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A tobacco rattle virus-induced gene silencing system for a soil-borne vascular pathogen *Ralstonia solanacearum*

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ABSTRACT. Bacterial wilt (BW), caused by *Ralstonia solanacearum*, is a devastating soil-borne vascular disease of solanaceous crops worldwide. However, information on the defense mechanisms of Solanaceae against this bacterium is limited. In this study, we optimized a virus-induced gene silencing (VIGS) system to broaden the application of VIGS for the study of plant response to soil-borne vascular pathogens such as *R. solanacearum*. Due to the soil-borne and rapid xylem-routed infection features of BW, factors involved in *Tobacco rattle virus*-based VIGS system, such as *Agrobacterium tumefaciens* strains, plant ages, inoculation methods and plant genotypes, were evaluated. An optimized cotyledon agroinfiltration method was developed using phytoene desaturase gene as the marker. All tomato cultivars, but a few pepper and no eggplant cultivars tested showed good competence in gene silencing using the optimized VIGS protocol. Because tomato responses at spatial and temporal levels are critical for the outcome of *R. solanacearum* infection, spatial and temporal assessments of VIGS efficiency were carried out. The results showed that silencing of *TGA2.2* in 9-day-old tomato plants initiated 5 days post inoculation in stembases and later in roots and then in young leaves. Moreover, a chimeric approach was shown to be effective for multi-gene silencing than mixing multiple *Agrobacterium* strains carrying individual target genes. With the established protocol, we were able to show that silencing *COI1*, an essential key player in the jasmonic acid (JA) signaling pathway, led to increase of *R. solanacearum* proliferation in stembases and mid-stems of 'Hawaii 7996', a tomato cultivar with durable resistance to BW. Our study provides the first demonstration for a positive role of the JA signaling pathway in tomato resistance to BW, and notably, is inconsistent with the reports in *Arabidopsis*. The involvement and possible interplays of other known defense signaling pathways in the tomato BW-defense network to *R. solanacearum* are discussed.

Keywords: Bacterial wilt; Jasmonic acid defense signaling pathway; *Ralstonia solanacearum*; Virus-induced gene silencing.

Abbreviations: BW, bacterial wilt; **VIGS,** virus-induced gene silencing; **JA,** jasmonic acid; **CFU,** colony-forming unit; **QTL,** quantitative trait loci; **ET,** ethylene; **ABA,** abscisic acid; **siRNAs,** small interfering RNAs; **TRV,** *tobacco rattle virus*; **JAR1,** jasmonate resistant 1; **JAZ,** jasmonate ZIM-domain; **RT-PCR,** reverse transcriptase-PCR; **PDS,** phytoene desaturase; **sqRT-PCR,** semi-quantitative RT-PCR; **DPI,** days post inoculation; **MAPK,** mitogen-activated protein kinase.

INTRODUCTION

Bacterial wilt (BW), caused by *Ralstonia solanacearum*, is a severe soil-borne vascular disease commonly occurring in the warm, humid tropics. This pathogenic bacterium has a wide host range; solanaceous vegetables, including tomatoes, eggplants, and peppers, are among the crops that suffer the most severe yield loss (Denny, 2006). Host resistance is the best control strategy due to the pathogen's soil-borne nature and wide host range. However, breeding for durable resistance in tomato plants has been hindered by the presence of diverse pathogen strains, and only a few resistant sources and commercial cultivars are available (Scott et al., 2005). Thus, gaining insight into the natural defense mechanism against *R. solanacearum* could benefit and facilitate the development of materials with durable resistance. In the natural infection process, *R. solanacearum* invades tomato plants at the emergence sites of secondary roots or at root tips, propagates intercellularly, and then enters the xylem (Vasse et al., 1995). The infection then progresses

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systemically, accompanied by bacterial propagation and the secretion of a large amount of extracellular polysaccharide, leading to complete wilting (Denny, 2006). The known resistant genotypes in tomatoes, eggplants, and peppers are not immune to the pathogen (Denny, 2006). Latent infections occur in resistant cultivars (Prior et al., 1994). The visual wilting symptom caused by *R. solanacearum* in tomato plants is correlated with the pathogen distribution and density in the stem. In resistant genotypes, the pathogen is suppressed and cannot multiply to the high-enough population level (10^9-10^{10} CFU g^{-1}) required in plant tissues to cause the wilting symptom (Grimault et al., 1994; Wang and Lin, 2005). It has been shown that the pathogen is largely confined to the primary xylem and is a poor colonizer in the resistant tomato variety Hawaii 7996 (H7996) (Nakaho et al., 2004). The resistance in H7996 involved several quantitative trait loci (QTLs) and appeared to be complex (Wang et al., 2000; Cameille et al., 2006).

Very little is known about plant defense mechanism in solanaceous plants against *R. solanacearum*. Current knowledge about defense mechanisms against *R. solanacearum* is mostly derived from the studies carried out in *Arabidopsis*. It has been demonstrated that jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) related defense signaling pathways are involved in *Arabidopsis* response to the bacterium's attack (Deslandes et al., 2002; Hernandez-Blanco et al., 2007; Hirsch et al., 2002). However, the information gained from the *Arabidopsis* system cannot be applied directly to the tomato system, as several studies have revealed that the molecular mechanisms and responses of solanaceous plants to *R. solanacearum* or other pathogens could be different from those in *Arabidopsis* (Robatzek et al., 2007; Wei et al., 2007; Lin et al., 2008). In this study, we established an effective gene silencing tool which would facilitate rapid gene functional assays to reveal the complex resistance mechanism in solanaceous hosts against *R. solanacearum*.

Virus-induced gene silencing (VIGS) (Burch-Smith et al., 2004) has been widely used as a powerful reverse-genetics tool in functional genomic studies. The advantages of VIGS include the transient nature and high efficiency in rapid knockdown of specific genes or gene families with functional redundancy (Burch-Smith et al., 2004). After plants are infected with the genetically modified viruses that carry a short sequence of a target gene in their genomes, specific small interfering RNAs (siRNAs) are generated resulting from enzyme-mediated degradation of the virus transcripts (Ossowski et al., 2008). The siRNAs would then bind to other RNA molecules containing their complementary regions to trigger further rounds of cleavage of target transcripts and consequently result in effectively silence the corresponding endogenous genes in host plants. VIGS has been successfully employed to study roles of genes in various aspects of plant function in a variety of species, including disease defense (Ekengren et al., 2003), abiotic response (Yoshioka et al., 2003; Senthil-Kumar et al., 2007a), and growth and development (Ahn et al., 2006). Among VIGS systems established to date, *Tobacco rattle virus* (TRV)-based vectors are the most commonly used vectors for its special properties, including: (1) TRV-induced symptoms in plants are usually very mild and thus do not disturb the phenotypic alterations caused by silencing of the target genes; (2) Unlike most plant viruses, TRV is capable of invading most plant tissues and cells, including meristems (Liu et al., 2002); (3) The efficacy of TRV-VIGS systems seems to be beyond its reputed host range of our knowledge (reviewed in Ekengre et al., 2003). TRV-VIGS systems have been used for functional genetic studies carried out in a range of species, particularly in Solanaceae (Ekengre et al., 2003; Senthil-Kumar et al., 2007).

