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Differential Space-Time Expression of *StLTPb1* Gene Between Resistant and Susceptible Potato Genotypes in Response to *Ralstonia solanacearum*

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Abstract

This study is to investigate the role of lipid transfer protein (*LTP1*) gene of potato (*Solanum tuberosum*) in bacterial wilt (*Ralstonia solanacearum*) resistance. A novel cDNA clone encoding nsLTP was isolated from cultivated potato (*Solanum tuberosum*) infected with *R. solanacearum* by 5´-rapid amplification of cDNA ends (RACE). The temporal and spatial expression of *StLTPb1* was studied during the early stages of potato-*R. solanacearum* interaction by reverse transcriptase PCR (RT-PCR) and Northern blotting. The sequence analysis of the cloned cDNA, named *StLTPb1*, showed 691 bp which encoded a type 1 nsLTP of 91 amino acids. Construction of a phylogenic tree showed that *StLTPb1* is well conserved in the coding region with high identity at the amino acid level with other *Solanaceae* nsLTPs. The temporal and spatial expression of *StLTPb1* was studied during the early stages of potato-*R. solanacearum* interaction. *StLTPb1* transcription is induced faster and transcripts accumulate to higher concentrations in resistant compared with susceptible genotypes by the pathogen. Dominant differences in the pathogen-induced gene expression pattern between the upper and lower leaves and stems were observed within the same genotypes. *In situ* hybridization results showed that the *StLTPb1* mRNA was localized in phloem cells of vascular tissues in potato leaf and stem tissues after pathogen infection. Salicylic acid, methyl jasmonate and abscisic acid could induce *StLTPb1* gene expression without significant difference between the upper and lower tissues. These abiotic elicitors could produce a long-lasting effect on the *StLTPb1* during early stages of potato-*R. solanacearum* interaction. Differential expression of *StLTPb1* gene between resistance and susceptible potato genotypes in response to *R. solanacearum* suggests that this gene plays a key role in plant defense mechanisms.

Key words: *Solanum tuberosum*, nsLTP, gene expression, *Ralstonia solanacearum*

INTRODUCTION

Plant non-specific lipid transfer proteins (nsLTPs) are abundant, small, lipid binding proteins that are capable of exchanging lipids between membranes *in vitro*. Despite their name, a role in intracellular lipid transport is considered unlikely, based on their extracellular localization (Yeats and Rose 2008). Until now the small cysteine-rich proteins are found throughout the plant kingdom, comprising over 100 potential members from up to 50 different species (Jose-Estanyol *et al*. 2004). nsLTPs 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). nsLTPs are small peptides encoded by a multigene family

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and comprise three types according to their primary structure (Boutrot *et al*. 2007). The nsLTPs that form the first and second family have molecular masses of approximately 9 and 7 kDa respectively and are basic, presenting isoeletric points (*p*I) of between 8.8 and 10 (Kader 1997). Type 3 nsLTPs also display a 7 kDa molecular mass and all type 3 non-specific lipid transfer protein (nsLTP) genes show anther-specific expression (Lauga *et al*. 2000). Plant nsLTPs are often purified as several isoforms with very similar sequences (Chae *et al*. 2007).

The three-dimensional structures of nsLTPs from many plant species such as rice (*Oryza sativa*), maize (*Zea mays*), and barley (*Hordeum uhulgare*) show a globular molecule, composed of four α-helices, which are stabilized by four disulfide bridges, and a C-terminal tail (Salcedo *et al*. 2007). The four helices form a hydrophobic cavity, which is the binding site for fatty acids, acyl-CoA, or phospholipids. The most outstanding feature of this hydrophilic molecule is a hydrophobic cavity that runs through the whole molecule and the cavity can adapt its volume to bind one or two monoacyl lipids, diacylated lipids, or a wide variety of hydrophobic molecules (Salcedo *et al*. 2007; Carvalho and Gomes 2007). Although many LTPs can bind lipids *in vitro*, no LTP has been shown to do so *in vivo*. The interaction between wheat (*Triticum aestivum*) nsLTP and phospholipids indicated 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 demonstrated for most of their suggested functions and the *in vivo* role of these proteins remains unclear.

More lines of evidence point to the role of nsLTPs in plant defence mechanisms developed by plants against the attack of bacteria, fungi and, possibly, viruses (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 with the induction by these allergens of severe symptoms in many patients (Salcedo *et al*. 2007; Yeats and Rose 2008). The genetic structures of nsLTPs indicate that different physiological processes demand the presence of different expression patterns. A variety of transcription levels of *nsLTP* genes have been shown in different plant tissues during diverse developmental stages and physiological conditions. Several signal molecules, such as abscisic acid, salicylic acid, ethylene, and methyl jasmonate, are involved in the signaling pathway responsible for the expression of nsLTP genes (Jung *et al*. 2003, 2005, 2006; Lu *et al*. 2006). It has been reported that LTP is involved in long-distance signaling during acquisition of systemic acquired resistance (SAR) in *Arabidopsis thaliana* (Maldonado *et al*. 2002). Added to these, a role of nsLTP as a signal mediator was suggested to be involved in control of plant defense responses (Blein *et al*. 2002).

