The potato *StLTPa7* gene displays a complex Ca^{2+} -associated pattern of expression during the early stage of potato–*Ralstonia solanacearum* interaction

GANG GAO^{1,2}, LI PING JIN¹, KAI YUN XIE¹ AND DONG YU QU^{1*}

¹Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China

²College of Life Science, Shanxi Normal University, Linfen 041004, China

SUMMARY

Although nonspecific lipid transfer proteins (nsLTPs) are widely expressed during plant defence responses to pathogens, their functions and regulation are not fully understood. In this article, we report the isolation of a cDNA for the new nsLTP, *StLTPa7*, from cultivated potato (*Solanum tuberosum*) infected with *Ralstonia solanacearum*. The cDNA was predicted to encode a type 1 nsLTP containing an N-terminal signal sequence and possessing the characteristic features of nsLTPs. A phylogenetic analysis showed that the encoded amino acid sequence of the nsLTP was similar to those of other previously reported plant nsLTPs, which contain a putative calmodulin-binding site consisting of approximately 12 highly conserved amino acid residues. The expression of the *StLTPa7* gene was studied during the early stages of potato–*R. solanacearum* interaction using real-time quantitative polymerase chain reaction (qRT-PCR) and Northern analyses, and a complex calcium (Ca^{2+})-associated pattern of expression was observed with the following features: (i) transcripts of the *StLTPa7* gene were systemically up-regulated by infection with *R. solanacearum*; (ii) the *StLTPa7* gene was stimulated by salicylic acid, methyl jasmonate, abscisic acid and Ca^{2+} ; (iii) qRT-PCR showed that, during the early stage of *R. solanacearum* infection, nsLTP transcripts accumulated over a time course that paralleled that of Ca^{2+} accumulation, detected using environmental scanning electron microscopy and energy-dispersive X-ray (EDAX) spectrometry; and (iv) the Ca^{2+} channel blocker, ruthenium red, partially blocked *R. solanacearum*-induced *StLTPa7* expression. This report represents the first use of EDAX analysis to establish a synchrony between Ca^{2+} accumulation and nsLTP expression in response to potato–*R. solanacearum* interactions. Collectively, these results suggest that *StLTPa7* may be a pathogen- and Ca^{2+} -responsive plant defence gene.

INTRODUCTION

Plant nonspecific lipid transfer proteins (nsLTPs), which were originally defined by their capacity to facilitate the transfer of phospholipids between a donor and an acceptor membrane *in vitro* (Kader *et al.*, 1984), are found throughout the plant kingdom, and are represented by more than 100 potential members from as many as 50 different species (Jose-Estanyol *et al.*, 2004). nsLTPs are small, cysteine-rich peptides encoded by a multigene family, and can be subdivided into three subfamilies on the basis of their primary structure (Boutrot *et al.*, 2007; Douliez *et al.*, 2000). Type 1 (nsLTP1) and type 2 (nsLTP2) nsLTPs have molecular masses of approximately 9 and 7 kDa, respectively, and are basic, with isoelectric points (pI) between 8.8 and 10 (Kader, 1997). Type 3 nsLTPs also have a molecular mass of approximately 7 kDa, but their genes are expressed in an anther-specific manner (Lauga *et al.*, 2000). Plant nsLTPs are often purified as several isoforms with very similar sequences (Capocchi *et al.*, 2005; Chae *et al.*, 2007). Unlike individual LTPs, nsLTPs isolated as a mixture exhibit a broad range of substrate specificities, and are capable of transferring several classes of phospholipids and/or glycolipids (for a review, see Carvalho and Gomes, 2007; Salcedo *et al.*, 2007).

The three-dimensional structures of nsLTPs from many plant species, including rice (*Oryza sativa*), maize (*Zea mays*) and barley (*Hordeum vulgare*), show a globular molecule composed of four α -helices and a C-terminal tail (Heinemann *et al.*, 1996; Samuel *et al.*, 2002; Shin *et al.*, 1995). The four helices, which are stabilized by four disulphide bridges, form a hydrophobic cavity that is the binding site for fatty acids, acyl-coenzyme A (acyl-CoA) and phospholipids. This hydrophobic cavity runs throughout the otherwise hydrophilic molecule and is a remarkable feature, capable of adapting its volume to bind one or two monoacyl lipids, diacylated lipids and a wide variety of hydrophobic molecules (Carvalho and Gomes, 2007; Hamilton, 2004; Salcedo *et al.*, 2007). Although many LTPs can bind lipids *in vitro*, no LTP has been shown to do so *in vivo*. The interaction between a wheat (*Triticum aestivum*) nsLTP and phospholipids has indicated

*Correspondence: E-mail: ggniche@126.com

that nsLTPs cannot load lipids from intact membranes under normal physiological conditions (Blein *et al.*, 2002).

Despite the many studies on nsLTPs, no direct evidence has been provided for most of the suggested functions for this class of proteins, and their *in vivo* role remains unclear. Accumulating evidence, however, points to a role for nsLTPs in plant defence mechanisms against the attack of bacteria, fungi and, possibly, viruses (for a review, see Salcedo *et al.*, 2007). In addition, several members of the nsLTP family have been identified as relevant allergens in plant foods and pollens. Their high resistance to both heat treatment and digestive proteolytic attack has been related to the ability of these allergens to induce severe symptoms in many patients (Borges *et al.*, 2006; Salcedo *et al.*, 2007). It has been reported that LTP is involved in long-distance signalling during the acquisition of systemic acquired resistance in *Arabidopsis thaliana* (Maldonado *et al.*, 2002). In addition, a role for nsLTP as a signal mediator involved in the control of plant defence responses has been suggested (Blein *et al.*, 2002; Buhot *et al.*, 2001, 2004).

nsLTP genes are expressed at different levels in different plant tissues during diverse developmental stages and under various physiological conditions. In potato (*Solanum tuberosum*), nsLTP family genes are expressed throughout the developing plant in a highly tissue-specific manner. Several plant hormones, such as abscisic acid (ABA), salicylic acid (SA), ethylene and methyl jasmonate (MJ), are involved in the signalling pathway responsible for the regulation of the expression of nsLTP genes (Jung *et al.*, 2003, 2005, 2006; Lu *et al.*, 2006). Recent reports have identified two transcript-derived fragments of nsLTPs in potato that display modulated expression profiles associated with the development of new tissues, exhibiting a peak of transcription around the time of tuberization and just prior to sprout development, at dormancy breakage (Horvath *et al.*, 2002). One of these nsLTP fragments was selectively expressed in the phloem surrounding the nodes of young plants and in the same tissue of tuber-related organs, whereas the other fragment showed little tissue or organ specificity. These expression profiles suggest that nsLTPs have multiple functions in the developing tubers. Three drought-responsive members of the nsLTP gene family in *Lycopersicon pennellii* (*LpLtp1*, *LpLtp2* and *LpLtp3*) show differential developmental expression patterns (Treviño and O'Connell, 1998). All of these genes are expressed exclusively in the aerial tissues of the plant and are inducible by drought and ABA. However, each gene shows a different pattern of expression in fruit, and *LpLtp1* and *LpLtp2*, unlike *LpLtp3*, are both primarily developmentally regulated in leaf tissue. Putative ABA-responsive elements are found in the proximal promoter regions of *LpLtp1* and *LpLtp2*.

