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RESEARCH PAPERS

Gene Transcription Analysis during Interaction between Potato and *Ralstonia solanacearum*[1]

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Abstract—Bacterial wilt (BW) caused by *Ralstonia solanacearum* (*Rs*) is an important quarantine disease that spreads worldwide and infects hundreds of plant species. The BW defense response of potato is a complicated continuous process, which involves transcription of a battery of genes. The molecular mechanisms of potato–*Rs* interactions are poorly understood. In this study, we combined suppression subtractive hybridization and macroarray hybridization to identify genes that are differentially expressed during the incompatible interaction between *Rs* and potato. In total, 302 differentially expressed genes were identified and classified into 12 groups according to their putative biological functions. Of 302 genes, 81 were considered as *Rs* resistance-related genes based on the homology to genes of known function, and they have putative roles in pathogen recognition, signal transduction, transcription factor functioning, hypersensitive response, systemic acquired resistance, and cell rescue and protection. Additionally, 50 out of 302 genes had no match or low similarity in the NCBI databases, and they may represent novel genes. Of seven interesting genes analyzed via RNA gel blot and semi-quantitative RT-PCR, six were induced, one was suppressed, and all had different transcription patterns. The results demonstrate that the response of potato against *Rs* is rapid and involves the induction of numerous various genes. The genes identified in this study add to our knowledge of potato resistance to *Rs*.

Key words: Solanum tuberosum, Ralstonia solanacearum, bacterial wilt, suppression subtractive hybridization, cDNA macroarray, EST.

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INTRODUCTION

The cultivated potato (*Solanum tuberosum* L.) is one of the most important food crops in the world. It is ranked fourth in world food production but is continually threatened by many pathogens and pests, which annually result in the losses of billions of US dollars. Bacterial wilt (BW) caused by *Ralstonia solanacearum* (*Rs*) is a devastating disease of potato. This vascular pathogen infects hundreds of plant species, including potato, tomato, banana, pepper, and even trees [1]. Although BW is regarded as a quarantine disease in Europe, recently this serious potato disease outbreak had been reported from various European countries [2, 3].

Over the past decade, attempts had been made to isolate and identify resistance genes and quantitative trait loci (QTLs), and to transfer defense-related genes into potato. To date at least 10 *R* genes and QTL for resistance to *Rs* have been identified from various plant species, including *RRS1* gene from *Arabidopsis thaliana* [4], *AP1* gene from potato [5], and QTL *BW-1, BW-3, BW-4, BW-5, Bwr-3, Bwr-4, Bwr-6,* and *Bwr-8* from tomato [6, 7]. Among transgenic potato plants containing foreign genes, such as genes encoding chitinase or defensins, only a few genes from potato, such as *snakin-1* and *snakin-2*, were reported to increase resistance to *Rs* [8]. Although some functional genes have been cloned and characterized, little is known about genes that are involved in incompatible interactions between potato and *Rs*.

Large-scale analysis of gene transcription during plant–pathogen interactions is a highly effective strategy for gaining insight into molecular events involved in this process. Suppression subtractive hybridization (SSH) is a powerful method to construct normalized cDNA libraries, which are enriched in differentially

[1] This text was submitted by the authors in English.

Abbreviations: BW—bacterial wilt; HR—hypersensitive response; JA—jasmonic acid; PCPI—potato cysteine proteinase inhibitors; QTL—quantitative trait loci; *Rs*—*Ralstonia solanacearum;* SAR—systemic acquired resistance; SSH—suppression subtractive hybridization; TF—transcription factors.

expressed genes [9]. Although this method has its disadvantages in generating false positives in the library, the combination of Mirror Orientation Selection and macroarray analysis with SSH can effectively decrease the number of false positive clones. In the present study, the combination of SSH and cDNA macroarrays was used to investigate transcription of genes involved in potato resistance to *Rs* infection.

MATERIALS AND METHODS

Plant material and Ralstonia solanacearum strain. The potato genotypes ED13 and ED25 (progeny of 772102.37[E] × USW7589.2[D]), originating from Wageningen University (the Netherlands) were provided by Professor Dongyu Qu (Institute of Vegetable and Flowers, Chinese Academy of Agricultural Sciences). The parent E, carrying resistance genes against *Rs*, originates from *Solanum phureja* and *S. vernei*. Potato plants were grown in a greenhouse under the condition of 25/18°C (day/night), and 14/10 h light/dark cycle.

Rs strain PO41 (race 3, biovar 2) was grown on NA medium (1% glucose, 0.5% trypton, 0.3% beef extract, 0.05% yeast extract, 1.5% agar, and 0.005% red tetrazoline, pH 7.0), cultured at 28°C for 24–48 h to get single colonies, picked out single colony, propagated on fresh NA medium under the same cultural condition, and then rinsed with sterile water. The concentration modulated to ~10^7 cfu/ml according to the OD value.

Inoculation of potato genotypes ED13 and ED25. Rs infection experiments were carried out using a modified version (designated as stem-bacterial co-culture method) of the traditional stem-inoculation method [10]. In brief, 20 potato plant stems (designated as cuttings) containing 6–7 leaves were collected from four equally aged potato ED13 plants and 6 cuttings from two ED25 plants, then directly co-cultured with *Rs* solution (10^7 cfu/ml) under a 14-h photoperiod, at 28°C, and 100% relative humidity. Another 26 cuttings from the same ED13 and ED25 plants were co-cultured with water as control. Fourteen *Rs*-treated and 14 control plant cuttings from ED13 were used to harvest leaves at different time points after treatments, then frozen in liquid nitrogen, and stored at –80°C until RNA extraction. Extra plant cuttings were kept for more than 7 days to confirm successful inoculation.