In potato (*Solanum tuberosum*), nsLTPs are encoded by a complex family of genes that are expressed throughout the developing plant in a highly tissue-specific manner. Recently, some transcript-derived fragments of nsLTPs were reported and these fragments displayed modulated expression profiles related to the development of new tissues, with a peak of transcription around the time of tuberization and just prior to sprout development, at dormancy breakage (Horvath *et al*. 2002). One of the nsLTPs fragments had a highly specific pattern of expression detected in the phloem surrounding the nodes of young plants and in the same tissue of tuber related organs, whereas the other showed little tissue or organ specificity. The expression profiles suggest that nsLTPs have multiple functions in the developing nightshade. Three drought-responsive members of the nonspecific lipid-transfer protein gene family in *Lycopersicon pennellii* show different developmental patterns of expression (Treviño and Connell 1998), all of the three genes (namely *LpLtp1*, *2*, *3*) were exclusively expressed in the aerial tissues of the plant and all were drought and ABA inducible. Each gene had a different pattern of expression in fruit, and *LpLtp1* and *LpLtp2*, unlike *LpLtp3*, were both primarily developmentally regulated in leaf tissue. Putative ABA-responsive elements were found in the proximal promoter regions of *LpLtp1* and *LpLtp2*.

In order to understand the increasing complexity of the proposed functions of nsLTPs, it is necessary to characterize each nsLTP gene expression. In this study, the expression pattern of the *StLTPb1* in potato was investigated in early response to *Ralstonia solanacearum*. We found the differences of space-time expression of *StLTPb1* gene between resistant and

susceptible genotypes during early stages of potato-*R. solanacearum* interaction.

MATERIALS AND METHODS

Plant materials, pathogen maintenance and inoculation

A clone of susceptible teraploid *S. tuberosum* L. cultivar Zhongshu 3 and a resistant genotype MS42.3 was used. Potato seedlings grown from seed tubers and under standard greenhouse conditions (at 22°C day and 18°C night temperature with 12 h of light per 24 h) were transplanted in 10-cm pots containing peat/vermiculite (3:1, v/v). *R. solanacearum* strain PO41 (potato, race 3 biovar 2) were cultured as described (He *et al*. 1983). Plants at the nine-leaf stage (three-week-old plants) were inoculated. Negative controls plants were inoculated with water. The experiments had been done from spring 2006 to spring 2008.

Chemical treatment

Chemical treatments were applied to both inoculated and non-inoculated treatments at the nine-leaf stage. Salicylic acid (SA) was dissolved in water to final concentration of 5 mM. Methyl-jasmonate (MJ) was first dissolved in 100% ethanol to a stock concentration of 100 mM, and then diluted in water to final concentration of 100 μM. Tween 20 was added (0.01%) as a surfactant. Final solutions were sprayed onto leaves until droplets formed. Control plants were sprayed with either Tween 20/water mixture for SA or Tween 20/water plus 0.1% ethanol for MJ and allowed to dry in the same manner as the corresponding treatment. All treated and control plants were immediately placed in a large black plastic bags for 24 h and maintained in the dark at room temperature. Application of chemicals and incubations were repeated 24 h later. ABA (abscisic acid, Sigma, St. Louis, USA) was dissolved to 20 mM in 100% ethanol. This stock solution was then diluted with water to a final concentration of 100 μM and sprayed evenly over the plants before sealing as described for the MJ treatment. Control plants were sprayed evenly with 0.1% (w/v) ethanol solution (Anderson *et al*. 2004). Leaf and stem tissues were then sampled at various time intervals after treatments, immediately frozen in liquid nitrogen, and the RNA extracted.

RNA and DNA preparation

For gene expression analysis, RNA was isolated from infected internode tissues at different dpi (day post inoculation), respectively, to proceed to RT-PCR. Total RNA (1 μg) was reverse transcribed at 42°C for 1 h in the presence of both cDNA synthesis (CDS) primer and SMART II oligonucleotide using the Clontech SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA). For SSH, Northern blotting, and rapid amplification of cDNA ends (RACE), RNA was isolated from inoculated and non-inoculated control plant internode stem tissues at 1-7 dpi by using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. DNA was extracted from leaves by grinding in liquid nitrogen with 10% (w/w) PVP, adding lysis buffer [2% (w/v) CTAB, 75 mM Tris-HCl (pH 8), 15 mM EDTA (pH 8), 1.05 M NaCl, 4% (v/v) β-mercaptoethanol], preheating to 65°C, heating for 30 min at 65°C, and centrifuging for 5 min at 12 000 r/min. The supernatant was mixed with an equal volume of chloroform/isoamyl alcohol (24:1, v/v) and blended gently upside down until milkiness appeared in the lower bottle-green liquid. After centrifugation, DNA was precipitated from the colorless supernatant with 0.1 vol. of 10 M ammonium acetate and 2 vols. of 95% (v/v) alcohol for 10 min at room temperature, and centrifuged for 10 min at 13 000 r/min. The pellet was washed with 70% (v/v) ethanol, dried in air, and resuspended in sterile distilled deionized water.

