

Expression of calmodulin and lipid transfer protein genes in *Prunus incisa* × *serrula* under different stress conditions

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Summary Calcium-binding proteins and pathogenesis-related (PR) proteins are crucial components of the inducible repertoire of plant stress and defence. Considering the important role played by calmodulin (CaM) and lipid transfer protein (LTP) in mediating plant signal transduction, the present study investigated the expression of *Ltp* and *CaM* genes in *Prunus incisa* × *serrula* (PIS) under various abiotic stress conditions. The aim of this study is to find out whether expression of these proteins is regulated in parallel or independently and to compare the expression profiles of *CaM* and allergenic proteins like *Ltp* under different stress conditions. Southern blot analyses indicated that *Ltp* and *CaM* are encoded by at least two to four genes, which might be indicative for the expected variability and presence of isoforms. Transcription levels of both genes were analysed in leaves and roots of micropropagated plantlets under low and high temperatures, salicylic acid and wounding stress, harvested after 0, 0.5, 1, 2, 4, 10, 24 and 72 h. Real Time qPCR data showed that both genes respond differently to various stresses. Furthermore, a high variation in transcription levels of both genes was observed in leaf tissues, while in roots both genes were expressed at a lower extent and down-regulated. Western blot analyses indicated that after 24 h the amount of CaM protein is higher, while the amount of LTP is lower in various stresses. Results obtained suggest that CaM and LTP are differentially regulated in response to different stresses in PIS plants, and additionally show tissue-specific expression, hinting at a potential role of different isoforms.

Keywords: allergens, gene expression, high temperature, low temperature, Real Time qPCR, salicylic acid, wounding.

Introduction

Plants have developed processes to overcome biotic and abiotic stresses by up- or down-regulating a number of genes/proteins, which are believed to have a role in different defence mechanisms (Cushman and Bohnert 2000, Hu et al. 2006). Among them, the production of calcium-binding and pathogenesis-related (PR) proteins in response to different stress stimuli makes them useful as marker genes/proteins for studying defence/stress responses and disease resistance in plants (Snedden and Fromm 2001, Phean-o-pas et al. 2005, Jwa et al. 2006, Jayaraj and Punja 2007).

Calmodulin (CaM), an ubiquitous Ca²⁺-binding protein, regulates the activity of many Ca²⁺-dependent cellular processes and target molecules involved in plant stress response. CaM is a small, 17 kDa, acidic protein, highly conserved and heat stable. It is expressed in many cell types and has different sub-cellular locations associated with the organelle membranes (Zielinski 1998, van der Luit et al. 1999, Lee et al. 2000, Camas et al. 2002, Bouché et al. 2005, Liu et al. 2005). In higher plants, unlike in animals, CaMs are present as multiple isoforms even within species. The CaM isoforms may activate selected subsets of target proteins and be induced by appropriate stimuli or physiological stresses in different tissues.

The PR proteins are classified into 17 families based on sequence, structure, function and biological activity (van Loon and van Strien 1999). Among them, the family of PR-14 proteins is characterized as non-specific lipid transfer proteins (nsLTPs), encoded by small multigene families in a diversity of plant species. Plant LTPs are small, soluble, basic proteins and are named due to their ability to transfer lipids through membranes (Arondel and Kader 1990, Kader 1996). These proteins, classified as plant panallergens resistant to protease and heat treatment (Asero et al. 2000, Herndl et al. 2007), are involved in plant defence and environmental stress response (Kader 1996, Arondel et al. 2000).

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So far, there have been few attempts to investigate *CaM* and *Ltp* genes in tree species. In fact, less information is available about the expression of both genes in trees compared to *Arabidopsis* or herbaceous crop species, which may be indicating the difficulties of molecular breeding and nucleic acid manipulation in trees. It is, therefore, tempting to find out whether expression of these two gene families in response to various stresses is regulated in parallel or independently, and whether expression levels vary in different tissues. Considering the important role of *CaM* and *LTP* in mediating plant stress responses, and based on the shortage of data in woody plants, *Prunus incisa* × *serrula* (PIS), Inmil^R was chosen as a model for fruit trees. PIS is a dwarfing cherry rootstock, with high commercial value, relatively small genome size (1C ~ 0.3 pg, diploid) and the close relationship to important *Prunus* species, allowing an understanding to be gained of the response of *Prunus* species to environmental stresses.

