Plant Biotechnology 24, 409–416 (2007)

Short Communication

Isolation and expression analysis of candidate genes related to *Ralstonia solanacearum*–tobacco interaction

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Received February 22, 2007; accepted July 11, 2007 (Edited by Y. Watanabe)

Abstract *Ralstonia solanacearum* is a causal agent of bacterial wilt, and the bacterium normally infects through wounds in roots. Since it is not possible to equally and simultaneously inoculate the host plants with bacteria via root inoculation, it has proven difficult to elucidate the molecular events in plants infected with *R. solanacearum*. To improve the efficiency of inoculation, we inoculated tobacco plants with *R. solanacearum* by leaf-infiltration. Differential display was carried out to isolate fragments of genes that are regulated in tobacco by virulent strains of *R. solanacearum* OE1-1 and by the avirulent mutant of *R. solanacearum*, 31b, which is mutated in the type III secreted PopA protein by transposon insertion. Nineteen *R. solanacearum*-responsive genes (RsRGs) were successfully isolated using equalized cDNA libraries constructed with water-, *R. solanacearum* OE1-1- and 31b-infiltrated tobacco leaves. From Northern blot analysis, RsRGs were divided into 3 groups; 1) responsive to both virulent and avirulent bacteria (8 RsRGs), 2) responsive to avirulent bacteria (3 RsRGs), and 3) responsive to virulent bacteria (3 RsRGs). These results suggest that the cDNA pool and method presented in this study provide a valuable resource for functional genomic analysis of *R. solanacearum*-plant interactions.

Key words: Differential display, leaf infiltration, *Nicotiana tabacum*, *Ralstonia solanacearum*.

Ralstonia solanacearum is a devastating, soil-borne pathogen with a global distribution and a wide host range (Hayward, 1991). It causes bacterial wilt in several economically important solanaceous crops and is well adapted to life in the soil in the absence of host plants. *R. solanacearum* generally invades through wounded roots or natural openings from which secondary roots subsequently emerge. It then proliferates in the intercellular spaces of the inner cortex and vascular parenchyma before invasion into xylem vessels (Hayward, 1991; Seile et al. 1997; Vasse et al. 1995). In tomato, resistance to *R. solanacearum* is controlled by several loci (Thoquet et al. 1996a, b), whereas in *Arabidopsis thaliana*, it is monogenic and conferred by the *RRS1-R* gene that encodes a novel resistance (R) protein. This resistance is dependent upon salicylic acid and the *NDR1* signaling pathway (Deslandes et al. 2002). Recently, PopP2, the cognate avirulence (Avr) protein for *RRS1-R*, was identified and shown to interact with the R protein (Deslandes et al. 2003). Although identification of R-genes has been extensively undertaken in order to analyze *R. solanacearum*-plant interactions, little is known regarding the molecular events in plants during the establishment of resistance or susceptibility to *R. solanacearum*. An important step in understanding the molecular basis of *R. solanacearum*-plant interactions is isolation and characterization of genes which are regulated in compatible or incompatible combinations. Godiard et al. (1991) reported that cDNA clones corresponding to mRNA accumulated during the early phase of the hypersensitive response (HR) in suspension-cultured tobacco cells challenged with a non-pathogenic strain of *R. solanacearum*. However, there have been few other informative reports of expression profiling and functional analysis of genes related to *R. solanacearum*-plant interactions.

The objectives of this study were the isolation and expression profiling of tobacco genes related to *R. solanacearum*-tobacco plant interactions. Many researchers employ root inoculation with the bacteria. However, since it is not possible to inoculate the host plants with the bacteria equally and simultaneously using root inoculation, it has proven difficult to elucidate the molecular events in plants infected with *R.*

Abbreviations: BSA, bovine serum albumin; DD, differential display; DIG, digoxigenin; EcDNA, equalized cDNA; HR, hypersensitive response; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RsRG, *Ralstonia solanacearum*-responsive gene.

The nucleotide sequence of RsRGs reported in this paper has been submitted to DDBJ, EMBL and Gene Bank under the accession numbers given in Table 3.

This article can be found at http://www.jspcmb.jp/

Table 1. Bacterial strains and their interactions with tobacco plants.