Taken together, these observations suggest that distinct biological functions of nsLTPs may be responsible for different biotic and abiotic stress conditions. An understanding of the increasingly

complex view of potential nsLTP functions requires a more complete characterization of the expression pattern of each nsLTP gene. In this study, we investigated the expression of *StLTPa7* in potato during the early phase of the response to *Ralstonia solanacearum* infection.

RESULTS

Identification and molecular cloning of *StLTPa7* cDNA

To identify the genes induced by *R. solanacearum* during the early stage of pathogen–potato interactions, a cDNA library was constructed by suppression subtractive hybridization using mRNA isolated from stems of inoculated and mock-inoculated cultivar 'zhong 3' as tester and driver, respectively. Of the clones obtained, that corresponding to an open reading frame coding for a plant nsLTP with a 3'-untranslated region was selected for further study. Using the 5' rapid amplification of cDNA ends (RACE) procedure, a clone encoding the full-length cDNA for *StLTPa7* (694 bp, accession no. EU057712) was obtained. The deduced amino acid sequence of *StLTPa7* revealed a 114-amino-acid protein that shared a number of features with the LTP1 family (Kader, 1996), including a high isoelectric point ($pI = 9.40$), a molecular mass of approximately 10 kDa and an N-terminal signal peptide (Fig. 1A). The mature *StLTPa7*, without the 23-amino-acid signal peptide (predicted by SignalP; <http://www.cbs.dtu.dk/services/SignalP>), was composed of 91 amino acids. Like *LpLTP1* and *LpLTP2* (Shin *et al.*, 1995; Yamada, 1992), *StLTPa7* has eight cysteines that are predicted to form four disulphide bridges, shown in other LTPs to be important for structure and function (Takishima *et al.*, 1988), and four proline residues that are highly conserved amongst LTPs. The deduced amino acid sequence of *StLTPa7* showed a similarity to the nsLTPs of other plants, including *Arabidopsis* and rice.

Recently, the calmodulin (CaM)-binding site in *Arabidopsis* nsLTP1 has been mapped to the C-terminal region of the protein, corresponding to amino acids 69–80. This region is highly conserved amongst plant nsLTPs, indicating that nsLTPs are a new family of CaM-binding proteins that may function through CaM-mediated signalling (Wang *et al.*, 2005). A comparison of the *StLTPa7* sequence with that of *Arabidopsis* nsLTP1 and other nsLTPs shows that *StLTPa7* has remarkable homology in this region, especially with respect to hydrophobic residues (Fig. 1B). The positively charged residues on the molecular surface of *StLTPa7* are structurally similar to those in other plant defence proteins, such as antifungal protein from radish and pokeweed (Samuel *et al.*, 2002). Although experimental evidence is lacking, the asymmetric distribution of basic residues in the *StLTPa7* three-dimensional structure strongly suggests a determinant for antimicrobial activity. The Corey–Pauling–Koltun model of *StLTPa7* shows a surface with a large concentration of positively

charged residues, very similar to the surface of some plant defence peptides (Fig. 1C), consistent with a role for *StLTPa7* as a defence against pathogens at the cell surface. One possible function of this protein would be to transport lipids to the plasma membrane, where they might remain to defend against cell surface pathogens.

A general view of the evolutionary history of the Solanaceae nsLTP family is provided by an unrooted phylogenetic tree constructed by the neighbour-joining method using the mature amino acid sequences available in GenBank (Fig. 2). The resulting tree shows three distinct phylogenetic groups for the LTP amino acid sequences. Grouping of nsLTPs into type 1 and type 2 is consistent with previous reports. Interestingly, potato nsLTPs are found in most branches of the phylogenetic tree, but are conspicuously absent from group II. They cluster into six distinct subgroups, with most potato nsLTP1 and nsLTP2 genes segregating into groups I and III, respectively. Notably, the nsLTP2 (BAC23052) sequence is located at a long phylogenetic distance from the others. The phylogenetic analysis reveals that at least five nsLTP1 gene subfamilies may exist in the potato genome. It also implies that the *StLTPa7* gene may have a common ancestor with *Solanum lycopersicum*.

StLTPa7 expression in response to pathogen infection

Root infection and systemic colonization were evaluated by environmental scanning electron microscopy (ESEM) and determination of the pathogen population size. The results showed that pathogenic bacteria were undetectable in the upper and lower stems and leaves up to 3 days post-infection (dpi; Fig. 3A–C), but a very small population of bacteria was detectable in the vascular bundles of the upper stems at 5 dpi (Fig. 3B), and a number of bacteria were present in lower tissues (Fig. 3D). To determine whether *StLTPa7* was systemically induced by pathogen infection, samples were collected from the lower (infection site, between second and third nodes) and upper (systemic site, between sixth and seventh nodes) stems and leaves at different times after infection for RNA gel blot analysis of *StLTPa7* gene transcription patterns. As shown in Fig. 3E, F, *StLTPa7* transcripts were induced by 3 dpi. The *StLTPa7* transcripts accumulated rapidly and strongly in the total samples of both stems and leaves collected at 3–5 dpi (Fig. 3G). The levels of *StLTPa7* mRNA were similarly increased in the lower and upper stems (Fig. 3E,F). These results indicate that *StLTPa7* induction is systemic, with considerable increases in expression occurring in

Fig. 2 Phylogenetic relationships among Solanaceae nonspecific lipid transfer proteins (nsLTPs) based on sequence alignments of the mature encoded proteins. The analysis was based on the alignment of amino acid sequences indexed in GENBANK databases. The accession numbers for the protein sequences used to compile the tree are shown following the specific name. The evolutionary tree was constructed by the neighbour-joining method and drawn by the MEGA program. The scale bar corresponds to 0.1 substitution per amino acid.

sites far removed from the local site of pathogen colonization. Although *StLTPa7* transcripts accumulated in the upper tissues by 3 dpi, there was no bacterial colonization of the vascular system by this point, suggesting that the expression of the *StLTPa7* gene was not a direct response to colonization of the bacteria in stem vascular tissue (Fig. 3B). This raises the possibility that the signal that mediates the activation of the *StLTPa7* gene in the upper stems and leaves is communicated at a distance.

