cell wall architecture. Overexpression of *TPX2* had no effect on wild-type development, but greatly increased the germination rate under high salt or osmotic stress. Differential scanning calorimetry showed that transgenic seeds were able to retain more water available for germination than wild-type seeds. Thermoporometry calculations indicated that this could be due to a lower mean pore size in the walls of transgenic seeds. Therefore, the higher capacity of transgenic seeds in retaining water could result in higher germination rates in conditions where the availability of water is restricted.

Key words: Agricultural biotechnology; Osmotic stress; Cell wall; Peroxidase

1. Introduction

The cultivation of many plant species is spatially and seasonally restricted by various environmental stresses affecting several developmental processes, that in turn affect yield, viability and quality of crops. Osmotic stress, caused by drought and high salinity, is one of the most important factors limiting crop productivity [1]. Generation of plants with enhanced adaptation to these adverse environmental conditions would not only extend their cultivation to different areas but also improve yield.

Several studies have revealed some of the mechanisms evolved by plants to adjust their physiology and metabolism to osmotic stress [1]. This has allowed the engineering of plants with increased tolerance to osmotic stress. For example, accumulation of metabolites such as mannitol and proline [2,3] or increasing the glutathione pool [4] in transgenic plants has been sufficient to provide some degree of osmotic stress tolerance.

Plant cell walls have been shown to be involved in the plant response to osmotic stress [5]. Adaptation of tomato cells to high NaCl in the culture medium correlated with modifications in the cell walls [6]. These modifications were further correlated with the appearance of *TPX2* transcripts of a tomato peroxidase gene [7]. *TPX2* is not induced by NaCl and only after the cells were adapted to NaCl in the culture media were *TPX2* transcripts detected [7].

Peroxidases are found throughout the plant kingdom, and most species contain numerous genes [8]. These enzymes catalyze the oxidation of a variety of organic and inorganic compounds with the oxidant hydrogen peroxide [9]. Some genes are expressed constitutively in specific tissues, whereas others show an induced or enhanced expression under different biotic and/or abiotic stresses [10,11]. Peroxidases have been shown to function in cell growth and expansion [12], auxin catabolism [13], and lignification [14], although a specific function for a particular isoenzyme remains to be established.

We now show a causal effect of increased TPX2 peroxidase activity in cell wall and adaptation to osmotic stress. Overexpression in tobacco plants of *TPX2* increases germination frequency under osmotic and salt stress. This increased germination could be explained by modifications of the cell wall architecture induced by TPX2.

2. Materials and methods

2.1. Overexpression of TPX2 in transgenic plants

The *TPX2* cDNA was transferred from Bluescript pBSII (Stratagene) cloning vector to binary plasmid pKYLX71 [15] by polymerase chain reaction amplification, using specific primers with targeted restriction sites. The pKYLX71-TPX2 vector was transferred to the LBA4404 strain of *Agrobacterium tumefaciens* by electroporation. Tobacco transgenic plants were generated following standard procedures [16].

2.2. Peroxidase activity and Northern analysis

Extraction was performed from leaves of primary transformants (Fig. 1B) as previously described [10]. The extraction from the seedlings (Table 1) distinguished the soluble fraction from the cell wall-bound fraction (with 1 M NaCl in the extraction buffer). Plant tissue was macerated in a grinder with 0.05 M phosphate buffer pH 6 (tissue:buffer ratio, 1:4 w/v), filtered through four layers of cheesecloth and the filtrate incubated overnight at 4°C with polyvinylpyrrolidone (PVP, PVP:tissue ratio, 2:1 w/v). The supernatant was used for determination of soluble peroxidase. The residue remaining in the cheesecloth was washed three times with the extraction buffer and then extracted overnight at 4°C with 1 M KCl in 0.05 M phosphate buffer pH 6. The extract was then filtered and the filtrate centrifuged for 40 min at 25 000 × *g* and exhaustively dialyzed against 0.025 M phosphate and assayed for enzyme activity. Peroxidase activity was measured using *o*-dianisidine as substrate as described previously [6]. A unit represents one increment increase in absorbance (460 nm) per minute at the assay conditions. Northern analysis were performed in samples from leaf tissue as described previously [10].

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2.3. Stress treatments and analysis

Sterilized mature seeds were placed on top of petri dishes containing Murashige and Skoog (MS) medium [17] with 7% agar and 30 g/l sucrose. Prior to gelation and pH 5.7 adjustment, NaCl, KCl or mannitol were added to the final concentrations indicated in the text. Three petri dishes with a mean of 40–50 seeds per dish were used per treatment in every experiment. The complete experiment was repeated twice. Seed germination and seedling growth at 20–22°C was followed. Seeds were considered to germinate after radicle and green cotyledon emergence occurred.

