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Cloning and molecular characterization of the potato RING finger protein gene *StRFP1* and its function in potato broad-spectrum resistance against *Phytophthora infestans*

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ABSTRACT

Really interesting new gene (RING) finger proteins function as ubiquitin ligase and play key roles in biotic and abiotic stresses. A new RING-H2 finger protein gene, *StRFP1*, was cloned from *Phytophthora infestans*-inoculated leaves of potato (*Solanum tuberosum*) clone 386209.10, which is free of *R1-R11* genes. The deduced amino acid sequence was characterized by an N-terminal transmembrane domain, a GLD region and a RING-H2 finger signature. StRFP1 is homologous to the tobacco NtACRE132 protein and belongs to the ATL family. The DNA gel blot analysis and mapping revealed that *StRFP1*, an intron-free gene, had one to two copies in the potato genome and was located on chromosome 3. RT-PCR assays showed that *StRFP1* was constitutively expressed in potato plants and significantly induced in detached potato leaves by *P. infestans* and plant defense-related signal molecules, abscisic acid, salicylic acid and methyl jasmonate. Transient expression studies revealed that *StRFP1* fused with GFP localized to the plasma membrane or out of that in onion epidermal cells. The function of *StRFP1* in potato resistance against late blight was further investigated by constructing overexpression and RNA interference (RNAi) vectors, which were introduced into potato cv. E-potato 3, respectively. By challenging the detached leaves with mixture races of *P. infestans*, all of the *StRFP1*-overexpressing plants displayed slower disease development than non-transformed controls in terms of the lesion growth rate (LGR). In contrast, *StRFP1*-silencing plants through RNAi were more susceptible to pathogen infection. The present results demonstrate that *StRFP1* contributes to broad-spectrum resistance against *P. infestans* in potato.

Introduction

The ubiquitin/26S proteasome pathway functions as the primary proteolytic system for degradation of cellular proteins among eukaryotes. Importantly, this system also serves as a control/regulation mechanism by eliminating normal proteins, such as rate-limiting enzymes and key regulators in different pathways (Moon et al., 2004; Dreher and Callis, 2007). The biochemical process of this system basically starts with ubiquitination of target proteins (covalent attachment of ubiquitin molecules to substrates), achieved through the iterative action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), which are then directed to the 26S proteasome for degradation with the concomitant release of ubiquitin moieties for reuse (Hershko and Ciechanover, 1998). The specificity of ubiquitination is largely determined by E3 for recruiting appropriate substrate(s) (Mazzucotelli et al., 2006).

In the *Arabidopsis* (*Arabidopsis thaliana*) genome, approximately 1300 genes are predicted to be involved in the ubiquitin/26S proteasome pathway, and almost 1200 genes encode diverse classes of E3 ligases (Smalle and Vierstra, 2004), 469 of which belong to the really interesting new gene (RING) finger class (Stone et al., 2005). RING-H2 finger proteins, forming the *ATL* gene family, were originally identified in *Arabidopsis* (Salinas-Mondragon et al., 1999) and later found to be distributed widely in plant species, for example, the *Arabidopsis* and rice (*Oryza sativa*) genomes contain 80 and 121 members of this family, respectively (Serrano et al., 2006). In addition to a

Abbreviations: ABA, abscisic acid; ACRE, Avr9/Cf-9 rapidly elicited; CaMV, cauliflower mosaic virus; EST, expressed sequence tag; ETH, ethylene; GFP, green fluorescent protein; HR, hypersensitive response; MeJA, methyl jasmonate; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RFP, RING finger protein; RING, really interesting new gene; RNAi, RNA interference; RT-PCR, reverse transcription PCR; SA, salicylic acid; UTR, untranslated region

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conserved RING-H2 finger domain, a single hydrophobic region of at least 18 residues and a GLD domain (denotes the first three conserved residues of the sequence, comprises about 16 residues where a glycine and a proline residue are highly conserved and the distance between them is almost invariable) have been predicted in ATLs (Serrano et al., 2006). This type of gene may function as the E3 ubiquitin ligases involved in the plant defense response (Kawasaki et al., 2005; Zeng et al., 2006). For example, the *Arabidopsis ATL2* and *ATL6* genes are rapidly and transiently induced 15–30 min after treatment with chitin (Salinas-Mondragon et al., 1999) and the mutants with constitutive expression of the *ATL2* gene exhibit upregulated expression of defense-related genes and SA- and JA-responsive genes (Serrano and Guzman, 2004), suggesting that ATL2 may be involved in the early defense response of plants to pathogen attack (Salinas-Mondragon et al., 1999). A T-DNA insertion mutant of *ATL9* results in increased sensitivity to powdery mildew disease (Ramonell et al., 2005). Similarly, two members of the rice *ATL* gene family have been studied in detail for their biochemical and biological function. One is an elicitor-responsive 5 (*EL5*) gene, upregulated in suspension-cultured rice cells under the treatment of *N*-acetylchitooligosaccharide elicitor at the early stage (Takai et al., 2001, 2002). Another is *OsBIRF1*, which plays important roles in growth and defense responses against biotic and abiotic stresses (Liu et al., 2008). The tobacco (*Nicotiana tabacum*) Avr9/Cf-9 rapidly elicited 132 (*NtACRE132*) gene is involved in disease resistance, especially in the specific hypersensitive response (HR) (Durrant et al., 2000; Kawasaki et al., 2005).

Having quantitative and durable characteristics, broad-spectrum resistance to diverse races of the late blight pathogen *Phytophthora infestans* is a major objective of potato breeding programs to overcome a rapid loss of R-gene governed resistance. As an initial effort to elucidate the molecular basis of potato quantitative resistance to late blight, the suppression subtractive hybridization (SSH) strategy was used to construct the cDNA library rich in *P. infestans*-upregulated genes obtained from the potato (*Solanum tuberosum*) leaves of the clone eliminated *R1-R11* genes (Tian et al., 2003). A total of 348 *P. infestans* responsive genes were further identified through the cDNA microarray, of which one expressed sequence tag (EST), 10-A12, had 50% identity to *NtACRE132* and was rapidly induced by *P. infestance* (Wang et al., 2005). We examined whether the gene has the structural features of ATL and how it responds to t pathogen infection, especially when diverse pathogen races are involved. To examine these questions, we isolated the *StRFP1* gene and investigated its expression pattern in response to *P. infestans* and defense-related signal molecules. We also analyzed its function through genetic transformations. Our results indicate that *StRFP1* is a RING-H2 type RING finger protein (RFP) gene in potato and enhances potato resistance to mixture races of *P. infestans*. These results provide insights for approaching the mechanisms of potato quantitative resistance to late blight.

