### Virus-induced gene silencing reveals the involvement of ethylene-, salicylic acid- and mitogen-activated protein kinase-related defense pathways in the resistance of tomato to bacterial wilt

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Bacterial wilt (BW), caused by Ralstonia solanacearum, is a devastating vascular disease of tomato worldwide. However, information on tomato's defense mechanism against infection by this soil-borne bacterium is limited. In this study, virus-induced gene silencing (VIGS) was employed to decipher signaling pathways involved in the resistance of tomato to this pathogen. Defined sequence fragments derived from a group of genes known or predicted to be involved in ethylene (ET) and salicylic acid (SA) signaling transduction pathways and mitogen-activated protein kinase (MAPK) cascades were subjected to VIGS in 'Hawaii 7996', a tomato cultivar with stable resistance to BW, and their effect on resistance was determined. The results indicated that silencing of ACO1/3, EIN2, ERF3, NPR1, TGA2.2, TGA1a, MKK2, MPK1/2 and MPK3 caused significant increase in bacterial proliferation in stembases and/or mid-stems. Partial wilting symptoms appeared on plants in which TGA2.2, TGA2.1a, MKK2 and MPK1/2 were silenced. These results suggested that ET-, SA- and MAPK-related defense signaling pathways are involved in the resistance of tomato to BW. This is the first report elucidating the multiple layers of defense governing the resistance of tomato to BW. The results are discussed to enlighten an important and complex interaction between tomato and a soil-borne vascular pathogen.

### Introduction

Plants have developed subtle mechanisms to rapidly sense and fend off pest and pathogen attacks. The key roles various hormones and signaling pathways play in plant defense mechanisms have been demonstrated. Major defense signaling pathways that have been intensively studied include jasmonic acid (JA), ethylene (ET), salicylic acid (SA) and mitogen-activated protein kinase (MAPK) cascades (Koornneef and Pieterse 2008).

Abbreviations – ABA, abscisic acid; BW, bacterial wilt; CFU, colony-forming unit; DPI, days post-inoculation; EST, expression sequence tag; ET, ethylene; H7996, Hawaii 7996; JA, jasmonic acid; LR recombination, recombination of attL sites with attR sites; MAPK, mitogen-activated protein kinase; MAPKKK, MAPK kinase kinase; *R* gene, *RESISTANCE* gene; RT-PCR, reverse transcriptase-PCR; SA, salicylic acid; TRV, tobacco rattle virus; VIGS, virus-induced gene silencing.

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In nature, these pathways are not completely separate. To minimize ineffective defenses and achieve the proper response to specific stress, plants need to temporally and spatially integrate and orchestrate various defense signaling pathways. Certain cross talks and regulation, both positive and negative, occur between these pathways in plants (Bostock 2005, Koornneef and Pieterse 2008).

Bacterial wilt (BW), caused by Ralstonia solanacearum, is a complex and serious soil-borne vascular disease affecting tomato and many other crops worldwide. Because of the lack of effective chemical controls, the soil-borne nature of the pathogen and the wide range of its hosts, host resistance is the main strategy for control (Scott et al. 2005). However, the diverse strains of *R. solanacearum* make the development of stable resistance a challenge. A few tomato cultivars have displayed stable resistance over locations or against various R. solanacearum strains (Wang et al. 1998) with cultivar 'Hawaii 7996' (H7996), the most studied. Although several quantitative trait loci associated with resistance in H7996 have been mapped (Carmeille et al. 2006, Wang et al. 2000), no information on their identity and possible function is currently available. It should be noted that none of the resistance to BW in tomato is immune. Latent infections occur in resistance cultivars of tomato (Prior et al. 1994). The visual wilting symptom caused by R. solanacerum in tomato is correlated with the pathogen distribution and bacterial density in the stem. A high bacterial density at the level of  $10^9 - 10^{10}$ CFU  $g^{-1}$  plant tissue is required for the appearance of wilting (Grimault et al. 1994, Wang and Lin 2005). In H7996, R. solanacearum is largely confined to theprimary xylem and is a poor colonizer in this host (Nakaho et al. 2004). It has been hypothesized that polyphenols produced by H7996 might restrict pathogen spreading through the root (Vasse et al. 2005). Information about the resistance mechanism in H7996 is limited.

Current knowledge about natural plant defense mechanisms against *R. solanacearum* is mostly derived from the studies with *Arabidopsis*. It has been demonstrated that JA-, 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). Additionally, the only *RESISTANCE* gene (*R* gene) against the pathogen isolated to date is *Arabidopsis RRS1*, which recognizes an avirulence protein of *R. solanacearum* strain GMI1000 (Deslandes et al. 2002, 2003, Lahaye 2004). 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 response of solanaceous plants to *R. solanacearum* or other pathogens could be different from those in *Arabidopsis* (Lin et al. 2008a, Robatzek et al. 2007, Wei et al. 2007). Thus, gaining insight into the natural defense mechanism of tomato to BW is crucial; the knowledge would contribute to breeding for more stable resistance as well as overall disease control.

