Expression of a highly basic peroxidase gene in NaCl-adapted tomato cell suspensions

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Abstract A tomato peroxidase gene, TPX2, that is only weakly expressed in the roots of young tomato seedlings is highly expressed in tomato suspension cells adapted to high external NaCl concentration. The protein encoded by this gene, with an isolectric point value of ≈9.6, is found in the culture medium of the growing cells. Our data suggest that the expression of TPX2 in the salt-adapted cells is not the result of the elicitation imposed by the in vitro culture or the presence of high NaCl concentration in the medium.

Key words: Peroxidase; Cell suspension; Salt adaptation; *Lycopersicon esculentum*

1. Introduction

Peroxidases (EC 1.11.1.7; donor:hydrogen-peroxide oxidoreductase) are enzymes that may accept a broad range of substrates in vitro but the identity of their in vivo electron donors remains unclear. In most plant species studied, a number of peroxidase isoforms have been identified which differ in their structures and catalytic properties [1]. Peroxidase isoforms are encoded by a multigene family and frequently undergo post-translational modification of the gene products [2]. Despite the abundant information on peroxidases in many plant systems, only in few cases the naturally catalysed reaction and, accordingly, the physiological function has been determined [3–5].

Peroxidases are implicated in many diverse aspects of plant growth and development [2] as well as in various plant responses to external signals [6]. Their involvement in these processes has been established by a combination of biochemical studies of the purified enzymes [5] and exhaustive expression studies [3,7]. Many of the cloned peroxidases encode a signal peptide that target them to the secretory pathway [1], being the cell wall the final destination of some of them. Thus, culture-grown plant cells release cell wall-targeted isoperoxidases to the medium as reported in peanut [8], tobacco [9] and tomato [10].

In tomato, seven peroxidase genes have been mapped and some of them have been characterized [11–13]. We report here the characterization of one of those genes, TPX2, in tomato cell suspensions adapted to grow in 256 mM NaCl.

2. Materials and methods

2.1. Plant material

Tomato (*Lycopersicon esculentum* cv. Pera) cell suspension cultures were obtained and maintained as previously described [10]. Callus was induced from leaf tissue and exposed to sodium chloride; the calli capable of growing in the presence of 256 mM NaCl were selected. Cell suspension cultures were obtained from friable calli. Control cell suspensions were also obtained from leaf-derived callus that had never been exposed to NaCl. Routinely, suspensions were maintained on liquid medium containing Murashige and Skoog salts [14], B5 vitamins [15], and 5.4 μM NAA, 0.45 μM 2,4-D and 0.46 μM kinetin. Additionally, a 256 mM supplement of NaCl was added to the medium of adapted cells. In all cases, the final pH was adjusted to 5.7. Stock cultures were maintained by transferring cells, in the stationary phase, to fresh medium. Cultures were incubated on gyratory shakers (110 rpm) at 26°C, with a 16 : 8 light : dark regime. Cells were sampled by vacuum filtration, frozen in liquid nitrogen and stored at −80°C until used, culture media was also stored at −80°C. Tomato seeds were germinated in Petri dishes, seedlings were grown for 2 weeks in vermiculite and finally transferred to hydroponic culture in the greenhouse under a 14 h daily photoperiod. Two-month-old plants were used for adult tissue sampling.

2.2. RNA preparation and analysis

RNA was extracted from samples using the acid guanidinium thiocyanate-phenol-chloroform extraction protocol [16]. The procedure was modified to include two, 2 M LiCl and ethanol, precipitation steps as described [12]. RNA transfer and hybridization were carried out as previously described [12].

2.3. Peroxidase extraction, assay and isoelectric focusing

Protein extraction and isoelectric focusing of isoenzymes were performed as previously described [10,17]. The peroxidase activity was measured as previously described [17] using *o*-dianisidine as substrate. One arbitrary unit of enzyme activity corresponds to an absorbance increase/min at 460 nm under the assay conditions.

3. Results

Northern blot analysis of tomato seedlings and healthy adult tomato plants detected a 1.3 kb homologous transcript only in seedling root tissue but not in adult plant root tissue (Fig. 1A). Although TPX2 was cloned from a cDNA library constructed from salt stressed plants [18] we found no effect of salt treatment in TPX2 transcript levels in root tissue (Fig. 1B).