Energy-dispersive X-ray (EDAX) analysis and induction of *StLTPa7* expression by CaCl_2

As earlier research on cabbage (*Brassica oleracea*) has shown that the cryoprotective activity of nsLTPs is strictly dependent on

Ca^{2+} (Hinch *et al.*, 2001), we investigated the changes in Ca^{2+} concentration ($[\text{Ca}^{2+}]$) by microscopic analysis and X-ray microanalysis. The advent of ESEM has revolutionized the observation of biological samples, enabling the direct examination of biological specimens in an aqueous environment (Cohen *et al.*, 2003). The chemical elements in living specimens were analysed by ESEM using an associated energy-dispersive microanalyser (Quanta 200FEG, FEI), with the electron beam focused on the location at which the elemental composition was to be determined. Because of the interaction between the primary electron and the sample, X-ray signals were collected with an EDAX analysis spectrometer, connected to ESEM. Stem tissues of living potato plants inoculated with *R. solanacearum* were used to obtain an area-restricted EDAX spectrum. Figure 4 shows that Ca^{2+}

Fig. 3 *StLTPa7* gene expression patterns induced in potato by *Ralstonia solanacearum* (virulent isolate PO41, race 3, biovar 2) at different stages of infection. (A, B) Population sizes of strain PO41 in the lower (A) and upper (B) stem tissue at different stages of infection. Above-ground stem tissue was sampled by grinding and the effluent water was directly dilution plated. The statistical significance was calculated using Student's *t*-test ($P < 0.05$). Triplicate experiments were tested using three plants from each experiment. (C, D) Environmental scanning electron micrographs showing colonizing bacteria in the vascular tissue of the lower (D) but not upper (C) stems after 3 days post-injection (dpi) (vertical section). Arrows indicate bacteria. Scale bars, 10 μm . (E, F) Histograms indicating the mRNA levels in the lower (between second and third nodes, E) and upper (between sixth and seventh nodes, F) stem tissue at various times after inoculation, determined by real-time quantitative polymerase chain reaction (qRT-PCR). The values were normalized to *actin* at each time point. The statistical significance for the observed up-regulation was calculated using Student's *t*-test. The experiments were repeated three times with similar results. Error bars represent the mean $\pm$ standard error ($P < 0.01$). (G) Northern analysis of *StLTPa7* gene expression after inoculation with the pathogen. The constitutive gene *actin* was used as a control. Experiments were repeated twice. A representative experiment is shown.

accumulation occurred in synchrony with *R. solanacearum*-induced expression of the *StLTPa7* gene. EDAX showed that Ca^{2+} was localized predominantly in the phloem cells of *S. tuberosum*. The peak in $[\text{Ca}^{2+}]$ was coincident with the point at which the accumulation of *StLTPa7* transcripts started to reach a maximum (Fig. 3). A strikingly high $[\text{Ca}^{2+}]$ was found in the region of vascular cells at 3 dpi (Fig. 4C,D), but smaller $[\text{Ca}^{2+}]$ peaks were present at earlier time points. The fact that increases in $[\text{Ca}^{2+}]$ were apparent in vascular tissues from inoculated, but not

mock-inoculated, plants suggests that the pathogen may perturb Ca^{2+} metabolism.

The co-accumulation of Ca^{2+} and *StLTPa7* transcripts predominantly in phloem cells during the early stage of potato–*R. solanacearum* interaction suggests that there may be a relationship between Ca^{2+} levels and *StLTPa7* expression. Because the X-ray analysis method employed detects Ca^{2+} (for a review, see Ge *et al.*, 2007), it is expected that calcium is involved in this process, which is presumed to be regulated by the pathogen

Fig. 4 Energy-dispersive X-ray (EDAX) spectra and CaCl_2 treatment suggest a role for Ca^{2+} in *StLTPa7* gene expression in the early response to *Ralstonia solanacearum*. (A, B, C) EDAX spectra of inner phloem cells (see E) from a representative experiment indicating peak Ca^{2+} values at 0 (A), 1 (B) and 3 days post-infection (dpi) (C) with *R. solanacearum*. (D) Scatter plot of EDAX spectra peak Ca^{2+} values at different time points shows an increasing accumulation in inner phloem cells (see 'Experimental procedures'). (E) Environmental scanning electron micrographs of the inner phloem from inoculated potato stem illustrating the restricted area for the EDAX spectrum (vertical section). The scanned region marked by a black cross represents the restricted area. ip, inner phloem. (F) Exogenous CaCl_2 -induced *StLTPa7* gene expression. Real-time quantitative polymerase chain reaction (qRT-PCR) was used to quantify *StLTPa7* gene expression after treatment with CaCl_2 . *StLTPa7* levels are expressed relative to those of the constitutively expressed *actin* gene (control). The statistical significance is similar to that described in Fig. 3.

as an external stimulant. To address the possibility that the accumulation of nsLTP RNA is associated with intracellular or extracellular Ca^{2+} , we treated healthy potato plants with CaCl_2 to raise the external $[\text{Ca}^{2+}]$, and examined the pattern of *StLTPa7* accumulation (Fig. 4F). *StLTPa7* transcripts began to accumulate 24 h after treatment and peaked after 3 days. The period of *StLTPa7* accumulation corresponded to the EDAX Ca^{2+} spectra in the inner phloem cells. The accumulation of *StLTPa7* transcripts and the elevated Ca^{2+} EDAX spectrum induced by *R. solanacearum* coincided with that induced by extracellular CaCl_2 . Collectively, these results reveal a dramatic increase in the expression of *StLTPa7* in phloem cells, concomitant with increased Ca^{2+} deposition in vascular tissue. A reasonable interpretation of these results is that bacterial invasion of the potato root promotes a cellular Ca^{2+} burst during the early stage of the invasion, which leads to subsequent Ca^{2+} accumulation in these cells.

Effects of chemical treatments on *StLTPa7* gene expression

As shown in Fig. 5, SA, MJ and ABA are all capable of stimulating an increase in *StLTPa7* gene expression. *StLTPa7* transcripts in potato stems were found to increase slowly after 24 h of SA treatment (Fig. 5A); this elevated level was maintained until day 5, and then gradually decreased. A similar pattern of gene expression was observed in stems after MJ and ABA treatment before 7 dpi (Fig. 5B,C). Twenty-four-hour ABA treatment produced a somewhat different pattern, inducing a robust increase in *StLTPa7* transcripts in potato stems that reached a maximum of approximately threefold at 3 days, and then remained variably elevated at least until day 7. At day 3, the ABA-induced increase in *StLTPa7* expression was comparable with that observed in response to the pathogen at 5 dpi. These data suggest that SA

Fig. 5 Effects of chemical treatments on *StLTPa7* gene expression. (A, B, C) Time course of expression of *StLTPa7* transcripts in noninoculated potato treated with salicylic acid (SA) (A), methyl jasmonate (MJ) (B) or abscisic acid (ABA) (C). (D) Effect of ruthenium red on *Ralstonia solanacearum*-induced *StLTPa7* expression in inoculated potato. Starting at 3 days post-infection (dpi), inoculated seedlings were treated with different concentrations of ruthenium red for 8 h, and RNA from the upper tissues was used for real-time quantitative polymerase chain reaction (qRT-PCR). The statistical significance in three different experiments was measured, as described in Fig. 3.

induces *StLTPa7* expression through a different and less effective mechanism than that engaged by ABA and MJ.