Subtraction suppression 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). We also performed a reverse subtraction, 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 evaluation of the subtraction efficiency, these subtracted cDNAs were subcloned in pGEM-T plasmids and were used to transform *Escherichia coli* DH5α bacteria (Invitrogen). A total of 384 cDNA clones specifically expressed in the resistant samples were performed by differential screening of the subtractive libraries following the recommendations of the PCR-Select Differential Screening Kit user manual (Clontech). Four sets of membranes were prepared and hybridized with the radio-labeled tester (T), driver (D), forward-subtracted tester (FT) and reverse-subtracted driver (RD) probes, respectively. 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 Technologies (Shanghai, China). All the inserted sequences were checked for homology using the BLASTX program against the GenBank/EMBL/DDBJ non-redundant sequence database. The sequences sharing no homology hit using the BLASTX program were further searched against the data set for plant EST sequences of the GenBank dbEST. Protein sequences were aligned using CLUSTAL W and the alignments 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 by SSH contained a 3′-untranslated region (UTR); therefore, 5′-rapid amplification of cDNA ends (RACE) was used to obtain the full-length cDNA with a SMART-RACE cDNA Amplification Kit (Clontech) following the user manual. 5′-gene-specific primers (GSP1: 5′-GACCAGCGGCTTTTCCCGTATCAAT-3′ and GSP2: 5′-CATGCTGTTTTCCGGTCTTCTGGGG-3′) were designed based on the sequences of the isolate by SSH. The 5′-end of the cDNA was amplified by PCR using the universal primer mix (UPM) along with the antisense GSP2 oligonucleotide. The obtained cDNA ends were ligated into pCR 2.1 (Invitrogen) and sequenced as described above. The resultant sequences were aligned with the isolates to obtain full-length cDNA. The full-length gene was designated as *StLTPb1* and submitted to GenBank under accession numbers EU057715.

Real-time quantitative PCR and Northern blot analysis

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germany) followed by DNase I treatment to remove any genomic DNA contamination. The quantitative real-time PCR analysis was performed as described earlier (Jain *et al*. 2006). In brief, the cDNA samples synthesized from 3 μg of the total RNA using the high capacity cDNA Archive Kit (Applied Biosystems, USA) were used as template and mixed with 200 nM of each primer and SYBR green PCR master mix (Applied Biosystems) for real-time PCR analysis, using the iCycler iQ™ real-time PCR detection system (Bio-Rad, Richmond, CA) according to the manufacturer's instructions. The sequences of forward and reverse primers, designed and synthesized by Sangon Technologies (Shanghai, China), were as follows: primer-forward: 5′-TGGCTCCCTGCCTCCCTTATC-3′, primer-reverse: 5′-CATCTTATTCTTCATCTCCGC-3′. The transcript of potato *actin* (GenBank accession number X55747) was used to standardize each reaction run (primer-forward:5′-GCTTCCCGATGGTC AAGTCA-3′, primer-reverse: 5′-GGATTCCAGCT GCTTCCATTC-3′). 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 real-time PCR analysis in triplicate. Northern blot or reverse transcription polymerase chain reaction (RT-PCR) analysis were performed as described previously (Wawrzynska *et al*. 2005). The pairs of respective primers were as follows:

5´-TGGCTCCCTGCCTCCCTTATC-3´,5´-TCCATCTTATTCTTCATCTCC-3´.

In situ hybridization analysis

Paraffin-embedded potato stem and leaf sections were used for *in situ* hybridization with digoxigenin (DIG)-labeled RNA probes as described previously (Elorza *et al*. 2004). To obtain the *StLTPb1* gene specific probes, cDNA fragments were PCR amplified using the primer pairs 5´-GGCTCCCTGCCTCCCTTATC-3´ and 5´- TCCATCTTATTCTTCATCTCC-3´. The PCR products were cloned into a pGEM-T plasmid (Promega), and the recombinant plasmids were sequenced. Sense and antisense riboprobes were labeled with digoxigenin-UTP using the DIG RNA Labeling Kit (SP6/T7; Roche Diagnostics).

RESULTS

Identification and molecular cloning of nsLTP cDNA

In order to identify genes induced by *R. solanacearum* at early stage of the pathogen-potato interaction, a cDNA library was constructed by suppressive subtractive hybridization (SSH) with two mRNA populations of stem mRNA, in which the inoculated and mock inoculated cultivar Zhongshu 3 mRNA as the tester and driver respectively. Among the clones obtained by the subtraction one corresponding to an open reading frame (ORF) coding for plant *nsLTP* with a 3´-untranslated region (UTR) has been studied further. Full-length cDNA (495 bp, (accession no. EU057715, named *StLTPb1*) coding for this clone was obtained by 5´-RACE PCR. The amino acid sequence deduced from the ORF revealed that it encodes a protein of 114 amino acids which exhibits similarity to a group of non-specific LTPs from various plants. This sequence shared with the LTP1 family such as high isoeletric points (*p*I = 9.40), molecular masses of approximately 10 kDa and the signal peptide at the amino terminal region. Further, LpLTP1 and LpLTP2 have eight Cys and four Pro residues at highly conserved positions found in all other nsLTPs. The deduced amino acid sequence of *StLTPb1* showed similarity to the members of plant nsLTPs such as *Arabidopsis*, rice and other plant species. *StLTPb1* has eight conserved cysteine residues shown to form four disulfide bridges, shown in other LTPs to be important for structure and function. A signal peptide of 23 amino acids was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP). The mature predicted protein without the signal peptide was 91 aa (Fig.1).

A general view of the evolutionary history of the *Solanaceae* nsLTP family was provided by an unrooted phylogenetic tree (Fig.2) constructed with the neighbor-joining method using the mature amino acid sequences available in the GenBank. The tree obtained shows three distinct phylogenetic groups among the LTP amino acid sequences. All groups are in agreement with type 1 and type 2 nsLTPs usually reported. Interestingly, potato nsLTPs are found almost all over the phylogenetic tree, except in the second group. Most potato type 1 (nsLTP1) nsLTPs appears clustered together in group I, whereas the type 2 (nsLTP2) LTP genes are present in group III. In particular, the nsLTP2 (BAC23052) sequence has a long phylogenetic distance from others. The phylogenetic analysis implies that the *StLTPb1* gene might be diverged from the common ancestor with *S. lycopersicum*.

