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Functional characterization of the silenced potato cysteine proteinase inhibitor gene (*PCPI*) in *Phytophthora infestans* resistance

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ABSTRACT

Construct carrying dsRNA of the potato cysteine proteinase inhibitor *(PCPI)* was transiently expressed in *Phythophthor*a resistance potato cultivar White Lady. Expression was strongly reduced as indicated by a reduction of up to 93.94% in *PCPI* transcript levels. Silencing of *PCPI* resulted in a marked increase in lesion size and water soaking, indicating that cysteine proteinase inhibitor plays a role in limiting lesion expansion. This analysis sheds light on the transcriptional changes that accompany plant infection by *P. infestans*, and will aid in identifying potential gene targets that could be used to generate stable resistant transgenic plants of the sensitive genotypes.

1. Introduction

Potato is the world's most important non-grain food crop and plays a main role in global food security. However, genetic and genomic research of potato has lagged behind most major crops.

Functional discovery of genes in potato is still a lengthy process and is often hampered by the complex characteristics associated with the potato genome, including autotetraploidy, self-incompatibility, and high heterozygosity. *Phytophthora infestans*is the oomycete pathogen responsible for the late blight disease on its potato host (*Solanum tuberosum*) inciting the worldwide most severe potato losses [1,2]. Factors controlling quantitative resistance to *P. infestans* were found on every potato chromosome, confirming the truly polygenic nature of this trait [3]. Although intensively studied, the differences between a resistant and a susceptible response to *P. infestans* attack are not fully understood [4]. Publication of the sequence of potato genome in 2011 has provided new insights into potato genetics and breeding research [5]. On the other hand, the advances in sequencing technology have also enabled deep sequencing of complete transcriptomes (RNA-seq) from large collections of tissues, conditions and time points to study the temporal and spatial distribution of gene activity [6]. Through these developments the focus on DNA sequencing has shifted from a primary data generation tool for the production of genome sequences to functional genomics tools for both fundamental and applied researches. High-throughput transcriptomic sequencing is crucial to generate a large transcript sequence data set for gene discovery. In the present study, we utilized the transcript sequences in response to *Phytophthora infestans* to study the function of upregulated genes in response to the biotic stress in tetraploid potato. Genes potentially involved in the development and responses to biotic and abiotic stresses can now be rapidly identified by whole-genome transcript profiling experiments [7–10]. It is only recently that a group of proteins from potato tubers having molecular masses ranging from 20 to 25 kDa has been described. Some of these proteins have been found to be various Kunitz-type proteinase inhibitors such as trypsin and/or chymotrypsin inhibitors [11], subtilisin inhibitor [12], cathepsin D inhibitors [13,14] and papain and/or cathepsin L inhibitor [15]. These inhibitors may play significant role in the natural defense mechanisms of the potato plant against insect and phytopathogen attack [12–14,16]. Protease inhibitors are ubiquitously plentiful in tubers and plant seeds [17]. Proteinase inhibitors are found in plants belonging to a variety of systematic groups, although high levels of proteinase inhibitors are often found in many plants belonging to the *Solanaceae* family [18]. They generally present in storage

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tissues, but also detectable in leaves in response to the attack of many species of herbivorous insect as well as fungal pathogens [19,20]. Plant cysteine proteinases are found particularly in the vacuoles and are responsible for the mobilization of endosperm storage proteins during seeds germination. Cysteine proteinases are also found in the extracellular medium such as those from papaya and fig [21]. The role of the cysteine protease inhibitor as an insecticidal protein has been reported previously by Wolfson and Murdock [22]. Oryza cystatin (rice CPI) I and II have been found to be responsible for the growth retardation of different species of bean insect pest [23]. However, to obtain direct demonstration of the role of a gene in determining a specific trait, functional genomics approaches must be adopted. One of these methods is post-transcriptional gene silencing (PTGS), which is often used for quick assignment of gene functions in plants [24,25]. In plants, cytoplasmic RNA silencing can be induced efficiently by agro-infiltration, a strategy for transient expression of T-DNA vectors after delivery by *Agrobacterium tumefaciens*. The transiently expressed DNA encodes either a single- or double-stranded RNA, which is typically a hairpin (hp) RNA. Because they provide a rapid, versatile and convenient way for achieving a very high level of gene expression in a distinct and defined zone of a leaf, *Agrobacterium*-mediated transient expression systems have been useful for inducing silencing processes [26–28]. Transient cytoplasmic RNA silencing can be induced efficiently by agroinfiltration for transient expression of T-DNA encoding hpRNAs, which are double-stranded RNAs (dsRNAs) derived from hybridization of inverted repeats. Transient RNAi-induced silencing by agroinfiltration with a hpRNA transgene has been demonstrated in leaves of *Nicotiana* species [29], grapevine leaves [30] and strawberry fruits [31–33] and in *Dendrobium* [34]. RNA interference (RNAi)-based potato gene silencing has recently been reported by several laboratories [35–38]. However, the RNAi technique relies on the traditional transformation procedure and is a low throughput methodology. Developing of a transgenic potato line using RNAi constructs takes long time, therefore this technique can only be used to target a limited number of potato genes. Transient gene assays are convenient alternatives to stable transformation because such techniques allow a rapid analysis of gene function with the additional advantage that the same bacteria can be subsequently employed to generate stable transgenic lines. In this work we have generated a dsRNA construct directed against *PCPI*; this construct was used to test the possibility to obtain transient gene silencing in potato by agroinfiltration. Delivery of the construct in leaves of potato plants resulted in the specific down-regulation of *PCPI* and a reduced accumulation of the corresponding transcript. We demonstrated that the agroinfiltration technique can be used as a rapid gene assay tool to determine the role of candidate genes in gene mediated potato late blight resistance. Furthermore, our results revealed that the silencing of potato gene encodes a cysteine proteinase inhibitor in the *p. infestans* -resistant "White Lady" cultivar broke down its resistance.