RNA extraction and cDNA library construction. Total RNA was isolated from *Rs* and water-treated cutting leaves of ED13 plants using TRIzol reagent (Invitrogen, United States). Poly(A)RNA was isolated from total RNA according to the Oligotex® procedure (Qiagen, Holland). cDNA was synthesized based on SMART cDNA Synthesis Kit (Clontech, United States). The subtracted library was constructed according to the manual of the SSH Kit (Clontech). The tester cDNA synthesized from an mRNA mixture of *Rs*-inoculated cutting leaves treated for 6/12 h was subtracted twice by the driver cDNA synthesized from the mRNA mixture of control cutting leaves, following the SSH kit manual (forward subtraction). The reverse subtraction was performed with interchange of “driver” and “tester”. Subtracted cDNAs were used as templates for selective amplification, then ligated with the pMD-18T vector (Takara, Japan) and transferred into *E. coli* competent cells (Top10).

To ensure the success of the SSH library construction, the specific primers for *actin* gene (5'-GATGGTGTCAGCCACAC-3' and 5'-ATTCCAGCAGCTTCCATTCC-3') were designed according to the published sequence (GenBank accession no. X55749), and the *actin* gene was used as a monitor during SSH library construction process.

Sequence analysis and BLAST search. DNA sequence analysis was carried out using the ABI Prism 3730 DNA sequencer (Qingke Ltd., China). Sequences were manually trimmed of vector and adaptor sequences, and similarity search was carried out against the NCBI Nt and Nr databases using BLASTX (N).

Differential screening of subtracted cDNA clones. The cDNA inserts were amplified with plasmid DNA as a template using primers N1 and N2R (provided in SSH kit). Five microliters of single-band PCR products and 5 μl of 0.6 M NaOH were mixed. One microliter of each mixture was arrayed on four duplicate Nylon membranes (Hybond N^+, Amersham, United Kingdom). The membranes were neutralized in 0.5 M Tris–HCl (pH 7.5) for 5 min and rinsed in distilled water for 30 s, then baked for 30 min at 120°C.

To decrease the hybridization background, the adaptors of subtracted cDNAs were removed, and the removing efficiency was checked by PCR with *actin* as a monitor. The adaptor-removed forward and reverse subtracted cDNAs were labeled with DIG according to the manual of DIG kit (Roche, Switzerland). According to the gray scale scanning result of the hybridization signal, the clones that only had hybridization signal with forward (or reverse) probes or had stronger (twofold) signal than that with reverse (forward) probes were picked out, and the corresponding sequenced cDNAs were classified according to Gene Ontology (www.geneontology.org/).

Semi-quantitative RT-PCR and RNA gel blot. According to the EST sequence bioinformation, seven of them were selected based on their potential role in the potato–*Rs* interaction, and specific gene primers were designed according to their sequences. First-strand cDNAs were synthesized from 1 μg of total RNA of each sample in 20 μl of reaction medium using Superscript II polymerase (Invitrogen, United States), and the first-strand cDNAs were standardized for semi-quantitative RT-PCR using *actin* as a control. To maintain the initial difference of each analyzed gene, PCR reactions were stopped as early as possible to get unsaturated PCR product accumula-

Fig. 1. Phenotypes of different potato genotypes 4 DPI with *Ralstonia solanacearum* (*Rs*).
The inoculation *Rs* concentration was 1×10^7 cfu/ml. I: Susceptible Genotype ED25, II: Resistant genotype ED13.

tion. PCR reactions were subjected to 22–35 cycles at 94°C for 30 s, 52–58°C for 30 s, and 72°C for 30 s. After separation on 1.5% (w/v) agarose gel, the transcription level was estimated according to the intensity of DNA bands.

For RNA gel blot analysis, 35 μg of total RNA was fractionated on a 2% agarose formaldehyde gel in Mops buffer, then transferred to a Nylon membrane (Hybond N^+,) by alkaline transfer method, and baked for 30 min at 120°C. Probe preparation and hybridization were performed according to the manual of DIG Kit (Roche).

RESULTS

Potato ED13 Genotype Possesses High Resistance to Rs Infection

ED13 possesses a high resistance and ED25 is susceptible to *Rs* infection. ED25 plant cuttings showed typical *Rs* infection phenotypes with leaf-dropping 4 days post infection (DPI), whereas the ED13 plant cuttings still were healthy (Fig. 1).

Construction of Suppression Subtractive Hybridization cDNA Libraries

Using the SSH approach, the subtractive libraries were generated with cDNAs derived from a pool of mRNAs obtained from potato leaves collected in 6 and 12 h post inoculation (HPI). Ligation efficiency and subtraction efficiency were monitored with *actin* as a control. For the efficiency of ligation between adaptors and ds-cDNAs, combinations of the N1 primer and *actin* reverse primer, and also the *actin* specific forward and reverse primers were used to amplify adaptor-ligated cDNA samples in both cases. The result showed that the same strong band was obtained following 25 PCR cycles (data not shown). For the assessment of subtraction efficiency, subtracted and unsubtracted cDNA populations were used as templates; the PCR products of the *actin* gene were not detectable in the subtracted cDNA pool following 30 PCR cycles, whereas the unsubtracted cDNA pool gave a strong band already after 25 PCR cycles (data not shown), confirming that subtraction was successful. Finally, the SSH library was constructed, and more than 2000 clones from forward and reverse SSH libraries were obtained. The average insert size of the forward and reverse libraries was about 300 bp based on checking 25 randomly selected clones through PCR reaction, which is correlated well with the restriction enzyme *Rsa*I used in the library construction.