Materials and methods

Plant materials, growth conditions and treatments

Two potato (*Solanum tuberosum* L.) genotypes, clone 386209.10 (eliminated *R1-R11* genes, horizontal resistance to late blight, which was kindly provided by the International Potato Centre) and cv. E-potato 3 (vertical resistance to late blight) were used. E-potato 3 was used only for the genetic transformation and 386209.10 was used in all the other experiments.

In vitro plantlets were propagated in sterile culture boxes containing MS medium supplemented with 3% sucrose and 0.8% agar and raised in a growth chamber under controlled conditions (16 h light/8 h dark cycle at 20 °C). Four- to five-week-old plantlets were transplanted to a greenhouse under normal conditions.

Five-week-old leaves of greenhouse-grown 386209.10 plants were incubated on the surface of wet filtrate paper in plastic trays and sprayed with freshly produced sporangia of *P. infestans* at 5×10^4 mL^{-1} zoospores, 2 mM salicylic acid (SA, pH 6.5), 50 μM methyl jasmonate (MeJA), 100 μM abscisic acid (ABA) and 200 μL L^{-1} ethylene (ETH) (Sigma-Aldrich, St. Louis, USA), respectively. SA, MeJA and ABA were dissolved in 0.1% ethanol. The respective control samples were treated in the same way with 0.1% ethanol or distilled sterilized water according to the solvent used. MeJA and ETH-treated leaves were sealed tightly with plastic bags. The treated samples were collected at the indicated time points, frozen in liquid nitrogen and maintained at −70 °C until use.

Isolation and characterization of the StRFP1

A pair of gene-specific primers (5′-GACAGAATAGTGAAGAGG-CAGAAG-3′) and (5′-CTGTAAGAGAAGATAGATTCCCCTC-3′) were designed according to the sequence of a previously obtained EST fragment, 10-A12, which has 50% similarity to the *NtACRE132* (Tian et al., 2003). Combining the reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE), these primers were used to amplify the 5′- and 3′-ends of the *StRFP1* gene using the mRNA, which was isolated from potato 386209.10 leaves after inoculation with *P. infestans*, as template. Amplified PCR products were purified by the DNA Gel Purification Kit (Sangon, Shanghai, China) and cloned into pMD18-T vector (Takara, Japan) followed by sequencing.

Based on the nucleotide sequences of the 5′- and 3′-RACE products, a forward primer (5′-AAGGATCCTGTCTGAAA***ATG***G-GAAGTGGT-3′, *Bam*H I site underlined) and reverse primer (5′-TTGAGCTCCTGTAAGAGAAGATAGATTCCCCTC-3′, *Sac* I site underlined) were used for the amplification of full-length cDNA and gDNA sequence of *StRFP1*. The resulting fragments were cloned into the vector pMD18-T and designated as pMD-StRFP1. The confirmed isolates were subjected to database analysis at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the genomic sequence. Various tools from Expasy (http://www.expasy.org/tools) were used to deduce the translated product and compute theoretical p*I* and molecular weight. The putative domains were identified in the InterPro database (http://www.ebi.ac.uk/interproscan/). Multiple sequence alignments involved the use of ClustalW.

StRFP1 mapping and DNA gel blot analysis

For *StRFP1* mapping, a pair of specific primers (CATCAGCATC-CACAAGTA and GAAGATAGATTCCCCTC) was designed and a potato BCT segregation population (Bonierbale et al., 1994) was employed. Forty-one individual genomic DNAs of BCT, previously chosen as the most informative subset of the population, were kindly provided by the International Potato Centre (CIP). A PCR amplification length polymorphism was detected and the *StRFP1* marker was inserted into the RFLP linkage framework of the BCT population using the MAPMAKER 3.0 program (Lander et al., 1987).

Genomic DNA was prepared from young leaves of greenhouse-grown potato plants using cetyl-trimethyl-ammonium bromide (CTAB) (Dellaporta et al., 1983). About 50 μg per sample was completely digested with *Pst* I, *Eco*R V, *Hind* I I I and *Sal* I (Takara, Dalian, China) and separated on a 0.8% (w/v) agarose gel, followed by blotting onto a positively charged nylon membrane

(Boehringer, Mannheim, Germany) using standard methods (Sambrook et al., 1989), and then probed with a DIG-labeled full-length cDNA of *StRFP1* gene prepared from the DIG High Prime DNA Labeling and Detection Starter Kit (Roche Molecular Biochemicals, Germany).

Construction and subcellular localization of CaMV 35S::StRFP1-GFP fusion protein in onion epidermal cells

The coding region sequence of *StRFP1* without the stop codon was amplified by PCR from the full-length clone of pMD-StRFP1, then fused with the green fluorescent protein (GFP) in the C-terminus and cloned into the pBI121 vector under the control of the 35S promoter. Transient expression of the CaMV 35S::StRFP1-GFP fusion construct and the CaMV 35S::GFP control was performed by introducing the resultant plasmids into onion (*Allium cepa* L.) epidermal cells by means of the particle bombardment method according to the manufacturer's protocol (Bio-Rad, CA, USA). The transformed cells were cultured on MS medium at 25 °C for 24 h and observed under a Bio-Rad MRC-1024 confocal laser scanning microscope (Bio-Rad, CA, USA) for detecting the fluorescence.