Materials and methods

Plant material

Embryogenic calli of PIS were induced on isolated roots of in vitro plantlets following the protocol of Druart and De Wulf (1993). Repetitive embryogenesis occurred spontaneously and was maintained over several months. Cultures were kept at 23 ± 1 °C either with a 16-h photoperiod provided by cool white fluorescent tubes with an irradiance of 60 µmol m⁻² s⁻¹ or in complete darkness and sub-cultured monthly on the basal medium. Plantlets were propagated on DKV medium (Driver and Kuniyuki 1984) with 1.5 mg l⁻¹ 2 ip riboside, 0.4 mg l⁻¹ BAP and 0.05 mg l⁻¹ IBA. When shoots reached 3 cm in height, plants were transferred to a rooting medium (RM; DKV macro- and microelements with 0.65% agar and 0.5 mg IAA).

Genomic DNA isolation and gel blot analysis

Total genomic DNA was extracted from 1 g of leaf tissue using DNeasy[®] Plant Maxi Kit (Qiagen, Hilden, Germany) following the supplier's instructions. Gel electrophoresis and spectrophotometry determined the DNA quality and concentration, respectively.

The following pairs of primers were used for the detection of the integrated genes: *CaM*-F and *CaM*-R (5'-ATGGCCGATCAGCTCACAGACGACCAGATC-3' and 5'-CTCACTTGGCCATCATGACTTTGACGAAC-3'), amplifying a ~ 1200 bp fragment of the *CaM* gene (GenBank Accession No. AF292108); *LTP*-F and *LTP*-R (5'-ATGGCTTGCTCTGCAATGA-3' and 5'-TCACTT CACGGTGGCGCAGTTG-3'), amplifying a 354 bp fragment of the *Ltp* gene (GenBank Accession No. AF221501).

The PCR amplifications were conducted in a total volume of 25 µl using 1× PCR buffer (Qiagen), 2 mM MgCl₂, 0.2 mM dNTPs, 4 pmol of each primer, 0.6 units

HotStarTaq Polymerase (Qiagen HotStarTaq[™] PCR) and 20–30 ng of total genomic DNA. The PCR-cycling conditions (Biometra) consisted of an initial denaturation step of 95 °C for 15 min followed by 35 cycles of 60 s at 95 °C, 60 s at annealing temperature (60 °C), and 2 min at 72 °C. A final step of 10 min at 72 °C ended the cycle.

For Southern blot analyses, 3 µg of genomic DNA was digested with 40 units of the restriction endonucleases *Pst*I and *Eco*RV (Roche) according to the procedure elaborated and described previously (Maghuly et al. 2007).

Stress treatments and sample collection

Three-centimeter-high shoots were transferred to Phytatray II (P5929-50EA SIGMA, St. Louis, MO) boxes containing 100 ml rooting medium (Druart 1990). The bottom of each box was covered with foil to induce vigorous and vital roots in darkness. After 6 weeks, when plantlets were growing under balanced physiological conditions, they were subjected to various stress treatments.

The low-temperature stress was applied by transferring the plantlets to a cold room at 4 °C in the dark. For high temperature stress, the plantlets were exposed to 37 °C in the dark. The sterile 5 mM salicylic acid solution was sprayed onto PIS plantlets at room temperature. For wounding stress, two leaves (one at the top and one at the bottom) of each plantlet were wounded with a needle, 3–4 times along the blade. Leaves and roots of four to five stress treated plantlets were harvested at various time intervals at 0, 0.5, 1, 2, 4, 10, 24 and 72 h. They were removed, immediately frozen in liquid nitrogen and stored at –70 °C until the isolation of total RNA.