Differential expression of *StLTPb1* gene between resistance and susceptible potato cultivars in response to *R. solanacearum*

RNA gel blot analysis was used to examine the abundance of the *nsLTPb1* transcripts in potato stems and leaves at different time points after inoculation with *R. solanacearum* (Fig.3). Gene expression corresponding to disease course was detected in two steps. Firstly, Northern blot analysis carried out with the total RNA isolated from potato stems and leaves after the inoculation revealed that transcripts corresponding to the potato *nsLTP* gene accumulated preferentially in resistant genotype, indicating specific induction during the early response upon the bacterial infection (Fig.3-A). The *nsLTPb1* transcripts were rapidly and strongly accumulated in both total samples of stems and leaves at the stage of 3-5 days post inoculation (dpi). Secondly, to further determine whether the *StLTPb1* gene was

systemically induced in potato plants, transcription patterns of the *nsLTPb1* gene in lower (infection site) and upper (systemic site) stem and leaves were examined at different time points after inoculation. As the Fig.3-B and D showed, *StLTPb1* transcripts were weakly detected in compatible interaction from 1st to 3rd d post inoculation in susceptible genotype, but were strongly in resistant genotype. This induction after 3rd dpi was dropped tardily in the systemic upper stems and leaves, while a quick reduction following the sharp induction in the infected lower stems and leaves was observed in susceptible genotype. This suggested that *StLTPb1* is subjected to some turnover under the increased pathogen stress (Fig.3-F). In contrast to susceptible genotype, the transcripts elevated rapidly in both lower infection site and upper systemic site and accumulated persistently in resistant genotype. It suggested that *StLTPb1* was considerably induced in a systemic pattern, far away from the niche where the pathogen colonized.

Effects of chemical treatments on *StLTPb1* gene expression

As shown in Fig.4, all three of plant hormones can stimulate *StLTPb1* gene up-regulated expression. *StLTPb1* transcripts in potato stems were found to increase slowly after 24 h of SA treatment; this increase was preserved to 5th dpi then reduced gradually. Similar patterns in the expression of this gene were observed in stems after MJ treatment. However, this increased expression was observed to rise again at the end of the early stage of potato-*R. solanacearum* interaction. This phenomenon was more remarkable in the case of ABA treatment. After one day of ABA treatment, *StLTPb1* transcripts in potato stems were induced to nearly comparable levels to those observed in response to the pathogen at 5th dpi (Fig.4). This suggests that ABA treatment must activate a different and more shortcut and effective mechanism of gene induction than SA and MJ treatments, producing a long-lasting effect on the *StLTPb1* gene expression.

In situ localization of StLTP mRNA in potato tissues

To examine the localization of transcripts of *StLTPb1* in response to pathogen infection, *in situ* hybridization was performed using the potato leaf and stem tissues (Fig.5). Three days after inoculation with the virulent strain, a heavy deposit of *StLTPb1* mRNA occurred mainly within the inner phloem area of vascular tissue

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TATCGGCCATTACGGCCGGGGACCCAACACTCAAAACAAATACATTTGTAGTATTATATC  60
ATCCTCTACTATTTCTATCGATCTTTACTCAATTTTTGTAACAATGGAAATGTTTGGCAA 120
                                              M  E  M  F  G  K -18
AATCGCATGCTTTGTGCTTTTGTGCATGGTGGTGGTTGCACCCCGTGCAGAGGCACTGAG 180
  I  A  C  F  V  L  L  C  M  V  V  V  A  P  R  A  E  A  L  S   3
CTGCGGCGAGGTTACATCTGGCTTGGCTCCCTGCCTCCCTTATCTTCAAGGGCGCGGTCC 240
  C  G  E  V  T  S  G  L  A  P  C  L  P  Y  L  Q  G  R  G  P  23
TATTGGAGGGTGTTGTGGTGGTGTTAAGGGTCTTTTGGGTGCAGCCAAAACCCCAGAAGA 300
  I  G  G  C  C  G  G  V  K  G  L  L  G  A  A  K  T  P  E  D  43
CCGGAAAACAGCATGCACTTGCCTTAAATCGGCAGCTAATTCTATTAAGGGAATTGATAC 360
  R  K  T  A  C  T  C  L  K  S  A  A  N  S  I  K  G  I  D  T  63
GGGAAAAGCCGCTGGTCTCCCTGGAGTTTGTGGAGTCAATATTCCTTACAAGATCAGCCC 420
  G  K  A  A  G  L  P  G  V  C  G  V  N  I  P  Y  K  I  S  P  83
TTCGACTGATTGCTCCAAGGTCCAGTAAGGTTGATGAAATTAAAGCTAAAGTCTATATAT 480
  S  T  D  C  S  K  V  Q  *                                    91
AGCTTGCGGAGATGAAGAATAAGATGGATATATATCGATCGAGTGTGATCCATCATTATA 540
TATGTTGTCTCTTTTCTTTTGTATTTGTGTCTGTTGGAGTACTTATATATTGTAGTCTTG 600
TAATGAACATTGGTGGTTGCTTGTGTTACAACCCAATCTTCATATAGTAATACATGATAT 660
TTTCTTCTTCAAAAAAAAAAAAAAAAAAAA                               691
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Fig. 1 Composite nucleotide sequences of the cDNA for *StLTPb1* and the deduced amino acid sequence. The amino acids are numbered from the alanine residue found at the amino terminus of the mature protein; the regions of signal peptide are italic.

