FEBS Letters 347 (1994) 195–198

FEBS 14156

Induction of a tomato peroxidase gene in vascular tissue

Miguel A. Botella[a], Miguel A. Quesada[b], María I. Medina[a], Fernando Pliego[b], Victoriano Valpuesta[a,*]

[a]*Departamento de Bioquímica y Biología Molecular and* [b]*Departamento de Biología Vegetal, Universidad de Málaga, 29071 Málaga, Spain*

Received 28 February 1994; revised version received 11 May 1994

Abstract

Expression of a tomato peroxidase gene that is constitutively expressed only in roots was induced in stems and leaves as a result of mechanical wounding. However, wound-induction of TPX1 transcript accumulation in leaves was limited to the mid-rib. No TPX1 transcript was detected in the lamina of the leaf after wounding. Peroxidase isozyme studies indicated the presence of a unique basic isoform in stems after wounding.

Key words: Peroxidase; Wounding; Small subunit of ribulose-1,5-bisphosphate carboxylase; *Lycopersicon esculentum*

1. Introduction

Wounding mediates the regulation of gene expression in some genes not directly involved in the plant defense response being down-regulated [1,2], whereas other genes the products of which are involved in wound-healing or pathogen attack are up-regulated [3,4]. However, plants can perceive and respond in a different manner depending on the signals generated by wounding or pathogen attack and the biochemical barriers formed are different [5,6].

Peroxidase involvement in wound-healing has been explained by its catalytic role in the cross-linking of pectins and structural proteins in the cell wall [7] and/or the polymerization of the phenolic monomers of lignin [8] and suberin [9]. Wound-inducible peroxidase genes have been reported in potato [10], and tomato [11]. In tomato, two peroxidase genes, *tap1* and *tap2* encoding anionic isoforms are expressed as a result of wounding in fruit, leaf and stem tissues [11,12]. *Tap1* transcripts could be detected 48 h after wounding, increase gradually to a maximum at 84 h, and subsequently decreased thereafter [12].

We have characterized two other peroxidase genes from tomato [13], that by primary sequence of the protein and by expression pattern are different from *tap1* and *tap2*. One of these genes, TPX1, is only expressed in roots throughout the development of tomato plants and is presumably involved in the deposition of suberin in this tissue [14].

In order to gain a broader insight in gene expression upon wounding, we decided to analyze the transcripts of two other genes, the proteinase inhibitor-II (PI-II) and the small subunit of the ribulose-1,5-bisphosphate carboxylase (SSU). Potato and tomato plants accumulate proteinase inhibitor II (PI-II) in leaves as a direct consequence of mechanical wounding or insect damage and this is considered to be a defense mechanism of plants against attacking insects [15].

We report that although the TPX1 transcript is constitutively present in roots, the transcript accumulation is wound-inducible in stems and the mid-rib of tomato leaves. We also report on changes in the relative activities of isoperoxidases already present in unwounded tissue and the appearance of a new activity band in the stem. A transient decrease in SSU transcripts is also detected upon wounding followed by a further recovery to normal levels.

2. Materials and methods

2.1. Plant material

Tomato (*Lycopersicon esculentum* cv. Pera) seedlings were grown in the greenhouse under a 14 h daily photoperiod. Two-month-old plants were used for the experiments.

2.2. Tissue wounding experiment

Stems of tomato plants were wounded by rolling a circular file over the surface of the stem. Leaves were also wounded by using a circular file with a wooden block under the leaf blade for support. This method allows sufficient crushing to release wound signals leaving enough intact cells among the broken material to express the wound-induced genes [12]. In the experiment to determine transcription levels away from the wound site in the stem, the injury was made by excising 5-mm wedges of the stem [16].

