تشخیص مولکولی ژن‌های القاء شده گیاه سیب زمینی دخیل در دفاع علیه باکتری *Ralstonia solanacearum*

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چکیده

با استفاده از تکنیک cDNA-AFLP واکنش مولکولی رقم حساس (مارفونا) و رقم متحمل (الس) در گیاه سیب زمینی (*Solanum tuberosum*) و ژنوتیپ مقاوم (*S. phureja*) برای شناسایی ژن‌های دخیل در مکانیسم‌های دفاعی مورد بررسی قرار گرفت. بیان ژن‌های مرتبط با دفاع در چهار زمانهای ۰، ۲، ٤ و ۷ روز بعد از آلوده سازی ریشه گیاهان با باکتری *R. solanacearum* در شرایط درون شیشه‌ای بررسی گردید. حدود ۲۸۲۰ قطعه مشتق از رونوشت مشاهده شد که در این میان ۲۰٦ قطعه الگوهای بیان متفاوتی نشان دادند. در مجموع ۲۰ قطعه با بیشترین اختلاف، توالی‌یابی شدند و همولوژی آنها با ژن‌های شناخته شده قبلی در بانک ژن بررسی گردید. این ژن‌ها تشابه قابل توجهی با برخی ژن‌های درگیر در فرایندهای فیزیولوژیکی واکنش به استرس‌ها، دفاع، ژن‌های ساختاری وژن‌های دخیل در نسخه‌برداری، سنتز و بسته‌بندی پروتئین داشتند. تغییر بیان این ژن‌ها در *S. phureja* نسبت به *S. tuberosum* پس از آلودگی با *R. solanacearum* بیشتر بود.

کلیدواژه: پژمردگی باکتریایی، *Solanum tuberosum*، *S. phureja*، cDNA-AFLP

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Molecular characterization of defense-related genes, induced against *Ralstonia solanacearum* in potato

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Abstract

Molecular responses of susceptible (Marfona) and tolerant (Els) cultivars of *Solanum tuberosum*, and a resistant genotype of *S. phureja* were comparatively studied using a cDNA-AFLP technique to identify candidate genes involved in defense mechanisms against *Ralstonia solanacearum*. Transcriptional changes were studied at 0, 2, 4 and 7 days post inoculation of the plants by *R. solanacearum* through root inoculation under *in vitro* conditions. Of the 2820 transcript derived fragments (TDFs) detected, 206 TDFs showed variable patterns. In total 20 TDFs with stronger differential induction were sequenced. These TDFs showed significant homologies with the genes involved in physiological processes, stress responses, cell structure, defense, transcription and protein synthesis/folding. Approximately 5% of the sequenced TDFs were identified with unknown function. Induction of a higher number of genes was altered in response to *R. solanacearum* in *S. phureja* compared to *S. tuberosum*.

Keywords: Bacterial wilt, *Solanum tuberosum*, *S. phureja*, cDNA-AFLP

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Introduction

Ralstonia solanacearum is an important plant pathogen that attacks many plants in warm and humid climates (Denny 2006). Bacterial wilt caused by *R. solanacearum* is a limiting factor in production of potato in both tropical and sub subtropical regions (Allen *et al.* 2005). Among disease control methods used for the pathogen, planting disease-resistant cultivars is one of the most successful, economical and environmentally-friendly strategy (Boshou 2005). Therefore, the development of genetically resistant and commercial potato cultivars is important for management of bacterial wilt disease for potato production.

Research on molecular mechanism(s) governing potato-*Ralstonia* interactions is an important preliminary step for discovering and utilization of effective resistance genes for improvement of potato resistance against *R. solanacearum* (Zuluaga *et al.* 2015). Scientists have known that some plants, like animals, can also be immune against some pathogens (Chester 1933). For the most crops susceptible to bacterial wilt, high level, gene-for-gene type of resistance encoded by a single dominant gene has not been reported and the available sources of resistance in cultivated potato are often polygenic (Boshou 2005). The low levels of resistance against *R. solanacearum* in *Solanum tuberosum* cultivars are not sufficient to withstand infection under optimal conditions for disease development. Useful approach for understanding the molecular basis of interaction between *R. solanacearum* and potato plant is to characterize the genes differentially expressed under compatible or incompatible combinations.