As shown by real-time quantitative polymerase chain reaction (qRT-PCR) in Fig. 5D, the increase in *StLTPa7* expression induced by *R. solanacearum* was retained in the presence of low concentrations (5 and 10 μM) of the intracellular Ca^{2+} channel inhibitor, ruthenium red, but was reduced at high concentrations (20–50 μM). This incomplete inhibition of *R. solanacearum*-induced *StLTPa7* gene expression by ruthenium red implies that intracellular Ca^{2+} is not the only factor driving changes in *StLTPa7* gene expression, suggesting that the entry of extracellular Ca^{2+} may contribute to this process. Ruthenium red inhibits ryanodine receptors, but does not inhibit inositol 1,4,5-trisphosphate receptors. In animal cells, the intracellular calcium pools available to these two intracellular calcium release channels are at least partially distinct. There is also the possibility that a component of the *StLTPa7* up-regulation mechanism is calcium independent. Despite this, taken together with the effects of CaCl_2 treatment, these data suggest that both the influx of extracellular Ca^{2+} and the mobilization of intracellular Ca^{2+} stores are required for the accumulation of *StLTPa7* in inoculated potato seedlings.

In situ localization of *StLTPa7* mRNA in potato tissues

To localize *StLTPa7* mRNA expression in the leaf and stem tissues of potato seedlings infected with the pathogenic *R. solanacearum* strain PO41, *in situ* hybridization was used (Fig. 6). Digoxin (DIG)-labelled probes were detected using an

alkaline phosphatase-conjugated anti-DIG antibody and nitroblue tetrazolium containing 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP), and visualized under a light microscope. Three days after inoculation with the virulent strain, a heavy deposit of *StLTPa7* mRNA was evident primarily within the inner phloem area of vascular tissue and in the outer phloem of stems (Fig. 6E) and leaves (Fig. 6D,F). Weak hybridization signals could also be observed occasionally in the epidermal tissue, but not in xylem vessels. In control hybridizations using sense probes, no signals were detected in any organelles of uninfected healthy leaf, stem or root tissues (Fig. 6A–C).

DISCUSSION

We have isolated the novel nsLTP gene, *StLTPa7*, from cDNA libraries constructed from potato leaves and stems infected with the virulent PO41 strain of *R. solanacearum*. *StLTPa7* shares a number of features found in other plant nsLTPs, including a low molecular weight, eight cysteine residues, a basic pI and a signal peptide predicted to target the proteins to the secretory pathway. A nucleotide sequence analysis and comparisons with protein databases revealed that the deduced amino acid sequence of potato *StLTPa7* was highly homologous to LTP1 proteins from other Solanaceae plants, showing the highest degree of identity with nsLTPs from tomato, pepper and tobacco (Fleming *et al.*, 1992, Pelèse-Siebenbourg *et al.*, 1994, Treviño and Connell, 1998, White *et al.*, 1994). Our results suggest that *StLTPa7* belongs to the largest phylogenetic class of nsLTPs, which contains a number

Fig. 6 Spatial expression of potato *StLTPa7* gene visualized by *in situ* hybridization. Consecutive 10- μ m transverse sections of leaves (A, B and C) and stems were hybridized with *in vitro*-synthesized antisense (D, E and F) or sense (A and B) *StLTPa7* RNA. The dark blue or brown coloration indicates a positive hybridization signal. The arrowheads indicate hybridization signals in the outer and inner phloem cells. ip, inner phloem; LE, lower epidermis; op, outer phloem; UE, upper epidermis; Vs, vascular bundle; x, xylem. Bars, 10 μ m. Two separate analyses were performed with similar results.

of novel cDNA clones encoding different isoforms of the plant nsLTP (Fig. 2). The three-dimensional structure of nsLTP1 indicates that the CaM-binding region of *StLTPa7* nsLTP1 is conserved amongst nsLTPs. This implies that nsLTP1 may be involved in Ca^{2+} /CaM signalling, suggesting a new biological function for this class of proteins. Because of the large number of positively charged surface residues, nsLTP1 proteins have been thought to function as antibacterial peptides. However, as is the case with *StLTPa7*, these charges are evenly distributed over the entire molecular surface of nsLTP1. An examination of the antifungal proteins from pokeweed [Protein Data Bank (PDB) code 1dkc] and radish (PDB code 1ayj), and thionin from wheat (PDB code 1gps) and rice (PDB code 1l6h) nsLTP2, shows that these structures have a high concentration of basic residues on one molecular face; this asymmetric distribution of positive charges may be necessary for effective antimicrobial activity in defence proteins (Samuel *et al.*, 2002). These results indicate that *StLTPa7* may be a new member of the CaM-binding protein family.

StLTPa7, identified in this study, was strongly up-regulated in potato stems and leaves after pathogen infection. The biochemical function of the three previously identified LTP genes remains unknown, but our data provide experimental support for *StLTPa7* involvement in the response to *R. solanacearum* in potato. As shown in Fig. 5, the transcription of the *StLTPa7* gene was strongly induced in stems treated with exogenous SA, MJ or ABA, suggesting that plant hormones may function in the signal transduction pathway leading to increased *StLTPa7* gene expression. Interestingly, the effect of ABA on *StLTPa7* gene expression in stem tissues was stronger than that of SA and MJ.

In this study, we found evidence for a possible role of Ca^{2+} in regulating the abundance of *StLTPa7* at the early stage of

potato–*R. solanacearum* interaction. Using ESEM and EDAX to study Ca^{2+} accumulation and qRT-PCR to evaluate the accumulation of *StLTPa7*, it was found that *StLTPa7* gene transcripts accumulated with a time course that closely paralleled that of Ca^{2+} accumulation. This synchrony, which has not been described previously, occurred only in inoculated potato plants during the early stage of infection. Compared with the phloem of control plants, which did not accumulate elemental Ca^{2+} , the phloem of inoculated plants showed a rapid and substantial increase in elemental Ca^{2+} that was clearly evident at 3 dpi.

The coincident time courses of Ca^{2+} and *StLTPa7* accumulation demonstrate a correlation between these two events, but do not provide evidence of a causal relationship. Importantly, treatment with CaCl_2 also induced an increase in the level of *StLTPa7* transcripts, demonstrating that Ca^{2+} is capable of inducing *StLTPa7* expression, and providing support for a functional role for elevated Ca^{2+} in the transcriptional response to *R. solanacearum* infection. In addition, *R. solanacearum*-induced *StLTPa7* expression was partially blocked by the Ca^{2+} channel blocker, ruthenium red, suggesting that this process is dependent, at least in part, on the release of Ca^{2+} from ruthenium red-sensitive intracellular stores. Although it was not shown directly that increased cytosolic $[\text{Ca}^{2+}]$ induced *StLTPa7* and ruthenium red prevented it, these results suggest that *StLTPa7* is a pathogen- and Ca^{2+} -responsive gene involved in plant defences. Additional evidence, however, will be required to confirm this connection.