TRV-VIGS has been used to study defense mechanisms of foliar diseases such as tomato bacterial speck (Ekengren et al., 2003), and root diseases such as root knot nematode (Bhattarai et al., 2007). However, TRV-VIGS systems have not been used in studies of complex interactions involving plant host and soil-borne vascular pathogens such as *R. solanacearum*. Because of its nature of soil-borne and rapid xylem-routed infection, the use of an efficient and rapid system to dissect the complex mechanisms involved in plant responses to the infection of this destructive bacterium would be certainly desired. Therefore, there is a need to evaluate and optimize important parameters of the TRV-VIGS system, including *Agrobacterium* strains, inoculation methods, and host plant genotypes. Additionally, as *R. solanacearum* invades the host from roots and cause systemic infection, and because plant responses at spatial and temporal levels are critical for the outcome of *R. solanacearum* infection (Wang et al., 2000), it is thus important to confirm the temporal and spatial silencing efficacy when carrying out VIGS assays. Moreover, silencing multiple genes to discriminate the complex parallel signaling pathway is desired, as the interactions between *R. solanacearum* and solanaceous hosts are expected to be complex (Chen et al., 2009). Chen et al. (2004) demonstrated the effectiveness of co-silencing of two genes using TRV containing a tandem construct to study functions of floral-associated genes in petunia. Similar protocols would be valuable for the study roles of resistance-associated genes in BW-resistant genotypes in response to *R. solanacaerum* infection.

To use the optimized TRV-VIGS protocol developed in this study for determining roles of candidate genes in BW-defense mechanisms of solanaceous crops, a functional assay of *COI1*, a SCF-type E3-ubiquitin ligase, was conducted in a tomato cultivar resistant to *R. solanacearum*. Being the receptor of JA signaling, COI1 plays an essential function in the JA signaling pathway and controls most JA responses (Xie et al., 1998; Li et al., 2004). In response to the developmental cues or environmental stimuli, plants produce bioactive amino acid-conjugated JAs from free form of JA by Jasmonate Resistant 1 (JAR1) enzyme. Binding of conjugated JAs

to COI1 triggers further direct interaction of COI1 with Jasmonate ZIM-domain (JAZ) proteins. The subsequent ubiquitination and degradation of JAZs release the JAZ-mediated repression of early JA-responsive genes and JA response pathways is then switched on (Thines et al., 2007). The JA signaling pathway plays important roles in plant disease response; however its role varies among plant-pathogen systems (Kunkel and Brooks, 2002; Robert-Seilaniantz et al., 2007), and its role in tomato defense mechanism to *R. solanacearum* is not clear.

The aim of this study was to optimize the TRV-VIGS conditions for rapid and reliable gene functional analyses for the study of complex interactions between solanaceous plants and *R. solanacearum*. Factors which may affect TRV-VIGS efficiency were analyzed, including *A. tumeficiens* strains, plant ages, inoculation methods, and solanaceous plant species and cultivars. Additionally, spatial and temporal efficacy of this system in tomato plants was determined and key factors affecting multiple gene silencing were examined. Furthermore, this optimized VIGS protocol was used to study the role of *COI1* in tomato BW-defense mechanism. The optimization of the high-efficient TRV-VIGS system in tomato plants allowed a quick and the first demonstration for a positive role of the JA signaling pathway in tomato resistance to *R. solanacearum*.

MATERIALS AND METHODS

Plant materials and growth conditions

Solanaceous plants used in this study included reported resistant (R) and susceptible (S) cultivars to *R. solanacearum*. Pepper (*Capsicum annuum*) cultivars included: PBC384(R), PBC535(R), PBC066(R), PBC631A(R), PBC743(R), Early Calwonder (ECW; S), and PBC1367(S). Eggplant (*Solanum melongena*) cultivars included: EG219(R), EG192(R), EG203(R), S56B(R), S3(R), and EG048(S). Tomato (*Solanum lycopersicum*) cultivars included: H7996 (R), Hawaii 7997(R), CRA66(R), BF Okitsu 101(R), R3034(R), L390(S), and L305(S). Seeds were directly sown or after 2 day-priming in two-inch pots containing potting mixture (MOS-010; Known-You Seed Co.). Plants were grown in a growth chamber at 24ºC with relative humidity from 60% to 80% and 16 hours extended daylight. 1000X liquid fertilizer (nitrogen: phosphorus: potassium 20:19:19; COMPO GmbH & CO. KG) was applied once before *Agrobacterium* infiltration. Plants were inoculated with *Agrobacterium* suspensions on the fully expanded cotyledons (9-10 days after sowing) or on the first true leaf (14 days after sowing).

Plasmid constructs and *Agrobacterium tumefaciens* strains

pTRV1 and pTRV2 derivatives were used as the vectors for gene silencing (Liu et al., 2002). Fragments of *PDS*, *ACO5*, *TGA2.2* and *COI1* were obtained by reverse transcriptase-PCR (RT-PCR) from the tomato (H7996), eggplant (EG203) or pepper (ECW) complementary DNA were used for experiments carried out in the respective plant species. Fragments of additional tomato genes tested in this work were amplified from tomato H7996 complementary DNA using primers listed in Table 1. The PCR product was cloned into the pCR® 8/GW/TOPO® vector. To prepare a chimeric construct of *PDS, TGA2.2,* and *ACO5*, fragments of *PDS* and *TGA2.2* were first digested with *Xba*I and/or *Spe*I, respectively, and ligated into pCR®8/GW/TOPO® vector to generate pCR®8/*TGA2.2*/*PDS* construct. Then *Xba*I and *Spe*I digested *ACO5* fragment was cloned into pCR®8/*TGA2.2*/*PDS* to generate a chimeric construct of pCR®8/*TGA2.2*/*PDS*/*ACO5*. Finally, pCR®8/*TGA2.2*/*PDS*/*ACO5* was then recombined into a pTRV2 vector, pYL279, by carrying out an LR recombination reaction using the Gateway® system (Invitrogen Co., Carlsbad, CA, USA). pYL279-m, a modified pTRV2 empty vector, in which the selectable CHLORAMPHENICOL RESISTANCE gene and the counter-selectable *ccdB* gene had been removed, was constructed via an LR recombination reaction with the empty pCR®8/GW/TOPO® vector. This was used as the empty vector control. The pYL279 recombinant constructs obtained were electroporated into cells of *A. tumefaciens* by a MicroPulser (Bio-Rad, USA). Three commonly used laboratory strains of *A. tumefaciens*, GV3101, GV2260 and C58C1, were used for transformation of the cloned silencing constructs in the indicated VIGS experiments.

Virus infection by *Agrobacterium*-mediated infiltration and agrodrench

A 3-mL culture of *A. tumefaciens* containing each TRV derivative was grown at 28°C in the appropriate antibiotic selection YEP medium (0.5% peptone, 0.5% yeast extract, and 1% sodium chlorine) containing 50 µg/mL kanamycin, 50 µg/mL gentamycin, and 25 µg/mL rifamycin for overnight. The culture was then inoculated into a 25-mL YEP medium containing the antibiotics, 10 mM MES and 20 mM acetosyringone and grown overnight at 28°C with shaking. *Agrobacterium* cells were harvested and resuspended in an infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 200 mM acetosyringone, pH 5.6), adjusted to OD_{600} of 1.5-2.0. Suspensions of pTRV1 and pTRV2 were mixed in 1:1 ratio and incubated at room temperature for 3 h before inoculation. *Agrobacterium* suspension was infiltrated into two cotyledons or first true leaves using a 1-mL needleless syringe. For the agrodrench method, 20 mL of *Agrobacterium* suspension was drenched over the soil surface of each pot. *Agrobacterium*-infected plants were maintained at 22°C or 24°C in a growth chamber for the indicated periods of time. In experiments comparing the effects of *Agrobacterium* strains, inoculation methods, and plant cultivars, the experiment design used was randomized complete block design with 3 replications and 10 plants per replication. In the experiments involving *PDS* silencing, percentages of plants displaying the photo-bleaching phenotype were recorded periodically.

When conducting analysis of variance, percentage data were transformed with arcsine square root and the least significant difference at 5% was used for mean comparisons.