Although potentially higher levels of resistance against *R. solanacearum* have been reported in *S. phureja* (Fock *et al.* 2005; Moslemkhani *et al.* 2012, Virupaksh *et al.* 2012; Rowe *et al.* 1972), the mechanisms regulating defense and resistance in *S. phureja* are still unknown. Some genes such as receptor like kinase have been characterized during Interaction between potato and *Ralstonia solanacearum* (Li *et al.*, 2010). However, little is known about the genes involved in incompatible interaction of *S. phureja* and *R. solanacearum*. Recently, the interactions of *R. solanacearum* with *S. tuberosum*, *S. lycopersicum and S. commersonii* have been studied at transcriptomic and proteomic levels (Dahal *et al.* 2009; Dahal *et al.* 2010; Li *et al.* 2010; Esposito *et al.* 2008). The results demonstrated that the response of *Solanum* spp. against *R. solanacearum* is by elevating the expression of pathogenesis-related and other defense-related proteins (Dahal *et al.* 2010).

Gene expression analysis of the responses of potato cultivars against *R. solanacearum* provides useful information about the molecular mechanisms of potato-*Ralstonia* interactions and the development of molecular markers for research on potato genetic breeding. Therefore, we applied a cDNA-amplified fragment length polymorphism (cDNA-AFLP) approach to identify and compare the genes differentially-induced against *R. solanacearum* in *S. tuberosum* (susceptible host) and *S. phureja* (resistant host) over time.

Material and methods

Plant material and inoculation

The highly virulent *R. solanacearum* strain SH_{12} (race 3, biovar 2) was isolated from potato plants of Khuzestan province in South Western Iran. Isolation and characterization of bacteria from samples was carried out according to Schaad et al. (2001). Also PCR and pathogenicity test were used for diagnosis of isolated bacteria (moslemkhani *et al.*, 2005). The bacterium was grown for 24 h, at 28°C on yeast peptone glucose agar (YPGA) medium overnight. The resulting bacterial suspension of 10^8 CFU/ml (OD_{600}=0.1) was used for plant inoculation.

Supper Elit seed tuber classes of cultivars Els and Marfona of *S. tuberosum* and botanical seeds of *S. phureja* were obtained from National Plant Gene Bank of Iran. *S. tuberosum* and *S. phureja* were cultured on MS basal medium (Murashing & Skoog, 1962) under *in vitro* conditions and multiplied using axillary shoot tips. The potato clones were subcultured at four weeks interval and maintained under environmental conditions with 16 h day $^{-1}$ illumination at 55 μmol^{-2} s^{-1}, 22°C and 70% relative humidity. Four week-old *in vitro* plants were inoculated using root inoculation method (Fock *et al.* 2000). Inoculated plants were sampled 0, 2, 4 and 7 days after inoculation to evaluate the transcriptome of the potato genotypes in response to bacterial infection. The experiment was carried out with 4 replicates.

RNA isolation and cDNA synthesis

Total RNA was extracted from whole plantlet tissue (leaf, stem and root), using RNeasy® mini kit (Qiagen), and mRNA was purified from 200 µg of extracted total RNA using Qiagen Oligotex mRNA purification kit according to their manufacturer's instructions. The quantity of the extracted total RNA and mRNA were checked on 1% agarose gel. First strand cDNA was synthesized by using Fermentas First Strand cDNA synthesis kit according to the manufacturer's protocol. Double stranded cDNA was synthesized and purified (Moslemkhani *et al.*, 2014).