2.4. Differential scanning calorimetry

A minimum of 12 mg of dry seeds were maintained for 1 day in a sealed container at 100% relative humidity. Then, hydrated seeds were sealed in aluminum pans which were loaded into the calorimeter (DSC Shimadzu, model DSC-50). Each sample was introduced into the sample vessel and cooled at 5°C/min to −90°C with liquid nitrogen. Thermograms were recorded against an empty aluminum pan placed in the reference vessel. The final thermogram of the sample was obtained after the baseline was subtracted. Pore size assignment was made as previously described [18]. Water potential was calculated as formerly described [19].

3. Results

3.1. Overexpression of a tomato cell wall peroxidase in tobacco

Nicotiana tabacum cv. Wisconsin 38 plants were transformed via *A. tumefaciens* with the cDNA of the tomato basic peroxidase *TPX2* under control of the constitutive cauliflower mosaic virus 35S promoter. The predicted signal peptide was included for targeting of the protein to its normal location in the cell wall (Fig. 1A). Ten independent plants transformed with *TPX2* and a control plant transformed with the empty vector (pKY) were selected and Northern analysis in the primary transformants (T_1) showed a high level of expression in all *TPX2* lines (Fig. 1B). Total peroxidase activity generally correlated with the level of expression of the transgene (Fig. 1C). The three lines with highest mRNA concentration and peroxidase activity, II-10, II-24 and II-32, were selected for further analysis and self pollinated to generate homozygous lines in the T_2 generation.

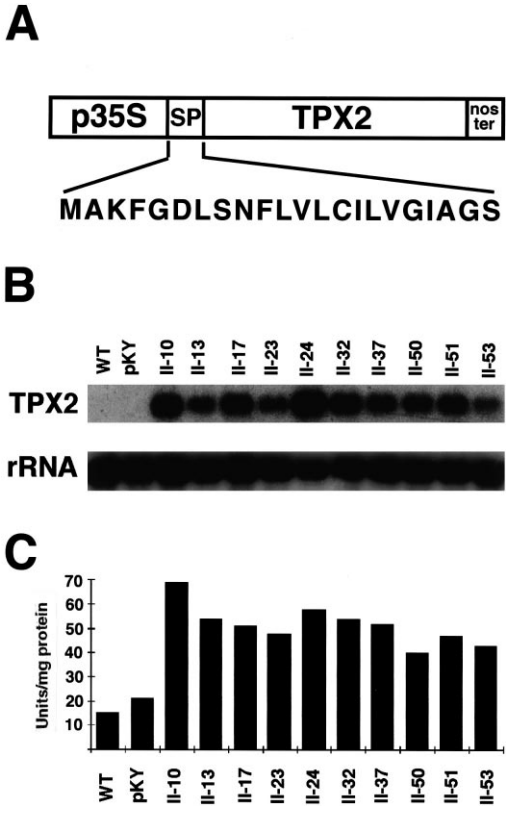

Fig. 1. Expression analysis in primary transformants. A: Diagram illustrating the structure of the *35S::TPX2* fusion. SP, predicted signal peptide for extracellular export with amino acid sequence shown below. B: Northern blot of wild-type, pKY and 10 *35S::TPX2* primary transformants. Total RNA extracted from leaves was probed with *TPX2* to check the level of expression of the transgene. Subsequently, a ribosomal probe was used to check the loading. C: Peroxidase activity in extracts from leaves of primary transformants and control plants.

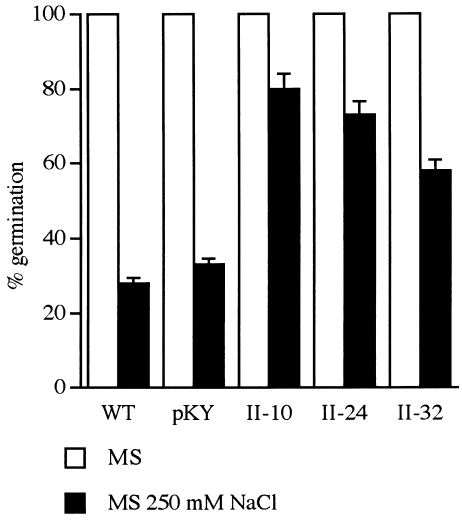

Fig. 2. Germination percentages in wild-type (WT), control (pKY) and *35S::TPX2* lines (II-10, II-24 and II-32). Seeds were germinated at room temperature either on normal MS medium or on MS containing 250 mM NaCl. Seed germination after 3 days on MS medium was used as control and compared to seed germination on high NaCl medium, evaluated after 13 days. Data are means (±S.E.M.) from three separate experiments.