Strain (Characteristics)	Symptom		Source or reference
	Root inoculation	Leaf infiltration	
Ralstonia solanacearum OE1-1 (Wild type : Compatible)	Systemic wilting	Necrotic lesions[1)] and systemic wilting	Hikichi et al. 1999
Ralstonia solanacearum 31b (Transposon inserted PopA mutant)	No systemic wilting	Necrotic lesions[1)] and no systemic wilting	In this study
Ralstonia solanacearum 8107 (Wild type: Incompatible)	No systemic wilting	Hyper sensitive response[2)] and no systemic wilting	Kiba et al. 2003

[1)] Necrotic lesions developed in tobacco leaves 72 h after infiltration with *R. solanacearum* OE1-1 or 31b. [2)] Hypersensitive response developed in tobacco leaves 24 h after infiltration with *R. solanacearum* 8107.

solanacearum. Our previous studies showed that a leaf-infiltration method produces the same phenotype in tobacco plants against *R. solanacearum* strains when compared to the root-inoculation method (Kanda et al. 2003a, b; Shinohara et al. 2005). A leaf-inoculation method is widely used for analysis of molecular events during plant-bacteria interactions. Reproducibility of plant responses is confirmed in many plant-bacteria pathosystems, including the tobacco-*R. solanacearum* interaction (Kiba et al. 2003). To improve the efficiency of inoculation, we inoculated tobacco leaves with *R. solanacearum* by leaf infiltration. In addition, we employed differential display (DD), originally reported by Liang and Pardee (1992), to isolate fragments from genes that are regulated in tobacco plants by inoculation with *R. solanacearum*. Since we expected to isolate not only abundant infection-specific genes, but also weakly expressed infection-specific genes, we used equalizing cDNA libraries (EcDNA) as templates for DD. We also applied reverse Northern blotting to effectively eliminate false-positive gene fragments. We identified *R. solanacearum*-responsive genes (RsRGs) involved in signal transduction, defense/stress responses and cellular metabolism, as well as genes with unknown functions.

The bacterial strains used in this study are listed in Table 1. *R. solanacearum* isolates OE1-1 (RsOE1-1) and 8107 (Rs8107) were grown for 16 h at 30°C in peptone yeast extract (PY) medium. *R. solanacearum* 31b (Rs31b), which has a mutated type III effector (PopA) as a result of transposon insertion, was cultured in PY medium containing 50 μg ml^{-1} of tetracycline. The bacterial population was measured spectrophotometrically at OD_{600}, and the suspension was adjusted to 0.1 at OD_{600} (10^8 cfu ml^{-1}) for inoculation. *Nicotiana tabacum* cv. Samsun NN was grown in pots containing a mixture of vermiculite/peat moss (3 : 1) in a growth room under 10000 lux at 25°C, and watered with Hoagland's solution diluted 1 : 4 with water (Hikichi et al. 1999). Inoculation with bacteria was carried out either by root inoculation or leaf infiltration using a syringe (Kanda et al. 2003a, b; Shinohara et al. 2005).

As shown in Figure 1, wilting symptoms were observed in tobacco plants inoculated with RsOE1-1 via the root, whereas tobacco plants did not show any wilt symptoms following inoculation with Rs31b or Rs8107. RsOE1-1 caused necrotic lesions in the infiltrated tobacco leaves, and the whole plants wilted. While Rs31b also induced necrotic lesions in the infiltrated tobacco leaves, there was never wilting of the whole plant. In Rs8107-infiltrated tobacco plants, HR was induced in infiltrated leaves and no visible wilt symptoms were observed in the whole tobacco plants. These results indicated that the tobacco plants showed the same phenotypes to respective bacterial strains using either the root-inoculation or leaf-infiltration method, as described previously with other mutant strains of *R. solanacearum* (Kanda et al. 2003a, b; Shinohara et al. 2005).

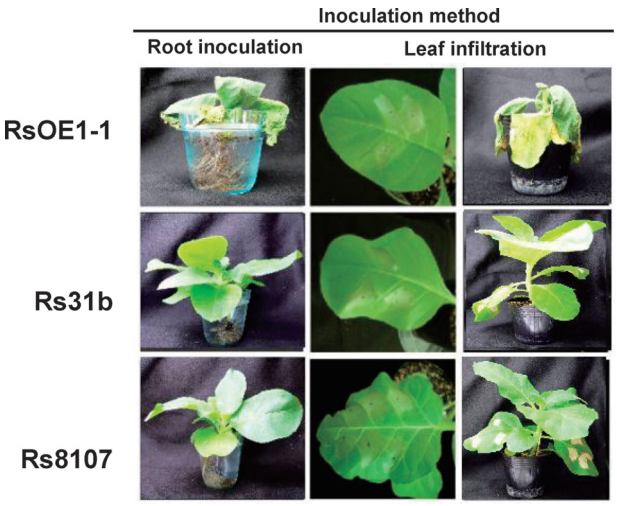

Figure 1. Phenotypic observation of tobacco plants inoculated with *Ralstonia solanacearum* by root inoculation and leaf infiltration. Tobacco plants were inoculated with *R. solanacearum* strain OE1-1 (RsOE1-1), 31b (Rs31b) and 8107 (Rs8107) by root inoculation and leaf inoculation. Wilt symptoms were photographed 10 and 14 days after the inoculation by root inoculation and leaf infiltration, respectively. Necrotic lesions and hypersensitive response were photographed 24 and 72 h after infiltration, respectively.

