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Identification and characterization of *defensin* genes conferring *Phytophthora infestans* resistance in tomato

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ABSTRACT

Defensins are widely distributed in all plants, and these proteins play important roles in the resistance to pathogens. In this study, two tomato defensin genes (*SlyDF1* and *SlyDF2*) were cloned and characterized from tomato *Solanum lycopersicum* Zaofen No.2. Phylogenetic analysis classified SlyDF1 and SlyDF2 into Group II of the defensin family, and the two proteins were shown to have a closer genetic relationship with MtDef4 and PDF2.5. Both *SlyDF1* and *SlyDF2* transcripts were observed in the root, stem, leaf, flower and mature fruit of the tomato plant, and were strongly upregulated 1 and 2 days after inoculation of the whole plant with *Phytophthora infestans*. The tomato plants that overexpressed *SlyDF1* displayed greater resistance to *P. infestans* infection, as evidenced by decreased *P. infestans* abundance, disease index, number of necrotic cells, lesion sizes, and number of sporangia per leaf compared to the control plants. SlyDF1 enhanced the activities of peroxidase (POD) and superoxide dismutase (SOD) to decrease the accumulation of H_2O_2, thereby preventing damage to the tomato cell membrane during resistance to *P. infestans* infection. The results suggest that tomato defensin might play a role of positive regulation in the response to *P. infestans* infection and could therefore be considered as a candidate gene for enhancing biotic stress-resistance in tomato.

1. Introduction

Plants are continuously exposed to various pathogens in nature, and therefore, they have developed a number of defense mechanisms to protect themselves against attack [1]. Such defenses include the production of a range of protective molecules, including beta-1,3-glucanase, chitinases, thaumatin-like, protease inhibitor, and plant defensins [2]. Among these, defensins seem to play the most important role in the direct inhibition of pathogen growth. Defensins are widely distributed in all plant families, and their presence in various plant tissues has been described [3]. They are a family of small, basic cationic peptides (with a length of approximately 45–54 amino acids) [4] composed of three β-sheets and one α-helix that are stabilized by four disulfide bonds, forming a cysteine-stabilized α-helix β-sheet motif (CSα/β). This structure confers great stability to the peptides to maintain their functions [5]. Plant defensins have been shown to inhibit the growth of pathogens [6,7], and they have also been proposed to act as protein synthesis inhibitors, α-amylase inhibitors, zinc tolerance mediators, and ion channel blockers [8–11]. The radish defensin RsAFP2 is an antifungal protein [12]. RsAFP2 recognizes and binds to GlcCer on the fungal membrane [13,14] and then activates the CWI pathway and MAP kinase signaling pathways [15,16]. This is followed by the production of reactive oxygen species (ROS) [16,17], induction of ion fluxes [17–19] and activation of caspases [20], eventually leading to fungal cell death. The defensin Psd1 from a pea seed can insert into the fugal membrane [21,22], causing cell cycle impairment and fungal cell death [23–26]. Other defensins, such as the *Arabidopsis thaliana* plant defensin AtPDF1.1, is involved in the response to biotic stress [27]. Overexpression of the defensin *J1-1* in pepper plants has been shown to provide strong resistance to the anthracnose fungus, significantly reducing lesion formation and fungal colonization [7]. Transgenic rice expressing a *Brassica rapa* defensin displays resistance to the brown planthopper (*Nilaparvata lugens*) [28]. Thus, defensin not only plays a direct role in killing pathogens, but also induces the production of ROS.

ROS play an important role in plant-pathogen interaction. Low levels of ROS act as signaling molecules in response to pathogen infection, but high concentrations of ROS are toxic to the cell because it can lead to peroxidation of lipids, destruction of cell membranes and ultimately cell death [29,30]. It is widely recognized that malonaldehyde (MDA) is an indicator of lipid peroxidation [31]. Excess ROS are usually

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scavenged by peroxidase (POD) and superoxide dismutase (SOD) [32].

Tomato is a horticultural plant and a model system for the study of plant -pathogen interaction. The tomato genome project has been completed [33]. Late blight (LB), caused by *Phytophthora infestans*, is one devastating diseases as it can cause severe loss of tomato crops [34,35]. Although LB can be effectively controlled through regular application of preventative fungicides, such measures are costly and damaging to the environment. Therefore, it is necessary to investigate the mechanism associated with tomato resistance to *P. infestans*. Several tomato genes that confer resistance against *P. infestans*, including *Ph-1-5*, have been reported and mapped [36–40]. In addition to tomato *Ph* genes, defensin has also been found to be involved in the resistance of other plants to *P. infestans*. For example, *lm-def*, a member of the defensin family isolated from maca, can be expressed in potato to help to control LB [41]. In addition, our previous RNA-Seq revealed differences in Fragments per Kilobase of exon model per Million mapped reads (FPKM) of *defensin* genes between tomato plants inoculated with and without *P. infestans*. Here, the coding sequences of two defensin genes in tomato, *SlyDF1* and *SlyDF2,* were cloned and characterized. Both *SlyDF1* and *SlyDF2* were found to be expressed in various tissues of the tomato plant and be induced by *P. infestans*. The *SlyDF1*-overexpressing tomato plants displayed fewer disease symptoms than wild-type (WT) tomato plants following infection with *P. infestans*.