and in the outer phloem. Very slight hybridization signals could be observed occasionally in the epidermal tissue. The *StLTPb1* transcripts were localized in the phloem cells of leave and stem tissues infected by the virulent isolate of *R. solanacearum*, but not in xylem vessels. No hybridization signals of *StLTPb1* mRNA were detected in any organelles of uninfected healthy leaf or stem tissues. No staining was observed in either the inoculated or the mock inoculated healthy plant root tissue. Similar results were obtained from two

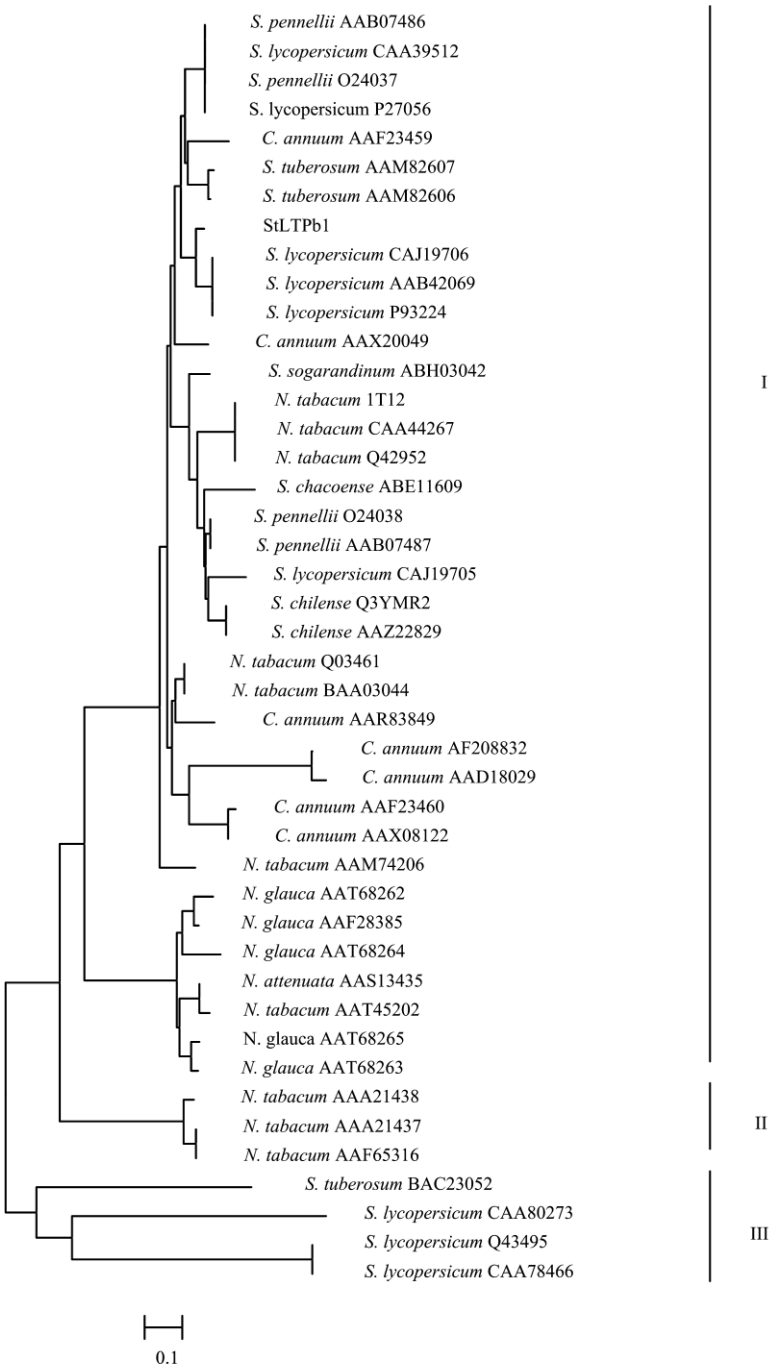

Fig. 2 Phylogenetic relationships of the *Solanaceae* nsLTPs based on sequence alignments of the mature encoded proteins. The analysis was based on alignment of forty-eight unique amino acid sequences. The primary accession numbers for the protein sequences used to compile the tree followed the specific name. The branch of StLTPb1 was showed separately from the tree. The scale bar corresponds to 0.1 substitution per amino acid. The evolutionary tree was constructed by the neighbor-joining method and drawn by the MEGA program.