Total RNA isolation and single-strand cDNA synthesis

Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) from 100 mg of leaf material according to the manufacturer's recommendations. Gel electrophoresis (1%, RNase-free) and spectrophotometry (260/280 nm) determined RNA quality and concentration.

The first-strand cDNA synthesis was performed on 2 µg total RNA treated with DNase I (Roche) using SuperScript[™] II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and oligo (dT)₁₈ according to the supplier's recommendation. The reverse transcription (RT) reaction was diluted 1/10 to reduce inhibitory effects of the RT buffer system.

Oligonucleotide primers and relative quantification of target genes using Real Time qPCR

Primers were designed according to the available sequences in the GenBank database using Lasergene 99 (DNASTAR Inc., Madison, WI). Primers ACTIN-Real-Left and ACTIN-Real-Right (5'-TCAATGTGCCTGCCATGTAT-3' and 5'-TGACTCACACCATCACCAGA-3'), amplifying a 103 bp fragment from different *Prunus* sp. (GenBank Accession No. AB188144, AF321852, AM491134) were used

as endogenous control. Primers CaM-Real-Left and CaM-Real-Right (5'-TCCGGGTGTTTGATAAGGAC-3' and 5'-CAGCCTCAGCAATCATCTCA-3') amplifying a 120 bp fragment were designed specific to the *CaM* gene from *Prunus avium* (GenBank Accession No. AF292108). Primers LTP-Real-Left and LTP-Real-Right (5'-GTGCCCATTGCTCAAGCC-3' and 5'-TCCACCGCCTCTCACGTAG-3') amplifying a 82 bp fragment were designed specific to the *Ltp* gene of different *Prunus* sp. (GenBank Accession Nos. BQ641140, X96714, AY792996, AY620230 and AF221501).

The Real Time PCR was carried out in the Rotor-Gene 2000 (Corbett Research) with a 72-well rotor. The amplification reactions were performed in 16 µl final volume containing 8 µl of a 2 × Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK), 0.96 µl of primer-pair mix (primer concentration: 300/300 nM) and 2 µl cDNA first-strand template. Thermal cycling conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The fluorescence was measured three times at the end of the extension step at 72 °C, 76 °C and 81 °C. Subsequently, melting curves were recorded between 65 and 99 °C with the hold every 2 s. All reactions were run in triplicate.

Statistical analysis

Quantitative PCR was carried out and analyzed with the software tool REST (Pfaffl et al. 2002). The mathematical model in REST used to calculate the relative expression ratio is

$$(E_{\text{target}})^{\Delta C_t \text{ target}(\text{control}-\text{sample})} / (E_{\text{ref}})^{\Delta C_t \text{ ref}(\text{control}-\text{sample})}$$

The relative expression ratio of a target gene is calculated based on its Real Time PCR efficiencies (E) and the crossing point (C_t) difference (Δ) of an unknown sample versus a control ($\Delta C_{t\text{control}-\text{sample}}$). The Real Time PCR efficiency was calculated from the slope of the standard curve, according to the established equation $E = 10^{[-1/\text{slope}]}$. Significance of expression ratios was tested by performing 10^4 randomization with the pair-wise reallocation test.

Protein extraction

Powdered leaf material was extracted by a one-step precipitation using 10% TCA/acetone and 1% PVPP incubated overnight at −20 °C. After precipitation, the protein pellet was mixed with 100% acetone and incubated for 1 h at −20 °C followed by a centrifugation step. The pellet was washed once again with 100% acetone and dried at room temperature. The obtained pellet was resolved in 8 M urea and the total protein content was determined using the BCA Protein Assay Kit (Pierce, Rockford), according to the manufacturer's instruction.