Gene expression analysis by RT-PCR

Total RNAs were extracted from various tissues, samples of different treatments and the transgenic lines with Trizol reagent and used for reverse transcription (RT) of the first-strand cDNA using the Super-Script III Kit (Invitrogen, CA, USA) according to the manufacturer's instructions. One microliter of resulting cDNA and 10 pM of each primer were used for RT-PCR in a total volume of 25 μL. PCR conditions were set as 94 °C for 30 s, 65 °C for 30 s and 72 °C for 30 s for 25–30 cycles based on the abundance of transcript for each gene, followed by 5 min of final extension at 72 °C. PCR products were electrophoresed through 1.0% agarose gel. Gene-specific primers (5′-ATGTCCTCTACGAAGTCCATCAT-3′ and 5′-CTTCTGCCTCTTCACTATTCTGTC-3′) were used to examine the expression level of *StRFP1*, and the *S. tuberosum Actin* gene (Genbank accession number: X55749.1) was amplified as an internal control to quantify the relative amounts of cDNA. Each experiment was repeated three times independently.

Generation and characterization of transgenic potato plants

Overexpression construct of *StRFP1* was made by doubly digesting the plasmid carrying the ORF sequence of pMD-StRFP1 cDNA with *Bam*H I and *Sac* I. The resultant DNA fragment was then inserted into the identical site of the plant binary vector pBI121 (Clontech) in a sense orientation between the *cauliflower mosaic virus* (CaMV) 35S promoter and the nopaline synthase (NOS) terminator by replacing the β-glucuronidase (GUS) reporter gene, yielding the p35S:StRFP1 construct.

For the construction of the *StRFP1* RNAi vector, a 327bp-fragment of cDNA was PCR amplified with the primers 5′-AAAAAGCAGGCT(CATGTCGATTGCATTGATATGTGG)-3′ and 5′-AGAAAGCTGGGT(TCTCAATCGTGTC GGTGTTGGTGT)-3′, containing two attB recombination sites. We sequentially subcloned the product into a donor vector by recombination *in vitro* (Gateway BP Clonase Enzyme Mix, Invitrogen) and created an entry vector to target the gene in both the sense and antisense orientations.

The resultant constructs were introduced into the *Agrobacterium tumefaciens* strain LBA4404 by a freeze–thaw procedure (Höfgen and Willmitzer, 1988) and then transformed cv. E-potato 3 according to *Agrobacterium*-mediated microtubers method (Si et al., 2003). Independent transgenic plants (27 overexpression and 6 RNAi) were isolated following selection on MS medium containing 200 μg mL^{-1} kanamycin and 250 μg mL^{-1} carbenicillin and conformed by PCR. Southern blot and RT-PCR analyses were also performed to confirm the stable integration and transcript levels of *StRFP1* in the transgenic lines.

Assays of potato transgenics for P. infestans resistance

A total of 33 morphologically normal and healthy transgenic plants were used for a late blight resistance test. The wild-type plants (WT) were used as negative controls and late blight susceptible cultivar Zhuanxinwu was used to test the pathogenecity of the *P. infestans* isolate. The methods for preparation of the culture medium for *P. infestans* growth and induction of zoospores have been described previously (Tian et al., 2006). The third to fifth fully expanded leaves were collected from the top of each 6-week-old greenhouse-grown plant. Only one site of each detached leaf was inoculated with 10 μL of 5×10^4 mL^{-1} zoospores on the back surface. They were then maintained in the culture room at 18 °C, 95–98% relative humidity and a 16 h light/8 h dark photoperiod provided by fluorescent lamps. The disease lesion dimensions were measured on days 3, 4, and 5 after inoculation. The lesion area (A) was calculated based on the following formula: $A = 1/4 \times \Pi \times \text{length} \times \text{width}$. If A was over 16 mm^2 at one time of the three measurements, the infection was considered successful. The lesion radius calculated from square-root transformation of the area was converted into lesion growth rate (LGR, mm d^{-1}) (Vleeshouwers et al., 1999). Three replicates with eight leaflets in each were set up.

Results

Isolation and characterization of StRFP1

In our previous studies aimed at elucidating the molecular biology of the potato defense response induced by *P. infestans*, an EST clone, 10-A12, was isolated and identified through the suppression SSH approach (Tian et al., 2003). Sequencing and BLAST similarity searching against the GenBank database revealed that 10-A12 is part of a gene homologous to the tobacco (*N. tabacum*) Avr9/Cf-9 rapidly elicited 132 (*NtACRE132*) gene. 5′ and 3′-RACE were performed to clone the full-length cDNA. The isolated cDNA is comprised of 1354-bp containing 203-bp 5′ and 362-bp 3′ untranslated region (UTR) sequences and a 786-bp open reading frame (ORF) (GenBank accession no. EF091877) (Fig. 1A). The ORF encodes a deduced protein of 262 amino acids with an estimated molecular mass of 29.1 kDa and a theoretical isoelectric point (p*I*) value of 7.57.

On the basis of the deduced amino acid sequence, analysis of the motif structure (Fig. 1B) revealed that it contains a characteristic RING-H2 finger signature (C-x2-C-x15-C-x-H-x2-H-x2-C-x10-C-x2-C) (Saurin et al., 1996). The gene was named as *Solanum tubrerosum* RING Finger Protein 1 (*StRFP1*). The N-terminal part of the protein (from 18-V to 36-L) is rich in hydrophobic residues (66%) and is a putative transmembrane domain confirmed with TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0). In addition, a putative GLD domain from 78-G to 93-D was identified.

Similarity searches and phylogenetic tree analysis indicated that StRFP1 was most similar to the tobacco NtACRE132 protein (accession no. AAG43550) with identity of 70%, followed by RcATL1M from *Ricinus communis* (accession no. XP_002533814),

with 47% identity. It also had sequence identity to *Arabidopsis thaliana* AtATL1M (accession no. At1g53820) and AtATL2 (accession no. At3g16720) with 38% and 35%, respectively (Fig. 1C).