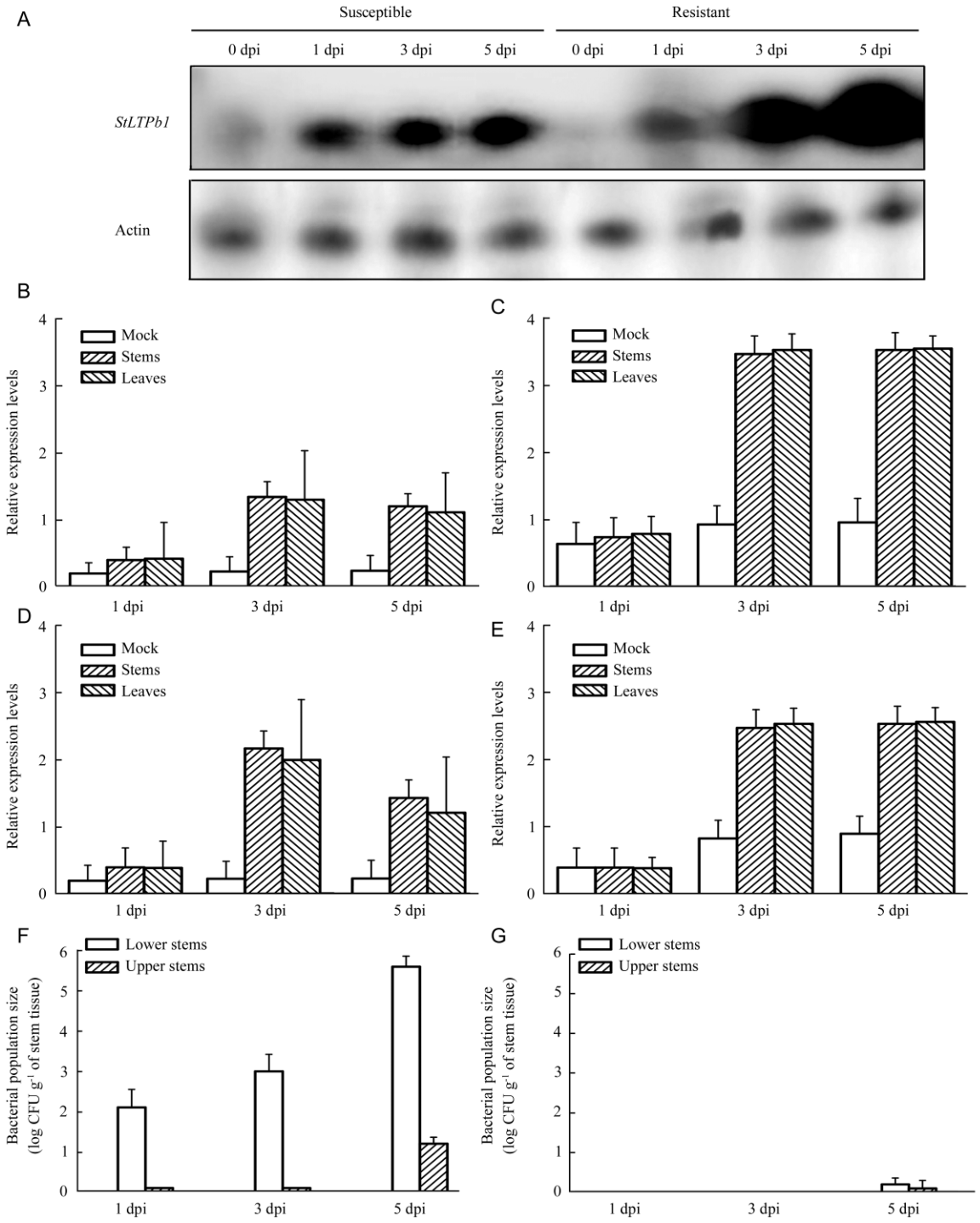

Fig. 3 Differential expression of *StLTPb1* gene in different potato genotypes during the early stage of *R. solanacearum*-potato interactions. A, Northern analysis of *StLTPb1* gene expression with the constitutive gene *actin* as inner control, total RNA were isolated from potato stems and leaves after the inoculation with virulent *R. solanacearum* (Race 3, Biovar 2, stain PO41) at indicated times and used for RNA gel-blot in a representative corresponding experiment. B and C, histograms indicating time courses of *StLTPb1* gene expression in the upper (between 6th-7th nodes) tissues of susceptible (B) and resistant (C) genotypes at various time intervals after inoculation by real-time PCR. The values were normalized to *actin* at each time-point. Statistical significance for the observed up-regulation was calculated with Students *t*-test: a P-value < 0.01. Error bars represent the mean ± standard error. As a control, healthy plants were mock-inoculated with water. D and E, relative transcript levels of *StLTPb1* gene in the lower (between 2nd-4th nodes) tissues of susceptible (D) and resistant (E) genotypes at various time intervals after inoculation. F and G, population sizes of strain PO41 in potato of susceptible (F) and resistant (G) genotypes at different stages of infection. Overground stem tissues were sampled by grinding and effluent water was dilution-plated directly. With Student's *t*-test, triplicate experiments were tested at the $P < 0.05$ significance level and 3 plants tested for each.

independent experiments.