SDS-PAGE and Western blotting of PIS CaM and LTP

Samples containing 21 µg of total protein were diluted in sample buffer containing 0.125 M Tris-HCl, pH 6.8,

20% v/v glycerol, 2% w/v SDS and 0.005% v/v bromophenol blue in the ratio 1:2 and denaturated for 1 h at room temperature. Samples were loaded on 4–20% gradient gels (4–20% Tris-glycine gel; Invitrogen, Carlsbad), and electrophoresis was carried out on the Novex System (Invitrogen). Electroblotting was performed on the XCell II Blot Module (Invitrogen) using a buffer containing 50 mM $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.1% w/v SDS and 20% v/v methanol. Nitrocellulose membranes (Biorad) were used as blotting membranes and were blocked overnight with 3% skim milk powder.

Western blot analyses were performed using commercial mAbs against CaM (Sigma) or a rabbit pAb against Mal d 3, the LTP in apple (Herndl et al. 2007) as primary antibodies. An AP-conjugated mouse anti-rabbit-IgG (Sigma) was used as a secondary antibody. Protein bands were visualized by bromochloroindolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Sigma) in alkaline phosphate buffer (100 mM NaCl, 5 mM MgCl_2 and 100 mM Tris). Pre-stained Precision Plus Protein Standard (Biorad) was used as a molecular weight marker. CaM isolated from bovine brain (Sigma) and apple LTP (Herndl et al. 2007) were used as positive controls.

Results

Genomic organization of CaM and Ltp genes

The PIS plants were analyzed by PCR for the detection of the *CaM* and the *Ltp* genes. All samples produced fragments of the expected sizes for both genes (data not shown). Southern blot analysis was performed to investigate the number of genes corresponding to each *CaM* and *Ltp* gene in the genome. Genomic DNA was digested with *Pst*I and *Eco*RV, and the resulting fragments were hybridized with the *CaM* and *Ltp* probes. *CaM* hybridized to two (with *Pst*I) or four fragments (with *Eco*RV) and *Ltp* hybridized to two fragments in the digested genomic DNA (Figure 1). These results suggest that each of the genes is present in several copies in the PIS genome.

Expression of CaM and Ltp genes under various stresses

To determine how mRNA levels of *CaM* and *Ltp* genes in PIS plants responded to different stresses, the expression on both genes was analyzed using Real Time qPCR in relation to the amount of *Actin* mRNA. Plantlets were subjected to four stress conditions (high and low temperature, salicylic acid and wounding) and were collected at eight different time intervals (0, 0.5, 1, 2, 4, 10, 24 and 72 h). The time point '0' represents unstressed plantlets. The PCR efficiency for *Actin* amplification was 90% with correlation coefficients (R^2) = 0.99, for *Ltp* 89% with R^2 = 0.997 and for *CaM* 100% with R^2 = 0.996.

All four stress conditions altered the expression of both genes. Relative gene expression analyses showed a lower *Ltp* expression, when compared to unstressed plantlets after

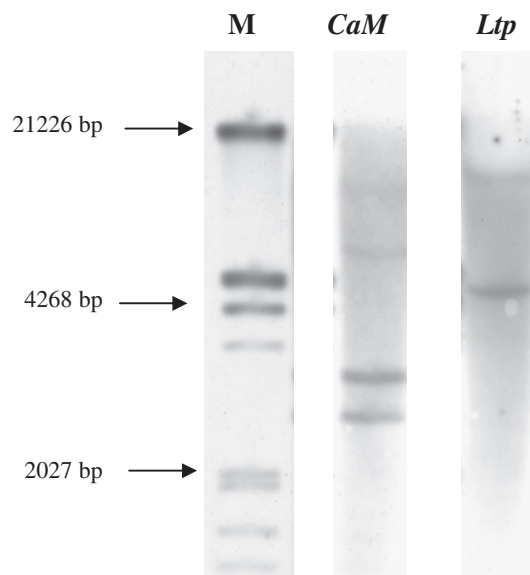


Figure 1. Southern blot analyses of DNA from PIS plants digested with *EcoRV*. *CaM*: DNA was probed with *CaM*. *Ltp*: DNA was probed with *Ltp*. M: DIG-labeled DNA Molecular weight Marker III (Roche).

different treatment times. *CaM* mRNA was significantly up-regulated in leaves (Figure 2A1–A4) and down-regulated in roots (data not shown), whereas *Ltp* mRNA was down-regulated in both leaves (Figure 2B1–B4) and roots (data not shown) under all stresses and at all time intervals. The obtained data indicate that both genes are differentially regulated by different stress conditions.