2. Materials and methods

2.1. Plant material

All experiments were performed using *Solanum tuberosum* "White Lady". The plants were cultivated in pots containing peat moss and maintained in greenhouse at 23 ± 1 °C under a 16-h photoperiod and 80% humidity.

2.2. Transcriptome analysis

To generate transcriptome profile of resistance by Next Generation Sequencing (NGS), the resistant cultivar "White Lady" was inoculated with *Phytophthora infestans* isolates in three replications in the greenhouse. After isolation of mRNA using RNAzol (Molecular Research Center) in different time points post inoculation, one pooled sample was prepared equally from the three replicates. Isolated mRNAs were sequenced using NGS, 5500 XL SOLiD Applied Biosystem and sequence reads were assembled into contigs, normalized and analyzed the fold change and the number of reads per thousand bases per million mapped reads [39] by CLC Genomics Workbench 4.8 (64 bit) software. According to the expression level of genes in comparison to control, some up regulated genes were selected and aligned with potato genome sequence in NCBI database using BLASTn.

2.3. Total RNA extraction, cDNA synthesis and cloning

Frozen leaves (100 mg) were homogenized in liquid nitrogen and total RNA was extracted using NucleoSpin RNA II Kit. Nucleic acid concentrations and purity were determined by Nano drop 1000 (Thermo Scientific) and the integrity was checked by electrophoresis in 1% agarose gel. cDNA was synthesized from the purified total RNA using In-Fusion® SMARTer™ Directional cDNA Library Construction Kit (Clontech) according the manufacturer's instructions. The product was used as a template for gene cloning and expression analysis. The672-bp corresponding to NM_001318627coding sequence (CDS) were amplified using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific), and a cloning primers designed to amplify the whole CDS as follows: PCPI F: 5-TGATGAAGTCGATTAATATTTTGAG-3 and PCPI R 5- GCTCC TAC GCC TT GATGAACACAGATG-3. The PCR reaction conditions were pre-heating at 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 40 s, then extension at 72 °C for 7 min. All PCR products were analyzed by electrophoresis through 1.2% (w/v) agarose TAE gels. These PCR products were cloned into the TA cloning vector using the pGEM-TEasy vector system (Promega, USA) and sequenced. The sequence verified PCR products in the pGEM-T Easy. The PCR products were transformed into DH5alpha *E. coli* and the plasmid DNA of the clones was sequenced to verify the insert. The sequence verified PCR identical to that of *PCPI* of *S. tuberosum.*

2.4. Construction of hpRNAi vectors

Full-length cDNA clones coding for *PCPI* (NM_001318627) gene were used as template for RNA synthesis. The plasmid DNA was isolated and used as templates for PCR reactions.500 bp fragment were chosen to be the target of RNA silencing in this study. Two 500-bp DNA fragments were amplified by polymerase chain reaction (PCR) from cloned DNA. Primers were designed to obtain two PCR products ("forward" and "reverse " fragments) that could be subsequently cloned in opposite orientation to obtain a hairpin construct. The primers used to amplify the sense fragment were PCPIXhoIF-5′-CGGCTCGAGATGAAGTCGATTAATATTTTG-3, PCPIKpn1R 5-AGCGGTACC TCATAATCTTGAAGATGTC-3 which included an *Xho1* and a *Kpn I* restriction site (underlined in the sequence), respectively, at their 5-end. The primers used to amplify the antisense fragment were, PCPI Xba1F 5′-GCGTCTAGA ATGAAGTCGATTAATATTTTGAG-3 and PCPI Cla1R 5- ACGATCGATT-CATAATCTTGAAGATGTC-3including *Xba1* and Cla1 site at the 5-end (underlined in the sequence), respectively. DNA amplification was carried out in 50 μl of total reaction volume. Five microliter of cDNA was mixed with 45 μl of the amplification mixture, consisting of 50 mM of each dNTP, 120 nM each primer, 1 mM MgCl2, 1 U Taq DNA polymerase and 10μlTaq buffer. The PCR cycling was as follows: 40 cycles of denaturation for 20 s at 94 °C, annealing for