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Identification and Expression of Stress-responsive Genes to *Fusarium solani* f. sp. *eumartii* Infection in Potato Tubers: Old and New Candidates

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ABSTRACT

In order to identify genes involved in the potato (*Solanum tuberosum* L.) tuber response to the pathogenic fungus *Fusarium solani* f. sp. *eumartii* (*F. eumartii*) a differential screening of infected-potato cDNA library was carried out. In this work, we identified potato cDNA clones coding for a heat shock protein, StHSP90 and four ribosomal proteins corresponding to small and large subunits, StS9, StS19 and StL3, StL36, respectively. Total RNA was extracted from non-wounding, wounding and wounding plus *F. eumartii*-infected tubers. The pattern of RNA accumulation for each gene was investigated by northern blot assays. Compared with control tubers, *StHSP90* and *StL36* genes were up-regulated by wounding and *F. eumartii* attack. StS9, StS19 and StL3 transcript levels showed a 2-fold increase upon *F. eumartii* infection. However, their expression profiles did not change by wounding. In addition, StS9 transcript level was accumulated in salicylic acid- and jasmonic acid-treated leaves. In conclusion, our findings bring evidence on new different kinds of stress-responsive genes that may counteract *F. eumartii* attack in potato tubers. Putative functions for HSP90 and ribosomal proteins are discussed.

Keywords: biotic stress, *Fusarium solani* f. sp. *eumartii*, heat shock protein, ribosomal protein, *Solanum tuberosum*, wounding
Abbreviations: JA, jasmonic acid; **SA**, salicylic acid; **StHSP90**, *Solanum tuberosum* heat shock protein 90; **StL3**, *Solanum tuberosum* ribosomal protein large subunit 3, **StL36**, *Solanum tuberosum* ribosomal protein large subunit 36; **StS1**, *Solanum tuberosum* ribosomal protein small subunit 19; **StS9**, *Solanum tuberosum* ribosomal protein small subunit 9

INTRODUCTION

Several species of the genus *Fusarium* are ubiquitous fungal pathogens in a wide variety of crops. Dry rot is caused by several species of *Fusarium* spp. in potato tubers. *F. eumartii* infects tubers at wounded sites causing lesions on the surface that extend deeply in the tuber tissue producing a visible rot (Carpenter 1915). Dry rot is an important post-harvest disease that affects tubers in storage and seed pieces after planting causing important crop losses. Nowadays, dry rot is combated in the fields with chemical fungicides. This therapeutic approach for killing organisms with toxic chemicals has been the prevailing pest control strategy for over 50 years. However, a fundamental shift to an integrated control management for crop protection is urgently needed to resolve escalating environmental and economical consequences. Major technological advances in molecular genetics and genetic engineering have resulted in an array of bio-rational products and tools that are not toxic and hazardous to humans and to the environment.

Plants respond to fungal invasion by activating an array of inducible defense mechanisms (Chen and Chen 2002; Nimchuk *et al.* 2003; Shah 2003; Katagiri 2004). Changes in gene expression play a critical role in the host plant response to biotic stress (Rojo *et al*. 2003). Analysis of the changes in transcript abundance during fungal establishment may provide insights into the complexity of this plant-pathogen interaction and lead to the discovery of novel strategies for the integrated pest management. In addition, the biological relevance of signaling molecules, such as salicylic acid (SA), jasmonic acid (JA) and ethylene has been described in several plant-microbial systems (Feys and Parker 2000).

In attempt to identify potato genes differentially induced by *F. eumartii* infection, a substractive screening of a potato cDNA library from 24 h *F. eumartii*-infected tuber has been previously carried out (Godoy *et al*. 2000). Several potato cDNA clones has been identified and further characterized: a potato cyclophilin gene *StCyp*, a transcription coactivator *StMBF1* and a cDNA clone coding for an ACC oxidase, *St*-ACO3 (Godoy *et al*. 2000, 2001; Zanetti *et al*. 2002).