Sequencing of cDNA Clones

More than one thousand and two hundred randomly chosen clones (688 from forward library, 576 from reverse library) carrying fragments ranging from 100 to 700 bp were sequenced, and finally 1161 ESTs (626 from forward library, 535 from reverse library) were sequenced successfully. After discarding redundant sequences, 495 unique cDNA sequences (272 from forward library, 197 from reverse library, 26 from both forward and reverse libraries) were obtained. Redundancy analysis revealed that 16 most abundant genes comprised 36.78% (427 clones) of the 1161 sequenced clones. Most of the sequenced genes were detected only once (data not shown).

Differential Screening

The 495 unique cDNAs were spotted onto nylon membranes and screened by differential hybridization against forward- and reverse-subtracted cDNA probes (Fig. 2). Before probe labeling, the efficiency of adaptor removal from subtracted cDNAs was checked by PCR using adaptorless cDNAs as templates. The N1 and *actin* reverse primer pairs gave no band, whereas the *actin* specific primer pairs gave a strong band after 35 PCR cycles (data not shown), confirming that the adaptors had been removed from the subtracted cDNAs successfully. As a final result, 302 differentially expressed clones (190 from forward library, 112 from reverse library) were obtained.

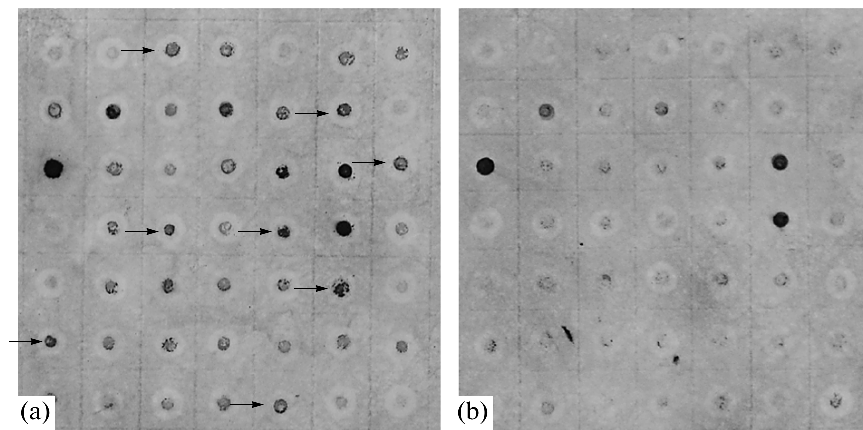

Fig. 2. Example of differential hybridization screening of potato cDNA clones from the subtractive libraries.
PCR products (about 100 ng cDNA) of specific single recombinant plasmids were arrayed on Nylon membranes and hybridized with complex cDNA probes obtained from forward- and reverse-subtracted cDNA pools left and right, respectively. Arrows indicate part of differential expressed clones.

Among 302 differentially expressed clones, 252 clones showed moderate to high homology ($0 < \text{E-value} < 10^{-10}$) to known plant sequences, 19 clones showed low similarity ($\text{E-value} \geq 10^{-10}$), and 31 clones demonstrated no match. According to their putative biological function, the 302 clones could be classified into 12 groups (Fig. 3), 19% of the clones being involved in cell rescue and defense, 4% in signal transduction, 5% are transcription factors, 12% participate in metabolism, 12% in protein synthesis and processing, 23% are proteins of unknown function, etc.

Fifty-three of 302 cDNA clones showed very strong hybridization signals to forward or reverse probes, most of them shared high similarity (E-value < e-30) with plant sequences, including genes encoding cell structure components, proteases, stress-related proteins, cell rescue and defense, transportation and energy related functions. Eighty-one of 302 clones have a putative function related to known resistance responses, e.g. signal transduction, transcription factors, hypersensitive response (HR), systemic acquired resistance (SAR), and cell rescue and protection (table).

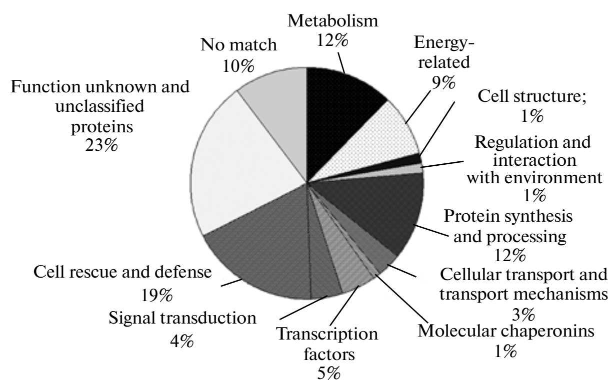

Fig. 3. EST classification according to their putative biological function.
Three hundred and two differentially expressed clones were classified into 12 groups. Percentages represent the proportions of genes belonging to a particular functional group of the total number of genes, including genes with known functions, unknown functions, and no match sequences.

RNA Gel Blot and Semi-Quantitative RT-PCR Confirmation for Various Expressed Genes

To confirm the results of the reverse RNA gel blot assay, transcription of seven identified genes was analyzed by RNA gel blot and semi-quantitative RT-PCR. RNA gel blot showed that *STB81* (protein phosphatase 2C, PP2C) was down-regulated (Fig. 4), suggesting that *Rs*-infected plants or *Rs* itself may produce specific inhibitors, which suppress *STB81.* transcription. Since the sensitivity of RNA gel blot was limited, semi-quantitative RT-PCR was used to analyze transcription of other six candidate genes and the results showed that they had different transcription patterns (Fig. 4).