cDNA-AFLP analysis

A cDNA-AFLP analysis was performed as described by Bachem et al. (1998) Transcript-derived fragments (TDFs) were generated by digestion of the ds cDNA with 5 u of *Mse*I and *EcoR*I (Invitogen). *EcoR*I and MseI adapters were then ligated to the digested cDNA. Ten µl of the ligation mix was used for pre-selective amplification using *EcoR*I+0 (5'-GACTGCGTACCAATTC-3') and *Mse*I+0 (5'-GATGAGTCCTGAGTAAC-3') primers. The pre-selective reaction was used for selective amplification. Standard *EcoR*I and *Mse*I AFLP primers were used with two selective nucleotides *EcoR*I CT, AC and *Mse*I CC, GG, AT, AC (Vos *et al.*, 1995). Selective amplification products were fractionated on a 6% denaturing polyacrylamide gel and visualized by silver staining (Bassam *et al.* 1991). TDFs of interest were excised from the gel, immersed in 50 µl of sterile water and incubated overnight at 4°C for DNA diffusion. After centrifugation of tubes contain excised fragment, the extracted DNA were re-amplified using AFLP selective primers under the same PCR conditions. The PCR products were resolved by agarose gel electrophoresis (Ausubel *et al.* 1992) and purified using Qiagen gel extraction kit (Qiagen, USA). The purified DNAs were sequenced by MWG Biothech, (Germany). The resulting nucleotide sequences were analyzed in the GenBank database using Basic Local Alignment Search Tool (BLAST) Program.

Results

The three genotypes of potato showed different response against *R. solanacearum*, as confirmed by bioassay. *S. phureja* was immune, while potato cv. Els showed high level of tolerance and cv. Marfona was susceptible against the pathogen (Moslemkhani *et al.*, 2011). Under *in vitro* conditions, sever wilt symptoms appeared on inoculated plantlets of cv. Marfona but not on cv. Els and *S. phureja*, ten days post inoculation. However, a mild wilting was observed at leaves around the inoculation site in cv. Els, 15 days after inoculation, while no symptom was observed on *S. phureja*, even one month post inoculation.

The result of cDNA-AFLP showed clear differences between infected and non-infected samples and among different sampling times post inoculation (Fig. 1). By using eight primers combinations, 2820 TDFs were amplified, ranging from 50 to 800 bp. Among them, 206 TDFs were differentially induced of which 7.28% and 74.27% corresponded to down-regulated and up-regulated transcripts, respectively. A considerable level of differentially TDFs (18.44%) were specific fragments to either *S. tuberosum* or *S. phureja* (Fig 2). Most of the up-regulated TDFs were presented in *S. phureja* 4 and 7 days after inoculation. Out of 324 TDFs obtained in *S. phureja*, 19/75% were up-regulated and 1/85% were down-regulated. Of the 284 TDFs in cv. Marfona transcriptom profile, 17/25% showed up-regulation and 1/76% showed down-regulation. Among 274 TDFs detected in cv. Els, 14/60% were up-regulated and 1/46% were down-regulated. Of the sequenced TDFs, 20 sequences showed similarity to those recorded in the GenBank databases (Table 1).

The functions of 20 sequenced TDFs were identified based on their similarities to the known proteins registered in centers such as NCBI. The sequences could be divided to eight categories from which 20% were involved in physiological processes, 15% in stress responses, 25% in cell defense, 15% in cell structure, 10% in signal transduction, 5% in transcription, and 5% in protein synthesis and folding. No homology was found for the remaining (5%) that corresponded to the proteins of known functions.

Discussion

Large scale analysis of gene transcription is a powerful approach for analysis of plant-pathogen interactions. In this study, cDNA-AFLP technique was used to identify differentially-expressed transcripts putatively associated with the infection