Total RNAs were isolated from *N. tabacum* cv. Samsun leaves and roots using the method previously described (Kiba et al. 2003). Frozen tissues were homogenized in RNA extraction buffer consisting

of 100 mM glycine (pH 9.5), 10 mM EDTA, 0.1 M NaCl, 1% (w/v) SDS, and 0.1% (w/v) bentonite. An equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) was added, the solution was vortexed and then centrifuged for 10 min at 3500×*g* at 4°C. The water-soluble phase was transferred to a new tube and an equal volume of chloroform/isoamyl alcohol (24 : 1) was added, and the solution was centrifuged again for 10 min at 3500×*g* at 4°C. The water-soluble phase was transferred to a new tube, a 1/3 volume of 10 M lithium chloride was added and the solution incubated at 20°C for 2 h. Centrifugation was then carried out at 20,000×*g*. The pellet was dissolved in 40 mM Tris/1 mM EDTA (TE) buffer (pH 8.0) and the solution was treated by phenol/chloroform/isoamyl alcohol extraction as described above. The resulting water-soluble phase was concentrated by ethanol precipitation and the precipitate was then dissolved in 100 μl of nuclease-free water. The quality and concentration of the RNA was determined by gel electrophoresis and OD at 260 nm.

Construction of equalized cDNA libraries was performed following the procedure described by Kouchi et al. (1995), with slight modifications. The mRNA was purified from 1 mg of total RNA obtained from tobacco leaves 3 and 9 h after infiltration with water, RsOE1-1 or Rs31b using the PolyATract System (Promega Corp., Madison, WI). The cDNA was synthesized using Reverse Transcriptase RAV-2 (Takara Shuzo, Shiga, Japan) with the oligodT primer (5′-CATGGGATG-GATCCTGCAGATCTTTTTTTTTTTTTTT-3′). Lone linkers (LL-SseIA; 5′-GAGATATTACCTGCAGGTA-CTC-3′ and LL-SseIB; 5′-GAGTACCTGCAGGTAA-TAT-3′) were annealed and ligated to both ends of the cDNA, and amplified by PCR using the LL-SseIA primer as follows: 25 cycles of 94°C for 2 min, 50°C for 2 min, and 72°C for 4 min, and 1 cycle of 94°C for 2 min, 50°C for 2 min, and 72°C for 10 min. The amplified cDNA library (20 μg ml^{-1}) was suspended in 50 μl of equalization buffer containing 0.3 M sodium phosphate buffer (pH 7.0), 0.4 mM EDTA and 0.04% SDS, denatured in boiling water for 5 min, and re-associated at 65°C for 24 h. Single-stranded cDNA was separated from the double-stranded cDNA by hydroxyapatite column chromatography (Bio-Rad Lab. Hercules, CA) at 65°C. It was then amplified by PCR under the same conditions as for cDNA amplification. This equalization cycle was repeated three times, thus creating the EcDNAs.

The random primers shown in Table 2 were used for DD using EcDNA as a template. PCR conditions were according to Yoshida et al. (1994). The PCR product was analyzed on a 2% agarose gel stained with ethidium bromide. To identify differentially-expressed gene fragments in response to each respective bacterial strain, DD products were then compared between water-, RsOE1-1- and Rs31b-infiltrated EcDNA. Amplified DNA fragments of interest were isolated from the gels and DNA was extracted from the gel using Quantum Prep® Freeze `N Squeeze DNA gel Extraction spin columns (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The PCR-amplified fragments were then cloned into a pGEM-T Easy vector (Promega, Madison, WI) according to the manufacturer's protocol.

Table 2. Primer sequences used for differential display.

