Journal of Plant Physiology 167 (2010) 488–496

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

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

Xuemei Ni, Zhendong Tian, Jun Liu, Botao Song, Conghua Xie *

Key Laboratory of Horticultural Plant Biology (Huazhong Agricultural University), Ministry of Education; National Centre for Vegetable Improvement (Central China), Potato Engineering and Technology Research Centre of Hubei Province, Huazhong Agricultural University, Wuhan, Hubei 430070, People's Republic of China

ARTICLE INFO

Article history:
Received 4 September 2009
Received in revised form
27 October 2009
Accepted 29 October 2009

Keywords:
E3 ubiquitin ligase
GFP
Phytophthora infestans
Potato
RING-H2 finger protein

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

* Corresponding author. Tel.: +86 27 87280969; fax: +86 27 87286939.
E-mail address: xiech09@yahoo.cn (C. Xie).

0176-1617/$ - see front matter © 2009 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2009.10.019

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