2.3. Preparation and analysis of RNA

RNA was extracted from tissue samples using the acid guanidinium thiocyanate–phenol–chloroform extraction protocol [14]. The procedure was modified and included an additional 2 M LiCl precipitation step followed by sodium acetate–ethanol precipitation. Poly(A)$^+$ RNA was separated from total RNA using oligo-dT cellulose [17]. RNA was estimated spectrophotometrically, separated on formaldehyde agarose gels, visualized with ethidium bromide, and transferred to a Hybond-N membrane (Amersham) by capillary transfer [17].

The ^{32}P-labeled probe was prepared by random primed labeling of the isolated cDNA inserts excised from TPX1 [13], PI-II [15] and 18S rRNA [18] clones. To obtain the SSU cDNA probe, poly(A)$^+$ extracted from tomato leaves was used as template and complementary DNAs

*Corresponding author.

SSDI 0014-5793(94)00542-4

were obtained using oligo-dT as a primer and MMLV reverse transcriptase. The SSU was amplified by PCR using a specific oligomer from the 5-terminus of the SSU and oligo-dT as primers [19], then filled with the Klenow fragment and cloned into the blunt end *Eco*RV site of the pBluescriptII SK(+) vector (Stratagene).

Filters were hybridized overnight in 6 × SSC, 2 × Denhardt's solution, and 0.1% SDS at 60°C. They were washed twice at room temperature for 15 min in 1 × SSC, 0.1% SDS and then 30 min in 0.2 × SSC, 0.1% SDS at 60°C.

2.4. Peroxidase extraction and isoelectric focusing

Leaf and stem tissues were frozen in liquid nitrogen and ground with a mortar and pestle, 0.75 g of the powder was extracted with 50 mM potassium phosphate buffer (fresh tissue/buffer ratio 1 : 3, w/v), pH 6.0, containing insoluble polyvinylpyrrolidone (0.08 g/ml) to immobilize phenolics and 1 M KCl to simultaneously extract soluble and ionically-bound peroxidases [20]. After dialysis the extracts were concentrated to ca. 200 μl by ultrafiltration.

Isoelectric focusing was performed in agarose plates in the pH range of 3 to 10 (FMC Bioproducts, Denmark). The samples were focused for 40–50 min following the manufacturer's instructions. The gels were then soaked for 20 min in 25 mM phosphate buffer, pH 6.0, containing 150 mM NaCl (PBS) to remove ampholines and equalize the pH [16]. Peroxidase isozymes were detected by soaking the gel in PBS wih 4-chloro-1-naphtol 3.3 mM, and 0.08% H_2O_2 as substrates. A similar pattern of bands was obtained using *o*-dianisidine as substrate. The volume of the electrophoresed sample corresponded to ca. 60 mg of fresh stem.

3. Results

A 1.3 kb band was detected in RNA from the wounded stem using the TPX1 insert (Fig. 1), whereas no transcript could be detected in tissues from unwounded or wound-induced leaves (Fig. 2). TPX1 transcript accumulation in stems was detected as early as 6 h after the injury. The levels of TPX1-specific transcripts reached a maximum by 24 h, then subsequently decreased and was undetectable 10 days after wounding.

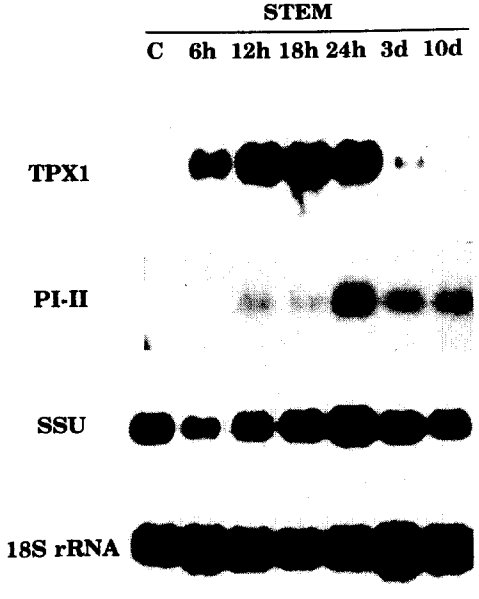

Fig. 1. Effect of wounding on TPX1, PI-II, and SSU transcript levels in tomato stem. Total RNA extracted from control unwounded (C), and wounded stem at 6 h, 12 h, 18 h, 24 h, 3 days (3 d), and 10 days (10 d) after the injury was analyzed by RNA blot hybridization with the probes indicated on the left.