The expression level of *CaM* strongly increased after 0.5 h (7.3 times, $P = 0.001$) under low-temperature stress (4 °C) treatments in leaves (Figure 2A2), while the level of *Ltp* mRNA strongly dropped during the first 0.5 h after salicylic acid and wounding stresses (4.4 and 6.9 times, respectively, $P = 0.001$) (Figure 2B3 and B4).

The expression of *CaM* mRNAs was strongly up-regulated at all time points and reached a maximal level after 4 h (9.8 times, $P = 0.001$) under low-temperature stress (4 °C), when compared to other stresses. However, gene expression decreased significantly at 24 and 72 h (8.1 and 7.8 times, respectively, $P = 0.001$) (Figure 2A1). In comparison, the expression of *Ltp* mRNAs was down-regulated at all time points and reached minimal levels after 24 h (24.4 times, $P = 0.001$) under low-temperature stress (Figure 2B1).

The expression patterns of *CaM* and *Ltp* mRNAs were investigated under high temperature (37 °C). Incubation of plantlets at 37 °C showed significant down-regulation of *CaM* mRNAs at 4 h (1.4 times, $P = 0.001$) and up-regulation at 72 h (1.3 times, $P = 0.001$) (Figure 2A2). In the same experiment, *Ltp* mRNA expression was significantly down-regulated between 0.5 to 4 h (1.6–20.8 times, $P = 0.001$) under high temperature. Incubation of the

plantlets at 37 °C induced only a slight change in *Ltp* gene expression during 4–72 h and reached minimal levels after 24 h (26.3 times, $P = 0.001$) (Figure 2B2).

CaM mRNA expression significantly increased during the first 24 h (from 1.3 times at 0.5 h to 3.2 times at 24 h, $P = 0.001$) after salicylic acid application. However, at 2 h and 10 h the *CaM* expression showed a significant decrease (2.2 and 2.4 times, $P = 0.001$ and 0.033, respectively) (Figure 2A3). On the other hand, the patterns of accumulation for *Ltp* mRNA showed significant down-regulation during the first 10 h (from 4.4 times after 0.5 h to 13.9 times after 10 h) and were slightly altered during 10–72 h (4.5 times, $P = 0.001$) (Figure 2B3).

As shown in Figure 2A4 wounding stress up-regulated the *CaM* mRNA level within the first 4 h (4.1 times, $P = 0.033$). However, *CaM* expression showed a significant decrease at 10 h (1.7 times, $P = 0.001$). In contrast, the *Ltp* mRNA level was strongly down-regulated at two time points after wounding stress: at 0.5 h (7 times, $P = 0.001$) and at 72 h (33.3 times, $P = 0.001$) and significantly changed at time points 1 and 2 h (1.3 and 1.35 times, respectively, $P = 0.001$) (Figure 2B4).

CaM and *LTP* protein levels under various stresses

Western blot analyses demonstrated the presence of the CaM and the LTP in leaves of PIS plants under normal physiological conditions. Western blot results indicate that the amount of CaM protein increased during 24 h and decreased after 72 h under low temperature, salicylic acid and wounding stresses when compared to unstressed plantlets (0 time) (Figure 3a, c and d). However, plantlets treated by high temperatures showed a very low expression (Figure 3b). The amount of LTP decreased during 24 h under low- and high-temperature and wounding stresses (Figure 3e, f and h), while after salicylic acid application a very low expression was found (Figure 3g). Furthermore, after 3 days, the amount of LTP increased upon low temperature and wounding (Figure 3e and g) and decreased under high temperature stress (Figure 3f).