2. Materials and methods

2.1. Plant material, growth conditions, and P. infestans inoculation

Tomato *Solanum lycopersicum* Zaofen No.2 seeds were germinated on petri dishes lined with moist filter paper at 25 ± 3 °C. After 5 days, the sprouting seeds were transferred to the soil and grown at 25 ± 3 °C under a 16-h light and 8-h dark photoperiod. The root, stem, leaf, flower and mature fruit were harvested for the tissue-specific *defensin* expression. Five-leaf stage tomato seedlings were treated with a suspension of *P. infestans* spores (10^6 zoospores/mL) and then placed in the dark at 20 ± 1 °C with 100% relative humidity to ensure spore germination. The leaves of each seedling were collected at t 0, 1, 2, 3, 4 and 5 days post inoculation (dpi). They were quickly frozen in liquid nitrogen and stored at - 80 °C until RNA isolation.

2.2. Isolation of total RNA, synthesis of first strand cDNA and cloning of tomato defensins gene

Isolation of total RNA from all samples was performed using RNAiso plus (TaKaRa, Dalian China). The first strand of cDNA was synthesized with a PrimeScript TM RT-PCR kit (TaKaRa, Dalian China).

To clone the coding sequences of the two tomato defensin genes, namely *SlyDF1* and *SlyDF2*, two pair of primers, namely c-SlyDF1-F/R and c-SlyDF2-F/R (Table S1), were designed according to available tomato genome information (Solyc07g007750.2.1 and Solyc07g007760.2.1) using the Primer 5 Software. Amplification was performed as follows: one cycle at 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, one cycle at 72 °C for 7 min. PCR product was isolated and cloned into pMD-19-T Vector (TaKaRa, Dalian China) and positive clones were confirmed by DNA sequencing.

2.3. Sequence analysis

To predict the characterization of defensin, BLAST was used to construct a sequence analysis with the default parameters at NCBI (www.ncbi.nlm.nih.gov/blast). The coding sequences of *SlyDF1* and *SlyDF2* and their predicted amino acid sequences were determined using BioEdit.

We analyzed the composition and physicochemical characteristics of the predicted protein sequences using ProtParam (http://web.expasy.org/protparam) with default parameters.

We used ClustalX 1.83 with the default settings to construct a multiple sequence alignment. We also used the neighbor-joining method (NJ, Bootstrapping = 1000) of MEGA6.06 software with the default parameters to produce a phylogenetic tree. The three-dimensional structural models of the conserved domains of SlyDF1 and SlyDF2 were built using SWISS-MODEL (https://swissmodel.expasy.org/).

2.4. Construction of plasmid for SlyDF1 overexpression and generation of SlyDF1- transgenic tomato

To decipher the function of *SlyDF1*, the coding sequence of *SlyDF1* was digested with *Bam*HI and *Sac*I, and cloned into *Bam*HI-*Sac*I digested pBI121. In the plasmid pBI121-*SlyDF1*, the *GUS* gene was removed and *SlyDF1* was placed under the control of the Cauliflower mosaic virus (CaMV) 35S promoter. The *Agrobacterium tumefaciens* strain GV3101 was transformed with the plasmid pBI121-*SlyDF1* by freeze-thaw method [42]. The transgenic tomato plants were constructed according to Li's method [42]. After obtaining kana-resistant plants, the presence of the transgene in the regenerated plantlets was further confirmed using PCR with gene-specific primers (Table S1). The expression level of *SlyDF1* in these selected positive transgenic lines was further examined.

2.5. Disease resistance analysis

To determine the function of *SlyDF1* in tomato plants infected with *P. infestans*, detached leaves from plants that overexpressed *SlyDF1* were inoculated with 20 μl *P. infestans* zoospore suspension (1×10^6 zoospores/ml) according to Li's method [42]. At seven days post infection with *P. infestans*, the leaf symptoms were scored, and the diameter of the lesions and sporangia per leaf were measured from the detached leaves.

The entire-plants were also inoculated with *P. infestans* and were kept in a greenhouse according to Cui's method [43]. Areas of necrosis surrounding the inoculation sites and disease indices were recorded at 7 dpi. Disease grades (DG) were evaluated according to Luan's method [44] and the disease index (DI) was calculated according to Li's method [42]. In addition, the *P. infestans actin* gene was used to quantify the abundance of *P. infestans*.

2.6. Histochemical assays and measurements of physiological parameters

Dead cells and H_2O_2 levels in the untreated and *P. infestans*-treated leaves were determined by trypan blue staining and DAB staining [42,43], respectively. We measured the POD and SOD activities according to a previously described protocol [45,46]. The content of MDA was also measured based on Cao's method [47].

2.7. Expression analysis

Tissue-specific expression of *Alydus* and the relative quantity of *SlyDF1*, *SlyDF2*, *SlyPOD*, *SlySOD* and *P. infestans actin* transcripts after treatment with *P. infestans* were measured by quantitative RT-PCR (qRT-PCR). qRT-PCR was performed with the SYBR Premix Ex TaqTM II kit (TaKaRa, Dalian China). The reaction was carried out in a Rotor Gene 3000 Real-time PCR cycler (Corbett Research, Mortlake, Vic., Australia). In addition, the expression level of *SlyDF1* in these transgenic lines was also examined by semi-quantitative PCR and qRT-PCR. The tomato *actin* gene was used as an internal housekeeping gene. The sequences of all primers are shown in Table S1. The *Ct* values of each gene were standardized, and the relative changes of each gene were analyzed by $2^{-\triangle\triangle Ct}$ method [44].