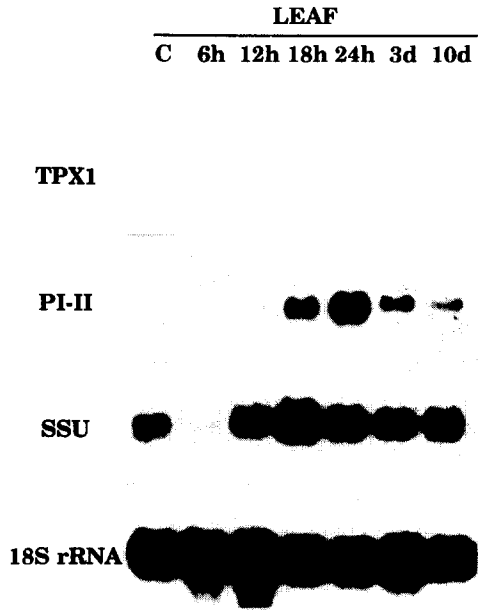

Fig. 2. Effect of wounding on TPX1, PI-II, and SSU transcript levels in tomato leaves. Total RNA extracted from control unwounded (C), and wounded leaf at 6 h, 12 h, 18 h, 24 h, 3 days (3 d), and 10 days (10 d) after the wounding was analyzed by RNA blot hybridization with the probes indicated on the left.

Reduced SSU mRNA levels were clearly detectable in leaves 6 h after wounding (Fig. 2). A slight decrease in the transcription level was also observed in the stem 6 h after the wounding (Fig. 1). The wounding-mediated initial decline in SSU message may constitute a common plant strategy of repressing those genes that are not directly involved in the plant defense response [1,2]. However, the latter higher level of transcription over the pre-wounding level, between 18 and 24 h after the physical damage, may constitute a plant strategy to recover a balanced cellular metabolism after the external stimuli have ceased.

PI-II mRNA was clearly detectable 12 h after wounding in leaves and its level gradually increased to a maximum by 24 h. Three and ten days after the wounding, PI-II mRNA was still clearly visible (Fig. 2). Transcripts from the wounded stems that hybridized to the PI-II probe were detected at 12 h and reached a maximum level after 24 h (Fig. 1).

Northern hybridization was also used to investigate the accumulation pattern of TPX1 transcripts at various distances from the site of physical damage. Tomato stems were wounded and 48 h later RNA was isolated from cross-sections of stem tissue harvested at 0, 1 and 2 cm from the wound site. The accumulation of TPX1 mRNA in the wound site was similar to that detected at increasing distances (1 and 2 cm) from the wound site (Fig. 3). As indicated above, there was no accumulation of TPX1 transcripts when the lamina of the leaf was

wounded. However, when the wound site of the leaf was the main vein, an accumulation of TPX1 mRNA was detected in similar amounts to that found in wounded stem (Fig. 3).

Isoelectric focusing of stem extracts after wounding revealed a decrease in neutral and weakly basic isozymes, pI range 6.5 to 8, followed by an increase in the acidic isoforms (Fig. 4). One unique peroxidase isozyme, pI close to 8.5, appeared 12 h after wounding and increased to a maximum at 24 h, being undetectable 10 days after the injury. The time course analysis of this isozyme correlates to the accumulation of TPX1 transcript and the pI value is also close to the theoretical pI deduced from the TPX1 sequence (7.5). Taken together, these data suggest that this new isozyme activity is the result of the wound-induction of the TPX1 gene.