Discussion

Southern blot analyses confirmed that two to four copies of *CaM* and two copies of *Ltp* are present in PIS plants. The obtained results are in agreement with previous findings that plants contain several isoforms of CaM and LTP encoded by multigene families (Kader 1996, Vignols et al. 1997, Arondel et al. 2000, Zielinski 2002, Folzer et al. 2005, Wang et al. 2008). Previous studies also revealed five *CaM* genes in soybean (Lee et al. 1995), eight genes in potato (Takezawa et al. 1995), 13 genes in tobacco (Yamakawa et al. 2001) and nine genes in *Arabidopsis thaliana* (L.) Heynh. (Zielinski 2002). At least three *Ltp* genes were reported in *Oryza sativa* L. and *Capsicum annuum* L. (Vignols et al. 1994, Jung et al.

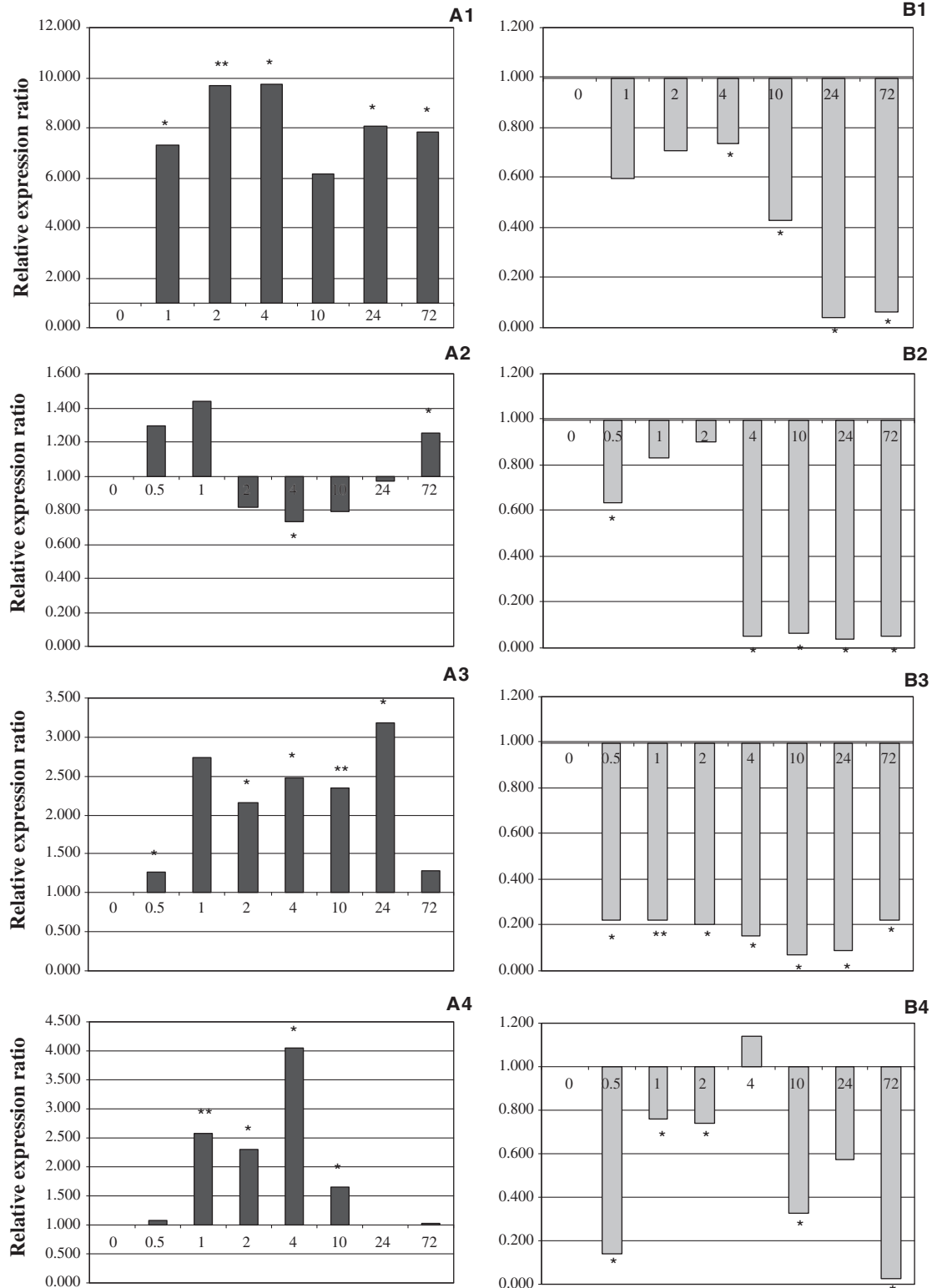


Figure 2. Expression profiles of (A1–4) *CaM* and (B1–4) *Ltp* mRNA from PIS plants assayed at 0, 0.5, 1, 2, 10, 24 and 72 h after the start of (A1–B1) low temperatures (4 °C), (A2–B2) high temperatures (37 °C), (A3–B3) salicylic acid and (A4–B4) wounding. Significance is indicated as * $P = 0.001$ and ** $P = 0.03$. All expression values are normalized to the expression value of *Actin*. Relative expression is calibrated based on the corresponding gene expression in plantlets collected at '0' time.

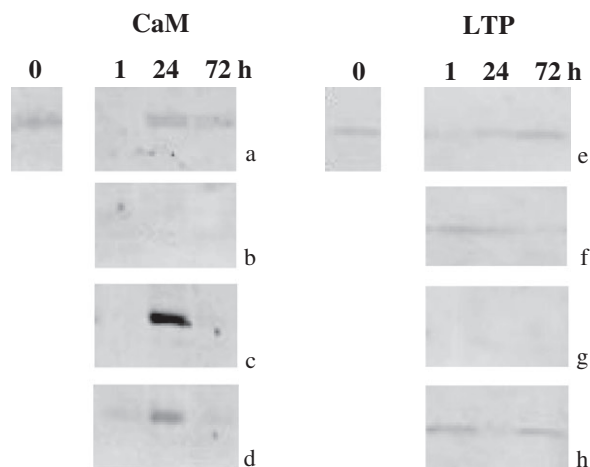


Figure 3. Immunoblot detection of CaM and LTP protein levels from PIS plants assayed at 0, 1, 24 and 72 h after the start of (a and e) low temperatures (4 °C), (b and f) high temperatures (37 °C), (c and g) salicylic acid and (d and h) wounding.

2003) and five genes in *Sorghum vulgare* Pers. (Pelèse-Siebenbourg et al. 1994).