Assessment of plant response to the infection of *R. solanacearum*

Ralstonia solanacearum strain Pss4 (phylotype I, race 1, biovar 3) was prepared to give an OD_{600} of 0.6 and used to challenge plants. The Pss4 suspension was poured over the pot soil surface of 3-week-old seedlings as described previously (Wang et al., 2000). The response of the inoculated plants were evaluated as the percentage of wilted plants 5 days after inoculation. When bacterial population in tomato plants were evaluated, stem were sterilized with 70% ethanol, and segments (about 1 cm in length) were removed from the base and mid-point of each plant, weighted and crushed in 1 mL of sterilized water, respectively. 100 μL of the tissue extract was subjected to 10-fold serial dilutions, and 10 μL of each diluted sample was spotted onto modified SM1 medium. The number of bacterial colonies developed was recorded two days after incubation at 28°C as described previously (Lin et al., 2008). The detection limit of this method is about 10^3 CFU/g tissues. Each assay included about 6 to 12 plants and repeated at least three times. Pair-wise mean comparisons of bacterial population at the stembases and mid-stems were conducted between TRV-infected and silenced plants with Student *t*-test.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from roots, collars or stems with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). One μg of total RNA was treated with RNase-free DNase I (Qiagen) followed by phenol/chloroform extraction to remove DNase. The first-strand cDNA was synthesized from 0.1 mg of total RNA with oligo (dT) primer and AMV reverse transcriptase (Promega, Madison, WI, USA). Semi-quantitative RT-PCR was performed as described (Ekengren et al., 2003). Primers used for RT-PCR to check transcript abundance of the silenced genes are listed in Table 1. Tomato ubiquitin gene *Ubi3* served as an internal control for equal cDNA use from control and silenced plants. To confirm lack of genomic DNA contamination, 200 ng of DNase I-treated RNA was also used as template for PCR reactions. The amplified products were analyzed on 1% (w/v) agarose gels stained with ethidium bromide.

RESULTS

Effects of inoculation methods, *Agrobacterium* strains and solanaceous species and cultivars on VIGS efficiency

VIGS efficacy of agroinfiltration on cotyledons and first true leaves was evaluated using *Agrobacterium tumefaciens* strains GV3101 or C58C1 as the carrier

Table 1. Primers used to amplify sequenced fragment used for VIGS tests and to evaluate transcript level of silenced genes.

Targeted tomato genes	GeneBank or unigene number[a]	Sequences used for VIGS tests		Evaluation of gene transcript levels	
		Primer sequences	Fragment length[b] (bp)	Primer sequences	Fragment length[b] (bp)
PDS	CAA42573	F: 5′-GGTCTAGACTGACGAGCTTTCGATGCAGTGCAT-3′[c] R: 5′-GGACTAGTATATATGGACATTTATCACAGGAAC-3′[c]	372	F: 5′-CGAATCAATGGGTCATAAGT-3′ R: 5′-CTCGTACCAATCTCCATCAT-3′	343
COI1	AAR82925	F: 5′-ATCAGGAGATGGAGGATGAAGAAGG-3′ R: 5′-TTCCATCGTTGGCAATAACTCG -3′	603	F: 5′-AGAAACTGTAGTCTCGGAGC-3′ R: 5′-GGCATACATATGGACAAGACA-3′	204
TGA2.2	SGN-U318626	F: 5′-GATGCTTCTTGTGGCTTGGTGG-3′ R: 5′-ACTAGTCAGTGATAGCAAGAAGAGCACG-3′	381	F: 5′-CAGTCGATGAGTGGAAATGG-3′ R: 5′-GCAGGAGTTTTCCACATGCC-3′	239
ACO5	CAG29395	F: 5′-GGTCTAGAGCAACACAGACTGGGAAAGC-3′ R: 5′-GGACTAGTATCCTGAAAGCGGAGGTG-3′	584	-	
UBI3	X58253	-	-	F: 5′-TGCAGATCTTCGTGAAAACC-3′ R: 5′-AGCGAGCTTAACCTTCTTCT-3′	302

[a]Accession numbers for tomato genes taken from http://www.ncbi.nlm.nih.gov or unigene numbers taken from http://www.sgn.cornell.edu/search/direct_search.pl?search=unigene.
[b]The length (measured in bp) of tomato cDNA fragments amplified with the corresponding primers.
[c]These primers were also used to amplify *PDS* sequences from pepper ECW and eggplant EG203.

bacterium and tomato phytoene desaturase (PDS) gene (Table 1) as a marker gene. The results showed that both methods and strains could efficiently cause the typical leaf bleaching phenotype on all silenced tomato plants and the silencing progression was similar among treatments (Figure 1A). Similar results were observed on two tomato cultivars, H7996 and Hawaii 7997. In addition, an agrodrench method, using *A. tumefaciens* strain GV2260 as reported previously (Ryu et al., 2004), resulted in leaf bleaching on 10% of the silenced plants at the end of the experiments (17 days after agrodrench), while agroinfiltration on cotyledons with *A. tumefaciens* strain GV3101 or C58C1 could lead to leaf bleaching on all of the silenced plants 14 days after agroinfiltration (Figure 1B). Considering the desired plant age for following inoculation with *R. solanacearum*, the cotyledon agroinfiltration method with strain GV3101 was used for further studies.

Silencing efficiency of the cotyledon agroinfiltration method was evaluated on a set of resistant and susceptible cultivars of tomatoes, peppers and eggplants using *PDS* as a marker gene (Table 2). *PDS* inserts originated from tomato H7996, pepper ECW, and eggplant EG203 were used in respective VIGS assays. The results showed that the seven tomato cultivars displayed similar competence for efficient VIGS, except L305 and R3034 had lower percentage of bleached plants. Among the tested pepper cultivars, the best silencing result was observed on PBC1367, followed by ECW and PBC535, while the other four cultivars were not competent for efficient VIGS. All of the six eggplant cultivars tested displayed poor or no silencing effect using the protocol.

Spatial and temporal analysis of VIGS efficiency

Because tomato responses at spatial and temporal levels are critical for the outcome of *R. solanacearum* infection,

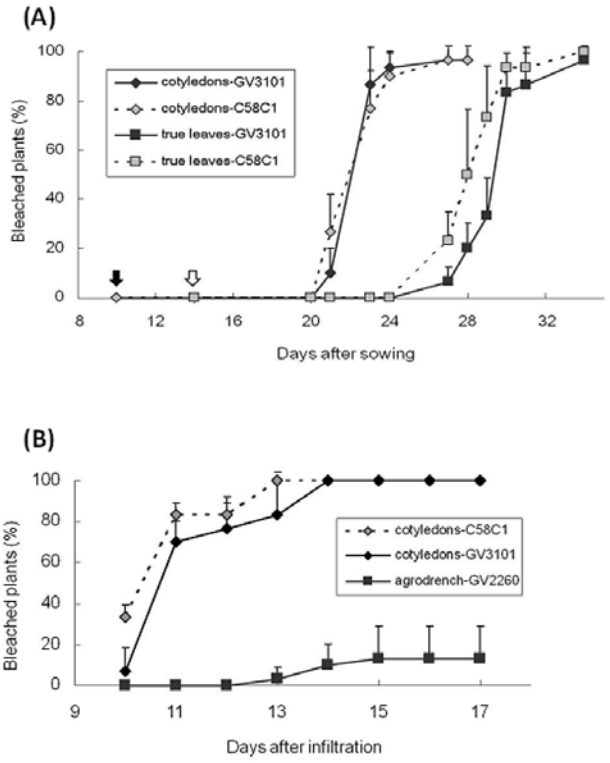

Figure 1. Effects of *A. tumefaciens* strains, plant ages, and inoculation methods on VIGS efficiency. Cotyledon infiltration was conducted on 10-day-old tomato H7996 plants (⬇) and first true leaf infiltration on 14-day-old plants (⇩) with *A. tumefaciens* strain GV3101 or C58C1 containing pTRV1 plus pTRV2-*PDS* (A); Cotyledon infiltration method was compared with agrodrench inoculation on 10-day-old plants with GV2260 strain (B). Silencing efficiency was evaluated based on the percentage of plants showing photo-bleaching phenotype in newborn leaves. The experiment was performed three times with 10 plants per treatment. Data presented were means over experiments.