Ruthenium red is a membrane-permeable diagnostic antagonist for intracellular endoplasmic reticular Ca^{2+} release channels, known as ryanodine receptors (Subbaiah *et al.*, 1994). The incomplete inhibition of *R. solanacearum*-induced *StLTPa7* expression by ruthenium red thus indicates that ryanodine receptor-mediated

intracellular Ca^{2+} release is not an obligate requirement for gene expression, suggesting the involvement of other Ca^{2+} sources; in particular, the influx of extracellular Ca^{2+} and stored calcium in the vacuole may contribute. It is worth noting that our data do not rule out the possibility that Ca^{2+} -independent signalling cascades are also involved.

Ca^{2+} homeostasis within the cytosol is tightly controlled by membrane-spanning Ca^{2+} -ATPases and $\text{H}^{+}/\text{Ca}^{2+}$ exchangers, which typically maintain low concentrations of Ca^{2+} in the cytosol and restore this concentration following signalling-related transient changes in Ca^{2+} levels (Deluc *et al.*, 2007; Grimplet *et al.*, 2007). Ca^{2+} has many essential roles in plant growth and development (Hepler, 2005), and is commonly involved as an intracellular messenger in the transduction of a wide range of biotic stimuli in plants, including signals from pathogenic and symbiotic fungi (Deluc *et al.*, 2007). In a previous study, EDAX revealed an increase in Ca^{2+} in the phloem, the cambium and the xylem elongation of poplar (*Populus tremula* $\times$ *Populus tremuloides*), suggesting a possible regulatory role for Ca^{2+} during xylem development (Lautner *et al.*, 2007). However, the role of Ca^{2+} signalling in the response to pathogenic bacteria remains largely unstudied. Recent investigations describing calcium influxes into the phloem by distant stimuli have provided new insights into the role of Ca^{2+} signalling in phloem cells (Furch *et al.*, 2007). Our current efforts are focused on the measurement of the intracellular free Ca^{2+} concentration during disease progression, and the determination of whether antibiotic activity is associated with or induced by Ca^{2+} . Further studies, especially those designed to identify substrates for Ca^{2+} , are required to understand the mechanisms underlying *R. solanacearum*-induced resistance. Likewise, the determination of what activates this signalling cascade will shed more light on the mechanisms that enable plants to defend themselves from pathogens.

The spatial patterns of *StLTPa7* gene expression imply a possibly significant role in vascular signalling networks. The plant vascular system, comprising the xylem and phloem, forms a continuous network throughout the plant body that transports water, nutrients and signalling molecules. Vascular cells differentiate by contact with each other, forming a continuous vasculature that can be colonized by the plant pathogen *R. solanacearum*. This feature has raised the possibility that some special inductive signals function to guide continuous responses to invading bacteria. Such inductive signals have not been defined, however, partly because of difficulties in analysing cell–cell interactions *in planta*. In general, nsLTPs interact with lipids and sterols and enhance their *in vitro* transfer between membranes (Kader, 1996; Yamada, 1992). Maldonado *et al.* (2002) have proposed that the nsLTP, DIR1, can interact with a lipid-derived molecule to promote long-distance signalling in *Arabidopsis thaliana*. DIR1 displays strong similarity to members of the LTP family in the *Arabidopsis* genome. Although the DIR1 LTP might, in principle, serve as the

mobile signal, it is more likely that it functions in co-operation with the mobile signal, because DIR1 over-expression alone is not sufficient to induce systemic acquired resistance; pathogen exposure is still required. Lipid molecules, such as oxylipins (jasmonic acid), phosphatidic acid and *N*-acylethanolamines, are synthesized or released from membranes on pathogen exposure and act as second messengers in plant defence signalling. Structural studies have predicted that plant LTPs contain an internal hydrophobic pocket that is sufficiently large to accommodate a fatty acid or lysophospholipid. Plant nsLTPs are predicted to contain signal peptides, some of which target them to the cell wall (Thoma *et al.*, 1993). Thus, an nsLTP, such as DIR1 LTP, may be a co-signal or, alternatively, may act as a translocator and/or chaperone responsible for releasing the mobile signal into the vascular system or conveying it through the plant. Our demonstration that the expression of *StLTPa7* may be induced at a site far removed from the infection site (Fig. 3) poses an interesting question: is the gene product involved in vascular signalling for systemic acquired resistance in potato plants? In this study, the response of *StLTPa7* in potato leaves and stems to plant pathogen infection is consistent with earlier reports on the expression of the pepper nsLTP, CALTP, and other plant pathogenesis-related genes (Gorlach *et al.*, 1996; Jung and Hwang, 2000; Jung *et al.*, 2003; Kim and Hwang, 2000).

Research into the breadth and complexity of genetic responses to *R. solanacearum* infection is in its early stages. Future studies should focus on the definition of the patterns of gene expression within the host plant at various locations and times during disease progression, and the characterization of the signals that affect it. The nature of these signals may not only help to identify the molecular targets of the invading pathogen during each stage of disease, but may also provide new opportunities for disease control, as disruption of the corresponding transduction pathways often leads to a loss of virulence. In conclusion, the pattern of expression of LTP genes in response to infection of potato by *R. solanacearum* is rather complex, as is the regulation of this response. Certain developmental and environmental signals are needed to determine the involvement of these proteins in the adaptation of plants to pathogen stress. Further isolation and characterization of LTP genes will provide insights into the role of the multifunctional LTP multigene family in potato.

EXPERIMENTAL PROCEDURES

Plant material, pathogen maintenance and inoculation

A clone of tetraploid *S. tuberosum* L. cv. Zh3, obtained from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China, was used. Potato seedlings grown from seed tubers under standard glasshouse conditions (22 °C day, 18 °C night, 12-h light/12-h dark cycle) were transplanted into

10-cm pots containing peat-vermiculite (3 : 1, v/v). *R. solanacearum* strain PO41 (potato, race 3 biovar 2) was cultured as described by He *et al.* (1983). Plants at the nine-leaf stage (3-week-old plants) were inoculated with either a virulent or an avirulent strain of PO41. Negative control plants were inoculated with water. Approximately 5 cm was cut from the bottom of the culture pot (10 × 10 cm), and the exposed roots of the plants were immersed for 10 min in a suspension containing 10⁸ bacteria/mL. An additional 30-mL bacterial suspension containing 10⁷ bacteria/mL was poured into the pot. The plants were then transferred to a growth chamber at 28 °C and maintained under a 16-h light (constant light at 500 µE/s/m²)/8-h dark cycle.