Clone *STA51* (methyl-CpG-binding domain-containing protein) was induced relatively quickly after inoculation and reached the highest level of transcription between 6 and 12 HPI, then leveled off. Transcription patterns of clones *STD62* (Pti1) and *STC48* (encoding DnaJ-like protein) were similar to *STA51*, but *STD62* needed longer time (about 24 h) to reach the highest transcription level, and this gene is normally constitutively expressed at low level; transcription of *STC48* decreased slowly after it showed the higher levels of transcript accumulation at about 12 HPI. Clones *STE96* (encoding Kunitz-type trypsin inhibitor) and *STA77* (RHG1) were induced relatively

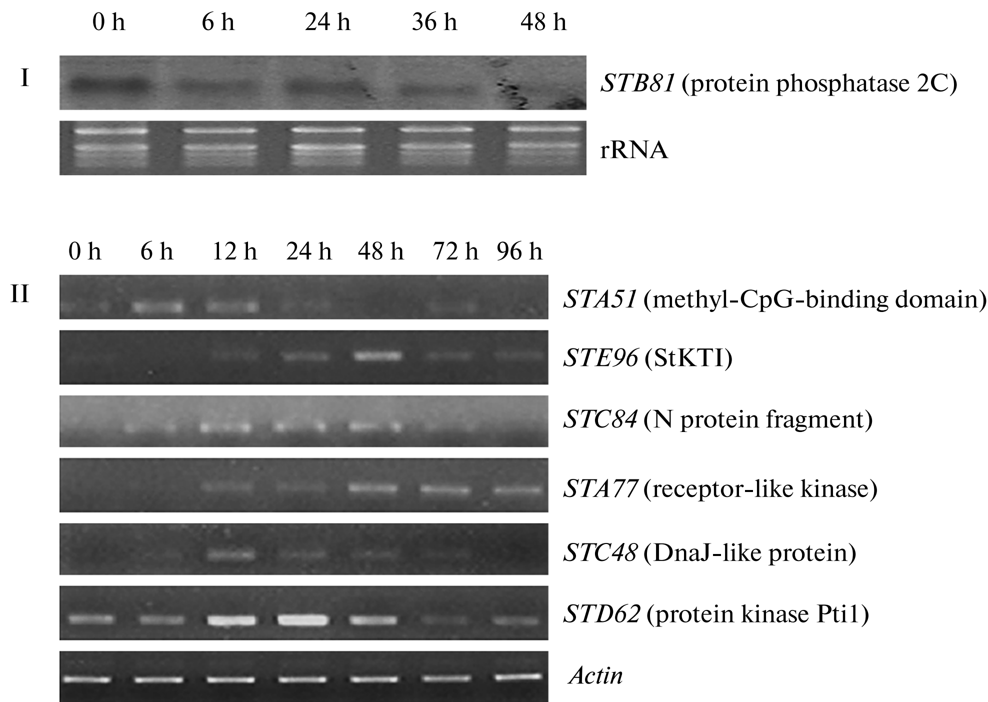

Fig. 4. I: RNA gel blot analysis of *STB81* upon *R. solanacearum* treatment. Total RNA was extracted from potato leaves harvested in 0, 6, 24, 36, and 48 HPI. The quantity of total RNA loaded on agarose gel lanes was modulated relative to rRNA concentration. II: RT-PCR analysis of six potato genes upon *R. solanacearum* treatment. cDNAs were synthesized from RNAs of potato leaves harvested in 0, 6, 12, 24, 48, 72, and 96 HPI. Actin gene was used to verify that equal quantities of cDNA templates were tested. Clone designation is indicated to the right of each panel.

slowly and reached an optimum at or later than 48 HPI. *STC84* had a unique transcription pattern and was also strongly induced. Its transcription level typically increased to a peak between 6 and 12 HPI, and persisted for more than 36 h, then decreased.

DISCUSSION

As we know, during diffusion of *Rs* from roots to stems, the propagating bacteria and their metabolites (such as polysaccharides) block the vascular bundles, and this affects water transfer and results in leaf wilting (BW). Therefore, in the present study, the stem-bacterial co-culture method was used for studying gene responses in leaves. This modified method may result in a little bit bigger wounding of samples than traditional methods, but previous studies clearly showed that it gave the same results as traditional methods in phenotype identification [10]. Additionally, the effect of wounding was also counteracted through the subtraction during the SSH library construction. At usage of this modified method, the symptoms emerged earlier than at traditional methods, which was helpful for confirming the time points in sample harvesting. Actually, the ED25 genotype cuttings wilted after 3 to 4 DPI, whereas the ED13 cuttings still kept healthy (Fig. 1).

SSH is a powerful method for identifying novel genes or low-abundance transcripts as compared to other transcript profiling technologies. In this study, 50 ESTs (low similarity with plant sequences or no match in NCBI) were identified, and they might represent novel genes. *Actin* gene was stable during biotic and abiotic stresses and is commonly used to normalize molecular transcription studies [9, 11]. The RNA gel blot and semi-quantitative RT-PCR results showed that the transcript levels of the seven genes were changed before 12 HPI, and some genes, such as *STA51, STC84*, and *STD62* were strongly induced as early as in 12 HPI, which correlated well with the results of reverse RNA gel blot and confirmed the reliability of the differential hybridization screening process. In the present study, the potato *Rs*-resistant gene *StSN2* (STM21) and many other resistance-related genes were identified. They are involved in pathogen recognition, signal transduction, transcription factor functioning, HR, SAR, etc. (table).