Primer	Sequence [5′-3′]	Primer	Sequence [5′-3′]
A00	ATCAGCGCACCA	A60	CAGGTGGGACCA
A01	AGCAGCGCCTAC	A62	TCGTCCGGAGAT
A03	TGCCTCGCACCA	A63	CAGGTTGGGTT
A04	GCCCCGTTAGCA	A67	GGCGTGGTTGTA
A06	ACTGGCCGAGGG	A70	GGTGACTGGTGG
A10	GCCTGCCTCACG	A71	GGTGCCGGAGCA
A12	CTCCTGCTGTTG	A81	GGCGAGGGAGGA
A13	CTCAGCGATACG	A83	ACTGGCCGGCAT
A17	GGTTGGGAATG	A86	TCCTGGGGCGTT
A18	GACCTGCGATCT	A87	GCCGCCAGAGGA
A19	AAGGCGCGAACG	A89	GTCGGTCGTGAA
A20	TTGCCGGGACCA	A97	GTGTGGAAGCCA
A21	GTGACCGATCCA	A99	GCGGTCAGCACA
A24	GACGGTTCAAGC	B01	AAGAAGCAGGCG
A25	GGTCAGGCACCA	B12	GGAGAGCGGACG
A30	CCTTTCCGACGT	F01	GGCATGGCCTTT
A40	GCGGAGGAACCA	F02	CGACGACGACGA
A45	TGGCCTCTTGGA	F05	TATCCTACCGGC
A46	TGGCCTATTGGC	F08	GGCCGACTTGGC
A47	GGTTTCCCAGGA	F10	CAGGCCAAGTC
A48	CCGCAGGGACCA	F12	ACGGGTCGTAAC
A56	AACATCTCCGGG	F14	TATCCACCGCTC
A58	GTCATGCCTGGA	F19	CCTTGGCGAAGC

Approximately 80 fragments were differentially amplified by DD with the respective EcDNAs. False positive clones were eliminated by reverse Northern hybridization with a DIG-labeled original cDNA library as probes, and 35 candidate RsRGs were isolated (data not shown). Among these RsRGs, 17 RsRGs, which showed quite different hybridization patterns with respective cDNA probes in reverse Northern hybridization, were subjected to further studies.

The RsRGs cloned into pGEM-T Easy were sequenced using an M4 primer (5′-GTTTTCCCAG-TCACGAC-3′) and a RV primer (5′-CAGGAAACAGC-TATGGAC-3′) with the reagents for the Big Dye Terminator Cycle Sequencing Kit and an Applied Biosystems 3100 Avant Automated Sequencer (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol. Sequence analysis was carried out using DNASIS (version 3.6; Hitachi, Yokohama, Japan) and the BLAST network service from the National Center for Biotechnology Information (Altschul et al. 1990).

Sequences of 8 of the 17 RsRGs corresponded to known genes that could be assigned a putative function

Table 3. Homologies of *Ralstonia solanacearum*-responsive genes (RsRGs) used in this study.

RsRGs	Accession	Homology (accession no.)	BLAST E value	Origin[1)]
3-2	AB293942	*Nicotiana plumb aginifolia* G protein beta-subunit-like protein (CAA96528)	9e-71	31b
3-3	AB293943	*Pisum sativum* MtN19 protein (AAU14999)	1e-27	31b
3-4	AB293944	*Nicotiana tabacum*e licitor-inducible LRR receptor like protein (BAA88636)	2e-52	31b
3-5	AB293945	*Nicotiana plumb aginifolia* G protein beta-subunit-like protein (CAA96528)	5e-78	31b
3-8	AB293946	*Nictiana tabacum* putative translationally controlled tumor protein (AAD42049)	2e-77	31b
3-9	AB293947	*Arabidopsis thaliana* putative hydroxyproline-rich glycoprotein (AKK92705)	2e-11	31b
3-10	AB293948	*Arabidopsis thaliana* receptor protein kinase-like protein (BAA98166)	5e-09	31b
3-11	AB293949	*Arabidopsis thaliana* putative protein (BAB10921)	1e-22	31b
3-13	AB293950	*Arabidopsis thaliana* putative hydroproline-rich glycoprotein (AKK92705)	4e-19	31b
9-12	AB293951	No significant similarity	—	OE
9-22	AB293952	No significant similarity	—	31b
9-29	AB293953	No significant similarity	—	31b
A1	AB293954	No significant similarity	—	OE
A16	AB293955	No significant similarity	—	OE
B8	AB293956	No significant similarity	—	31b
B11	AB293957	No significant similarity	—	31b
B16	AB293958	No significant similarity	—	31b

[1)] Each RsRG was amplified by differential display with cDNA derived from tobacco leaves inoculated with *Ralstonia solanacearum* OE1-1 (OE) or strain 31b (31b).