Chemical treatment

Chemical treatments were applied to both inoculated and noninoculated plants at the nine-leaf stage. SA (Sigma, St. Louis, MO, USA) was dissolved in water to a final concentration of 5 mM. MJ (Aldrich, Milwaukee, WI, USA) was prepared as a 100 mM stock solution in 100% ethanol and diluted in water to a final concentration of 100 µM. Tween 20 was added (0.01%) as a surfactant in SA and MJ solutions. ABA (Sigma) was prepared as a 20 mM stock solution in 100% ethanol and diluted with water to a final concentration of 100 µM. The final solutions were sprayed onto the leaves until droplets formed and were then allowed to dry, as described previously (Gabriel *et al.*, 2006). Control plants were sprayed with a Tween 20–water mixture (SA control), a Tween 20–water plus 0.1% ethanol mixture (MJ control) or a 0.1% (w/v) ethanol solution (ABA) (Anderson *et al.*, 2004), and allowed to dry in the same manner as the treated plants. All treated and control plants were immediately placed in large black plastic bags for 24 h and maintained in the dark at room temperature. The application of chemicals and incubations were repeated 24 h later. For CaCl₂ treatment, healthy seedlings were placed in a 0.5 mM CaCl₂ solution (pH 4.5) overnight. Leaf and stem tissues sampled at various time intervals after treatments were immediately frozen in liquid nitrogen and used for RNA extraction. A ruthenium red (Sigma) solution was freshly prepared in sterilized double-distilled water. Three days after inoculation, the infected potato seedling roots were immersed in ruthenium red solution (10, 20 or 50 µM) or distilled water (control) for 8 h. RNA was isolated from the upper stems and leaves for qRT-PCR analysis.

RNA and DNA preparation

For suppression subtractive hybridization, Northern blotting and RACE procedures, RNA was isolated from inoculated and noninoculated control plant internode stem tissues at 1–6 dpi using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. For gene expression analysis, total RNA was isolated from infected internode tissues at 0, 1, 2,

3, 4, 5 and 6 dpi. Total RNA (1 µg) was reverse transcribed at 42 °C for 1 h in the presence of cDNA synthesis (CDS) primer and SMART II oligonucleotides using the Clontech SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). DNA was extracted from the leaves by first grinding in liquid nitrogen with 10% (w/w) polyvinylpyrrolidone (PVP), followed by the addition of lysis buffer [2% (w/v) cetyltrimethylammonium bromide (CTAB), 75 mM Tris-HCl (pH 8), 15 mM ethylenediaminetetraacetic acid (EDTA) (pH 8), 1.05 M NaCl, 4% (v/v) β-mercaptoethanol], heating for 30 min at 65 °C, and centrifuging for 5 min at 13 400 g. The supernatant was mixed with an equal volume of chloroform–isoamyl alcohol (24 : 1, v/v) and gently blended upside down. After centrifugation, DNA was precipitated from the colourless supernatant by incubating for 10 min at room temperature with 0.1 vol of 10 M ammonium acetate and 2 vol of 95% (v/v) alcohol, and then centrifuged for 10 min at 17 900 g. The pellet was washed with 70% (v/v) ethanol, air dried and resuspended in sterile distilled deionized water.

Suppression subtractive hybridization

The subtractive cDNA libraries were obtained using the PCR Select cDNA Subtraction Kit (Clontech). The tester (inoculated) SMART-cDNA sample was subtracted twice by the driver (mock-inoculated) SMART-cDNA sample following the manufacturer's recommendations (forward subtraction). A reverse subtraction was also performed, in which the tester served as the driver and the driver as the tester, in order to produce complex cDNA probes for further differential screening of the subtracted clones. After the evaluation of subtraction efficiency, these subtracted cDNAs were subcloned into pGEM-T plasmids and used to transform *Escherichia coli* DH5α bacteria (Invitrogen). Four sets of membranes were prepared and hybridized with the radiolabelled tester (T), driver (D), forward-subtracted tester (FT) and reverse-subtracted driver (RD) probes. Only clones showing an intense hybridization signal with probes T and FT and a weak signal with probes D and RD were selected for sequencing and bioinformatics analysis.

Sequencing and bioinformatics analysis

Sequencing was performed by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). All inserted sequences were evaluated for homology against the GENBANK/EMBL/DDBJ nonredundant sequence database using the BLASTX program. Protein sequences were aligned using CLUSTAL W (Higgins *et al.*, 1994), and the alignments were manually refined using the BioEdit suite of programs (www.mbio.ncsu.edu/BioEdit). Phylogenetic and molecular evolutionary analyses were performed on amino acid sequences of selected plant nsLTPs using MEGA version 4 (Tamura *et al.*, 2007).

Isolation of full-length nsLTP cDNA

An isolate identified by suppression subtractive hybridization contained a 3'-untranslated region; thereafter, the 5' RACE procedure was used to obtain the full-length cDNA with a SMART-RACE cDNA Amplification Kit (Clontech), following the manufacturer's protocol. 5' Gene-specific primers (GSP1, 5'-GACCAGCGGCTTTCCCGTATCAAT-3'; GSP2, 5'-CATGCTGTTTCCGGTCTTCTGGGG-3') were designed based on the sequences of the suppression subtractive hybridization isolate. The cDNA 5'-end was amplified by PCR using the universal primer mix (UPM) and an antisense GSP2 oligonucleotide. The cDNA ends thus obtained were ligated into pCR 2.1 (Invitrogen) and sequenced as described above. The resulting sequences were aligned with that of the isolate to obtain a full-length cDNA. The full-length gene was designated *StLTPa7* and submitted to GENBANK under the accession number EU057712.

qRT-PCR and Northern blot analysis

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), followed by DNase I treatment to remove potentially contaminating genomic DNA. qRT-PCR analysis was performed as described previously (Jain *et al.*, 2006). Briefly, cDNA samples synthesized from 3 µg of total RNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) were used as a template and mixed with 200 nM of each primer and SYBR Green PCR Master Mix (Applied Biosystems). Analysis was carried out using the iCycler iQ™ real-time PCR detection system (Bio-Rad, Richmond, CA, USA), according to the manufacturer's instructions. The sequences of forward and reverse primers, designed and synthesized by Sangon Biological Engineering Technology & Services Co., Ltd., were 5'-TCCTTGCTCCTTATCTTCA-3' (forward) and 5'-CATCTTATTCTTATCTCCGC-3' (reverse). The transcript of *S. tuberosum* actin (GENBANK accession number X55747) was used to standardize each reaction employing the primers 5'-GCTTCCCGATGGTCAAGTCA-3' (forward) and 5'-GGATTCCAGCTGCTTCCATTC-3' (reverse). The specificity of the reactions was verified by melting curve analysis. Relative fold expression for each gene was calculated by the method of Pfaffl (2001). At least two independent RNA isolations were used for cDNA synthesis, and each cDNA sample was subjected to qRT-PCR analysis in triplicate. Northern blot and standard RT-PCR analyses were performed as described previously (Wawrzynska *et al.*, 2005). Primer pairs used for standard RT-PCR were 5'-TGGCTCCTGCCTCCCTTATC-3'/5'-CATCTTATTCTTATCTCCGC-3'.

In situ hybridization analysis

Paraffin-embedded potato stem and leaf sections were used for *in situ* hybridization with DIG-labelled RNA probes, as described

by Elorza *et al.* (2004). *StLTPa7* gene-specific probes were obtained by amplifying cDNA fragments by PCR using the primer pair 5'-TGGCTCCTGCCTCCCTTAT-3' and 5'-TCCATCTTATTCTCATCTCC-3'. The PCR products were cloned into a pGEM-T plasmid (Promega), and the recombinant plasmids were sequenced. Antisense and sense riboprobes were labelled with DIG-UTP using the DIG RNA Labelling Kit (SP6/T7; Roche Diagnostics).