The *STC84* shares high similarity with the 3’-end of TMV-resistant gene *N*. The *N* gene was strongly induced upon TMV infection, but not by other infections [12]. In this study, *STC84* was induced by *Rs*, indicating that whether or not the *STC84* is an *N* gene homologue should be confirmed further, but the induction pattern of *STC84* is similar to that of TMV-

Expression spectra of resistance-related genes in potato

Clone ID	Length, bp	Accession no.	Best match (BLASTN/BLASTX) and origin of species matching sequence	E-value
			Pathogen recognition	
STC84	421	AB120513.1	[*Nicotiana tabacum*] N protein fragment	2e-12
			Signal transduction and transcription factor	
STA77	301	AAM44274.1	[*Glycine max*] receptor-like kinase RHG1	2e-36
STB52	163	Y11688.1	[*Solanum tuberosum*] mRNA for 14-3-3 protein	3e-30
*STB81***	110	NP_180926.1	[*Arabidopsis thaliana*] catalytic/protein phosphatase type 2C	5e-08
STC44	92	ABB02651.1	[*S. tuberosum*] p34-annexin p34-like	2e-08
STD30	301	AAR83888.1	[*Capsicum annuum*] auxin-repressed protein ARP1	3e-25
STC93	109	TC117177	[*A. thaliana*] WD-40 repeat family protein/zfwd1 protein (ZFWD1) identical to zfwd1 protein (GI:12057164)	2.8e-13
STD49	137	AAD12777.1	[*S. tuberosum*] ethylene receptor homolog	7e-09
STD58	284	DQ228333.1	[*S. tuberosum*] ADP-ribosylation factor 1-like protein	1e-151
STD62	336	AAO92595.1	[*G. max*] protein kinase Pti1	8e-48
STF62	436	Z34518.1	[*Lycopersicum esculentum*] (cv. Retina)] 54-kD signal recognition particle (SRP) specific protein	2e-130
STF86	261	L29150.1	[*S. lycopersicum*] 14-3-3 protein	1e-134
STB60	434	AAR17080.1	[*N. tabacum*] heat shock protein 70-3	4e-53
STJ09	120	AF509339.1	[*Saussurea medusa*] heat shock protein 70 (HSP70)	3e-38
STC48	526	DQ885360.1	[*S. tuberosum*] DnaJ-like protein	0.0
STJ22	537	ABR46110.1	[*A. thaliana*] RPM1-interacting protein 4	7e-16
STK03	227	NM_130129.3	[*A. thaliana*] acyltransferase/calcium ion binding	1e-08
STO28	171	AM235387.2	[*Lupinus albus*]mRNA for auxin influx carrier (*aux1* gene)	8e-28
STB15	305	AAZ38969.1	[*G. max*] GAMYB-binding protein	2e-14
STC21	277	AAM34767.1	[*Petunia × hybrida*] nam-like protein 4	5e-47
STD32	134	CK716927	[*A. thaliana*] zinc-finger protein Lsd1	2.4e-20
STA55	285	ABB87130.1	[*S. tuberosum*] putative transcription factor BTF3-like	2e-45
STD73	193	TC120214	[*S. tuberosum*] transcription factor RAU1	8.6e-31
STG72	325	At4g00380	XH/XS domain-containing protein/XS zinc finger domain-containing protein, putative transcription factor	1e-24
STH46	216	NM_101532.1	[*A. thaliana*] transcription cofactor	4e-15

Table. (Contd.)

Clone ID	Length, bp	Accession no.	Best match (BLASTN/BLASTX) and origin of species matching sequence	E-value
STF90	190	EU294352.1	[*S. tuberosum*] pathogen-induced transcription factor NAC1-like mRNA	6e-88
STJ44	355	NM_128011.3	[*A. thaliana*] transcription factor (FZF)	5e-31
STL05	251	AJ630505.1	[*S. tuberosum*] MYC transcription factor (*jamyc2*)	1e-119
STM44	330	NM_180730.1	[*A. thaliana*] transcription factor (AT5G23090)	5e-12
STM05	344	AB079024.1	[*N. tabacum*] mRNA for RAS-related protein RAB8-5	1e-122
STM88	366	CT984568.1	[*Eucalyptus gunnii*] Lambda ZAP II normalized full-length cDNA library of differentiating xylem	2e-52
			Hypersensitive response	
*STA90***	618	BAA10929.1	[*N. tabacum*] cytochrome P450 like_TBP	2e-52
STB19	146	TC113130	[*S. tuberosum*] cytochrome P450	1.4e-19
STC63	468	BAD15331.1	[*Panax ginseng*] cytochrome P450	6e-40
STG50	456	X71657.1	[*S. melongena*] catalase (CAT2) mRNA	1e-160
STK81	260	AY529867.1	[*Capsicum annuum*] hypersensitive-induced reaction protein	5e-24
			Systemic acquired resistance	
STA51	362	TC127470	[S. *tuberosum*] methyl-CpG binding domain	1.2e-33
STG30	427	AY500290.1	[*S. tuberosum*] catalase (CAT2) mRNA	0.0
STB26	481	AAR97905.1	[*S. tuberosum*] catalase	2e-94
STA58	265	TC111772	[*S. tuberosum*] class 1 chitinase	1e-21
*STC51***	156	P52403	[*S. tuberosum*] endochitinase 1 precursor	8e-25
STK12	515	U02607.1	[*S. tuberosum*] chitinase (chtB3)	0.0
STM03	597	U02608.1	[*S. tuberosum*]chitinase (chtB4) mRNA	0.0
STK19	385	AF024538.1	[*S. tuberosum*] class II chitinase (ChtA4)	0.0
STO64	202	U49969.1	[*S. tuberosum*] class II chitinase (ChtA2)	6e-97
			Cell rescue and protection	
STB07	334	AF152172.1	[*S. tuberosum*] pectin methyl esterase	7e-174
STB58	341	BAD37413.1	[*Oryza sativa*] putative senescence associated protein 5	2e-18
STC22	405	BAA95794.1	[*Nicotiana tabacum*] DC1.2 homologue	7e-20
STD80	462	AAX20046.1	[*Capsicum annuum*] DC1.2-like protein	2e-62
STE80	387	BAC54828.1	[*N. tabacum*] vacuolar processing enzyme-1b	1e-36
STA13	253	X55691.1	[*L. esculentum*] glycine-rich protein (clone w1-8)	4e-18
STF35	289	X55688.1	[*L. esculentum*] glycine-rich protein	1e-46

Table. (Contd.)