DISCUSSION

Conservative property of *StLTPb1* in evolution showed by structural analysis

Plant LTPs participated in many physiological processes including plant disease resistance. Although nsLTP1s play an important role in plant response to pathogens, the mechanism of action is not known. It's important to the analyses of structure, homologies and evolution of potato nsLTPs, which may help predict their functions. In most plant species, nsLTPs are encoded by a small gene family and linkage of some members of the gene family has been observed, e.g., tobacco, sorghum, maize, and barley, among others and additional nsLTP isoforms have been reported to occur in several subfamily (Treviño and Connell 1998). In this study, a new non-specific lipid transfer protein genes *StLTPb1* was isolated from the cDNA libraries constructed from potato leaves and stems infected with virulent strain of *R. solanacearum*. *StLTPb1* was selected for expression

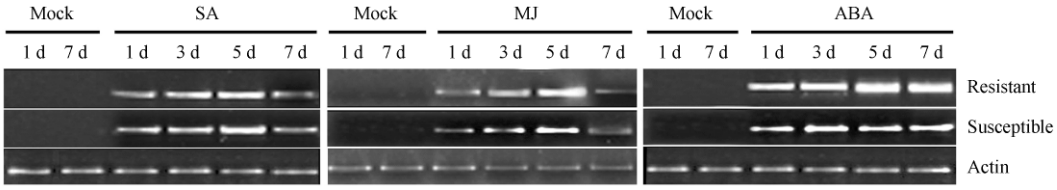

Fig. 4 Relative expression of the *StLTPb1* in potato stems at various time intervals after treatment with plant hormones or abiotic elicitors, such as SA (5 mM), MJ (100 μM) and ABA (100 μM) in a representative corresponding experiment. Healthy plants were mock-treated as control.

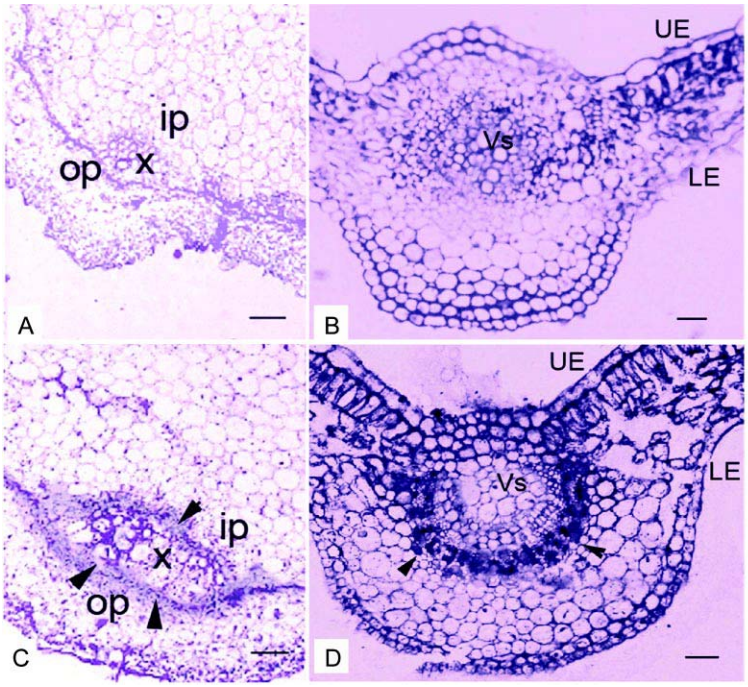

Fig. 5 *In situ* localization of *StLTPb1* mRNAs in potato stems and leaf tissues. Consecutive 10 μm transverse sections of the leaves (A and C) and stems (B and D) were hybridized with the sense (A and B) and antisense (C and D) *in vitro*-synthesized *StLTPb1* RNA. The arrowheads point out hybridization signal in the outer and inner phloem cells. UE, upper epidermis; LE, lower epidermis; op, outer phloem; ip, inner phloem; Vs, vascular bundle; x, xylem. Bars = 10 μm.

analysis. Similar to other plant nsLTPs, potato nsLTPs share the same features including a low molecular weight, eight cysteine residues, a basic *p*I and a signal peptide predicted to target the proteins to the secretary pathway (Kader 1996). Alignment showed the highest degree of amino acid sequence identity with nsLTPs from tomato, pepper and tobacco. Regarding the phylogenetic distribution of nsLTPs (Fig.2), our results suggest that *StLTPb1* belong to the biggest class and the other novel cDNA clones encoding different isoforms of the plant non-specific lipid transfer protein. The phylogenetic tree suggests that this gene be conservative in evolution in *Solanaceae*. When based on amino acid sequence comparisons of *StLTPb1* with other members from the gene family, the sequence of *StLTPb1* was much closely related to predict amino acid sequences of LTP genes isolated from potatoes and tomatoes. According to Wang *et al*. (2005), the conservative CaM-binding region of *StLTPb1* nsLTP1 could be identified at the N-terminal. This implied the nsLTP1 could be involvement of Ca^{2+}/CaM signaling for its biological function as a kind of antibacterial peptide. Further more, the nsLTP1 have been considered as a kind of antibacterial peptide because of a large number of positively charged residues on the face of the molecule, but these are evenly distributed over the entire molecular surface such as the *StLTPb1* nsLTP1. Examination of the charge distributions of structures of the antifungal protein from pokeweed (PDB code 1dkc), antifungal protein from radish (PDB code 1ayj), thionin from wheat (PDB code 1gps), and rice nsLTP2 (PDB code 1l6h) showed a high concentration of basic residues on one molecular face and the asymmetric distribution of positive charges may be necessary for effective antimicrobial activity in defense proteins (Salcedo *et al*. 2007). Taken together, these results emphasize to link the structure of this type 1 nsLTPs with its function in plant defense mechanisms.