Microscopy and EDAX

Stems were washed by dipping briefly in deionized water and longitudinal sections of individual vascular bundles (~4–6 mm thick) were excised from the appropriate internode of *R. solanacearum*-inoculated and control plants at different days post-infection using a dichloromethane-washed razor blade. Hydrated samples were viewed directly under a Quanta 200FEG (FEI) ESEM instrument with an EDAX EDS system at an accelerating voltage of 15 kV, using the wet mode at 5 °C, 3.6–6.4 Torr, 7.8 mm working distance and an internal relative humidity of 85%–90%. Two replicate plants were used for each analysis. Relative Ca²⁺ levels at given days post-infection were analysed using a multipoint detection method. Vascular bundles were first identified under low power, and then at least 10 phloem cells were randomly chosen from two randomly selected sections from each plant under high power. Several points of interest were scanned in a restricted area (three to five areas per cell). The information was collected and processed using the EDAX microanalyser software.

REFERENCES

- Anderson, J.P., Badruzsaufari, E., Schenk, P.M., Manners, J.M., Desmond, O.J., Ehler, C., Maclean, D.J., Ebert, P.R. and Kazan, K. (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. *Plant Cell*, **16**, 3460–3479.
- Blein, J.P., Coutos-Thevenot, P., Marion, D. and Ponchet, M. (2002) From elicitors to lipid-transfer proteins: a new insight in cell signalling involved in plant defence mechanisms. *Trends Plant Sci.*, **7**, 293–296.
- Borges, J.P., Jauneau, A., Brule, C., Culerrier, R., Barre, A., Didier, A. and Rouge, P. (2006) The lipid transfer protein (LTP) essentially concentrates in the skin of Rosaceae fruits as a cell-surface exposed allergen. *Plant Physiol. Biochem.*, **44**, 535–542.
- Boutrot, F., Meynard, D., Guiderdoni, E., Joudrier, P. and Gautier, M.F. (2007) The *Triticum aestivum* non-specific lipid transfer protein (*Taltp*) gene family: comparative promoter activity of six *Taltp* genes in transgenic rice. *Planta*, **225**, 843–862.
- Buhot, N., Douliez, J.P., Jacquemard, A., Marion, D., Tran, V., Maume, B.F., Milat, M.L., Ponchet, M., Mikes, V., Kader, J.C. and Blein, J.P. (2001) A lipid transfer protein binds to a receptor involved in the control of plant defence responses. *FEBS Lett.*, **509**, 27–30.
- Buhot, N., Gomes, E., Milat, M.-L., Ponchet, M., Marion, D., Lequeu, J., Delrot, S., Coutos-Thevenot, P. and Blein, J.-P. (2004)

- Modulation of the biological activity of a tobacco LTP1 by lipid complexation. *Mol. Biol. Cell*, **15**, 5047–5052.
- Capocchi, A., Fontanini, D., Muccilli, V., Cunsolo, V., Saviozzi, F., Saletti, R., Lorenzi, R., Foti, S. and Galleschi, L. (2005) NsLTP1 and NsLTP2 isoforms in *Triticum aestivum* cv. Centauro and *Triticum dicoccon* Schrank (farro) bran. *J. Agric. Food Chem.* **53**, 7976–7984.
- Carvalho, A.O. and Gomes, V.M. (2007) Role of plant lipid transfer proteins in plant cell physiology—a concise review. *Peptides*, **28**, 1144–1153.
- Chae, K., Zhang, K., Zhang, L., Morikis, D., Kim, S.T., Mollet, J.-C., de la Rosa, N., Tan, K. and Lord, E.M. (2007) Two SCA (stigma/style cysteine-rich adhesin) isoforms show structural differences that correlate with their levels of *in vitro* pollen tube adhesion activity. *J. Biol. Chem.* **282**, 33 845–33 858.
- Cohen, M., Klein, E., Geiger, B. and Addadi, L. (2003) Organization and adhesive properties of the hyaluronan pericellular coat of chondrocytes and epithelial cells. *Biophys. J.* **85**, 1996–2005.
- Deluc, L., Grimplet, J., Wheatley, M., Tillett, R., Quilici, D., Osborne, C., Schooley, D., Schlauch, K., Cushman, J. and Cramer, G. (2007) Transcriptomic and metabolite analyses of Cabernet Sauvignon grape berry development. *BMC Genomics*, **8**, 429.
- Doulliez, J.P., Michon, T., Elmorjani, K. and Marion, D. (2000) Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *J. Cereal Sci.* **32**, 1–20.
- Elorza, A., Leon, G., Gomez, I., Mouras, A., Holuigue, L., Araya, A. and Jordana, X. (2004) Nuclear SDH2-1 and SDH2-2 genes, encoding the iron–sulfur subunit of mitochondrial complex II in Arabidopsis, have distinct cell-specific expression patterns and promoter activities. *Plant Physiol.* **136**, 4072–4087.
- Fleming, A., Mandel, T., Hofmann, S., Sterk, P., de Vries, S. and Kuhlemeier, C. (1992) Expression pattern of a tobacco lipid transfer protein gene within the shoot apex. *Plant J.* **2**, 855–862.
- Furch, A.C.U., Hafke, J.B., Schulz, A. and van Bel, A.J.E. (2007) Ca²⁺-mediated remote control of reversible sieve tube occlusion in *Vicia faba*. *J. Exp. Bot.* **58**, 2827–2838.
- Gabriel, D.W., Allen, C., Schell, M.A., Denny, T.P., Greenberg, J.T., Duan, Y.P., Flores-Cruz, Z., Huang, Q., Clifford, J.M., Presting, G., González, E.T., Reddy, J., Elphinstone, J., Swanson, J., Yao, J., Mulholland, V., Liu, L., Farmerie, W., Patnaikuni, M., Balogh, B., Norman, D., Alvarez, A., Castillo, J.A., Jones, J., Saddler, G., Walunas, T., Zhukov, A. and Mikhailova, N. (2006) Identification of open reading frames unique to a select agent: *Ralstonia solanacearum* race 3 biovar 2. *Mol. Plant–Microbe Interact.* **19**, 69–79.
- Ge, L.L., Tian, H.Q. and Russell, S.D. (2007) Calcium function and distribution during fertilization in angiosperms. *Am. J. Bot.* **94**, 1046–1060.
- Gorlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H. and Ryals, J. (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell*, **8**, 629–643.
- Grimplet, J., Deluc, L., Tillett, R., Wheatley, M., Schlauch, K., Cramer, G. and Cushman, J. (2007) Tissue-specific mRNA expression profiling in grape berry tissues. *BMC Genomics*, **8**, 187.
- Hamilton, J.A. (2004) Fatty acid interaction with proteins: what X-ray crystal and NMR solution structures tell us. *Progr. Lipid Res.* **43**, 177–199.
- He, L.Y., Sequeira, L. and Kelman, S.L. (1983) Characteristics of strains of *Pseudomonas solanacearum* from China. *Plant Dis.* **67**, 1357–1361.
- Heinemann, B., Andersen, K.V., Nielsen, P.R., Bech, L.M. and Poulsen, F.M. (1996) Structure in solution of a four-helix lipid binding protein. *Protein Sci.* **5**, 13–23.
- Hepler, P.K. (2005) Calcium: a central regulator of plant growth and development. *Plant Cell*, **17**, 2142–2155.
- Higgins, D.G., Thompson, J.D. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **11**, 4673–4680.
- Hincha, D.K., Neukamm, B., Srór, H.A.M., Sieg, F., Weckwarth, W., Ruckels, M., Lullien-Pellerin, V., Schroder, W. and Schmitt, J.M. (2001) Cabbage cryoprotectin is a member of the nonspecific plant lipid transfer protein gene family. *Plant Physiol.* **125**, 835–846.
- Horvath, B.M., Bachem, C.W.B., Trindade, L.M.E.P., Oortwijn, M. and Visser, R.G.F. (2002) Expression analysis of a family of nsLTP genes tissue specifically expressed throughout the plant and during potato tuber life cycle. *Plant Physiol.* **129**, 1494–1506.
- Jain, M., Kaur, N., Tyagi, A.K. and Khurana, J.P. (2006) The auxin responsive GH3 gene family in rice (*Oryza sativa*). *Funct. Integr. Genomics*, **6**, 36–46.
- Jose-Estanyol, M., Gomis-Ruth, F.X. and Puigdomenech, P. (2004) The eight-cysteine motif, a versatile structure in plant proteins. *Plant Physiol. Biochem.* **42**, 355–365.
- Jung, H.W. and Hwang, B.K. (2000) Pepper gene encoding a basic b-1,3-glucanase is differentially expressed in pepper tissues upon pathogen infection and ethephon or methyl jasmonate treatment. *Plant Sci.* **159**, 97–106.
- Jung, H.W., Kim, W. and Hwang, B.K. (2003) Three pathogen-inducible genes encoding lipid transfer protein from pepper are differentially activated by pathogens, abiotic, and environmental stresses. *Plant Cell Environ.* **26**, 15–928.
- Jung, H.W., Kim, K.D. and Hwang, B.K. (2005) Identification of pathogen responsive regions in the promoter of a pepper lipid transfer protein gene (CALTPI) and the enhanced resistance of the CALTPI transgenic Arabidopsis against pathogen and environmental stresses. *Planta*, **221**, 361–373.
- Jung, H.W., Lim, C.W. and Hwang, B.K. (2006) Isolation and functional analysis of a pepper lipid transfer protein III (CALTPIII) gene promoter during signaling to pathogen, abiotic and environmental stresses. *Plant Sci.* **170**, 258–266.
- Kader, J.C. (1996) Lipid-transfer proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 627–654.
- Kader, J.C. (1997) Lipid-transfer proteins: a puzzling family of plant proteins. *Trends Plant Sci.* **2**, 66–70.
- Kader, J.C., Julienne, M. and Vergnolle, C. (1984) Purification and characterization of a spinach-leaf protein capable of transferring phospholipids from liposomes to mitochondria or chloroplasts. *Eur. J. Biochem.* **139**, 411–416.
- Kim, Y.J. and Hwang, B.K. (2000) Pepper gene encoding a basic pathogenesis-related 1 protein is pathogen and ethylene inducible. *Physiol. Plant.* **108**, 51–60.
- Lauga, B., Harbonnel-Campaa, L. and Combes, D. (2000) Characterization of MZm3-3, a *Zea mays* tapetum-specific transcript. *Plant Sci.* **157**, 65–75.
- Lautner, S., Ehltling, B., Windeisen, E., Rennenberg, H., Matyssek, R. and Fromm, J. (2007) Calcium nutrition has a significant influence on wood formation in poplar. *New Phytol.* **173**, 743–752.