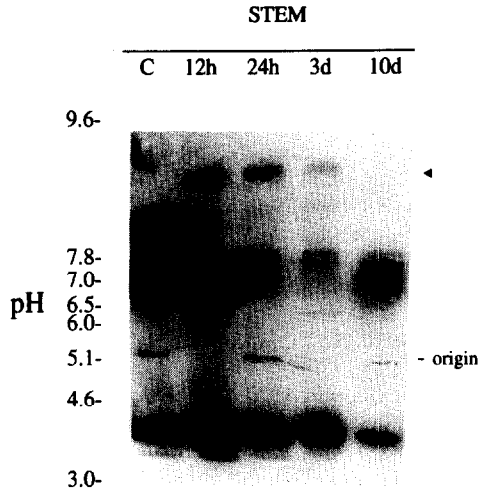

Fig. 4. Effect of wounding on isoperoxidase activity of tomato stem. Tissue extracts equivalent to 60 mg fresh weight tissue were obtained at 0, 12, 24 h, 3 d and 10 d after the wounding, applied to isoelectric focusing gels, and stained for peroxidase activity. Arrow indicates the new isozyme band detected after wounding.

4. Discussion

The peroxidase gene TPX1 exhibits an expression pattern which is subject to both developmental and environmental regulation. Under the developmental program TPX1 mRNA constitutively accumulates to detectable levels in tomato roots [14]. Accumulation occurs in the plant stem and main-vein of leaves only after mechanical wounding. Expression of TPX1 is likely the result of two separate signalling pathways similar to potato proteinase inhibitor [21,22].

The expression of defense genes is highly regulated in each tissue or cell type and specific members of a gene multifamily are differentially expressed in response to an external stimulus [23]. Tomato plants respond to wounding by inducing at least three different peroxidase genes, TPX1, *tap1* and *tap2*. TPX1 encodes for a cell-wall-targeted tomato peroxidase that is only 35 percent identical to *tap1* and *tap2* [13]. Moreover, pI values of proteins encoded by *tap1* and *tap2* are highly acidic [11]. Developmental and environmental regulation of TPX1 expression is also unique relative to *tap1* and *tap2*. Whereas TPX1 is constitutively expressed only in the roots [14] and in the stem in response to wounding (Fig. 2), *tap1* is constitutively expressed in the exocarp of maturing green tomato fruits [24] and both *tap1* and *tap2* are expressed in leaves, roots and fruits after wounding [12,25]. Furthermore, highest levels of TPX1 transcripts occurred around 24 h after wounding (Fig. 2) whereas maximum expression of *tap1* in the leaves of transgenic tobacco plants occurred 84 h after the physical damage [12,26]. These differences in both tissue expression and time course response to wounding between TPX1 and *tap1* and *tap2* may indicate a complex response of tomato plants to physical damage and the involvement of different signal transduction pathways.

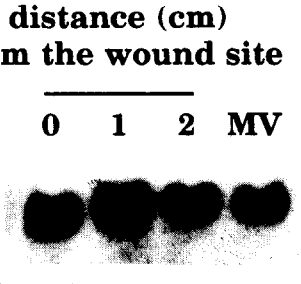

Fig. 3. Expression of TPX1 in the stem and the main vein of the leaf after wounding. One cm cross-sections of stem tissue at 0, 1, 2 cm from the wound site were harvested for RNA isolation two days after the injury. Total RNA was also extracted from the main vain (MV) of the leaf two days after wounding this tissue.

Basic information about the plant defense gene expression at the level of different isoforms must constitute an obligate step to further understand the whole plant response and its application to develop resistant organisms.

Acknowledgements: We wish to thank to Dr. Silvia Milrad de Forchetti and Ms. Iraida Amaya for collaboration in some experiments and Drs. Sánchez-Serrano and Josefa Pérez for providing us with the PI-II and SSU probes. We also would like to thank Dr. P.M. Hasegawa for critical reading of the manuscript and Mr. Rafael Cameselle for his technical assistance. M.A.B. was supported by a fellowship from the Dirección General de Investigación Científica y Técnica (DGICYT) Spain and M.I.M. by a postdoc fellowship from the CONICET, Argentina. Finantial support was obtained from DGICYT, grant No. AGR91-0858-C02-01.