Structural features of conserved domain organization and additional homology throughout the sequence of StRFP1 protein are similar to the characteristic regions present in the ATL family proteins (Salinas-Mondragon et al., 1999; Serrano et al., 2006), and therefore *StRFP1* encodes a potato RING-H2 finger protein

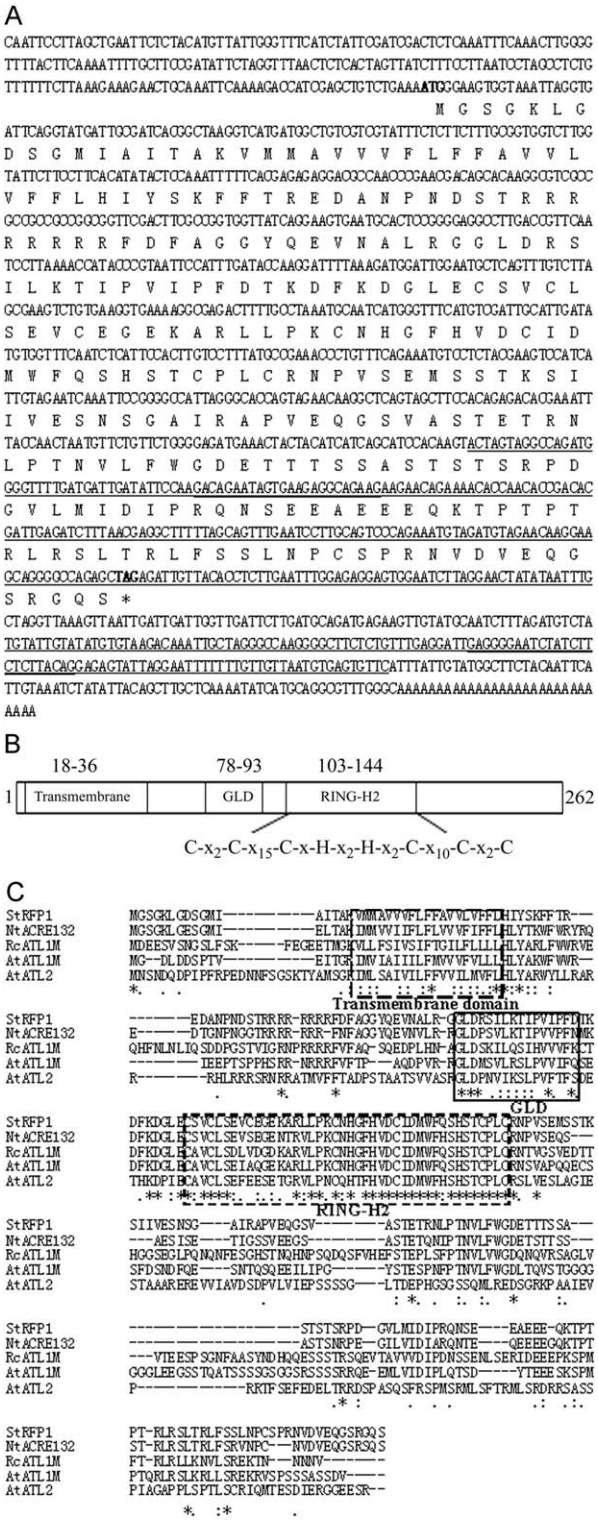

Fig. 1. Nucleotide and deduced amino acid sequences analysis of potato *StRFP1* gene: (A) nucleotide and deduced amino acid sequences of StRFP1. The deduced amino acid sequence is shown under the corresponding nucleotide sequence (GenBank accession no. EF091877). The start codon ATG and stop codon TAG are boldface; the stop codon is indicated by "*". The sequence of EST fragment is underlined, and gene-specific primers are double-underlined. (B) Schematic representation of primary structure of putative *StRFP1* gene. The numbers indicated the amino acid position of each conserved transmembrane, GLD and RING-H2 domains in *StRFP1* gene, respectively. The consensus amino acid sequences are shown below the RING-H2 sequence. G, glycine; L, leucine; D, aspartate; C, cysteine; H, histidine. (C) Homologous comparison of the StRFP1 protein and related ATL family. Conserved transmembrane, GLD and RING-H2 domains are marked by different black lines. Aligned from top to bottom are deduced amino acid sequences of StRFP1 and putative proteins from *Nicotiana tabacum* NtACRE132 (accession no. AAG43550), *Ricinus communis* RcATL1M (accession no. XP_002533814), *Arabidopsis thaliana* AtATL1M and AtATL2 (accession no. At1g53820 and At3g16720). The dashes "-" denote gaps introduced to maximize the alignment.

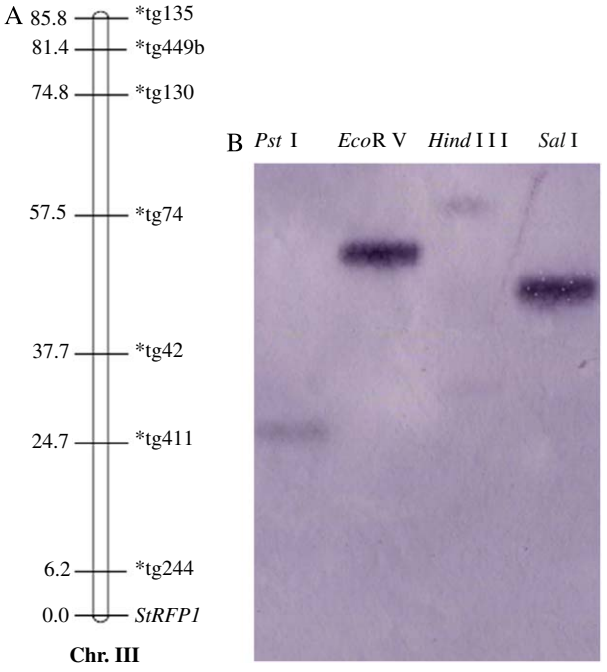

Fig. 2. Mapping and DNA gel blot analysis of *StRFP1* gene: (A) the chromosomal localization of *StRFP1* gene in potato BCT population. RFLP markers are shown on the potato genetic map. *StRFP1* was mapped on potato chromosome 3. (B) Genomic DNA of potato 386209.10 leaves (50 μg) was digested with the restriction enzymes *Pst* I, *Eco*R V, *Hind* I I I and *Sal* I as indicated, respectively, and fractionated on 0.8% agarose gel. The filter was hybridized with a probe of DIG-labeled potato *StRFP1* gene fragment.

that belongs to the ATL family described by Jensen et al. (1998). Furthermore, the intron–exon junction of the *StRFP1* gene established by comparison of the cDNA and the genomic sequences indicated that it consists of a single exon without an intron (data not shown).