Basic isoperoxidases have been reported in the culture medium of of tobacco [9] and peanut [8] cell suspensions, therefore TPX2 expression was studied in tomato cell suspensions with the results shown in Fig. 2. TPX2 transcripts were detected at measurable levels only in salt-adapted tomato cells. In addition, TPX2 transcripts in adapted cells showed a stage-dependent pattern with highest level at late-linear stage. The isoelectric profile of peroxidases extracted from cell suspension and culture medium of the salt-adapted cells at the stationary phase as well as non-stressed plant roots is shown in

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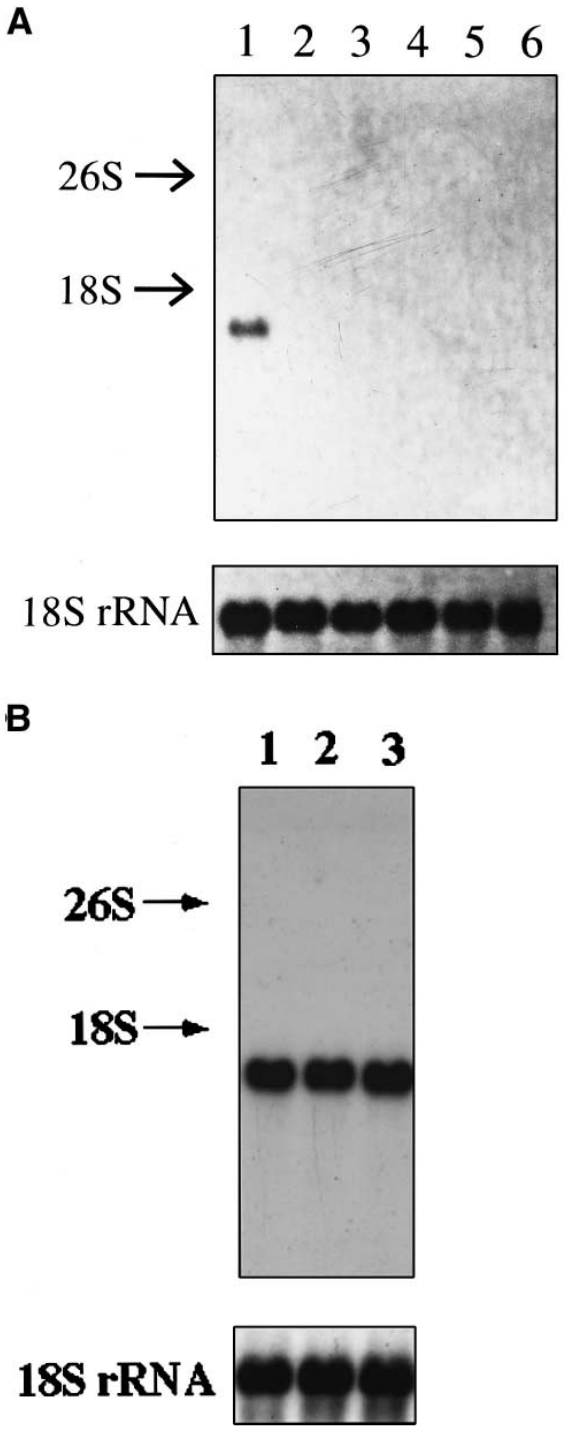

Fig. 1. TPX2 mRNA levels in different tomato plant tissues. Northern blots were performed using 10 μg of total RNA per lane extracted from: A, seedling roots (1), hytocotyls (2) and cotyledons (3), and roots (4), stems (5) and leaves (6) of adult plant; B, seedling roots treated with NaCl (256 mM) for 0 h (1), 24 h (2) and 72 h (3).

Fig. 3. Previous studies showed the presence of basic peroxidases in the culture medium of salt-adapted tomato cells with highest values at late-linear stage [10]. The TPX2 gene encodes a highly basic isoenzyme with a calculated pI value of 8.5 but the native protein pI value is ≈9.6 as deduced from its heterologous expression in transgenic tobacco plants (result not shown). It is apparent from our results that the isoperoxidase encoded by TPX2 is present in the culture medium but it is non-detectable in root of adult plants and cultured cells (Fig. 3).

To know whether TPX2 expression resulted from the cells, exposure to NaCl or was associated to the salt adaptation process, two experiments were performed. First, NaCl was gradually removed from the culture medium by subculture of the cells at decreasing salt concentrations (Fig. 4) and second, cells were directly subcultured at NaCl concentrations of >256 mM (Fig. 5). Northern analysis reveals no significant changes in TPX2 transcripts as a result of the decreased or increased NaCl concentrations in the culture media.

Finally, when studying a possible effect of ABA, we found that 10 μM concentration of ABA did not affect the TPX2 transcript level in salt-adapted cells when the hormone treatment was performed at the begining of a cell growth stage (Fig. 6), but levels of transcripts clearly decreased after a 24 h treatment with 100 μM ABA. Treatment of unadapted cells with ABA, in the same conditions, did not induce TP2 expression (results not shown).