- Lu, Z.X., Gaudet, D., Puchalski, B., Despins, T., Frick, M. and Laroche, A. (2006) Inducers of resistance reduce common bunt infection in wheat seedlings while differentially regulating defence-gene expression. *Physiol. Mol. Plant Pathol.* **67**, 138–148.
- Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C.J. and Cameron, R.K. (2002) A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. *Nature*, **419**, 399–403.
- Pelèse-Siebenbourg, F., Caelles, C., Kader, J.-C., Delseny, M. and Puigdomènech, P. (1994) A pair of genes coding for lipid-transfer proteins in *Sorghum vulgare*. *Gene*, **148**, 305–308.
- Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, 2002–2007.
- Salcedo, G., Sánchez-Monge, R., Barber, D. and Díaz-Perales, A. (2007) Plant non-specific lipid transfer proteins: an interface between plant defence and human allergy. *Biochim. Biophys. Acta*, **1771**, 781–791.
- Samuel, D., Liu, Y.J., Cheng, C.-S. and Lyu, P.-C. (2002) Solution structure of plant nonspecific lipid transfer protein-2 from rice (*Oryza sativa*). *J. Biol. Chem.* **277**, 35 267–35 273.
- Shin, D.H., Lee, J.Y., Hwang, K.Y., Kim, K.K. and Suh, S.W. (1995) High-resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings. *Structure*, **3**, 189–199.
- Subbaiah, C.C., Bush, D.S. and Sachs, M.M. (1994) Elevation of cytosolic calcium precedes anoxic gene expression in maize suspension-cultured cells. *Plant Cell*, **6**, 1747–1762.
- Takishima, K., Watanabe, S., Yamada, M., Suga, T. and Mamiya, G. (1988) Amino acid sequences of two nonspecific lipid-transfer proteins from germinated castor bean. *Eur. J. Biochem.* **177**, 241–249.
- Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**, 1596–1599.
- Thoma, S., Kaneko, Y. and Somerville, C. (1993) A non-specific lipid transfer protein from *Arabidopsis thaliana* is a cell wall protein). *Plant J.* **3**, 427–436.
- Treviño, M.B. and Connell, M.A.O. (1998) Three drought-responsive members of the nonspecific lipid-transfer protein gene family in *Lycopersicon pennellii* show different developmental patterns of expression. *Plant Physiol.* **116**, 1461–1468.
- Wang, Z., Xie W., Chi, F. and Li, C. (2005) Identification of non-specific lipid transfer protein-1 as a calmodulin-binding protein in *Arabidopsis*. *FEBS Lett.* **579**, 683–687.
- Wawrzynska, A., Lewandowska, M., Hawkesford, M.J. and Sirko, A. (2005) Using a suppression subtractive library-based approach to identify tobacco genes regulated in response to short-term sulphur deficit. *J. Exp. Bot.* **56**, 1575–1590.
- White, A.J., Alison Dunn, M., Brown, K. and Hughes, M.A. (1994) Comparative analysis of genomic sequence and expression of a lipid transfer protein gene family in winter barley. *J. Exp. Bot.* **45**, 1885–1892.
- Yamada, M. (1992) Lipid transfer proteins in plants and microorganisms. *Plant Cell Physiol.* **33**, 1–6.