Sequence analysis predicts that TPX2 is secreted to the cell wall [20,21]. Peroxidase activity in the extracts of tobacco seedlings, in both soluble and 1 M KCl-extracted fractions, was significantly higher in the three transgenic lines (Table 1). Increased peroxidase activity in the extracts corresponding to the enzymes ionically bound to the cell wall (1 M NaCl-extracted) confirmed the TPX2 targeting to the wall. The increased peroxidase activity in the soluble fraction could be explained by the existence of an equilibrium between the ionically bound and soluble fractions in the cell wall of transgenic plants, and/or an unprocessed TPX2. Isoelectric focusing profiles of these extracts revealed the presence of a new p*I* 9.6 isoperoxidase in the transgenic lines (data not shown).

Table 1
Specific peroxidase activities in the soluble fraction and ionically bound fraction from seedlings of wild-type (WT) and transgenic plants

Plant line	Peroxidase activity (U/mg protein)	
	Soluble	Ionically bound
WT	6.6	18.1
pKY	2.2	24.5
II-10	109	269
II-24	27	36
II-32	78.9	99.3

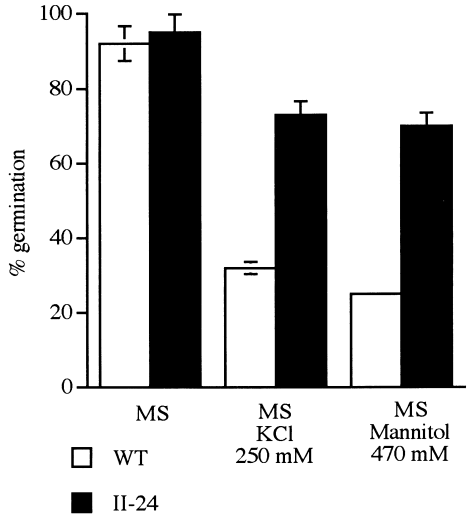

Fig. 3. Germination of wild-type and transgenic (II-24) tobacco seeds under different osmotic stress conditions. Seed germination after 3 days in MS medium was used as a control. Seed germination in KCl and mannitol media was evaluated after 13 days. Data are the means (±S.E.M.) from three independent petri dishes. The experiment was repeated twice and the results obtained were comparable.

3.2. Plants overexpressing TPX2 showed increased germination under salt stress

To investigate the effect of *TPX2* overexpression on germination under salt stress, seeds from the three selected lines and from the pKY control were germinated at NaCl concentrations that severely impaired seed germination in wild-type plants. At 250 mM NaCl, germination of seeds from the selected transgenic lines was significantly higher than that of the control plants (Fig. 2). Significant differences in germination were observed over the 1 month time assay, and they were highest between 10 and 15 days after initiating imbibition. Germination of the *TPX2* overexpressing seeds under non-stressful conditions was identical to wild-type plants. Furthermore, no differences were observed in terms of growth rate, flowering time and seed production between transgenic and control plants under comparable conditions. Increased germination in the three independent transgenic lines excluded any insertional effects and confirmed a role for the TPX2 peroxidase in the tolerance phenotype. Segregation analyses of the transgenic lines II-10, II-24 and II-32 revealed the presence of multiple copies of the transgene in lines II-10 and II-32, and therefore line II-24 was selected for further studies.

Table 2
Distribution (%) of the imbibed water in wild-type (WT) and transgenic seeds (II-10, II-24 and II-32), among the different stage-bound fractions calculated from the DSC cooling thermograms (±S.E.M.)

Plant line	Crystallized from −12 to −23°C[a]
WT	13.0±3.5
II-10	23.0±6.5
II-24	22.8±2.9
II-32	23.1±1.1

[a]Amount of water corresponding to the area from −12 to −23°C (as in Fig. 2B, shaded area). 100% corresponds to total water uptake (crystallized water corresponding to peaks I, II, III, and IV, plus the non-crystallizable water).