according to the BLAST results. One RsRG showed homology to a putative protein of *A. thaliana*, and the remaining six had no significant similarity to any known genes (Table 3). Intriguingly, RsRG3-2 and 3-5 all showed similarity to the *Nicotiana plumbaginifolia* G protein beta-subunit-like protein. RsRG3-9 and 3-13 also each showed similarity to the same gene; the *A. thaliana* putative hydroxyproline-rich glycoprotein. These results suggested that the procedures were reproducible. The RsRGs were classified into three subgroups: genes with functions related to signal transduction (RsRG3-2, 3-4, 3-5 and 3-10), defense and stress response (RsRG3-3, 3-8, 3-9 and 3-13), and those with unknown functions (RsRG3-11, 9-12, 9-22, 9-29, A1, A16, B8, B11 and B16).

Comparative analysis of gene expression in tobacco in response to virulent and avirulent strains is important in understanding the molecular events that take place during *R. solanacearum*-tobacco interactions. For this purpose, the 14 independent gene-coding RsRGs were subjected to further analysis by Northern blot (Figure 2). RNA samples were isolated from tobacco leaves infiltrated with water, RsOE1-1, Rs31b and Rs8107 as described above.

The total RNA (10 μg) was separated on a 1.5% agarose gel under denaturing conditions and blotted onto Hybond N+ membrane (GE healthcare, Co. Ltd., Uppsala, Sweden). The blot was hybridized with a DIG-labeled DNA probe for the respective RsRGs, in accordance with the procedures of the manual. Hybridization was performed at 68°C for 16 h in ULTRAhybTM (Ambion Inc. TX). The blots were washed in 2×SSPE with 0.1% SDS at 65°C, and 0.1×SSPE with 0.1% SDS at 65°C, and detection was performed using the alkaline phosphatase-conjugated anti-DIG antibody and CDP-star (Roche Diagnostics, Mannheim, Germany) according to the supplier's instructions.

Among the 15 RsRGs, no signals from RsRG3-2 were detected, perhaps due to weak expression of this RsRG (data not shown). Since a cDNA of a low abundance transcript was recovered by this method, we judged that the cDNA libraries were effectively equalized. From the results of Northern blot analysis, RsRGs were divided into 3 groups; 1) responsive to all bacterial strains, 2) regulated in tobacco by the avirulent strain (Rs8107) and/or the avirulent mutant (31b), and 3) drastically induced in tobacco by the virulent strain (RsOE1-1).

Group 1, in which the RsRGs respond to all bacterial strains, RsOE1-1, Rs8107 and Rs31b, included RsRG3-3 (Mt19N protein), 3-4 (receptor like protein kinase), RsRG3-8 (a tumor protein), 3-9 (hydroproline-rich glycoprotein), 3-10 (receptor like protein kinase), 3-11 (an *A. thaliana* putative protein) and 9-22 and 9-29 (no known similarity). Several genes showing homology to tumor proteins are listed in the database, however, little is known about their functional role(s) in plant cells. In mammalian cells, the role of tumor proteins is related to cell death and stress response (Rinnerthaler et al. 2006), and it can be speculated that tumor proteins may be related to stress response in plants. Receptor kinases have a role in recognition of invading pathogens and in triggering the plant defense responses (Morillo and Tax 2006). The Mt19N gene was originally isolated from *Medicago truncatula*, and its expression is up-regulated in recently formed root nodules (Gamas et al. 1996). Since rapid and strong up-regulation of Mt19N was reported in pea after exposure of pods to a Bruchin, it is likely that this gene plays an important role in the response of pea to these insect elicitors (Doss et al.