Differential expression and *in situ* localization of *StLTPb1* gene induced by *R. solanacearum*

The role of LTPs in the defense mechanisms of plants has been investigated either by studying the activity of purified proteins (Carvalho and Gomes 2007), or through the expression pattern of LTPs genes following the response to pathogen infection (Park *et al*. 2002). Generally, LTPs are known to be associated with the formation of the cutin and suberin layers and inhibition of fungal growth. The heterologous expression of a LTP from pepper (*Capsicum anuum*) in *Arabidopsis* was recently reported to increase the resistance to *B. cinerea* (Jung *et al*. 2005). Here, our data provided more experimental supports for their involvement in resistance to *R. solanacearum* in potato. Our results are in agreement with those observations and support the view that the action of the products of the nsLTPs contributes to the resistance of plants phloem, possibly forming a second protective shield against infection with *R. solanacearum* (Carvalho and Gomes 2007). Due to antibiotic properties of nsLTPs *in vitro*, several reports involving LTPs in plant defense mechanisms appeared in the literature. However, it does not appear to be a general feature of the peptides.

The temporal and spatial expressions of *StLTPb1* gene imply its possibility playing a significant role in the vascular networks. The plant vascular system, comprised of xylem and phloem, forms a continuous network throughout the plant body to transport water, nutrients and signaling molecules. Vascular cells differentiate by contact with each other to form a continuous strand of vasculature where the plant pathogen *R. solanacearum* could colonize. This systemic expression pattern of *StLPTb1* has raised the possibility that some special inductive signals function to guide continuous responses to the invasion of the bacteria. Such inductive signals have not been defined, however, in part because of difficulties in analyzing cell-cell interactions *in planta*. It's interesting that there exist differences between the lower infection site and upper systemic site of stem and leaves and between the susceptible and resistant genotypes. A possible explanation for the differences is that the pathogen regulates *StLPTb1* via some transcription factors. Maldonado *et al.* (2002) indicated that lipid transfer protein DIR1 in *Arabidopsis thaliana* could interact with a lipid-derived molecule to promote long distance signaling. The non-specific LTP DIR1 displays high similarity to members of LTP family in *Arabidopsis* genome. The DIR1/LTP might in principle be a mobile signal or functions in cooperation with the mobile signal

because over-expression of DIR1 is not sufficient to induce SAR and pathogen exposure is still required. Lipid molecules such as oxylipins (jasmonic acid), phosphatidic acid and N-acylethanolamines are synthesized or released from membranes upon pathogen. These act as second messengers in plant defense signaling. Structural studies predict that plant LTPs contain an internal hydrophobic pocket that is large enough to accommodate a fatty acid or lysophospholipid. It predicts that plant nsLTPs contain signal peptides, some of which target them to the cell wall (Garcia-Olmedo *et al*. 1995). So, nsLTPs like DIR1/LTP could be either a co-signal or act as a translocator for release of the mobile signal into the vascular system and/or chaperone the signal through the plant. Our result of the expression of *StLTPb1* far away from the infection site (Fig.3) suggested that the gene product be involved in vascular signaling for systemic expression.

Organ- and tissue-specific expression of LTP genes has been demonstrated in several plant species (Jung *et al*. 2003). The expression of the LTP genes was found to be localized in phloem cells of vascular tissues in aerial potato stem and leaf after pathogen infection, which suggested that the *StLTPb1* gene specifically expressed in certain tissues during the development of disease. The transcription of the *nsLTP* gene was strongly induced in the compatible interactions. This result suggested that increase in the nsLTP transcripts concomitant with bacterial infection play a significant role in the expression of response to *R. solanacearum* in potato vascular tissues. The responses of *StLTPb1* to plant pathogen infection in potato leaves and stems are consistent with the earlier observations of expression of pepper non-specific lipid transfer protein CALTP and other plant pathogenesis related genes (Jung and Hwang 2000; Jung *et al*. 2003; Kim *et al*. 2000).

Induction of *StLTPb1* gene by abiotic stimuli

Study of environmental regulation of gene expression during each stage of disease is very important. Recent evidences point towards a role in plant defense signaling pathway for some type 1 nsLTPs. A tobacco type 1 LTP (NtLTP1) binds the defense signaling molecule jasmonic acid (JA) and the resulting complex is able to interact with a plasma membrane-located receptor (Buhot *et al*. 2004). A grapevine LTP (VvLTP4) showed efficient interaction with oxylipin (Girault *et al*. 2008). Exogenous application of the VvLTP4-JA complex on grapevine plantlets induced a high level of tolerance towards *Botrytis cinerea*, as compared with control plants. These data point towards a LTP-JA or a LTP-oxylipin complex as a candidate for the SAR mobile signal (Yeats and Rose 2008). As shown in Fig.4, transcription of the *StLTPb1* gene was strongly induced in stem treated with exogenous SA, MJ or ABA, which suggests that plant hormones may function in the signal transduction pathway to activate the *StLTPb1* gene expression. In contrast, ABA had little more rapid effect in *StLTPb1* gene expression in the stem tissues. The accumulation of *StLTPb1* transcripts by these hormones may reflect the putative role of nsLTPs in vascular tissue, because this transport system plays a significant role in the water balance and signal transport. It can be concluded that the pattern of expression of LTP genes in response to infection of potato by *R. solanacearum* is rather complex. The regulation of the response of LTP-genes is also complex. Some 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. However, we are still in the early stages of understanding the extent and sophistication of gene expression involved in *R. solanacearum* interaction. Future research should focus more on gene expression inside the host plant at various locations and times during disease, and the nature of the signals that affect it. The nature of the remaining signals not only will explain what the pathogen looks for during each stage of disease, but also may provide new targets for disease control, since disruption of the transduction pathways often leads to loss of virulence.

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