In this work, we identified new potato cDNA clones coding for a heat shock protein StHSP90 for *Solanum tuberosum* heat shock protein 90 and four ribosomal proteins belonging to small (S) and large (L) families named StS9, StS19, and StL3, StL36 respectively. Their gene expression patterns suggest that a complex and still unknown mechanism may be acting in potato tubers upon *F. eumartii* infection.

MATERIALS AND METHODS

Plant and fungal material

Potato tubers from *Solanum tuberosum* (*S. tuberosum*) cv. 'Spunta' were harvested in the late summer and stored at 4°C in the dark. Potato plants were cultivated in a growth chamber at 25°C with fluorescent light (250 μmol m^{-1} s^{-1}) under 16 h photoperiod. *Fusarium solani* f. sp. *eumartii* was initially obtained from INTA Collection, Balcarce, Argentina and maintained in our laboratory. The fungus was grown on potato dextrose agar (PDA) medium at 25°C for 3 weeks in the dark.

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Wounding, inoculation and chemical treatments

Treatments were performed at 25°C using the hollow punch method described by Radtke and Escande (1973). This method causes mechanical injury or wounding to the tuber tissue. At different times after treatment, tissue samples from 0.5 cm around the inoculation site were collected, frozen in liquid nitrogen and stored at -80°C for RNA isolation. Mycelia and spores of *F. eumartii* grown on PDA were used to inoculate potato tubers (approximately 1×10^5 spores ml^{-1}). Wounding was made by placing sterile PDA disks in potato tubers. For treatments with 1 mM SA and 0.1 mM JA (Sigma) potato leaves were sprayed with each agent at the corresponding concentration. Controls were treated with sterile water or with methanol 0.1% for SA and JA treatments, respectively. At different times after onset of treatments tissue samples were collected, frozen in liquid nitrogen and total RNA was isolated.

Isolation and sequencing of potato cDNA clones

cDNA clones were isolated from a 24 h *F. eumartii* infected-tuber cDNA library. This library was differentially screened with *F. eumartii*-inoculated and mock-inoculated subtracted probes (Godoy *et al.* 2000). StHSP90, StS9, StS19, StL3 and StL36 cDNA clones were amplified by PCR using T3 and T7 universal primers from UNI ZAP XR vector (Stratagene). PCR steps were performed for 40 cycles at the following times and temperatures: 50 s at 94°C, 50 s at 53°C, and 1 min at 72°C. PCR products were purified by using PureLink® PCR Purification and PureLink® Quick Gel Extraction (Invitrogen) and sequenced with T3 universal primer using a ABI 377 automated DNA Sequencer (Applied Biosystem USA) in High-Throughput Genomics Unit, Department of Genome Sciences, Washington University, USA.

Bioinformatics analysis

Potato cDNA clones were identified through simultaneous database searches using BLASTn algorithm at the NCBI network service to the GenBank nr database (http://www.ncbi.nlm.nih.gov/BLAST/) and at the Solanaceae Genomics Network (SGN, http://sgn.cornell.edu/). Similarities with expected value (E) smaller to 10^{-20} were considered significant.

RNA isolation and northern blot analysis

Total RNA was isolated from potato tubers or leaves using guanidine-HCl for extraction and LiCl for precipitation according to Laxalt *et al.* (1996). Total RNA was isolated from healthy, wounded, and wounded plus *F. eumartii*-inoculated tubers. RNA (10 µg $lane^{-1}$) was separated on denaturating 1% agarose gel and transferred onto a nylon membrane (Hybond N^+, Amersham Biosciences, USA) according to the manufacturer's indications and hybridized with the indicated radioactive probe. cDNA probes were labeled with [α-^{32}P] dCTP by random priming using Megaprime DNA labeling system® (Amersham Biosciences, USA). Nylon membranes were pre-hybridized with 20 ml of pre-hybridization solution (0.5 M buffer phosphate pH 7.2, 7% SDS and 10 mM EDTA, pH 8.0) for 20 min at 65°C. Hybridization was performed in the same pre-hybridization solution at 65°C for 24 h. The membranes were washed twice in 2 × sodium salt citrate buffer (SSC), 0.1% SDS for 15 min at 65°C, followed by 1 × SSC, 0.1% SDS for 30 min at 65°C and 0.5 × SSC, 0.1% SDS for additional 15 min at 65°C. Membranes were exposed in an Imaging Plate (Fujifilm) at room temperature for 4 days. Images were obtained from a Storm Scanner (Amersham Biosciences, USA).