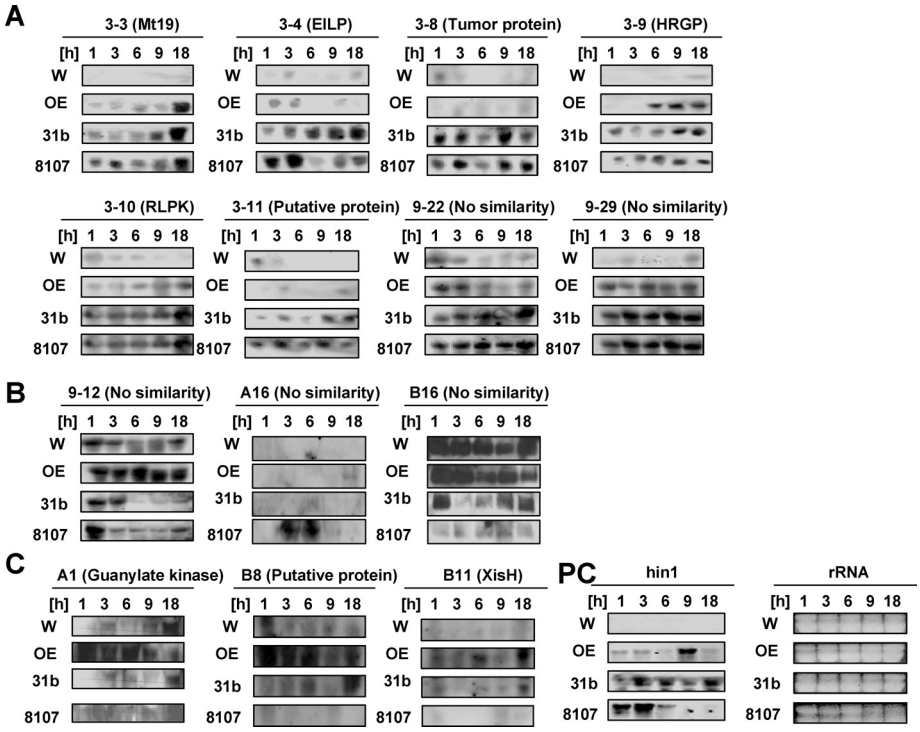

Figure 2. Expression patterns of RsRGs in tobacco leaves in response to inoculation with *R. solanacearum*. Total RNA was isolated from *Nicotiana tabacum* cv. Samsun NN leaves infiltrated with water (W), RsOE1-1 (OE), Rs31b (31b) and Rs8107 (8107), after incubation at 25°C for indicated times. (A) Expression profiles of group 1 RsRGs, which respond to all bacteria strains. (B) Expression profiles of group 2 RsRGs, which were regulated in tobacco by the avirulent strain (Rs8107) and/or avirulent mutant (Rs31b). (C). Expression profiles of group 3 RsRGs, which induced in tobacco by the virulent strain (RsOE1-1). Expression of the hin1 gene was determined as a marker gene for the plant defense response as a positive control (PC). Northern blot analysis was performed with DIG-labeled cDNA probes for the respective RsRGs. Equal loading of rRNA was estimated by ethidium bromide staining (rRNA).

2005). It has been reported that a hydroxyproline-rich glycoprotein cross-links with the plant cell wall in pathogen-challenged plants, and acts as a physical barrier against pathogen invasion (Bradley et al. 1992). These results suggested that this group contained defense-related genes. In addition, overall these results showed that these RsRGs tend to be more inducible in tobacco plants challenged with the avirulent bacteria (Rs8107 and Rs31b) than that with virulent bacteria (RsOE1-1). Greenberg and Yao (2004) found that the responses and features of early incompatible interactions were similar to those of late compatible interactions, with most differences being quantitative. Large-scale mRNA expression profile analysis of *A. thaliana* challenged with *Pseudomonas syringae* also comprehensively revealed an overall similarity between compatible and incompatible interactions (Tao et al. 2003). Moreover, our previous study showed that defense-related responses and induction of programmed cell death are observed during the HR and disease development of bacterial necrotic leaf spots in eggplants, but the timing or level of these responses was different between the two interactions (Kiba et al. 2006). Therefore, modulation of the level of gene expression, including that of group 1 RsRGs, may be one of the critical factors determining disease resistance or susceptibility.

Group 2, in which RsRGs are responsive to the avirulent bacteria (Rs8107 and/or 31b) included RsRG9-12, A16 and B16 (no known similarity to genes reported in databases). Among these RsRGs, RsRGA16 was up-regulated in tobacco plants challenged with Rs8107, suggesting a defensive role of these RsRGs in tobacco. In contrast, RsRG9-12 and B16 were continuously expressed in water- and RsOE1-1-infiltrated tobacco, whereas the expression was suppressed in Rs31b- and Rs8107-inoculated tobacco. These results suggested that suppression of these types of genes may be required for induction of defense. Although the genes belonging to this group did not provide any functional information in plants from sequence homologies, it is possible that there are defense-related genes in plants.

Group 3, in which the RsRGs respond to the virulent strain, RsOE1-1, contained RsRGA1, B8 and B11 (no known similarity). These results suggested that there may be host genes controlling disease susceptibility. We previously showed that there are not only quantitative, but also qualitative, differences in the induction mechanisms of HR and disease development (Kiba et al. 2006). Therefore, there may be novel and/or unidentified

host responses related to disease susceptibility.