Table 2. *PDS* silencing efficiency on tomato, pepper or eggplant cultivars resistant or susceptible to *R. solanacearum*.

Tomato		Pepper		Eggplant	
Cultivar[a]	Bleached plant (%)[b]	Cultivar[a]	Bleached plant (%)[b]	Cultivar[a]	Bleached plant (%)[b]
L390 (S)	100.0 (89.7)	PBC1367 (S)	96.7 (83.6)	S3 (R)	6.7 (9.1)
BF Okitsu 101 (R)	100.0 (89.7)	ECW (S)	76.7 (66.0)	S56B (R)	3.3 (6.3)
H7996 (R)	96.7 (83.6)	PBC535 (R)	53.3 (46.9)	EG219 (R)	0.0 (0.3)
CRA66 (R)	96.7 (83.6)	PBC384 (R)	10.0 (15.1)	EG192 (R)	0.0 (0.3)
H7997 (R)	93.3 (77.6)	PBC066 (R)	6.7 (12.4)	EG203 (R)	0.0 (0.3)
L305 (S)	76.7 (64.1)	PBC631A (R)	0.0 (0.3)	EG048 (S)	0.0 (0.3)
R3034 (R)	73.3 (61.2)	PBC743 (R)	0.0 (0.3)		
LSD 0.05	(22.4)	LSD 0.05	(19.4)	LSD 0.05	(12.3)

[a] Reported resistant (R) or susceptible (S) cultivars. Cotyledons of 10-day-old plants were agroinfiltrated with *A. tumefaciens* strain GV3101 containing pTRV1 plus pTRV2-*PDS*. *PDS* inserts originated from tomato H7996, pepper ECW and eggplant EG203 were used in respective VIGS assays.

[b] Data of the percentage of bleached plants recorded 23, 26, 30 days after agroinfiltration for the tomato, pepper and eggplant cultivars, respectively, were transformed by arcsin square root for ANOVA. Transformed data were presented in the parentheses. $LSD_{0.05}$ was calculated using the transformed data.

whether and when the cotyledon agroinfiltration method could effectively trigger systemic gene silencing effect upward and downward at the transcriptional level was further examined. Results of semi-quantitative RT-PCR (sqRT-PCR) illustrated that silencing of tomato *PDS* in roots and young leaves was efficiently achieved using our TRV-VIGS protocol (data not shown). Because we are interested in studying plant defense response to pathogen infection, tomato *TGA2.2* (Table 1) was used as a marker gene for further analysis. TGA2.2 is a key transcription factor downstream of NPR1, the central regulator of salicylic acid and JA defense signaling pathway (Ekengren et al., 2003). The results revealed that the reduction of *TGA2.2* transcript level in 10-day-old plants initiated 5 days post inoculation (DPI) in stembases (Figure 2A), 7 DPI in roots (Figure 2B) and 10 DPI in young leaves (Figure 2C).

VIGS-mediated simultaneous silencing of multiple genes

Factors involved in TRV-based VIGS were tested further to determine how VIGS-based multiple-gene silencing can be efficiently achieved. In general, two approaches can be used to silence multiple genes simultaneously by VIGS. One approach would be inoculation of plants with a mixture of several *Agrobacterium* lines, in which each line carries individual target genes. Therefore, the effect of *Agrobacterium* titer used for agroinfiltration was first assessed. As shown in Figure 3, when *A. tumefaciens* strain GV3101 containing pTRV1 plus pTRV2-*PDS* was diluted with the inoculation buffer, the VIGS efficacy of diluted *Agrobacterium* samples, even at an 8-fold dilution, remained the same as that of the original dose. However, when *A. tumefaciens* strain GV3101 containing pTRV1 plus pTRV2-*PDS* was diluted with *Agrobacterium* carrying empty TRV vector, the VIGS efficacy significantly reduced.

Another approach to achieve multiple-gene VIGS would be agroinfiltration of plants with a single *Agrobacterium* carrying a pTRV2 vector containing chimeric sequences from the targeted genes. We hence compared the efficiency of the mixing approach and the chimeric approach for multiple-gene VIGS. In this analysis, three genes with distinct functions were included (Table 1): *PDS* (a commonly used marker gene for VIGS and functioning in pigmentation), *ACO5* (involved in ethylene biosynthesis), and *TGA2.2* (playing an important role in disease defense). The results showed that *PDS* alone and the chimeric construct caused efficient leaf bleaching on all of the silenced tomato plants 17 DPI, while only 23% of the silenced plants showed bleaching phenotype when a mixing approach was used (Figure 4A). When the VIGS efficacy was analyzed spatially at the transcriptional level, sqRT-PCR results illustrated the reduced level of *PDS* transcripts caused by VIGS with a chimeric construct was not apparently different from *PDS* alone in stembases, roots and young leaves (Figure 4B); however, VIGS by mixing multiple *Agrobacterium* strains failed to reduce the *PDS* transcript level in any of the tissues. Together, these results indicate that the chimeric approach is more efficient for multiple-gene silencing compared to the mixing approach.

Silencing of *COI1* resulted in increase of *R. solanacearum* proliferation in tomato H7996 plants

The cotyledon agroinfiltration protocol developed above was used to evaluate whether *COI1* is involved

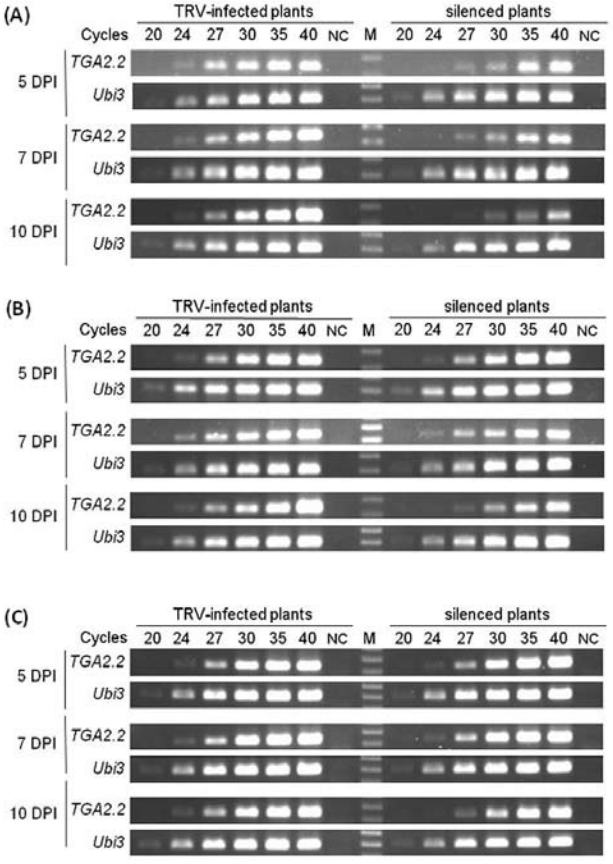

Figure 2. Spatial and temporal analysis of VIGS efficiency. Cotyledons of 9-day-old tomato H7996 plants were agroinfiltrated with *A. tumefaciens* strain GV3101 containing pTRV1, pTRV2-*TGA2.2*, or pTRV2 empty vector. pTRV1 plus pTRV2-*TGA2.2* is referred to as silenced plants and pTRV1 plus pTRV2 empty vector is referred to as TRV-infected plants. cDNA was synthesized from total RNA isolated from pooled samples of stembases (A), roots (B) or young leaves (C) collected from two plants agroinfiltrated with TRV or TRV-*TGA2.2* after 5, 7 or 10 days. Primers used for *TGA2.2*-specific amplification are listed in Table 1. The experiment was performed twice with similar results. PCR amplification from cDNA from a single representative sample is presented. Amplification of tomato ubiquitin gene *Ubi3* was used as an internal control for equal cDNA use from control and silenced plants. PCR cycles are indicated on the top of the sections. Lane M indicates DNA ladder and NC indicates negative control where RNA was used as template for PCR amplification in the absence of reverse transcriptase.