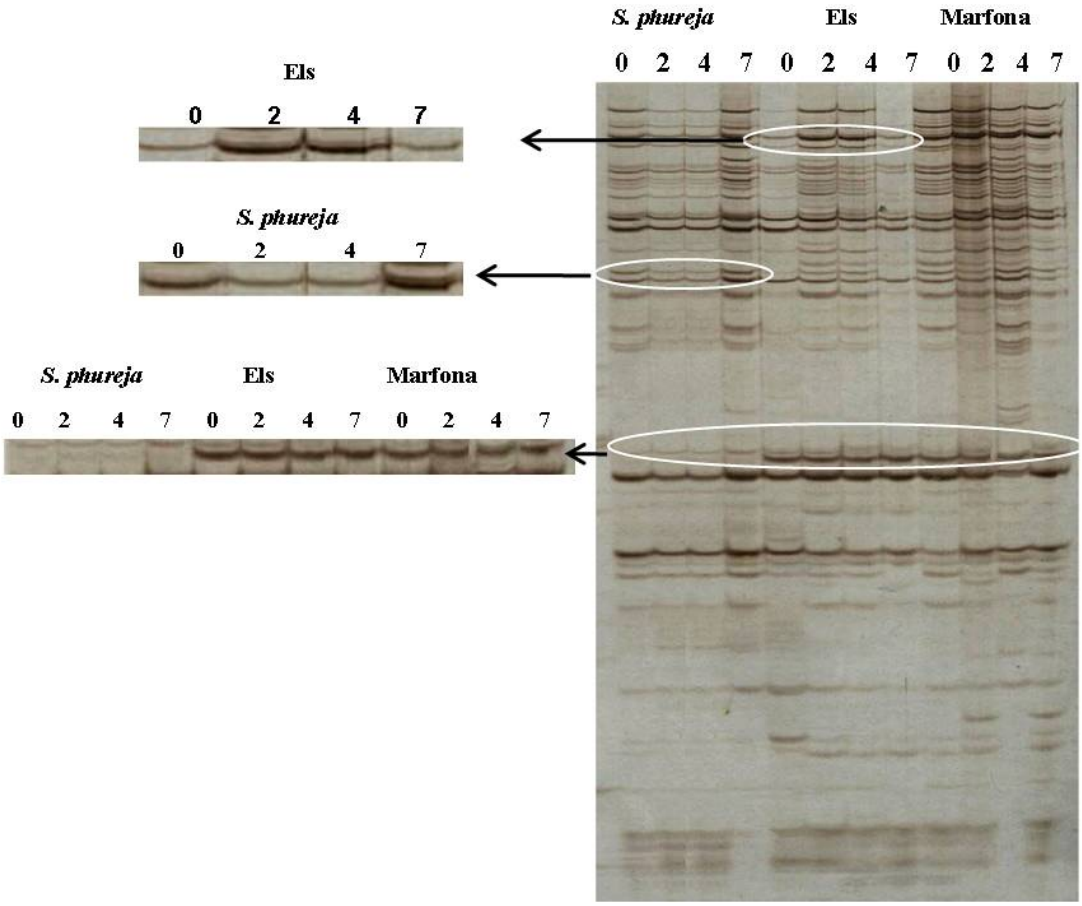

Fig. 1. Part of results obtained from cDNA-AFLP analysis of *Solanum phureja*, *Solanum tuberosum* cvs. Els and Marfona after infection by *Ralstonia solanacearum*. TDFs were obtained from selective amplification using Mse-GG, EcoR-CT primer combinations. Each row corresponds to three potato genotypes including control (0), and inoculated plantlet sampled 2, 4 and 7 days post inoculation.

شکل ۱. قسمتی از نتایج حاصل از آنالیز cDNA-AFLP در *Solanum phureja* و ارقام الس و مارفونا در گونه *Solanum tuberosum* بعد از آلوده سازی با *Ralstonia solanacearum* . قطعات مشتق از رونوشت که با استفاده از ترکیب پرایمری Mse-GG, EcoR-CT به دست آمده‌اند. هر ردیف مربوط به سه ژنوتیپ سیب زمینی است که شامل (۰) : گیاه سالم، (۲، ٤ و ۷): گیاهان نمونه برداری شـده در دو، چهار و هفت روز پس از آلوده سازی.

process and resistance response during the interaction of *S. tuberosum* (cv. Els and Marfona) and *S. phureja* against race 3, biovar 2 of *R. solanacearum* under *in vitro* conditions. Results from the present and our previous studies (Moslemkhani *et al.* 2011) indicate that potato cv. Marfona is susceptible and cv. Els is relatively tolerate against *R. solanacearum*. The highest level of resistance was found in *S. phureja*. *In vitro* assessment of plant-pathogen interaction has advantages as compared to the greenhouse assessment and eliminates confounded effects of other variables that may occur under greenhouse conditions (Moslemkhani *et al.* 2011).