4. Discussion

Although seven peroxidase genes have been mapped in tomato [19], their tissue, developmental, and environmentally induced expression is still unknown for some of them. One of these genes, TPX2, was cloned from a cDNA library constructed from salt-stressed seedlings [18]. Sequence identity of the mature protein at amino acid level was highest to tomato peroxidase genes TPX1 (72%) and cevi-1 (54%) whereas among peroxidases from other species the homology was highest to the basic peroxidases from tobacco (58%) [9] and *Stylosanthes* (57%) [20]. TPX2 transcripts were barely detected in the root of young seedlings, with no effect of salt stress or wounding on its expression as it was the case of the tomato TPX1 gene [12]. As a first step to establish the role of TPX2 we performed expression studies in both unadapted and salt-adapted tomato cell suspensions.

Cells in suspension represent a plant system that is continuously stressed. Therefore, a high level of expression of stress-induced genes can be expected [21]. In fact, basic peroxidases are released to the medium in tobacco [9] and peanut [8] cell suspensions. However, TPX2 transcripts were not detected in unadapted tomato cell suspensions even after exposure to 256

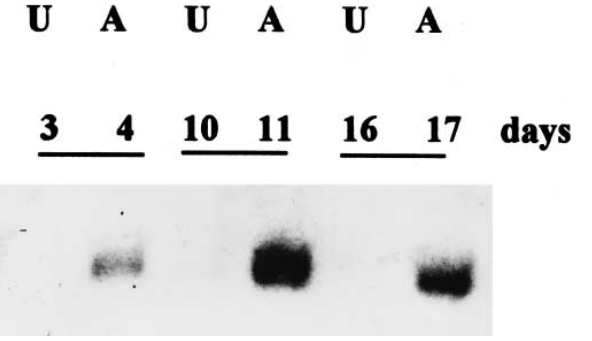

Fig. 2. TPX2 mRNA levels in unadapted (U) and salt-adapted (A) tomato cells during the growth cycle. Total RNA (10 μg) extracted from cultured cells at lag (3, 4 days), exponential (10, 11 days) and stationary (16, 17 days) phases of the growth cycle, was electrophoresed, blotted and hybridized with ^{32}P-labeled TPX2 probe.

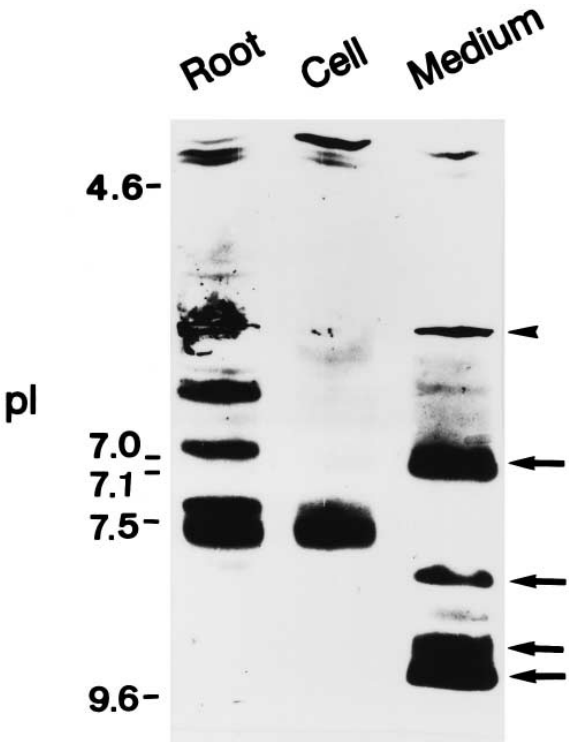

Fig. 3. Peroxidase activity bands in the isoelectric focusing gel of crude extracts of tomato roots, cells and culture medium. Crude extracts from tomato roots (20 mg fresh weight; 1.35 arbitrary units of peroxidase activity), cells (16 mg fresh weight; 0.7 arbitrary units of peroxidase activity) and medium (corresponding to 12.5 mg fresh weight; 3.3 arbitrary units) of salt-adapted cultured cells at stationary phase were separated in agarose plates by isoelectric focusing and assayed for peroxidase activity using 4-chloro-1-naphtol and H_2O_2 as substrates. Arrows indicate the most relevant isoenzyme bands detected in the medium (arrowhead indicates the sample loading site).

mM NaCl in the medium (result not shown). The uniqueness of the TPX2 expression is the presence of transcripts only in the salt-adapted cells and the appearance of the encoded isoperoxidase in the culture medium. The fact that withdrawal or addition of salt to the medium do not alter TPX2 transcript levels excludes the possibility that TPX2 expression results only from the elicitation of the cells by the presence of NaCl. Thus, the clear difference between TPX2 and other