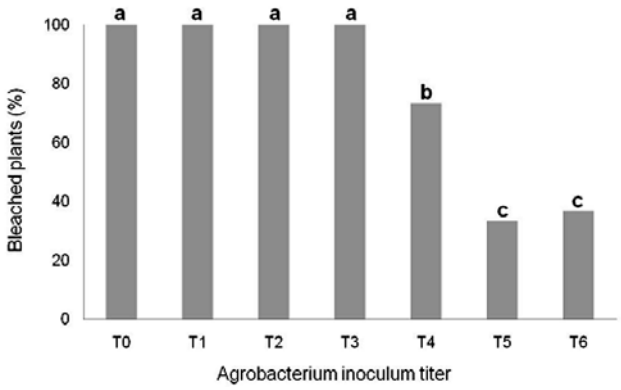

Figure 3. Effects of *Agrobacterium* inoculum titer on VIGS efficiency. Cotyledons of 10-day-old H7996 plants were agroinfiltrated with *A. tumefaciens* strain GV3101 containing pTRV1 plus pTRV2-*PDS* diluted at various folds with the inoculation buffer or *Agrobacterium* carrying the pTRV2 empty vector. T0: original concentration of pTRV2-*PDS*. T1, T2 and T3: pTRV2-*PDS* was diluted at 2-, 4- and 8-fold with the inoculation buffer. T4, T5 and T6: pTRV2-*PDS* was diluted at 2-, 4- and 8-fold with *Agrobacterium* carrying the pTRV2 empty vector. The silencing efficiency was evaluated based on the percentage of plants showing photo-bleaching phenotype in newborn leaves. The experiment was performed three times with 10 plants per treatment. The percentage data were transformed by arcsine square root for mean comparisons. Letters indicate significant differences on mean comparison by using Fisher's LSD test at p =0.05.

in the disease-defense mechanism of tomato H7996. As illustrated in Figure 5A, the accumulation of *COI1* transcripts in stembases of *COI1*-silenced plants had been reduced 15 days post *Agrobacterium* infiltration. No noteworthy sequence homology (21 continuous identical nucleotides) was found between the *COI1* fragment and ESTs or genes currently available in tomato sequence databases (Thomas et al., 2001; Ekengren et al., 2003; Bhattarai et al., 2007; Senthil-Kumar et al., 2007a). No apparent alteration in plant morphology was observed.

Resistance to *R. solanacearum* in tomato plants is usually correlated with the pathogen distribution and density in the stem (Grimault et al., 1994, Wang and Lin 2005). Therefore, both visual symptom development and internal bacterial density in stems were determined in this experiment. *COI1*-silenced H7996 plants, along with plants agroinfiltrated with the empty TRV vector, were inoculated with *R. solanacearum* by root drenching 10 days post agroinfiltration, when VIGS efficacy already has started in roots, the stembases, and even in young leaves. Tomato cultivar L390 was used as the susceptible control to confirm the success of pathogen inoculation. Under the defined experimental condition, we observed that TRV infection slightly slowed plant growth of H7996 and L390, but their response to the infection of *R. solanacearum* was not different from the control plants without VIGS treatment (data not shown). L390 plants first showed wilting symptoms 3 DPI and completely wilted 5 DPI, when the internal *R. solanacearum* density reached a mean greater than 10^9 CFU g^{-1} in midstems. The *COI1*-silenced H7996 plants did not develop apparent wilting symptoms 12 days after *R. solanacearum* inoculation. However, when compared to the control plants, a much higher proportion of *COI-1*-silenced plants were detected with the presence of the bacterium in stembases (60.0% vs. 27.3%) and midstems (23.3% vs. 0.0%). Large variations in the bacterial density of assayed H7996 plants were observed. This is due to several reasons, including the high detection limit of the direct plating method ($\geq 10^3$ CFU g^{-1} plant tissues), the effect of plant physiological status and environment on the progress of the disease, and the uneven gene silencing degree over tested plants and even within

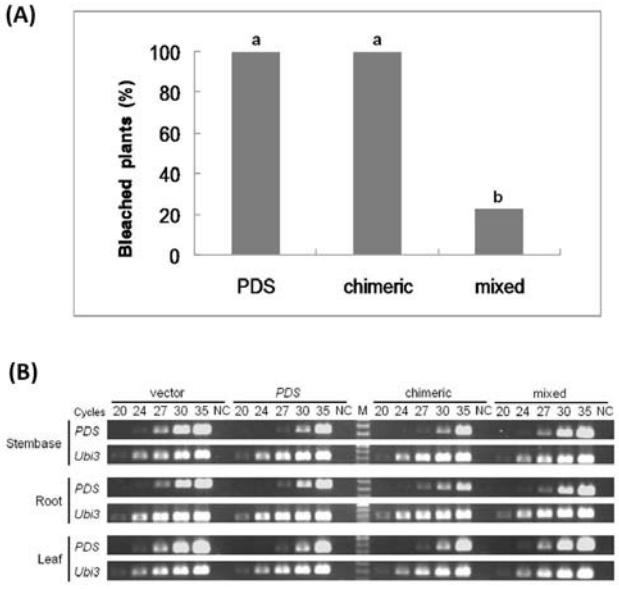

Figure 4. VIGS-mediated simultaneous silencing of multiple genes. H7996 plants were agroinfiltrated with *A. tumefaciens* strain GV3101 containing pTRV1 plus various pTRV2 vectors. *PDS*, pTRV1 plus pTRV2-*PDS*; chimeric, pTRV1 plus chimeric pTRV2-*PDS-TGA2.2-ACO5*; mixed, pTRV1 plus a mixture containing equal amount of pTRV2-*PDS*, TRV2-*TGA2.2* and pTRV2-*ACO5*. (A) Leaf photobleaching caused by *PDS* gene silencing. The VIGS efficiency was evaluated based on the percentage of plants showing photo-bleaching phenotype in newborn leaves. The experiment was performed three times with 10 plants for each treatment in each independent experiment. The percentage data were transformed by arcsine square root for mean comparisons. Letters indicate significant differences on mean comparison by using Fisher's LSD test at p =0.05; (B) VIGS-mediated reduction of *PDS* transcript levels. cDNA was synthesized from total RNA isolated from pooled samples of roots, stembases, or young leaves collected from two plants 15 days after agroinfiltration. Primers used for *PDS*-specific amplification are listed in Table 1. Amplification of tomato ubiquitin gene *Ubi3* was used as an internal control for equal cDNA use from control and silenced plants. PCR cycles are indicated on the top of the sections. Lane M indicates DNA ladder and NC indicates negative control where RNA was used as template for PCR amplification in the absence of reverse transcriptase.

the same plant. Nevertheless, pair-wise t-test showed that the *COI-1*-silenced plants had significantly higher bacterial population in stembases (4.38 vs. 1.89 log CFU g^{-1} tissue) and mid-stems (1.90 vs. 0.00 log CFU g^{-1} tissue) compared to that of H7996 plants inoculated with the empty vector (Figure 5B). These results indicate that silencing the JA signaling pathway increased the population of *R. solanacearum* in the resistant tomato plants.