Due to the missing genomic DNA sequence of PIS and considering the high similarities between different *Prunus* sp., novel primers for gene expression studies were designed. Furthermore, based on the conserved regions of *CaM* and *Ltp* available in the GenBank, most gene family members could be recognized. Results indicate that both genes are expressed at significant levels in all vegetative tissues, under various stress conditions and at different time intervals. In general, the expression of *CaM* genes in leaves is up-regulated, while *Ltp* genes are down-regulated, whereas, in roots both genes are down-regulated. Although *CaM* isoforms are ubiquitously expressed, their tissue-specific function can be achieved through interacting proteins, accumulating in a tissue-specific pattern (Reddy et al. 2002). Expression levels of *CaM* were described to differ in various plant tissues, indicating different functions (Zielinski 1998, Lee et al. 2000, Camas et al. 2002, McCormack et al. 2005). The levels of *CaM* mRNA increased in the meristematic zones of barley leaves compared with mature leaves and roots (Zielinski 1998). The expression of *Ltp* genes was demonstrated to be organ and tissue specific in several plant species (Sterk et al. 1991, Clark and Bohnert 1999, Jung et al. 2003). The expression of *Ltp* genes was found in certain tissues during the development of aerial parts and in some root tips, but was not detected in entire roots of different plants (Sterk et al. 1991, Thoma et al. 1994, Kader 1996, Vignols et al. 1997, Sohal et al. 1999). Soufleri et al. (1996) reported that three genes encoding *LTP*₁ in *Brassica napus* were barely detected in seedling roots and not detected in roots of mature plants. In *C. annuum*, *CALTPH* was detected in leaves, while *CALTPI* was detected in flowers, and neither of them was expressed in roots (Jung et al. 2003). Suelves and Puigdomènech (1997) also reported that three *Ltp* genes