StRFP1 mapping and DNA gel blot analysis

The potato segregating population BCT was used for mapping the *StRFP1* gene. A PCR amplification length polymorphism was detected in the female but absent in the male, and the individuals showed a segregation ratio of 1:1. The *StRFP1* gene was located on chromosome 3 of the potato genome, at a map position 6.2 cM to the existed marker tg244 (Fig. 2A).

The DNA gel blot analyses of potato genomic DNA probed with the full-length *StRFP1* cDNA clone are shown in Fig. 2B.

Hybridization under high stringency conditions revealed that *StRFP1* had one to two copies in the potato genome.

Subcellular localization of StRFP1 protein in onion epidermal cells

According to analyses using the TMHMM and TMPRED programs, the sequence between amino acids 18 and 36 of StRFP1 contains a putative transmembrane domain signal. To determine the cellular localization of the StRFP1 protein, the ORF of *StRFP1* was fused into the pBI121 vector, downstream of a constitutive CaMV35S promoter and upstream of a *GFP* gene to create a CaMV 35S::StRFP1-GFP fusion construct. It was subsequently introduced into the onion epidermal cells by particle bombardment. After incubation for 24 h, GFP fluorescence was detected and revealed that the fluorescence signal of CaMV 35S::StRFP1-GFP fusion protein was predominantly localized on the plasma membranes or out of that in onion epidermal cells, whereas the control, transformed with CaMV 35S::GFP construct alone, exhibited fluorescence spread throughout the entire cell (Fig. 3).

Organ-specificity and response of StRFP1 to the biotic and abiotic stresses

In order to characterize the expression pattern of the *StRFP1* gene in potato in more detail, the expression level of corresponding mRNA was investigated by RT-PCR analysis. As a first step, total RNAs from different organs were isolated from healthy potato plants (Fig. 4A). It was shown that *StRFP1* was constitutively expressed in almost all organs examined, and exhibited much at higher levels in leaves than in roots, stems, flowers, shoot tips, stolons and tubers.

Because *StRFP1* was initially identified in *P. infestans*-induced leaves, it is possible that the expression of *StRFP1* is modulated by this biotic stress. To test this possibility, its mRNA accumulation profile during *P. infestans* infection was monitored in potato detached leaves. RT-PCR analysis showed that the expression of *StRFP1* was induced at 24 h and then increased rapidly at 48 h (Fig. 4B), indicating that *StRFP1* is an early *P. infestans*-induced gene in potato that is activated at early stages of pathogen infection.

To further understand the effect of defense signal molecules on *StRFP1* expression, SA, MeJA, ETH and ABA were applied to potato detached leaves (Fig. 4C). Expression of the *StRFP1* gene was activated at 8 h after ABA treatment, peaked at 24 h, but then decreased to the initial expression level. Low levels of induction of the *StRFP1* gene were also detected after SA and MeJA treatment, whereas there were no discernable changes in the expression of *StRFP1* in response to ETH treatment.

Generation of StRFP1 overexpressing and silencing transgenic potato plants

To explore the biological function of *StRFP1*, transgenic potato plants were generated by overexpressing and silencing *StRFP1* under the control of the CaMV 35S promoter (Fig. 5A and B). Thirty-three (27 overexpression and 6 RNAi repression) independent transformants were obtained with 13 copies of the gene insertion confirmed by DNA gel blot analysis using full-length *NPT* as a probe (data not shown).

The expression level of *StRFP1* in transgenic plants was analyzed by RT-PCR. Some overexpression transgenic lines had higher transcription levels, while others showed no obvious difference from the wild-type plants (Fig. 5C). Line Brg20, with high expression levels and one copy of the insertion, was selected for further experiments.

StRFP1 expression was reduced in all of the RNAi lines relative to non-transformed controls, and the average interference efficiency was 84.6%. Ba3 and Ba6 in particular showed the most suppression in-abundance of *StRFP1* compared to the control (Fig. 5D and E). Ba3 with one copy of the insertion was selected for further evaluation of the gene function.

StRFP1 positively regulated the resistance to P. infestans in potato

As the *StRFP1* gene could be markedly induced by the infection of *P. infestans* (as shown in Fig. 4B), the disease resistance against

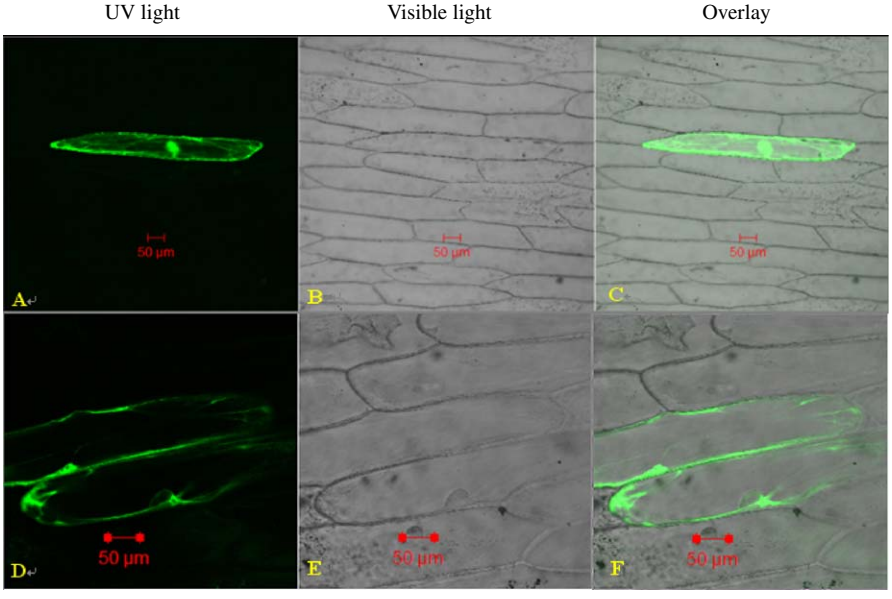

Fig. 3. Subcellular localization of CaMV 35S::GFP and CaMV 35S::StRFP1-GFP fusion constructs in onion epidermal cells by transient expression. The GFP coding region was fused in-frame to the 3′-end of the StRFP1 coding region and introduced into onion (*Allium cepa*) epidermis cells. The expression of introduced proteins was visualized with laser confocal-scanning fluorescence microscopy 24 h after particle bombardment with a gene gun. The photographs were taken in the dark field for green fluorescence (A and D), under bright light for the morphology of the cell (B and E) and overlay images (C and F), respectively, for p35S::GFP control plasmid (A-C) and p35S::StRFP1-GFP plasmid (D-F). Bars = 50 μm.