DISCUSSION

TRV-based VIGS systems have been widely used in functional genomics studies for plants; however, it has not been used for studies of plant defense mechanisms to soil-borne vascular pathogens, such as *R. solanacearum*. To establish an optimized method for such studies, we first evaluated factors that may affect TRV-VIGS efficiency, and confirmed the system's spatial and temporal efficacy in tomato plants.

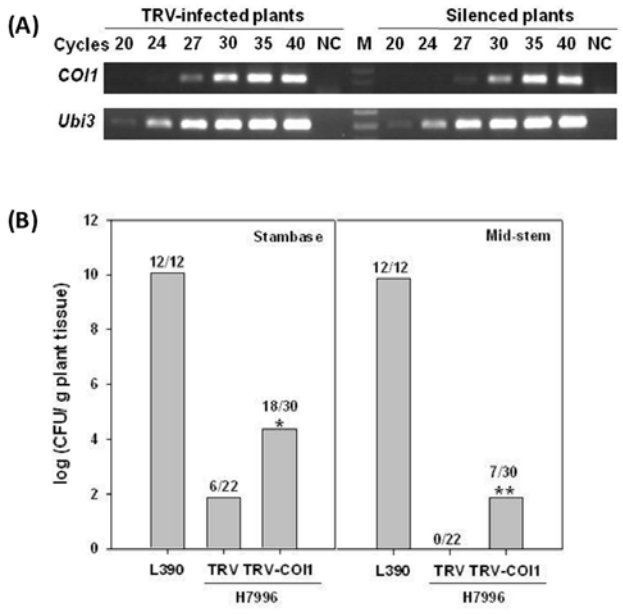

Figure 5. Silencing of *COI1* resulted in increase of *R. solanacearum* proliferation in tomato H7996 plants. Resistant cultivar H7996 and susceptible cultivar L390 were agroinfiltrated with *A. tumefaciens* strain GV3101 containing pTRV1 plus pTRV2 containing *COI1* genes (silenced plants), or plus pTRV2 empty vector (TRV-infected plants). (A) VIGS-mediated reduction of *COI1* transcript level. cDNA was synthesized from total RNA isolated from pooled samples of stembases collected from two plants 15 days after agroinfiltration and used for PCR amplification. Amplification of the internal control *Ubi3* was performed. Primers used for *COI1*-specific amplification are listed in Table 1. The experiment was performed twice with similar results. PCR amplification from cDNA from a single representative sample is presented. PCR cycles are indicated on the top of the sections. Lane M indicates DNA ladder and NC indicates negative control where RNA was used as template for PCR amplification in the absence of reverse transcriptase; (B) *R. solanacearum* population in L390 and H7996 infected with TRV or pTRV-*COI1*. Assessment was carried out 5 DPI by a method described in Materials and Methods. Numbers of positively detected plants over total assayed plants were indicated above each column. Means were calculated from data of all assayed plants and pair-wise mean comparisons were made between TRV-infected and silenced plants of H7996 with Student *t*-test method (*, significant at 0.05 level; **, significant at 0.01 level).

Plant age is a main concern when establishing the TRV-VIGS protocol, as it is known that plants become more resistant to *R. solanacearum* as they aged (Hwang et al., 2004; Zhang et al., 2004). Twenty-day old plants (with three fully expanded true leaves) are suitable to differentiate resistant and susceptible interactions as shown by Wang et al. (2000). However, the reported procedures for TRV-VIGS studies in Solanaceae mostly conduct agroinoculation on 14-days-old or older seedlings, and then perform with pathogen challenge several days afterward (Ekengren et al., 2003; Brigneti et al., 2004; Bhattarai et al., 2007; Senthil-Kumar et al., 2007a). Therefore, a cotyledon agroinfiltration method using 9 or 10-day-old seedlings were developed for our needs after evaluating several important factors. Systemic gene silencing effect was observed 5, 7 and 10 days post *Agrobacterium* infiltration in stembases, roots, and young leaves of tomato plants. In our established VIGS protocol, we challenged the silenced plants with *R. solanacearum* 10 days after agroinfiltration when systemic silencing effect has reached and the plants are about 20 days old. Currently, there are only two reports describing procedures applicable on younger seedlings: agrodrench (Ryu et al., 2004) and vacuum infiltration (Hartl et al., 2008). Our comparative study clearly revealed that agroinfiltration on cotyledons is much more efficient than agrodrench to trigger systemic gene silencing in young seedlings. Additionally, our preliminary experiments indicated that vacuum infiltration not only is much more time consuming, also would cause damage on tomato true leaves. The cotyledon agroinfiltration method established in this study would be applicable for the studies of other soil-born vascular pathogens.

Consistent with previous reports (Brigneti et al., 2004; Senthil-Kumar et al., 2007b), VIGS efficiency of the developed cotyledon agroinfiltration method varied among Solanaceous species and cultivars. All tested tomato cultivars were similarly susceptible to the method and efficiently revealed the effects of gene silencing (Table 2). However, only three of the seven tested pepper cultivars and none of the test eggplant cultivars displayed gene silencing results. It is possible that peppers and eggplants require a longer time to achieve full gene silencing than tomatoes, similar to the observation noted in some species of potatoes (Brigneti et al., 2004). Alternatively, this may reflect host preference of TRV and/or *A. tumefaciens* strains among solanaceous species (Burch-Smith et al., 2004; Ghazala and Varrelmann 2007; Hartl et al., 2008), in which the level of *Agrobacterium* infection and viral replication/movement in various plant species might vary and consequently affect VIGS efficiency. Besides, it is also plausible that the PDS sequences used did not have

sufficient homology in other cultivars of peppers and eggplants. Additionally, manipulation of environmental conditions, such as temperatures for plant growth, might enable desired gene silencing effects in peppers and eggplants. Future studies would help clarify the causes for the low VIGS efficiency observed in peppers and eggplants, and lead to further optimization of the system in these plants.

As plant traits often are controlled by multiple genes and pathways acting redundantly or synergistically, simultaneous reduction of the expression of multiple genes would be necessary to reveal complicated mechanisms, such as tomato resistance to *R. solanacearum*. Previously, Chen et al. (2004) demonstrated the effectiveness of co-silencing of two genes using TRV containing a tandem construct in petunia. Their design was to co-silence a gene of unknown function linked to silencing of a gene with a visible phenotype. Our study is the first report demonstrating the co-silencing of three genes. We showed that a chimeric construct of *PDS* with the other two genes was as efficient as a single *PDS* fragment in silencing *PDS* in roots, stembases, and leaves. More tests constructing different gene combinations would be needed to determine the limit of the chimeric approach to co-silence three genes. We also demonstrated the apparent reduction of the gene silencing effect caused by inoculation of multiple *Agrobacterium* lines either carrying empty RNA2 vector or vectors containing additional targeted genes. This implied that more than one TRV vector could not be co-expressed in a plant cell. A similar observation has been made by Giritch et al. (2006), showing that two different TMV vectors failed to co-express in cells of *Nicotiana benthamiana* via agroinfiltration. Our accumulated studies have demonstrated that more than one defense signaling pathways contribute to the resistance to BW in tomato plants, and that these pathways may interact in an additive mode (Lin et al., 2004; Chen et al., 2009). The multiple-gene silencing protocol developed in this study could be used to clarify the interactions and contributing level of different signaling pathways.