were strongly expressed in flowers, while undetectable in roots and leaves of *Prunus amygdalus*. In rice, three *Ltp* genes presented faint bands in roots (Vignols et al. 1997).

The high variations in expression of *CaM* under different stress conditions suggest an important protective role and functions for *CaM* in PIS plants. Highly conserved *CaM* isoforms may actually modulate their target proteins differentially. In *Arabidopsis*, *CaM2* was shown to stimulate NAD kinase more effectively than *CaM4* and *CaM6* (Liao et al. 1996). Previous results indicate that although the expression of genes encoding different *CaM* isoforms is ubiquitous, they are differentially regulated by various stress signals (Heo et al. 1999, Yamakawa et al. 2001, Liu et al. 2005, Phean-o-pas et al. 2005). The PIS *CaM* transcript was also highly expressed under different stress conditions at 24 h, which might be correlated to an established plant protection mechanism. Competition among *CaM* isoforms for target proteins may occur, and therefore different transcriptional regulation can significantly affect the ratio of *CaM* isoforms, which means more 'active' *CaM* isoforms may exist in a cell responding to a particular stimulus (Phean-o-pas et al. 2005).

Although a regulation of *Ltp* gene expression by biotic and abiotic stimuli was reported, scarce information on the effects of important environmental factors like wounding, cold and high stress on *Ltp* expression is available (Sohal et al. 1999, Yubero-Serrano et al. 2003). In this study, the expression of *Ltp* in leaves of PIS plantlets was down-regulated after several hours under various stress treatments. In comparison, *LTP* transcripts were observed at a lower level under different stress conditions, especially after salicylic acid treatment, which corresponds to the down-regulation of mRNAs. Previous works indicate that certain *nsLTP* genes are inducible by salinity in tomato, barley and pepper, by drought in tomato and pepper and by wounding in pepper. However, no response to cold stress has been observed in barley *nsLTP* (Torres-Schumann et al. 1992, Molina and Garcia-Olmedo 1993, Trevino and O'Connell 1998, Jung et al. 2003). In leaves of cowpea seedlings the expression of the *VULTP* gene underwent small variation under different stress conditions (Carvalho et al. 2006). In *C. annuum* plants inoculated with *Xanthomonas campestris* pv. *vesicatoria* and TMV the *Ltp* gene expression increased after 12 and 24 h, reached a maximal level at 24 and 48 h, but decreased at 36 and 72 h, respectively (Park et al. 2002).

The wide distribution of LTPs in plants suggests a potential role of these proteins as plant panallergens (Ballmer-Weber 2002). Kader (1996) found that LTP-like proteins present in wheat, barley, pine and *Petunia* are the substrate for Ca^{2+} -dependent protein kinases. Recently, in the effort to characterize plant *CaM*-binding proteins, Liu et al. (2001) and Wang et al. (2005, 2008) demonstrated that some *nsLTP* and LTP-like proteins (*CaMBP-10*) in Chinese cabbage and *Arabidopsis* may have *CaM*-binding properties.

The different expression levels of *CaM* and *Ltp* genes observed suggest that these two genes may play an important but independent role in the adaptation of plants to environmental stresses. However, the expression of *Ltp* is regulated by different factors than *CaM* under stress-response conditions in *Prunus*. However, further studies will be performed to assess the full role of CaM and LTP, as well as to understand the function of individual isoforms.

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