Quantification of transcript levels

Densitometry analysis of transcript levels revealed by northern blot assays was performed using Image J Program (version 1.42q, National Institute of Health, USA); values were normalized to the level of 18S ribosomal RNA (18S rRNA) in each sample.

Statistical analysis

The data were subjected to analysis of variance (one- way ANOVA) and post hoc comparisons were done with Tukey's multiple range test at $P<0.05$ level. The statistical software program used was SigmaStat 3.1.

RESULTS AND DISCUSSION

Isolation of subtractive cDNA clones and sequencing analysis

StHSP90 cDNA clone isolated by a differential screening of the potato cDNA library exhibited 99% identity at the amino acid level to *Solanum lycopersicum HSP90-2* (Genbank: ABB55365). In addition, StS9, StS19, StL3 and StL36 cDNA clones were isolated and identified by single-pass sequencing. In these cases, all cDNA sequences corresponded to unidentified *S. tuberosum* genes. StS9, StS19, StL3 and StL36 cDNA clones matched with SGN-U268670, SGN-U269740, SGN-U268285 and SGN-U287282 unigenes, respectively, from Sol Genomics Database (http://sgn.cornell.edu). Predicted amino acid of all clones revealed high similarity with ribosomal proteins. StS9 exhibited 99% identity to *Solanum demissum* 40S ribosomal protein S9, StS19 showed 86% identity with *S. tuberosum* 40S ribosomal protein S19-like, StL3 presented 96% identity to *S. tuberosum* ribosomal protein L3-like while StL36 showed homology with *Arabidopsis thaliana* 60S ribosomal protein L36 (92%).

Up-regulation of potato genes upon wounding and *Fusarium eumartii* infection

Gene expression profiles of *StHSP90*, *StL36*, *StS9*, *StS19* and *StL3* genes upon wounding and *F. eumartii* infection were analyzed by northern blot assays. A transcript of approximately 2.4 kb corresponding to StHSP90 was barely detectable in non-wounded tubers. However, it was accumulated upon wounding and *F. eumartii* infection. The transcript level of StHSP90 was maintained up from 4 to 72 h after initial treatment (**Fig. 1**). Thus, wounding as well as *F. eumartii* infection stimulated StHSP90 mRNA accumulation in potato tubers, suggesting that the putative StHSP90 protein might be involved in folding and assembly of newly synthesized proteins by wounding and fungal infection. Swindell *et al.* (2007) described that *Phytophthora infestans* elicits gene expression of members belonging to HSP70, HSP90, and HSP100 families in *A. thaliana* leaves. Certain members of HSP70 family are expressed only when the organism is challenged by environmental assaults (Wang *et al*. 2004). A tobacco HSP90 interacts with proteins which confer resistance to tobacco mosaic virus (Liu *et al*. 2004). In different organisms, cytoplasmic members of HSP90 protein family, as well as other HSPs are thought to act as molecular chaperones since they stabilize the target protein during processing and transport (Ellis *et al.* 1991; Gething *et al* 1992). All these data contribute to speculate that StHSP90 may act as a chaperone protein, i.e. preventing aggregation and assisting in the refolding of non-native proteins under stress conditions and/or play a regulatory role in stress-associated gene expression.