The majority of the differential transcription rate in the present study (74.27%) were up-regulated in response to *R. solanacerarum* infection in potato indicating a drastic change in the biochemical composition of plant cells. (Dahal *et al.* 2010; Li *et al.* 2010; Esposito *et al.* 2008). Number of the up-regulated genes in resistant potato (*S. phureja*, incompatible interaction) was significantly higher than those in *S. tuberosum* cultivars Els (partial tolerant) and Marfona (susceptible) in compatible systems. Similar results have been reported in other incompatible plant-pathogen interactions (Kiba *et al.* 2003)

We identified various TDFs from cv. Marfona,

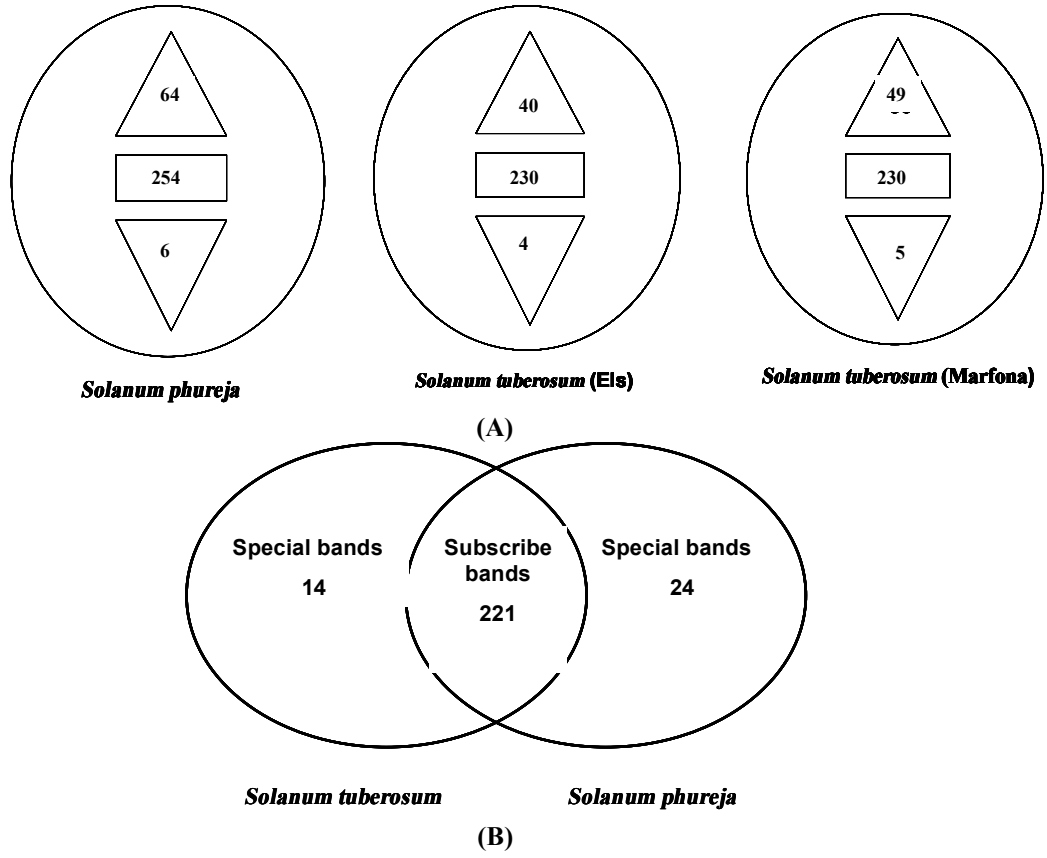

Fig. 2. The induction rate of TDFs detected by cDNA-AFLP analysis in Els and Marfona (*Solanum tuberosum*) and *Solanum phureja* using eight primer combinations. A) Number of TDFs up-regulated (△), down regulated (▽) or not changed (▭) in inoculated plants compared to mock-inoculated plants. B). TDFs transcribed in one (left or right zone) and in both (middle zone) Solanum species