Our study is the first report revealing that the tomato defense mechanism against BW involves the JA signaling pathway. Prior to this study, limited information was available, primarily on *Arabidopsis*. *COI1* mutant of susceptible *Arabidopsis* Col0 ecotype displayed milder disease symptoms compared with the wild-type plant in response to *R. solanacearum* infection (Hernandez-Blanco et al., 2007), which suggested the JA signaling pathway may play a negative role in *Arabidopsis* defense to *R. solanacearum*. However, our results showed that silencing *COI1* in the bacterial wilt-resistant tomato cultivar H7996 resulted in systemic increase of *R. solanacearum* proliferation, suggesting the JA signaling pathway is positively involved in tomato defense mechanism against this bacterium. Therefore, our findings provide additional support for differences in *R. solanacearum* interactions with tomato plants and *Arabidopsis* (Lin et al., 2008), although knocking down the target genes by taking a transgenic RNAi approach would further help fortify these results.

In nature, plants orchestrate multiple defense signaling pathways to achieve proper response to various pathogen attacks (Pieterse and Dicke, 2007). In this study, we demonstrated the involvement of the JA signaling pathway in tomato defense against BW. Studies in tobacco have demonstrated that silencing players of mitogen-activated protein kinase (MAPK) cascades led to altered JA level, and thus resulted in changes of SA accumulation (Seo et al., 2007) and the JA-dependent signaling pathway (Kandoth et al., 2007). In *Arabidopsis*, MAPK cascades also are suggested to be involved in the ET signaling transduction pathway (Yoo et al., 2008). Additional results obtained from our VIGS assays on three SA-related candidate defense genes further suggested a positive role of SA related defense signaling pathways in tomato resistance to BW (Chen et al., 2009). Therefore, the protocol established in this study will be an effective tool to systemically evaluate the interplays of MAPK cascades, JA/ET and SA pathways in the tomato BW-defense network to *R. solanacearum*.

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LITERATURE CITED

Ahn, C.S., J.H. Lee, A. Hwang Reum, W.T. Kim, and H.S. Pai. 2006. Prohibitin is involved in mitochondrial biogenesis in plants. Plant J. **46:** 658-667.

Bhattarai, K.K., Q. Li, Y. Liu, S.P. Dinesh-Kumar, and I. Kaloshian. 2007. The *MI-1*-mediated pest resistance requires *Hsp90* and *Sgt1*. Plant Physiol. **144:** 312-323.

Brigneti, G., A.M. Martin-Hernandez, H. Jin, J. Chen, D.C. Baulcombe, B. Baker, and J.D. Jones. 2004. Virus-induced gene silencing in Solanum species. Plant J. **39:** 264-272.

Burch-Smith, T.M., J.C. Anderson, G.B. Martin, and S.P. Dinesh-Kumar. 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J. **39:** 734-746.

Carmeille, A., C. Caranta, J. Dintinger, P. Prior, J. Luisetti, and P. Besse. 2006. Identification of QTLs for *Ralstonia solanacearum* race 3-phylotype II resistance in tomato. Theor. Appl. Genet. **113:** 110-121.

Chen, J.C., C.Z. Jiang, T.E. Gookin, D.A. Hunter, D.G. Clark, and M.S. Reid. 2004. Chalcone synthase as a reporter in virus-induced gene silencing studies of flower senescence. Plant Mol. Biol. **55:** 521-530.

Chen, Y.Y., Y.M. Lin, T.C. Chao, J.F. Wang, A.C. Liu, F.I. Ho,

and C.P. Cheng. 2009. Tomato resistance to bacterial wilt involves ethylene, salicylic acid and mitogen-associated protein kinase related defense pathways. Physiol. Plant. (In press)

Denny, T.P. 2006. Plant pathogenic *Ralstonia* species. *In* S.S. Gnanamanickam (ed.), Plant-Associated Bacteria. Springer, Dordrecht, The Netherlands, pp. 573-644.

Deslandes, L., J. Olivier, F. Theulieres, J. Hirsch, D.X. Feng, P. Bittner-Eddy, J. Beynon, and Y. Marco. 2002. Resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* is conferred by the recessive *RRS1-R* gene, a member of a novel family of resistance genes. Proc. Natl. Acad. Sci. USA **99:** 2404-2409.

Ekengren, S.K., Y. Liu, M. Schiff, S.P. Dinesh-Kumar, and G.B. Martin. 2003. Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. Plant J. **36:** 905-917.

Ghazala, W. and M. Varrelmann. 2007. *Tobacco rattle virus* 29K movement protein is the elicitor of extreme and hypersensitive-like resistance in two cultivars of *Solanum tuberosum*. Mol. Plant-Microbe Interact. **20:** 1396-1405.

Giritch, A., S. Marillonnet, C. Engler, G. van Eldik, J. Botterman, V. Klimyuk, and Y. Gleba. 2006. Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. Proc. Natl. Acad. Sci. USA **103:** 14701-14706.

Grimault, V., G. Anais, and P. Prior. 1994. Distribution of *Pseudomonas solanacearum* in the stem tissues of tomato plants with different levels of resistance to bacterial wilt. Plant Pathol. **43:** 663-668.

Hartl, M., H. Merker, D.D. Schmidt, and I.T. Baldwin. 2008. Optimized virus-induced gene silencing in *Solanum nigrum* reveals the defensive function of leucine aminopeptidase against herbivores and the shortcomings of empty vector controls. New Phytol. **179:** 356-365.

Hernandez-Blanco, C., D.X. Feng, J. Hu, A. Sanchez-Vallet, L. Deslandes, F. Llorente, M. Berrocal-Lobo, H. Keller, X. Barlet, C. Sanchez-Rodriguez, L.K. Anderson, S. Somerville, Y. Marco, and A. Molina. 2007. Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance. Plant Cell **19:** 890-903.

Hirsch, J., L. Deslandes, D.X. Feng, C. Balague, and Y. Marco. 2002. Delayed symptom development in *ein2-1*, an *Arabidopsis* ethylene-insensitive mutant, in response to bacterial wilt caused by *Ralstonia solanacearum*. Phytopathology **92:** 1142-1148.

Hwang, K.Y., I.C. Chou, Y.M. Lin, and C.P. Cheng. 2004. Plant age-related resistance to *Ralstonia solanacearum*, the causal agent of bacterial wilt. J. Gene Mol. Biol. **15:** 108-115.

Kunkel, B.N. and D.M. Brooks. 2002. Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol. **5:** 325-331.

Kandoth, P.K., S. Ranf, S.S. Pancholi, S. Jayanty, M.D. Walla, W. Miller, G.A. Howe, D.E. Lincoln, and J.W. Stratmann. 2007. Tomato MAPKs LeMPK1, LeMPK2, and LeMPK3 function in the systemin-mediated defense response against herbivorous insects. Proc. Natl. Acad. Sci. USA **104:** 12205-12210.

Li, L., Y. Zhao, B.C. McCaig, B.A. Wingerd, J. Wang, M.E. Whalon, E. Pichersky, and G.A. Howe. 2004. The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16:** 126-143.

Lin, W.C., C.F. Lu, J.W. Wu, M.L. Cheng, Y.M. Lin, N.S. Yang, L. Black, S.K. Green, J.F. Wang, and C.P. Cheng. 2004. Transgenic tomato plants expressing the *Arabidopsis NPR1* gene display enhanced resistance to a spectrum of fungal and bacterial diseases. Transgenic Res. **13:** 567-581.