Next, to determine the response of selected RsRGs to *R. solanacearum* infection in roots, we carried out expression analysis of RsRGs by reverse transcription-polymerase chain reaction (RT-PCR). When *R. solanacearum* was inoculated via roots, the bacterium colonized intercellular space in the root approximately 1 day after inoculation, and systemic movement was observed about 5 days after inoculation (Hikichi et al. 1999). Since we focused on the early stage of interaction

Table 4. Primer sequences used for RT-PCR.

RsRGs	Upper primer [5′-3′]	Lower primer [5′-3′]
3-3	GAGGATGGACGCGTCATATG	CAAGTAGAGCAATTCCAAACAC
3-4	TGAAGTCAAAGCCGGGTCTG	ACATCCTCTTATATGACATC
3-8	GATGAGCTCCTTTCGGATTC	TCTTTTTAACACTTGACCTCC
3-9	GGTACGGGATAAGCATATGGTCGGTCAT	CCACAGAATTATGTCAATGAGAAACAGCTG
3-10	ATGAGTGAAGTGGTGAAAGAGA	CCTCCAGAACACTTTTCCTT
3-11	GCTGCAGTTTTTGGTATAATC	GACTACTTGGCTGGTTGTGT
9-12	GGCTGCGGGTTTCCTCTTATC	GCGCACCATTCTCTTGGACTTT
9-22	TAGAGGTACAATATCAATTCTC	TAACAAATAAAGGTCTAATTACC
9-29	CCAATGCTATGGCAAAAGATGCATCAAATG	GGCATATCGAACAGCAATGCAAACTAACTG
A1	GGCAATCGTCGATTTGCCCGCC	CAGGAACCATATTGGCTTTCGCGCGC
A16	CACAATACAGGAGTTTACATAAACAACCCC	GCCTATGTCAACCACACCAAGTGATG
B8	CCAGATCCTTGAACACGGTGTCGCGCG	GGGGCTGACGCTCAAGGAAGCGGGGC
B11	GCATTTACGACCAGAGCATGGTACAGATCC	TCCCAAGCCACCCATCATGGCGGG
B16	CCAGCTCATTTTGCAGTAGATTTAGAAGAG	ATAATTACAGCAGAACAGCAAATAGAAGCC
hin1	TCCAACTTGAACGGAGCCTATTATGG	TTGGTTTTATTGGGAGATAGAGAGAAA
Actin	TTTTCCAGCCATCTATGATTGGAATGGA	TCTGGTGGAGCCACAACCTTAATCTTCAT