In addition, the expression of a small set of ribosomal genes was analyzed. In particular, *StS9*, *StS19* and *StL3* genes were differentially up-regulated by *F. eumartii* infection but they did not change by wounding (**Fig. 2A-C**). Compared with non-wounded tubers (C), StS9, StS19 and StL3 transcript levels increased approximately 3-fold in fungal infected tubers. However, StL36 transcript level increased approximately 2-fold by wounding and *F. eumartii* infection (**Fig. 2D**). No cross-hybridization between probes was evident, suggesting that each cDNA probe was specific for each mRNA. Coincident with our results, Kruger *et al.* (2001) described that five genes (Genbank: BM134651, BM136009, BM136722, BM140473 and BM136113) en-

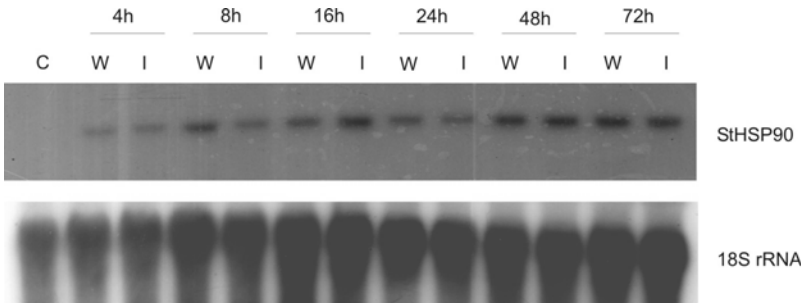

Fig. 1 StHSP90 transcript level increases in potato tubers upon wounding and *Fusarium eumartii* infection. Total RNA was isolated from non-wounded (C), wounded (W) or wounded plus-*Fusarium eumartii* inoculated (I) tubers as indicated. Membranes were hybridized with StHSP90 probe (upper panel) or with rRNA probe to test the loading of the RNA in each lane (lower panel).