Lin, Y.M., I.C. Chou, J.F. Wang, F.I. Ho, Y.J. Chu, P.C. Huang, D.K. Lu, H.L. Shen, M. Elbaz, S.M. Huang, and C.P. Cheng. 2008. Transposon mutagenesis reveals differential pathogenesis of *Ralstonia solanacearum* on tomato and *Arabidopsis*. Mol. Plant-Microbe Interact. **21:** 1261-1270.

Liu, Y., M. Schiff, and S.P. Dinesh-Kumar. 2002. Virus-induced gene silencing in tomato. Plant J. **31:** 777-786.

Ossowski, S., R. Schwab, and D. Weigel. 2008. Gene silencing in plants using artificial microRNAs and other small RNAs. Plant J. **53:** 674-690.

Pieterse, C.M. and M. Dicke. 2007. Plant interactions with microbes and insects: from molecular mechanisms to ecology. Trends Plant Sci. **12:** 564-569.

Prior, P., V. Grimault, and J. Schmit. 1994. Resistance to bacterial wilt (*Pseudomonas solanacearum*) in tomato: present status and prospects. *In* A.C. Hayward and G.L. Hartman (eds.), Bacterial Wilt: The disease and its causative agent, *Pseudomonas solanacearum*. CAB International, Wallingford, pp. 209-223.

Robatzek, S., P. Bittel, D. Chinchilla, P. Kochner, G. Felix, S.H. Shiu, and T. Boller. 2007. Molecular identification and characterization of the tomato flagellin receptor LeFLS2, an orthologue of *Arabidopsis* FLS2 exhibiting characteristically different perception specificities. Plant Mol. Biol. **64:** 539-547.

Robert-Seilaniantz, A., L. Navarro, R. Bari, and J.D. Jones. 2007. Pathological hormone imbalances. Curr. Opin. Plant Biol. **10:** 372-379.

Ryu, C.M., A. Anand, L. Kang, and K.S Mysore. 2004. Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species. Plant J. **40:** 322-331.

Scott, J.W., J.F. Wang, and P.M. Hanson. 2005. Breeding tomatoes for resistance to bacterial wilt, a global view. *In* T. Momol, P. Ji, and J.B. Jones (eds.), Proceeding of 1st International Symposium on Tomato Diseases, Acta Hort., pp. 161-172.

Senthil-Kumar, M., G. Govind, L. Kang, K.S. Mysore, and M. Udayakumar. 2007a. Functional characterization of *Nicotiana benthamiana* homologs of peanut water deficit-

induced genes by virus-induced gene silencing. Planta **225:** 523-539.

Senthil-Kumar, M., R. Hema, A. Anand, L. Kang, M. Udaya-kumar, and K.S. Mysore. 2007b. A systematic study to determine the extent of gene silencing in *Nicotiana benthamiana* and other Solanaceae species when heterologous gene sequences are used for virus-induced gene silencing. New Phytol. **176:** 782-791.

Seo, S., S. Katou, H. Seto, K. Gomi, and Y. Ohashi. 2007. The mitogen-activated protein kinases WIPK and SIPK regulate the levels of jasmonic and salicylic acids in wounded tobacco plants. Plant J. **49:** 899-909.

Thines, B., L. Katsir, M. Melotto, Y. Niu, A. Mandaokar, G. Liu, K. Nomura, S.Y. He, G.A Howe, and J. Browse. 2007. JAZ repressor proteins are targets of the $SCF^{(COI1)}$ complex during jasmonate signalling. Nature **448:** 661-665.

Thomas, C.L., L. Jones, D.C. Baulcombe, and A.J. Maule. 2001. Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector. Plant J. **25:** 417-425.

Vasse, J., S. Danoun, and A. Trigalet. 2005. Microscopic studies of root infection in resistant tomato cv. Hawaii7996. *In* C. Allen and A.C. Hayward (eds.), Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex, APS Press, MN, pp. 285-291.

Wang, J.F. and C.H. Lin. 2005. Colonization capacity of *Ralstonia solanacearum* tomato strains differing in aggressiveness on tomatoes and weeds. *In* C. Allen, P. Prior, and A.C. Hayward (eds.), Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. St Paul: APS Press, pp. 73-79.

Wang, J.F., J. Olivier, P. Thoquet, B. Mangin, L. Sauviac, and N.H. Grimsley. 2000. Resistance of tomato line Hawaii7996 to *Ralstonia solanacearum* Pss4 in Taiwan is controlled mainly by a major strain-specific locus. Mol. Plant-Microbe Interact. **13:** 6-13.

Wei, C.F., B.H. Kvitko, R. Shimizu, E. Crabill, J.R. Alfano, N.C. Lin, G.B. Martin, H.C. Huang, and A. Collmer. 2007. A *Pseudomonas syringae* pv. *tomato* DC3000 mutant lacking the type III effector HopQ1-1 is able to cause disease in the model plant *Nicotiana benthamiana*. Plant J. **51:** 32-46.

Xie, D.X., B.F. Feys, S. James, M. Nieto-Rostro, and J.G. Turner. 1998. *COI1*: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280:** 1091-1094.

Yoshioka, H., N. Numata, K. Nakajima, S. Katou, K. Kawakita, O. Rowland, J.D. Jones, and N. Doke. 2003. *Nicotiana benthamiana* $gp91^{phox}$ homologs *NbrbohA* and *NbrbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. Plant Cell **15:** 706-718.

Yoo, S.D., Y.H. Cho, G. Tena, Y. Xiong, and J. Sheen. 2008. Dual control of nuclear EIN3 by bifurcate MAPK cascades in C_2H_4 signaling. Nature **451:** 789-795.

Zhang, H., D. Zhang, J. Chen, Y. Yang, Z. Huang, D. Huang, X.C. Wang, and R. Huang. 2004. Tomato stress-responsive factor TSRF1 interacts with ethylene responsive element GCC box and regulates pathogen resistance to *Ralstonia solanacearum*. Plant Mol. Biol. **55:** 825-834.

利用煙草脆裂病毒基因靜默系統研究土壤傳播性維管束病原菌 *Ralstonia solanacearum* 引起之病害

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由 *Ralstonia solanacearum* 引起的茄科作物青枯病是一具全球重要性的土壤傳播性維管束病害，然而茄科作物對青枯病菌防禦機制的研究及資料卻尚闕如。在此研究中，為了應用病毒誘導基因靜默系統 (VIGS) 於青枯病及其他土壤傳播性維管束病害相關研究，我們首先評估農桿菌菌株、植物株齡、接種方法及茄科植物品種對 phytoene desaturase (*PDS*) 基因靜默效率的影響，將煙草脆裂病毒的 VIGS 系統最佳化，並發展出子葉注射法。以此方法靜默 *PDS*，發現在所有供試番茄品種上均有良好靜默效果，而部分番椒及所有茄子品種卻無明顯靜默效果。於 9 天大的番茄幼苗靜默植物抗病相關基因 *TGA2*.2 的測試發現，靜默效應在接種 5 天後已經在莖基部開始發生，接著在根部而後在幼葉呈現；同時將不同標的基因之片段建構於同一載體中所達到之多基因靜默效果，比混合數個各自帶有不同標的基因片段農桿菌菌株的方式更佳。利用本文建立之靜默系統進行茉莉酸防禦訊息傳遞途徑關鍵基因 *COI1* 之靜默，結果發現青枯病菌在番茄抗病品種‘Hawaii 7996’莖基部及中段內的菌量顯著增加，顯示茉莉酸防禦訊息傳遞途徑在番茄對青枯病的防禦機制中扮演正面角色，此發現與阿拉伯芥對青枯病的防禦機制研究所知的相反；文中並討論其他防禦訊息傳遞途徑可能如何共同參與番茄青枯病防禦網絡。

關鍵詞：青枯病；茉莉酸防禦訊息傳遞途徑；青枯病菌；病毒誘導基因靜默。