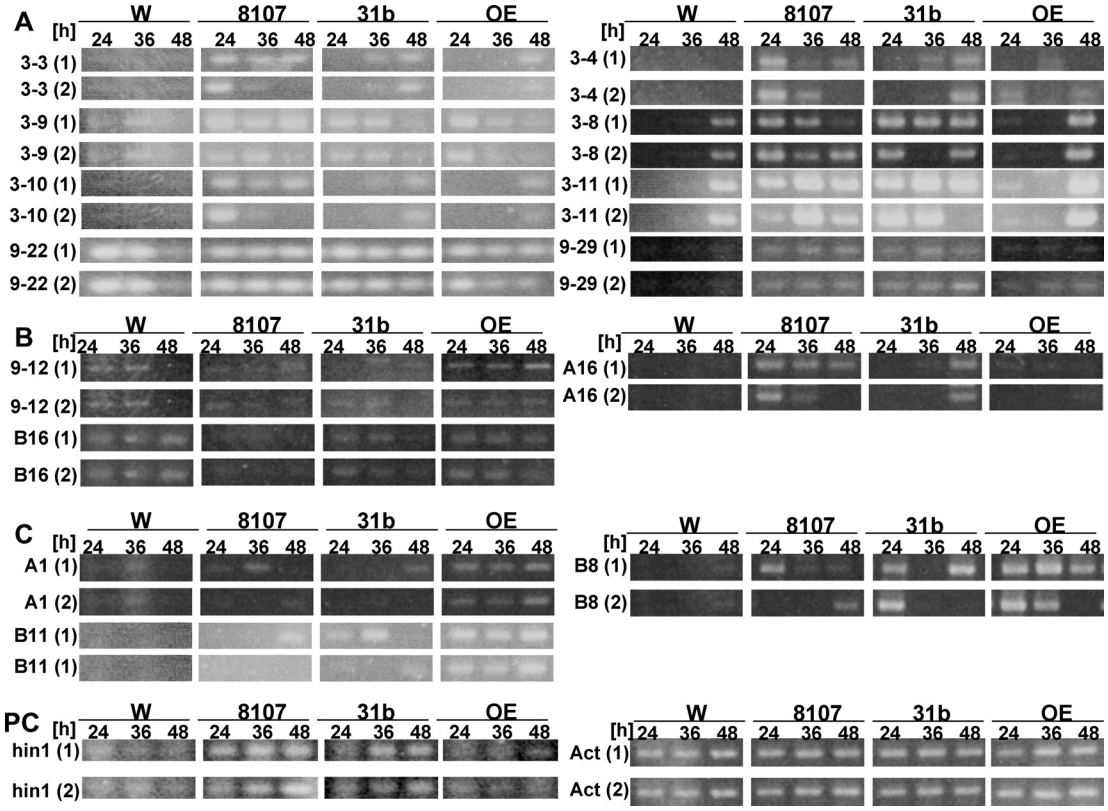

Figure 3. Expression patterns of RsRGs in tobacco roots in response to inoculation with *R. solanacearum*. Total RNA was isolated from *N. tabacum* cv. Samsun NN roots treated with water (W), Rs8107 (8107), Rs31b (31b) and RsOE1-1 (OE) after incubation at 25°C for indicated times. Numbers indicated immediately following clone numbers ((1) and (2)) indicate two biological replicates using two independent total RNA samples. (A) Expression profiles of group 1 RsRGs, which respond to all bacteria strains. (B) Expression profiles of group 2 RsRGs, which were regulated in tobacco by the avirulent strain (Rs8107) and/or avirulent mutant (Rs31b). (C) Expression profiles of group 3 RsRGs, which was drastically induced in tobacco by the virulent strain (RsOE1-1). RT-PCR analysis was performed with specific primer combinations for the respective RsRGs (Table 4). Expression of the hin1 gene was determined as a marker gene for the plant defense response as a positive control (PC). Amplification of actin genes (Act) was carried out as an internal standard.

between tobacco and *R. solanacearum*, we isolated total RNA from tobacco roots 24, 36 and 48 h after inoculation of the bacterium as described above. The cDNA was synthesized with oligodT primer and used as templates for RT-PCR. The PCR primers used for RT-PCR are shown in Table 4. Cycling parameters were as follows; 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. PCR products were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. To determine reproducibility of the expression pattern, the analysis was carried out with two biological replications. As shown Figure 3, all RsRGs responded to inoculation with each strain of *R. solanacearum* in roots. The expression patterns of all RsRGs could be divided into three groups in a similar manner to leaf samples. However, in root samples we observed a lack of reproducibility in expression patterns, such as the levels and kinetics of expression. Since the RsRGs were actually expressed in root samples, expression changes of RsRGs in the bacterium-inoculated leaves may reflect natural plant-*Ralstonia* interactions in roots. Furthermore, the leaf-infiltration method is thought to be a suitable model system for quantitative and qualitative analysis of molecular events during plant-*R. solanacearum* interactions.

The studies on plant-bacteria interactions have focused on a limited number of plant-bacteria pathosystems based on gene-for-gene interactions, such as *A. thaliana*-*P. syringae*, tomato-*P. syringae* pv. *tomato* (Maleck et al. 2000; Mysore et al. 2002; Schulze-Lefert 2004). Although the information obtained from these model systems has advanced our understanding of the molecular events in these plants, certain plant-bacteria interactions may involve unique response mechanisms and regulation of gene expression, especially in gene-for-gene independent interactions. In addition, little information is available for resistance- or susceptibility-related genes in *R. solanacearum*-tobacco interactions *in planta*. In the present study, we identified several genes that did not show significant similarity to any other genes reported in databases. Therefore, there may be novel genes related to *R. solanacearum*-plant interactions. Further study to isolate full-length cDNAs will be required for characterization and functional analysis of these RsRGs. We were also able to isolate several genes showing similarity to defense-related genes. Therefore, the cDNA pool and method used in this study provide a valuable resource and large number of genes for functional genomic analysis of tobacco plants in *R. solanacearum*-plant interactions. Functional analysis of the respective RsRGs is currently in progress, using virus-induced gene silencing and the creation of transgenic plants.

Acknowledgements

This work was supported by Grants-in Aid for Scientific Research to AK (16780031 and 18780029) and to YH (15028214 and 16380037) from the Ministry of Education, Science, Sports and Culture, Japan. AK is also grateful for financial support from the Asahi Glass Foundation.

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