3.3. Plants overexpressing TPX2 showed increased germination under osmotic stress

The detrimental effects of salinity can be caused by (1) the osmotic stress due to insufficient water availability, or (2) the ionic stress resulting from a high concentration of toxic salt ions. To determine which mechanism was operating in the overexpression of *TPX2*, the transgenic plants were studied under 250 mM KCl or 470 mM mannitol (osmotically equivalent to 250 mM NaCl). Under both conditions, germination of II-24 seeds was greater than wild-type (Fig. 3). About 70%

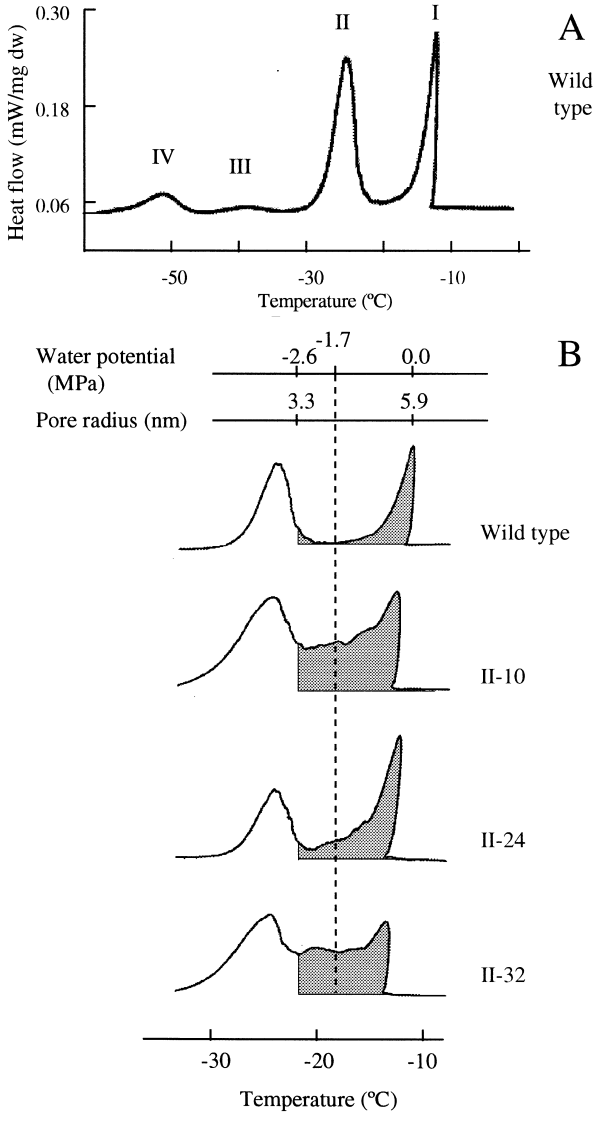

Fig. 4. DSC thermograms from wild-type and transgenic tobacco seeds. Seeds of wild-type and transgenic plants, equilibrated for 1 day in a closed container at 100% relative humidity, were cooled from 0 to −90°C, and calorimetric changes were recorded. A: Thermogram of wild-type seeds in the temperature range from 0 to −70°C. B: Thermograms of wild-type and transgenic seeds (II-10, II-24 and II-32) in the temperature range from −10 to −35°C. Shaded area corresponds to the heat flow in the temperature range from −12 to −23°C. Water potential of −1.7 MPa (dashed line) was estimated for the germination medium (250 mM NaCl, MS salts and sucrose, and agar). The other water potential value (−2.6 MPa) was calculated from the transition temperatures in the thermogram according to Nobel [19].

of the transgenic seeds germinated under KCl or mannitol, while only 32 or 23% germinated for the control seeds. These data together point to an improved performance of the transgenic lines under osmotic stress rather than to an ionic, or sodium, effect.

3.4. Transgenic seeds show differential water uptake capability

Differential scanning calorimetry (DSC) has been extensively used to estimate the physicochemical properties of polymeric materials and their interaction with water [22]. DSC detects thermal events as the temperature is gradually changed. Thus, for cooling water adsorbed on polymers the downward freezing temperature shift is directly related to the binding of the water to the polymer [22]. The lower the freezing temperature, the higher the binding strength of the sorbed water. Although seed is a highly heterogeneous polymeric material, the state of the sorbed water could be studied by this technique. The cooling thermogram obtained for wild-type seeds shows four exothermic peaks at −12, −26, −41 and −54°C (I, II, III and IV) (Fig. 4A). The area below each peak represents the amount of imbibed water that crystallizes as the temperature decreases. Peak I corresponds to free water since it has the same transition temperature as pure water [23]. Peak II corresponds to water that interacts with highly polar groups such as hydroxyl groups of cellulose whose transition temperature has been reported to be between −23 and −40°C. Peaks III and IV correspond to imbibed water with strong interaction with the seed components. In addition, there is a portion of water that does not crystallize even at −90°C whose interaction with the seed components is very strong. DSC thermograms for transgenic seeds showed an increased area from peak I to peak II (Fig. 4B). The amount of water in this region (shaded area, from −12 to −23°C) is significantly higher in transgenic seeds (Table 2). This indicates a higher percentage of sorbed water in transgenic seeds whose interaction with the seed components is higher than with itself (pure water) and lower than with polar compounds in comparison to wild-type seeds. DSC performed on dry seeds showed no thermal events in either wild-type or transgenics (data not shown). Water uptake by seeds was slightly higher in transgenic seeds (0.38 mg water/mg dry seed) than for wild-type (0.34 mg water/mg dry seed).