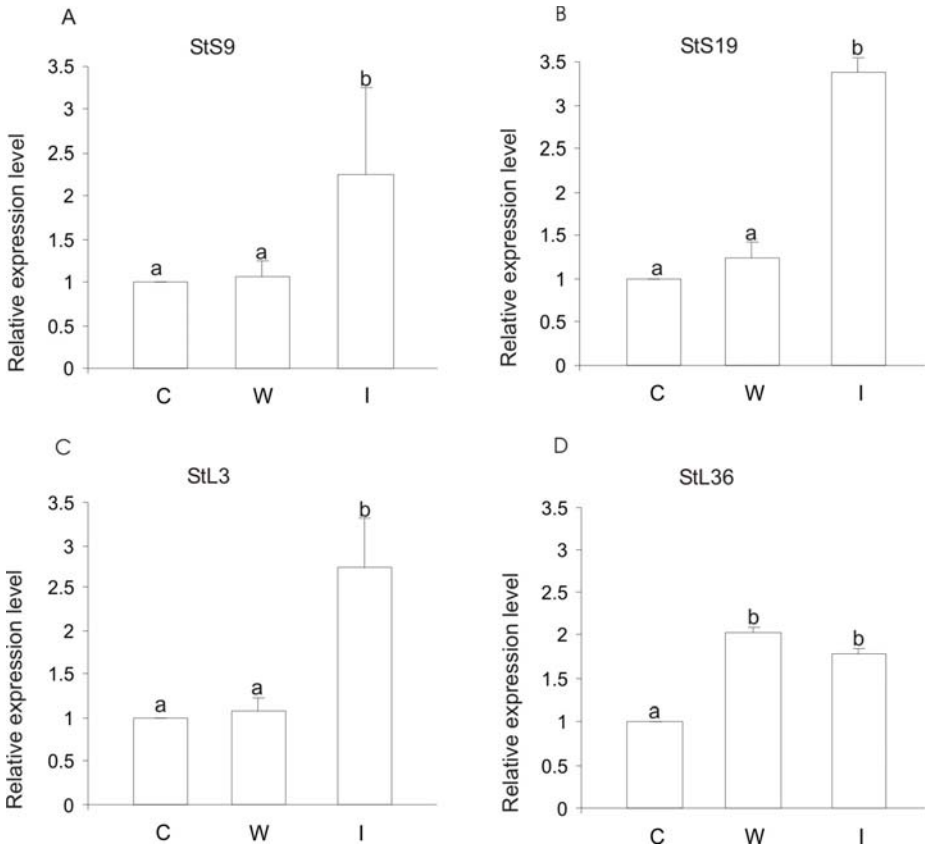

Fig. 2 Gene expression pattern of *StS9, StS19, StL3* and *StL36* in potato tubers. Total RNA was isolated from non-wounded (C), wounded (W) or wounded plus-*Fusarium eumartii* inoculated (I) tubers at 24 h after treatment. Northern blot assays were carried out using different probes: StS9 (**A**), StS19 (**B**), StL36 (**C**) and StL3 (**D**). The histogram represents the average of at least three independent experiments. Error bars represent the ± SD. Letters among bars indicate statistically different values at $P < 0.05$. The amount of hybridizing RNA measured in control tubers has been taken as the 100% value.

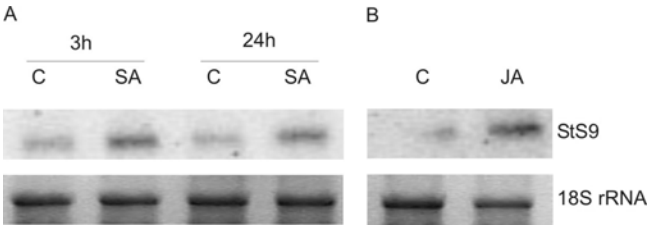

Fig. 3 StS9 transcript level increases in SA- and JA-treated leaves. (**A**) Total RNA was isolated either from control (C) and SA-treated leaves at the indicated times. (**B**) Total RNA was isolated either from control (C) or JA-treated leaves at 24 h after treatment. Membranes were hybridized with StS9 probe (upper panel). Ethidium bromide-stained ribosomal RNA band was used as the loading control (lower panel).

coding for different ribosomal proteins are expressed during wheat-*Fusarium graminearum* interaction. Adomas *et al.* (2007) detected a 2-fold increase of a putative ribosomal protein in *Gremmeniella abietina*-inoculated *Pinus sylvestris*.

In order to extend the analysis of *StS9* gene expression, StS9 transcript level was measured in SA- and JA-treated leaves. As shown in **Fig. 3A**, a transcript of 1.3 Kb corresponding to the expected for StS9 was increased by SA treatment. StS9 transcript level was maintained up at 3 h and 24 h after initial treatment. In addition, JA induced the accumulation of StS9 mRNA at 24 after treatment (**Fig. 3B**). SA and JA are very important signaling molecules involved in the defense response against different pathogens (Liechti *et al.* 2002). SA has been associated with the hypersensitive response (HR) and the establishment of systemic acquired resistance (SAR) (Delaney *et al*. 1994; Ryals *et al*. 1996; Mur *et al*. 1997, 2000). Studies in *A. thaliana* and tomato mutants deficient in JA synthesis and perception demonstrated that JA is essential for defense against some insects and mites (Howe *et al*. 1996; Reymond *et al*. 2000; Ament

et al. 2004). Thus, it is reasonable to assume that both, SA and JA could be involved in the pathway of *StS9* activation as part of different regulatory levels of the potato inducible defense response against *F. eumartii* infection.

In conclusion, the identification and characterization of potato genes atypically related with the plant defense mechanisms to biotic stress may indicate the existence of a still incomprehensive potato defense response to *F. eumartii* infection. Although many genes encoding potato ribosomal proteins were identified, their physiological relevance in the potato defense response to fungal infection is still unknown. Further studies on this subject are required to provide direct evidence for the ribosomal protein roles and to identify the relay of potato signaling pathways triggered by *F. eumartii* attack.

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