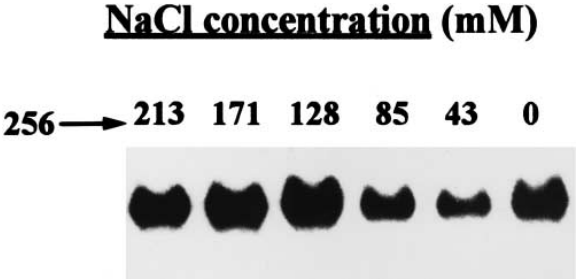

Fig. 4. Effect of decreasing NaCl concentrations on the TPX2 transcript level of salt-adapted cells. Salt-adapted (256 mM NaCl concentration) tomato cells at stationary phase were sucessively subcultured every 3–4 days in media with decreasing NaCl concentration (213, 171, 128, 85, 43 and 0 mM). Total RNA (10 μg) was extracted just before the initiation of the following subculture, electrophoresed, blotted and hybridized with a ^{32}P-labeled TPX2 probe.

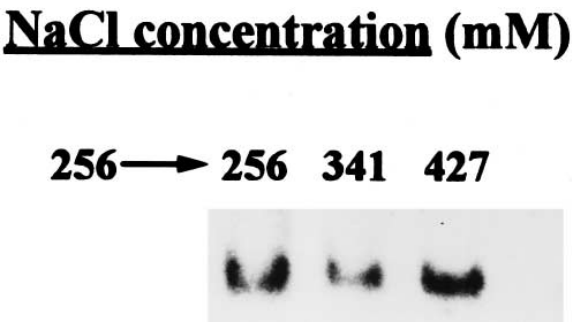

Fig. 5. Effect of increasing NaCl concentrations on the TPX2 transcript level of salt-adapted cells. Salt-adapted cells (256 mM NaCl concentration) were subcultured in media with 341 and 427 mM NaCl concentration. Total RNA (10 μg) was extracted 4 days after initiation of the subculture, electrophoresed, blotted and hybridized with a ^{32}P-labeled TPX2 probe.

peroxidase genes is that TPX2 expression is specifically associated to the salt adaptation process and not only the result of cell elicitation in the culture. Other possible explanation that can not be discarded is that the high salt in the adaptation process selected cell types expressing TPX2.

The plant hormone abscisic acid (ABA) has often been involved in the plant response to salt stress [22]. Furthermore, varietal tolerance in rice to salt stress has been related to this hormone's levels and associated plant molecular responses [23]. Previous studies in tomato callus have found that 100 μM concentration of ABA enhanced the transcript level of the tomato peroxidase gene tap1 [11]. TPX2 expression in cell suspensions of salt-adapted cells is not affected by low levels of external ABA, and 100 μM concentration causes a decrease of transcripts.

Cross-linking of proteins [4,5] and lignin biosynthesis [3] are the main roles assigned to peroxidases in the cell wall. TPX2 encodes a putative cell wall-targeted protein and its expression pattern during the cell growth stage shows that transcript level is higher during cell enlargement [24] as occurs for other cell wall peroxidase genes [9]. Previous studies on peroxidases excreted to the culture medium of tomato cell suspensions identified an acidic isoenzyme involved in the cross-linking of cell wall proteins and a basic isoenzyme that did not contribute to this process [25]. However, based on the amino acid content analysis, this basic peroxidase is not the TPX2 gene product. The results presented here suggest a role for the TPX2 gene product in the salt adaptation of tomato cells, probably by modifying the cell wall. There are many indications that this

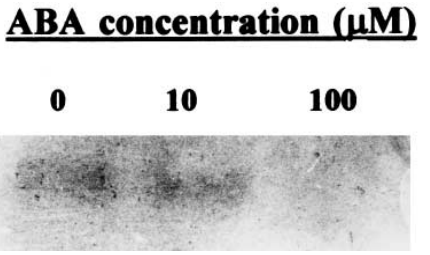

Fig. 6. Effect of different concentrations of ABA on TPX2 mRNA levels in salt-adapted tomato cells. Cells at stationary phase were subcultured in a medium containing 10 or 100 μM ABA, thus initiating a new cell growth stage. After 24 h treatment, total RNA (10 μg) was extracted from cells, electrophoresed, blotted and hybridized with a ^{32}P-labeled TPX2 probe.

cell compartment is involved in the plant cell adaptation to salt stress [26]. We had previously shown that filtered salt-adapted cells contained >50-fold higher amount of lignin-like compounds than unadapted cells [10]. However, the specific contribution of the TPX2 product, whether in lignin formation or protein cross-linking, remains unclear and it is presently under study.

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