DSC data of water sorbed at a liquid-solid interface can be used to estimate the mean pore size of the solid [18]. The difference between temperature transition and the triple point of water is related to the radius of the solid phase pore [18]. Calculations for the −12 and −23 °C transition temperatures estimated pore radii of 5.9 and 3.3 nm, respectively (Fig. 4B). Thus, while control plants have an estimated pore radius of 5.9 nm, the DSC of transgenic seeds would indicate the presence of intermediate pore sizes within this range.

Water potential of the different fractions in the DSC thermograms can be inferred from the transition temperature. Thus, whereas pure water has a transition temperature of −12°C, a PEG solution with water potential equivalent to −2.6 MPa has a transition temperature in the DSC thermogram of −23°C. Under osmotic stress, a significant amount of the water retained by the transgenic seeds has a water potential between −1.7 MPa (the estimated value for the germination medium containing 250 mM NaCl, MS salts, sucrose, and solid agar) and −2.6 MPa (Fig. 4B), and it is most likely available for the embryo.

4. Discussion

Overexpression of the cell wall peroxidase TPX2 does not affect the normal growth and development of tobacco plants but greatly enhances germination capacity under high osmotic conditions. TPX2 could modify the architecture of the cell wall where specific peroxidase activity may be limiting. Therefore, despite the ability of peroxidases to oxidize a range of substrates in vitro, overexpression in plants can lead to changes that are specific to one compartment. This most likely occurs through peptide targeting to the extracellular space.

Seed germination occurs when enzyme activity is restored after hydration of the proteins [24]. Imbibition of the seed is the event that triggers all the subsequent steps leading to radicle emergence through the seed coat. In transgenic seeds a significantly higher amount of imbibed water with a freezing temperature over −23°C is available in comparison to the control (Table 2). A higher amount of water is expected for a complete hydration of proteins in seeds and subsequent cell growth. However, the differences already apparent after only 1 day imbibition (Fig. 4, Table 2) may explain the higher germination displayed by the three independent lines after 13 days of imbibition (Fig. 2). Therefore, it is likely that the DSC data indicate an enhanced capacity for water uptake of the transgenic seeds under unfavorable conditions. This would be a purely physical process that makes water available to the embryo prior to the resumption of metabolic activity [25].

Water potential has four components defined as osmotic, gravitational, hydrostatic and matric potential [19,26]. The matric potential is a measure of the tendency of the matrix to adsorb additional water molecules [19,26]. Water uptake in the seeds is at first largely a function of matric forces; the surface of proteins and some cell wall polysaccharides must become hydrated before germination occurs [26]. Protein contents are not different in transgenic and control seeds. However, DSC results strongly support a specific effect of overexpression of TPX2 on the cell wall architecture. The main contributor to the matric potential in the cell wall is often the negative hydrostatic pressure arising from surface tension effects in the air-liquid interfaces of the cell wall pores [19]. The magnitude of the hydrostatic component is related to the pore size and contact angle [19]. We therefore hypothesize that the different DSC displayed by the transgenic seeds in comparison to the control could be explained by the negative hydrostatic component caused by differences in the cell wall pore size.

The cell wall structure is largely defined by the pectin matrix and the cross-linking of pectins and non-cellulosic polysaccharides by ester linkage with dihydroxycinnamic acids [27]. These cross-links are catalyzed by cell wall peroxidases [9]. Therefore, increased extracellular TPX2 activity could modify the cell wall by affecting cross-linking in the pectin matrix which, in turn, could alter the pore size in transgenic seeds. This modification of the cell wall could increase the amount of water retained in the seed cell wall at a water potential available for the embryo. This transgenic approach provides a significant potential for the germination of seeds and plant survival under a range of environmental stresses, with no other plant traits noticeably affected.

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