شکل ۲. نرخ القاء قطعات مشتق از رونوشت شناسایی شده با استفاده از تکنیک cDNA-AFLP در رقم الس و مارفونا (*Solanum tuberosum*) و *Solanum phureja* با استفاده از هشت ترکیب پرایمری مختلف. A: تعداد قطعات مشتق از رونوشت که در گیاهان آلوده در مقایسه با گیاهان سالم افزایش بیان (△) یا کاهش بیان (▽) نشان دادند یا بدون تغییر (▭باقی ماندند. B: تعداد قطعات مشتق از رونوشت که تنها در یکی از گونه‌های Solanum نسخه برداری شده است (قسمت سمت راست یا چپ تصویر) یا در هر دو گونه وجود دارد.

some of which corresponding to stress responding and senescence/dehydration-associated proteins. Dehydration-related genes are expressed under water deficiency and low temperature stresses (Riechmann *et al.* 2000). Wilting is depended on the density of the pathogen in vascular system, preventing sufficient water exchange and resulting in dehydration stress (Denny 2006). Dehydration-associated proteins are involved in many developmental and stress-related processes (Choudhary *et al.* 2009). Probably, early dehydration responses play a role in scavenging stress-induced generation of reactive oxygen species (Choudhary *et al.* 2009).

Small heat shock protein genes are also stress-related genes that were up-regulated in *S. phureja* after inoculation by *R. solanacearum*. These genes are induced during exposure to elevated temperature and a variety of cellular stresses such as oxidative stress (Banzet *et al.* 1998; Byth *et al.* 2001). The synthesis of these stress proteins can be considered as an adaptive mechanism in which mitochondrial protection could be essential (Banzet *et al.* 1998).

Gene encodes a DnaJ-like protein was up-regulated in potato cv. Marfona two days post

Table 1: Homologies of the sequences of differential fragments of potato genotypes against *R. solanacearum* with sequences registered in the GenBank databases

جدول ۱: همولوژی توالی‌های قطعات متمایز در ژنوتیپ‌های گیاه سیب زمینی در واکنش به آلودگی *R. solanacearum* با ژن‌های ثبت شده در پایگاه داده‌های بانک ژن

TDF	Species	Primer comb	Length (bp)	Blast score	Accession number	Function	Annotation
B8 (M.2,4)	*S.tuberosum*	AT-AC	129	1e-58	JZ531628	Phosphatase activity	NIL interacting factor protein
H8 (M.2)	*S.tuberosum*	CT-CA	117	3e-52	JZ772715	Molecular chaperone	DnaJ- like protein
B7 (E.4)	*S.tuberosum*	AT-AC	85	1e-05	JZ772716	Signal transduction	Leucin rich repeat family protein
B9 (M.2)	*S.tuberosum*	AT-AC	310	1e-146	JZ772717	Stress response	Senescence/ dehydration associated protein-related
B10 (S. ph. 4)	*S. phureja*	AT-AC	90	6e-35	JZ772722	oxidation-reduction process	putative peroxisomal (S) -2-hydroxy acid oxidase 2
C16 (S. ph. 4)	*S. phureja*	GG-AC	116	6e-48	JZ772723	–	Un known protein
E2 (S. ph. 4)	*S. phureja*	AT-CT	214	2e-85	JZ772724	structural	Plastid lipid associated protein
E3 (S. ph. 4)	*S. phureja*	AT-CT	174	1e-66	JZ772725	physiology	1-deoxy-D-xylulose 5 phosphate synthase
E4 (E.4)	*S.tuberosum*	AT-CT	223	4e-99	JZ772721	structural	Plastid lipid associated protein
G33 (S. ph.2)	*S. phureja*	AC-CA	125	2e-56	JZ772733	defense	chitinase
F5 (S. ph. 4,7)	*S. phureja*	GG-CT	94	7e-40	JZ772726	defense	Plastidic pyruvate kinase beta subunit 1
B11 (S. ph. 7)	*S. phureja*	AT-AC	116	2e-47	JZ772727	transferase activity	Uracil phosphoribosyl transferase putative
C5 (S. ph. 2)	*S. phureja*	GG-AC	77	9e-31	JZ772728	Stress response	Mitochondrion localized small heat shock protein
C17 (S. ph. 4)	*S. phureja*	GG-AC	149	8e-68	JZ772729	Signal transduction	Serine/threonine-protein kinase
B2 (S. ph. 4)	*S. phureja*	AT-AC	128	4e-47	JZ772730	structural	Pentatricopeptid repeat containing protein
H6 (E. 2,4)	*S.tuberosum*	CT-CA	100	4e-43	JZ772718	Stress response	Mitochondrial small heat shock protein
H5 (E.sh)	*S.tuberosum*	CT-CA	129	2e-48	JZ772719	defense	Lypoxygenase
H7 (E.7)	*S.tuberosum*	CT-CA	123	2e-55	JZ772720	defense	Polygalactronase inhibiting protein
C8 (S. ph. 2,4)	*S. phureja*	GG-AC	118	9e-53	JZ772731	transcription	Myb transcription factor
B3 (S.ph.4,7)	*S. phureja*	AT-AC	116	1e-51	JZ772732	defense	Putative aspartic protease