Clone ID	Length, bp	Accession no.	Best match (BLASTN/BLASTX) and origin of species matching sequence	E-value
*STC29***	352	CAA92243.1	[*L. esculentum*] metallothionein-like protein	1e-10
*STJ67***	445	Z68138.1	[*L. esculentum*] metallothionein-like protein	1e-133
STG70	255	DQ167250.1	[*G. max*] enzymatic resistance protein	2e-38
STE96	111	AB061247.1	[*S. tuberosum*] Kunitz-type trypsin inhibitor	2e-45
STG06	537	DQ168317.1	[*S. tuberosum*] Kunitz-type protease inhibitor precursor	0.0
STB29	128	DQ168314.1	[*S. tuberosum*] proteinase inhibitor I precursor	5e-50
STO59	280	AY517498.1	[*S. phureja*] proteinase inhibitor 2 protein (Pin-II2x)	1e-148
STM21	304	AJ312904.1	[*S. tuberosum*] mRNA for snakin2 (*sn2* gene)	1e-154
STM18	678	AY737315.1	[*S. tuberosum*] thaumatin-like proteinl, PR-5/319	0.0
STM68	352	X58548.1	[L. *esculentum*] pathogenesis-related protein P2	1e-127
STP30	183	AF004878.1	[*L. esculentum*] resistance complex protein I2C-1	1e-09
			Other related pathogen-resistance genes	
STA20	322	BAD07011.1	[*Coffea arabica*] peroxidase	2e-39
STH80	359	AY032674.1	[*N. tabacum*] peroxidase (PER9-6)	4e-96
*STB18***	424	CAA63482.1	[*L. esculentum*] glycolate oxidase	1e-68
STE09	307	NP_194457.2	[*A. thaliana*] FMN binding/oxidoreductase	2e-37
STB55	253	CAD29291.1	[S. *tuberosum*] alcohol $NADP^+$ oxidoreductase	1e-31
STC24	118	Z48736.1	[*L. esculentum*] *cyp*-3 gene for pre-pro-cysteine proteinase	1e-22
STC83	396	CAB53515.1	[*S. tuberosum*] cysteine protease	2e-54
*STA84***	422	BAA96501.1	[*N. tabacum*] cysteine protease	1e-62
STP66	454	AY662995.1	[*Petunia* × *hybrid*a] cysteine proteinase (CP9)	5e-53
STG63	367	X95269.1	[*L. esculentum*] LRP gene	2e-45
STC92	96	AF243378	[*G. max*] glutathione S-transferase GST 23	2e-05
STB61	203	TC120079	[*S. tuberosum*] similar to TIGR_Ath1|At1g15740.1 68414.m01888 leucine-rich repeat family protein	9.8e-30
STH15	292	CT025841.2	[*Ipomoea batatas*] putative serine/threonine protein kinase	2e-08
STH43	179	AF290617.1	[*N. glauca*] putative delta TIP (MIP1)	1e-42
STJ56	446	X96405.1	[*S. tuberosum*] mRNA for 13-lipoxygenase, clone *H1*	0.0
STJ01	257	AB010717.1	[*N. tabacum*] gene for sulfite reductase	3e-28
*STL63***	362	U12439.1	[*S. chacoense*] PI 320287 abscisic stress ripening protein	2e-98
STP54	214	AJ133422.1	[*N. tabacum*] glyceraldehyde-3-phosphate dehydrogenase	1e-70

Notes: * TC is TIGR accession no., others are Gene bank accession number.
** Emerged both in forward and reverse libraries (8 clones). Clones named as STA-STH are from forward library (56 clones). Clones named as STJ-STQ are from reverse library (25 clones).

induced *N* gene. Suggesting that *Rs*-induced plant response may share components with the TMV-induced signaling pathway, the exact function of *STC84* should be studied further.

Pti1 (*STD62*), a potential downstream components of the Pto (a resistant gene in tomato) signaling pathway, interacts with Pto and is phosphorylated by Pto in vitro. Overexpression of *Pti1* in tobacco enhanced the HR to *P. syringage* pv. *tabaci* [13]. Overexpression of *OsPti1a*, a tomato *Pti1* homolog, in rice impaired resistance in both compatible and incompatible interactions, indicating that *OsPti1a* plays a role in the negative regulation of both *R* protein-mediated defense signaling and basal resistance [14]. The *STD62* was strongly induced by *Rs* and might participate in the potato–*Rs* interaction.

As important members of RLK/Pelle family, plant receptor-like kinases play important roles in plant development, signal transduction, and disease resistance [15]. Receptor-like kinases exist in various plant species, e.g. at least 600 receptor-like kinases were identified in *Arabidopsis* [16]. In the present study, the receptor-like kinase (STA77) may be a receptor to participate in signal recognition and the *Rs*-resistance response.

DnaJ-like protein (STC48) is a co-chaperone, which functionally cooperates with DnaK-like chaperones. Members of the DnaK/Hsp70 family (STB60, STJ09) are essential components of the plant defense signal transduction pathway [17]. Additionally, transmembrane domains of DnaJ-like protein may play an important role in transport and signal transduction, as well as in protein folding and subunit assembly [18]. The *DnaJ*-like gene (*STC48*) was induced by *Rs* as well as up-regulated by jasmonic acid (JA), and *STC48* may play a role in recovering the activity of protein destroyed by *Rs* or JA stress [19].

PP2C (*STB81*) plays an important role in the mitogen-activated protein kinase pathway. It was specifically induced in seeds upon ABA treatment and reduced upon GA treatment, but did not response to drought stress [20]. In this study, *STB81* was down-regulated upon *Rs* treatment, indicating that *Rs* and GA may use the same or similar pathways in regulating PP2C.