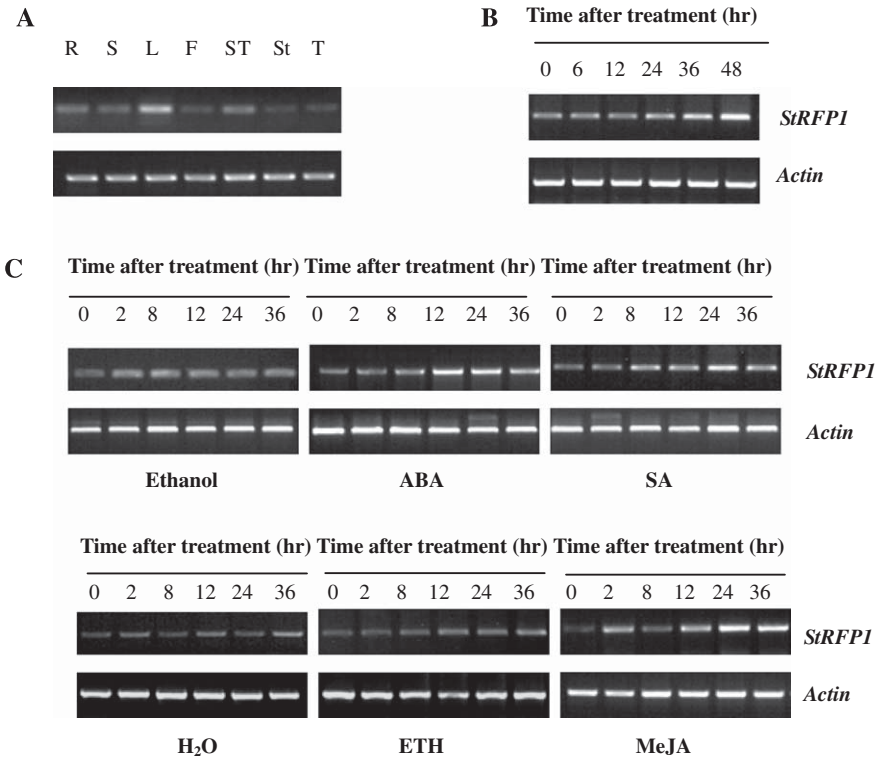

Fig. 4. The expression patterns of *StRFP1* in different tissues of potato 386209.10 and in response to biotic and abiotic stresses: (A) the transcript accumulation of StRFP1 gene in potato different organs. Total RNAs were extracted from roots (R), stems (S), leaves (L), flowers (F), shoot tips (ST), stolons (St) and tubers (T) of healthy potato 386209.10 plants, respectively. (B) Expression of *StRFP1* gene induced by *P. infestans* in potato 386209.10 detached leaves spraying with 5×10^4 mL^{-1} zoospores. (C) Induction of the *StRFP1* gene by 2 mM SA, 50 μM MeJA, 100 μM ABA and 200 μL L^{-1} ETH in potato 386209.10 detached leaves. Samples were collected at the time points indicated on the top and the transcript accumulation was investigated by RT-PCR analysis. The *Actin* gene was used as the internal control to show the normalization of templates (lower panel).

P. infestans of transgenic potato plants was estimated by the mean value of the lesion growth rate (LGR) after challenging with the pathogen. Two days after the zoospore inoculation, water soaked-like necrosis developed on the *StRFP1*-RNAi lines and non-transformed control leaves (Fig. 6A). Larger LGR were obtained from RNAi transgenic plants (Ba3) compared to controls and the difference was statistically significant ($P < 0.05$). In contrast, the disease necrosis developed more slowly in transgenic lines that overexpressed *StRFP1*. The LGR values of this line (Brg20) were smaller than controls and reached significant levels (Fig. 6B).

The mRNA accumulation of *StRFP1* was examined by RT-PCR analyses. As shown in Fig. 6C, expression of *StRFP1* was obviously suppressed in the RNAi line Ba3, whereas overexpression of *StRFP1* resulted in a marked accumulation of its transcripts, showing a pattern of continuous increase during the course of the pathogen infection. These results illustrate that the expression of *StRFP1* was inversely proportional with the disease development, revealing that *StRFP1* positively regulates the resistance in potato against late blight.

Discussion

StRFP1 is a member of ATL gene family in potato

In this study, a new RING-H2 type RING finger domain-containing gene, *StRFP1*, was isolated from potato leaves. In addition to a highly conserved RING-H2 finger domain, the deduced amino acid sequence of *StRFP1* contains an amino-terminal transmembrane domain and a 16-amino-acid residues-conserved GLD region (Fig. 1B and C), which are typical features of the *ATL* gene family (Salinas-Mondragon et al., 1999; Serrano et al., 2006). Therefore, StRFP1 is further assigned to an ATL protein, a subgroup of RFPs. Similar to the previously identified structures that 90% of the *Arabidopsis ATL* genes are intronless (Serrano et al., 2006), *StRFP1* has no intron and constitutively expresses at low levels in various potato organs under normal growth conditions (Fig. 4A). Together, characteristics of *StRFP1* in sequence demonstrate that it is a member of the *ATL* gene family in potato.