inoculation. This protein may play an important role in transport and signal transduction of the molecules induced by *R. solanacearum* (Li *et al.* 2010). Recent study by semi-quantitative RT-PCR has indicated that the gene is induced by infection by *Ralstonia solanacearum* as well as by treatment

with jasmonic acid, suggesting that DnaJ-gene may play a role in recovering the activity of proteins destroyed by *R. solanacearum* or jasmonic acid stresses (Li *et al.* 2007).

Polygalactronase-inhibiting protein (PGIP) coding gene was up-regulated in potato cv. Els with considerable level of resistance. PGIPs are plant cell wall proteins that protect plants from pathogen invasion (Matteo *et al.* 2003). They inhibit bacterial polygalactronases, the effective enzymes in multiplication and distribution of the pathogen in plant tissues. it seems that PGIPs contribute to the development of defense responses and delaying the appearance of disease symptoms (Schacht *et al.* 2011).

Putative aspartic protease (AP) coding gene was severely up-regulated in *S. phureja* 4 and 7 dpi as compared to *S. tuberosum* cultivars, which implies that these genes are involved in resistance responses against *R. solanacearum*. It has been reported that aspartic protease APs are involved in senescence, defense and stress responses (Guevara *et al.* 2004).

The DNA sequence corresponding to TDF C_8 in *S. phureja* had a high homology with the gene encoding for Myb transcription factor that participate in regulation of defense-related genes (Rushton & Somssich 1998).TDF H_5 showed homology to lipoxygenase (LOX) gene that was down regulated in the cv. Els over times post inoculation. LOX genes are transcriptionally activated by wounding, pathogen infection and elicitors (Kolomiets *et al.* 2000). Contrary to several reports, our results showed that transcription rate of lipoxygenase gene was down-regulated in relatively resistant cv. Els. This could be due to the fact that lipoxygenase gene are expressed in early stage of potato infection by *R. solanacearum*. These observations need to be further confirmed by additional experiments. The analysis of transcriptome data in this study provides new insights to dissection and understanding the molecular mechanisms involved in susceptibility, tolerance and immunity responses of potato plant against *R. solanacearum*.

This study provides useful information on differential gene induction in potato plantlet after pathogen challenge in compatible and incompatible interactions. The identified TDFs, showed significant homologies with the genes involved in physiological processes, stress responses, cell structure, defense, transcription and protein synthesis/folding, suggesting their direct or indirect involvement in plant response to the pathogen and supporting the complexity and the polygenic regulation of plant response against *Ralstonia solanacearum*. Our data indicated in *S. phureja* more transcriptional changes involves against *R. solanacearum*. The TDFs, showed significant homologies with the Myb transcription factor is one of the excellent candidate for study defense responses of *S. phureja*. Myb transcription factor has central role in controlling the synthesis of the phenylpropanoid-derived compounds that contributes to the enhancement of plant defense response. We suggest future investigation to learn how Myb transcription factor involve in resistance of *S. phureja* and focusing research efforts on engineering these transcription factors to promote resistance in *Solanum tuberosum*.

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