Transcription factors (TFs) participate in the regulation of defense-related genes, and some are induced by pathogens or treatment with plant hormones. NAC1 (*STF90*) is a pathogen-induced TF. Transcription of *CaNAC1* was rapidly and specifically induced during incompatible interactions between pepper and bacterial or viral pathogens [21]. MYC2 (*STL05*) is a Leu zipper TF, which might modulate JA responses via differential regulation of an intermediate specter of TFs with activating or repressing roles in JA signaling [22].

Hypersensitive response is one of the responses of plant cell protection against the pathogen attack. The cytochrome P450 genes (*STA90, STB19*, and *STC63*) may participate in the biosynthesis of defense-related compounds and are involved in HR. Apart from the HR, SAR, and the cell rescue and protection reactions are also triggered, which provide nonspecific resistance to a wide range of pathogens throughout the plant. In this study, at least nine SAR-related ESTs, 18 cell rescue- and protection-related ESTs, and 18 other pathogen-resistance-related ESTs were identified (table).

Plant chitinases (STA58, STC51, STK12, STM03, and others) are characterized as pathogenesis-related proteins and classified into five classes. Expression of chitinase transgene in *Arabidopsis* and wheat resulted in enhanced resistance to *Pseudomonas syringae* pv. and *Fusarium graminearum*, respectively [23, 24].

DNA methylation is the major modification of eukaryotic genomes and plays an important role in plant growth and development [25]. DNA methylation and demethylation regulate gene expression, and methyl CpG-binding proteins are considered to play critical roles in epigenetic control of gene expression in eukaryotes [26]. Clone *STA51* (methyl-CpG-binding domain-containing protein) was induced by *Rs* and reached the highest level of transcription between 6 and 12 HPI (Fig. 4). But the function of this clone should be studied further.

Potato cysteine proteinase inhibitors (PCPIs) represent a distinct group of cysteine protease inhibitors and belong to the family of Kunitz-type trypsin inhibitors. PCPI genes do not possess any introns, and their expression products accumulate in vacuoles of stems after treatment with JA, indicating that these inhibitors participate in potato defense against insects and pathogens [27]. Intronless genes may be able to respond more rapidly to various exogenous signals. In this study, *STE96* (Kunitz-type trypsin inhibitor) was induced in 12 HPI, which supports the above hypothesis.

This investigation, although far from being exhaustive, has provided new molecular insights into the nature of the potato–*Rs* interaction, and it is the first study aimed at specifically targeting transcripts participating in resistance to *Rs* in scale. The ESTs reported here may provide useful data for improving our knowledge of potato resistance to pathogens. Some ESTs identified in the present study, such as *STM21*, a high homolog of potato *snakin2*, which is regarded as *Rs*-resistance gene [8], may also be used as candidate genes for developing molecular markers to help potato genetics breeding.