StRFP1 is a positive regulator in defense responses to P. infestans infection

In addition to protein synthesis, increasing attention has been focused on protein degradation thereby and the proteolytic pathway involving the ubiquitination of target proteins for subsequent degradation by the 26S proteasome (Hershko and Ciechanover, 1998). Ubiquitination is emerging as a common regulatory mechanism that controls a range of cellular processes in plants (Smalle and Vierstra, 2004; Mazzucotelli et al., 2006), and exciting discoveries from several laboratories suggest that E3 ubiquitin ligases containing RING-H2 finger motifs play important roles in different plant defense responses, including gene-for-gene resistance, early defense reactions and induced disease resistance (Salinas-Mondragon et al., 1999; Stone et al., 2005; Liu et al., 2008). The *ATL* gene family belongs to a subgroup of RING-H2 finger-type E3 ubiquitin ligases, originally isolated as members rapidly induced in response to elicitors (Salinas-Mondragon et al., 1999). Among the members of the *ATL* gene family in *Arabidopsis*

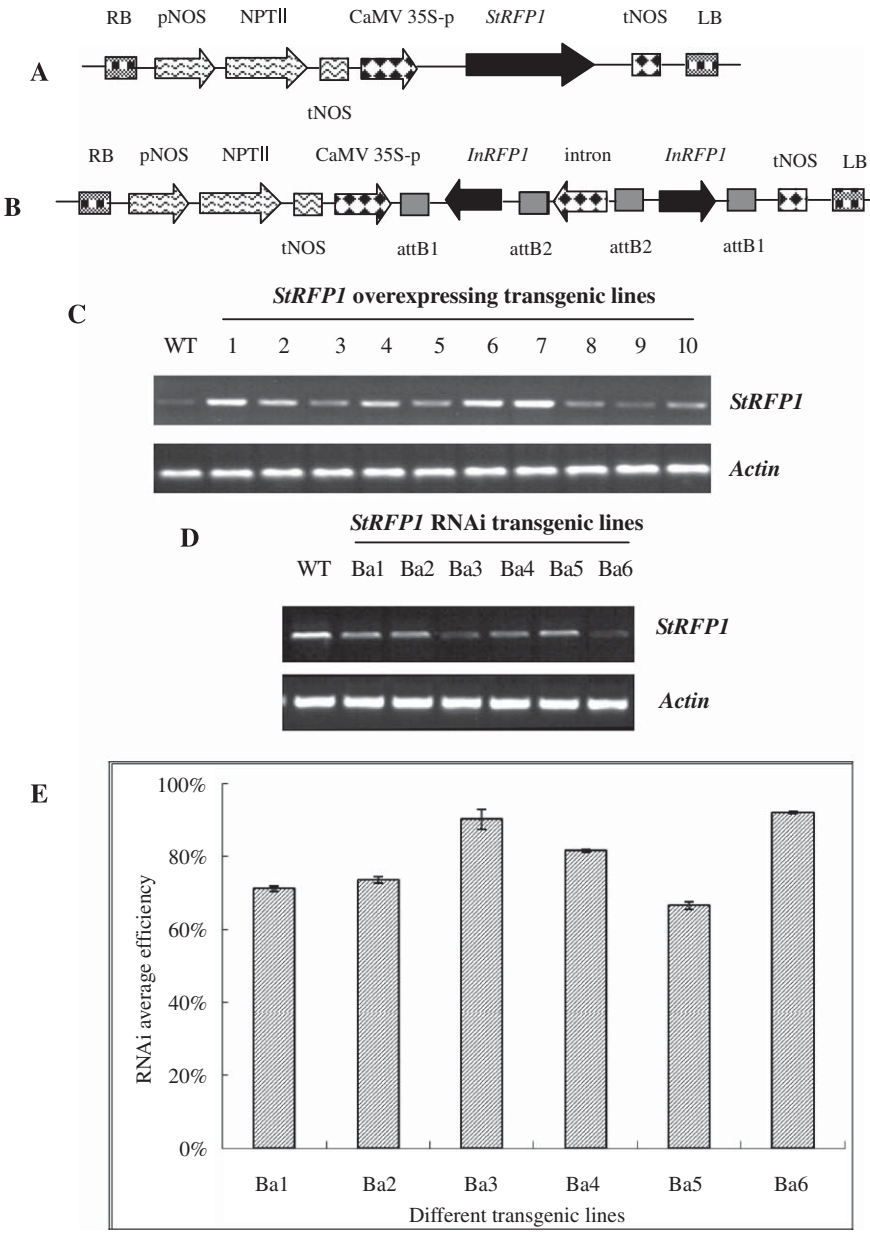

Fig. 5. Generation and characterization of the transgenic potato plants: (A) the scheme of *StRFP1* overexpression plant vector, the *GUS* on pBI121 vector was replaced by *StRFP1* under the control of CaMV 35S promoter. (B) The sketch map of *StRFP1* RNAi expression vector, the black arrowheads denoted the silenced fragment of *StRFP1*. (C) Expression pattern of *StRFP1* in overexpression transgenic lines by RT-PCR with gene-specific primers. WT: wild-type plants; 1 10: transgenic potato lines of *StRFP1*-overexpressed. (D) RNAi efficiency analysis of transgenic potato lines by RT-PCR with gene-specific primers. The *Actin* gene was represented as the internal control (lower panel). (E) Quantification of the *StRFP1* transcript amounts in different transgenic potato lines. The relative expression levels of each line are considered, shown with normalized intensity and represented by black bars. WT: wild-type plants; Ba1 Ba6: transgenic potato lines of *StRFP1*-silenced. Vertical bars indicate the mean ± SE of three replications.

thaliana, the *ATL2* and *ATL6* genes are rapidly and transiently induced after treatment with a chitin elicitor (Salinas-Mondragon et al., 1999; Serrano and Guzman, 2004). In rice, the expression of an ortholog of the *ATL* gene family, namely the elicitor-responsive 5 (*EL5*) gene, is also upregulated at the early stage of elicitor treatment (Durrant et al., 2000; Takai et al., 2001). In addition, results from a number if research groups have also indicated that the *ATL* gene family and related protein breakdown play important roles in the signal transduction pathways leading to defense responses against biotic and abiotic stresses (Dreher and Callis, 2007; Hondo et al., 2007). For example, the expression of *LeATL6*, an ortholog of *Arabidopsis ATL6* that encodes a RING-H2 finger protein, was induced in tomato roots treated with a cell wall protein fraction (CWP) or mycelia homogenate of the biocontrol agent *Pythium oligandrum* (Hondo et al., 2007). Moreover, the tobacco ortholog of *StRFP1*, *NtACRE132*, was rapidly induced within 30 min upon addition of the *C. fulvum*-derived fungal effector Avr9 in the presence of cycloheximide (Durrant et al., 2000). In our experiments, *StRFP1* was significantly upregulated in potato leaves by *P. infestans* and was inducible by the defense-related signal molecules SA and MeJA (Fig. 4C). Importantly, *StRFP1* is also rapidly activated at early stages of *P. infestans* infection in potato cultivars with different late blight resistance types, implying that *StRFP1* may be involved in potato early defense response to *P. infestans* attack in SA- and JA-dependent pathways.