References

[1] Peña-Cortés, H., Sánchez-Serrano, J., Rocha-Sosa, M. and Willmitzer, L. (1988) Planta 174, 84–89.
[2] Kombrink, E. and Hahlbrock, K. (1990) Planta 181, 216–219.
[3] Bowles, D. (1990) Annu. Rev. Biochem. 59, 873–907.
[4] Showalter, A.M. (1993) Plant Cell 5, 9–23.

[5] Choi, D., Bostock, R.M., Avdiushko, S. and Hildebrand, D.F. (1994) Proc. Natl. Acad. Sci. USA 91, 2329–2333.
[6] Pautot, V., Holzer, F.M., Reisch, B. and Walling, L. (1993) Proc. Natl. Acad. Sci. USA 90, 9906–9910.
[7] Fry, S.C. (1986) Annu. Rev. Plant Physiol. 37, 165–186.
[8] Lagrimini, L.M., Burkhart, W., Moyer, M. and Rothstein, S. (1987) Proc. Natl. Acad. Sci. USA 84, 7542–7546.
[9] Kolattukudy, P.E. (1980) Science 208, 990–1000.
[10] Espelie, K.E., Francheschi, V.R. and Kolattukudy, P.E. (1986) Plant Physiol. 81, 487–492.
[11] Roberts, E. and Kolattukudy, P.E. (1989) Mol. Gen. Genet. 217, 223–231.
[12] Mohan, R., Bajar, A.M. and Kolattukudy, P.E. (1993) Plant Mol. Biol. 21, 341–354.
[13] Botella, M.A., Quesada, M.A., Hasegawa, P.M. and Valpuesta, V. (1993) Plant Physiol. 103, 665–666.
[14] Botella, M.A., Quesada, M.A., Kononowicz, A.K., Bressan, R.A., Pliego, F., Hasegawa, P.M. and Valpuesta, V. (1994) Plant Mol. Biol., in press.
[15] Sánchez-Serrano, J., Schmidt, R., Schell, J. and Willmitzer, L. (1986) Mol. Gen. Genet. 203, 15–20.
[16] Lagrimini, L.M. and Rothstein, S. (1987) Plant Physiol. 84, 438–442.
[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Delcasso-Tremousaygue, D., Grellet, F., Panabieres, F., Ananiev, E.D. and Delseny, M. (1988) Eur. J. Biochem. 172, 767–776.
[19] Pichersky, E., Bernatzky, R., Tanksley, S.D. and Cashmore, A.R. (1986) Proc. Natl. Acad. Sci. USA 83, 3880–3884.
[20] MacAdam, J.W., Nelson, C.J. and Sharp, R.E. (1992) Plant Physiol. 99, 872–878.
[21] Sánchez-Serrano, J., Peña-Cortés, H., Willmitzer, L. and Prat, S. (1990) Proc. Natl. Acad. Sci. USA 87, 7205–7209.
[22] Lorberth, R., Dammann, C., Ebneth, M., Amati, S. and Sánchez-Serrano J. (1992) Plant J. 2, 477–486.
[23] Niebel, A., Almeida-Engler, J., Tiré, C., Engler, G., Van Montagu, M. and Gheysen, G. (1993) Plant Cell 5, 1697–1710.
[24] Sherf, B.A. and Kolattukudy, P.E. (1993) Plant J. 3, 829–833.
[25] Roberts, E., Kutchan T., and Kolattukudy, P.E. (1988) Plant Mol. Biol. 11, 15–26.
[26] Mohan, R., Vijayan, P. and Kolattukudy, P.E. (1993) Plant Mol. Biol. 22, 475–490.