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REFERENCES

1. Hayward, A.C., Biology and Epidemiology of Bacterial Wilt Caused by *Pseudomonas solanacearum, Annu. Rev. Phytopathol.*, 1991, vol. 29, pp. 65–87.
2. Genin, S. and Boucher, C., Lessons Learned from the Genome Analysis of *Ralstonia solanacearum, Annu. Rev. Phytopathol.*, 2004, vol. 42, pp. 107–134.
3. Van der Wolf, J., Bonants, P., Smith, J., Hagenaar, M., Nijhuis, E., van Beckhoven, J., and Saddler, G., Genetic Diversity of *Ralstonia solanacearum* Race 3 in Western Europe Determined by AFLP, RC-PFGE, and Rep-PCR, *Bacterial Wilt Disease: Molecular and Ecological Aspects*, Prior, P., Allen, C., Elphinstone, J., Eds., Berlin: Springer-Verlag, INRA, 1998, pp. 44–49.
4. Deslandes, L., Pileur, F., Liaubet, L., Camut, S., Can, C., Williams, K., Holub, E., Beynon, J., Arlat, M., and Marco, Y., Genetic Characterization of *RRS1*, a Recessive Locus in *Arabidopsis thaliana* That Confers Resistance to the Bacterial Soilborne Pathogen *Ralstonia solanacearum, Mol. Plant–Microbe Interact.*, 1998, vol. 11, pp. 659–667.
5. Feng, J., Yuan, F., Gao, Y., Liang, C., Xu, J., Zhang, C., and He, L., A Novel Antimicrobial Protein Isolated from Potato (*Solanum tuberosum*) Shares Homology with an Acid Phosphatase, *Biochem. J.*, 2003, vol. 376, pp. 481–487.
6. Carmeille, A., Prior, P., Kodja, H., Chiroleu, F., Luisetti, J., and Besse, P., Evaluation of Resistance to Race 3, Biovar 2 of *Ralstonia solanacearum* in Tomato Germplasm, *J. Phytopathol.*, 2006, vol. 154, pp. 398–402.
7. Thoquet, P., Olivier, J., Sperisen, C., Rogowsky, P., Prior, P., Anais, G., Mangin, B., Bazin, B., Nazer, R., and Grimsley, N., Polygenic Resistance of Tomato Plants to Bacterial Wilt in the French West Indies, *Mol. Plant–Microbe Interact.*, 1996, vol. 9, pp. 837–842.
8. Berrocal-Lobo, M., Segura, A., Moreno, M., Lopez, G., Garcia-Olmedo, F., and Molina, A., Snakin-2, an Antimicrobial Peptide from Potato Whose Gene Is Locally Induced by Wounding and Responds to Pathogen Infection, *Plant Physiol.*, 2002, vol. 128, pp. 951–961.
9. Liu, X., Zhang, M., Duan, J., and Wu, K., Gene Expression Analysis of Germinating Rice Seeds Responding to High Hydrostatic Pressure, *J. Plant Physiol.*, 2008, vol. 165, pp. 1855–1864.
10. Li, G.C., Jin, L.P., Xie, K.Y., Pang, W.F., Bian, C.S., Duan, S.G., and Qu, D.Y., A New Identification Method for *Ralstonia solanacearum* Resistance in Potato (*Solanum tuberosum* L.), *Chin. Potato J.*, 2006, vol. 20, pp. 129–134.
11. Olbrich, M., Gerstner, E., Welzl, G., Fleischmann, F., Osswald, W., Bahnweg, G., and Ernst, D., Quantification of mRNAs and Housekeeping Gene Selection for Quantitative Real-Time RT-PCR Normalization in European Beech (*Fagus sylvatica* L.) during Abiotic and Biotic Stress, *Z. Naturforsch.*, 2008, vol. 63, pp. 574–582.
12. Levy, M., Edelbaum, O., and Sela, I., Tobacco Mosaic Virus Regulates the Expression of Its Own Resistance Gene *N, Plant Physiol.*, 2004, vol. 135, pp. 2392–2397.
13. Zhou, J., Loh, Y.T., Bressan, R.A., and Martin, G.B., The Tomato Gene *Pti1* Encodes a Serine/Threonine Kinase That Is Phosphorylated by Pto and Is Involved in the Hypersensitive Response, *Cell*, 1995, vol. 83, pp. 925–935.
14. Takahashi, A., Agrawal, G.K., Yamazaki, M., Onosato, K., Miyao, A., Kawasaki, T., Shimamoto, K., and Hirochika, H., Rice Pti1a Negatively Regulates RAR1-Dependent Defense Responses, *Plant Cell*, 2007, vol. 19, pp. 2940–2951.
15. Becraft, P.W., Receptor Kinase Signaling in Plant Development, *Annu. Rev. Cell Dev. Biol.*, 2002, vol. 18, P. 163–192.
16. Shiu, S.H., Karlowski, W.M., Pan, R., Tzeng, Y.H., Mayer, K.F.X., and Lia, W.H., Comparative Analysis of the Receptor-Like Kinase Family in Arabidopsis and Rice, *Plant Cell*, 2004, vol. 16, pp. 1220–1234.
17. Kanzaki, H., Saitoh, H., Ito, A., Fujisawa, S., Kamoun, S., Katou, S., Yoshioka, H., and Terauchi, R., Cytosolic HSP90 and HSP70 Are Essential Components of INF1-Mediated Hypersensitive Response and Non-Host Resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana, Mol. Plant Pathol.*, 2003, vol. 4, pp. 383–391.
18. Toutain, C.M., Clarke, D.J., Leeds, J.A., Kuhn, J., Beckwith, J., Holland, I.B., and Jacq, A., The Transmembrane Domain of the DnaJ-Like Protein DjlA Is a Dimerisation Domain, *Mol. Gen. Genom.,* 2003, vol. 268, pp. 761–770.
19. Li, G.C., Jin, L.P., Wang, X.W., Xie, K.Y., Xie, B.Y., and Qu, D.Y., Cloning of *DnaJ*-Like Gene cDNA in Diploid Potato Using RACE Methods Combined with cDNA Library, *Acta Hortic. Sinica*, 2007, vol. 34, pp. 649–654.
20. Lorenzo, O., Rodriguez, D., Nicolas, G., Rodriguez, P.L., and Nicolas, C., A New Protein Phosphatase 2C (FsPP2C1) Induced by Abscisic Acid Is Specifically Expressed in Dormant Beechnut Seeds, *Plant Physiol.*, 2001, vol. 125, pp. 1949–1956.
21. Oh, S.K., Lee, S., Yu, S.H., and Choi, D., Expression of a Novel NAC Domain-Containing Transcription Factor (CaNAC1) Is Preferentially Associated with Incompatible Interactions between Chili Pepper and Pathogens, *Planta*, 2005, vol. 222, pp. 876–887.
22. Dombrecht, B., Xue, G., Sprague, S., Kirkegaard, J., Ross, J., Reid, J., Fitt, G., Sewelam, N., Schenk, P., Manners, J., and Kazana, K., MYC2 Differentially Modulates Diverse Jasmonate-Dependent Functions in Arabidopsis, *Plant Cell*, 2007, vol. 19, pp. 2225–2245.

23. Hong, J. and Hwang, B., Promoter Activation of Pepper Class II Basic Chitinase Gene, CAChi2, and Enhanced Bacterial Disease Resistance and Osmotic Stress Tolerance in the CAChi2-Overexpressing Arabidopsis, *Planta*, 2006, vol. 223, pp. 433–448.
24. Shin, S., Mackintosh, C., Lewis, J., Heinen, S., Radmer, L., Dill-Macky, R., Baldridge, G., Zeyen, R., and Muehlbauer, G., Transgenic Wheat Expressing a Barley Class II Chitinase Gene Has Enhanced Resistance against *Fusarium graminearum, J. Exp. Bot.*, 2008, vol. 59, pp. 2371–2378.
25. Grafi, G., Zemach, A., and Pitto, L., Methyl-CpG-Binding Domain (MBD) Proteins in Plants, *Biochim. Biophys. Acta*, 2007, vol. 1769, pp. 287–294.
26. Noh, E.J., Lim, D.S., and Lee, J.S., A Novel Role for Methyl CpG-Binding Domain Protein 3, a Component of the Histone Deacetylase Complex, in Regulation of Cell Cycle Progression and Cell Death, *Biochem. Biophys. Res. Commun.*, 2009, vol. 378, pp. 332–337.
27. Gruden, K., Strukelj, B., Ravnikar, M., Poljsak-Prijatelj, M., Mavric, I., Brzin, J., Pungercar, J., and Kregar, I., Potato Cysteine Proteinase Inhibitor Gene Family: Molecular Cloning, Characterisation, and Immunocytochemical Localisation Studies, *Plant Mol. Biol.*, 1997, vol. 34, pp. 317–323.