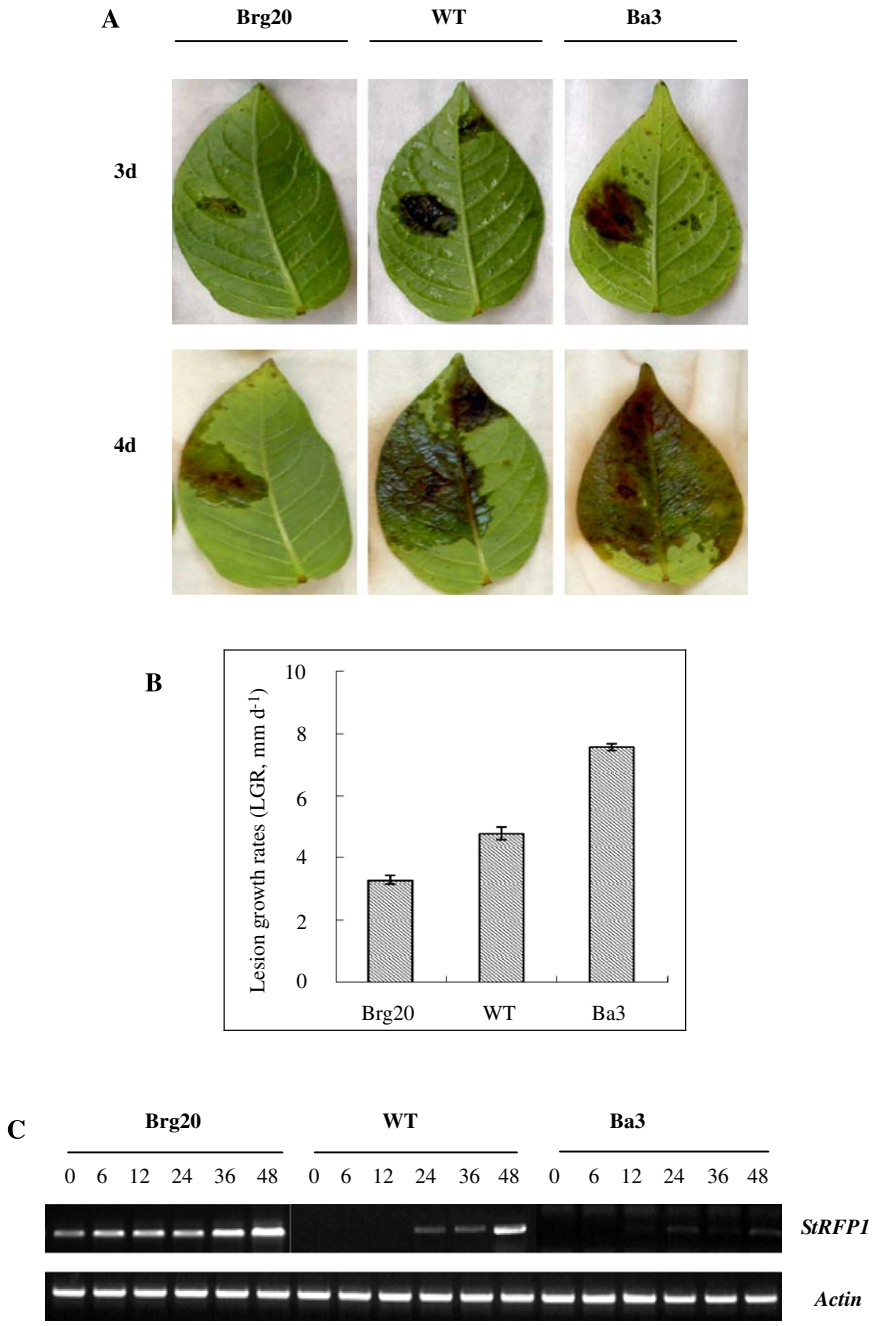

Fig. 6. Response of selected *StRFP1* transgenic lines and WT to *P. infestans* in detached leaves: (A) symptom developed on the leaves of *StRFP1* transgenic lines and wild-type plants after inoculation with *P. infestans* zoospores for 3 (higher panel) and 4 (lower panel) d. (B) Lesion growth rates (LGR, mm d^{-1}) of *P. infestans* on detached leaves of selected *StRFP1* transgenic lines and control plants. Vertical bars indicate the mean ± SE of three replications. (C) The mRNA expression analysis of *StRFP1* in transgenic lines and WT in response to pathogen infection was investigated by RT-PCR using *StRFP1* specific primer. Samples were collected at various time points indicated on the top after inoculation with the *P. infestans*. The *Actin* gene was used as the internal control (lower panel). WT: wild-type plants; Ba3: RNAi transgenic lines; Brg20: overexpressing transgenic lines.

Direct evidence upholding this conclusion comes from the functional analysis of *StRFP1* in transgenic potato plants. As *StREP* was identified and cloned from potato clone 386209.10, which lacks the *R* gene and possesses a feature of the partial resistance to late blight, *StRFP1* is suggested to be a horizontal resistance-related gene to *P. infestans*. Under the experimental conditions, disease symptoms caused by the mixture of various races of *P. infestans* appeared later in the transgenic leaves overexpressing *StRFP1* and earlier in lines suppressed by RNAi compared with non-transgenic controls (Fig. 6A). This phenomenon was in accordance with the expression of the *StRFP1* gene in corresponding plants (Fig. 6C). The disease development, as indicated by the lesion growth rate (LGR), was obviously slower in *StRFP1* overexpressing plants and the reverse was true when *StRFP1* was silenced by RNAi (Fig. 6B). Our results demonstrate that *StRFP1* is an important gene in potato horizontal resistance to *P. infestans* and its expression is largely associated with the regulation of the early defense response and resistance level of potato plants against late blight. The mechanism of *StRFP1* in potato late blight defense is worth investigating through its E3 ubiquitin ligase function in response to the disease development.

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