Virus-induced gene silencing (VIGS) has been used in high-throughput reverse and forward genetic screens to study roles ofgenes in various aspects of plant function (Burch-Smith et al. 2004). Among the vectors developed for VIGS, the *tobacco rattle virus* (TRV)-based vector is the most commonly used in a range of plant species, particularly in Solanaceae (Burch-Smith et al. 2004). However, VIGS has not been used to study interactions involving plant and soil-borne vascular pathogens in a systematic manner.

In this study, we employed TRV-based VIGS, combined with an inoculation system mimicking natural infection developed previously (our unpublished data), to explore the role of ET-, SA- and MAPK-related defense signaling pathways in the molecularmechanisms governing BW resistance in tomato cultivar H7996. The results suggest that tomato fends off the bacterium by multiple layers of defense mechanisms, which involve ET-, SA- and MAPK-related defense signaling pathways.

### **Materials and methods**

#### Plant material and growth conditions

Tomato (*Solanum lycopersicum*) cultivars used in this study included H7996 (BW resistant) and L390 (BW susceptible). After soaking in water with gentle shaking for 2 days, the germinated seeds were sown in 2.5-inch pots containing potting mixture. Plants were grown in a growth chamber with 25°C day temperature and 20°C night temperature, a 12 h light period and relative humidity of 75%. Nine to ten days after sowing in soil, when cotyledons were fully expanded, plants were subjected to VIGS tests by inoculation with *Agrobacterium* suspensions (see below).

### Plasmid constructs and Agrobacterium tumefaciens strains

pTRV1 and pTRV2 derivatives were used as the vectors for gene silencing (Liu et al. 2002). Fragments of *ACO1*, *ACO5*, *CTR1*, *EIN2*, *ERF3*, *TSRF1*, *NPR1*, *TGA2.2*, *TGA1a*, *MP3Kα*, *MKK2*, *MKK3*, *MKK4*, *MPK1*, *MPK3* and *MPK4* were obtained by reverse transcriptasepolymerase chain reaction (RT-PCR) from tomato H7996 complementary DNA using primers listed in Appendix S1. The PCR product was cloned into the pCR<sup>®</sup> 8/GW/TOPO<sup>®</sup> vector, and then recombined into a pTRV2 vector, pYL279, by carrying out an LR recombination reaction using the Gateway<sup>®</sup> system (Invitrogen Co., Carlsbad, CA). 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<sup>®</sup> 8/GW/TOPO<sup>®</sup> vector. This was used as the empty vector control. The pYL279 recombinant constructs obtained were electroporated into cells of *A. tumefaciens* GV3101 by a MicroPulser (Bio-Rad, Hercules, CA). The resultant *Agrobacterium* strains were used in the indicated VIGS experiments.

# Virus infection by *Agrobacterium*-mediated infiltration

*A. tumefaciens* GV3101 strains carrying each TRV derivative were grown at  $28^{\circ}$ C in the selection YEP medium (0.5% peptone, 0.5% yeast extract and 1% sodium chloride) containing appropriate antibiotics, and bacterial suspensions (OD<sub>600</sub> = 2) were prepared for infiltration on cotyledons following a similar procedure as described in Liu et al. (2002). Agroinfiltrated plants were maintained at  $22^{\circ}$ C in a growth chamber with a 12 h light period for the indicated periods of time.

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

Inoculation of R. solanacearum on tomato plants was conducted as described in Lin et al. (2008a); the procedure is outlined briefly here. A suspension of strain Pss4 (phylotype I, race 1, biovar 3) was prepared to give an  $OD_{600}$  of 0.6 and used to challenge plants. Ten days after agroinfiltration of the VIGS constructs, the Pss4 suspension was poured over the pot soil surface. A lower inoculum dose  $(5 \times 10^5 \text{ CFU ml}^{-1})$  was used when gene-silencing experiments were carried out in L390 to determine the role of CTR1 in tomato susceptibility to R. solanacearum. The percentages of wilted plants were recorded 5 days after inoculation. Afterward, bacterial density in tomato plants was determined by taking surface-sterilized segments (about 1 cm in length) of stembase and mid-stem of eachplant. The stem segments were weighed, crushed in 1 ml sterilized water, diluted and plated on SM1 medium. The number of bacterial colonies developed was recorded 2 days after incubation at 28°C. For the assay of each gene, 6-12 plants were used in each experiment, and at least three independent experiments were performed. Pair-wise mean comparisons of bacterial density at the stembases and mid-stems were conducted between TRV-infected and silenced plants with Student's *t*-test.

#### Semiquantitative RT-PCR analysis

Total RNA was isolated from the indicated tissues of plants with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Fiveto ten micrograms of total RNA was treated with RNase-free DNase I (Promega, Madison, WI) followed by phenol/chloroform extraction to remove DNase. The first-strand cDNA was synthesized from 1  $\mu$ g total RNA with oligo (dT) primer and AMV reverse transcriptase (Promega). Semiquantitative 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 Appendix S1.

### Results

Silencing of genes involved in the ET signaling transduction pathway decreased resistance of H7996 to BW and enhanced tolerance of L390.

Defined sequence fragments (Table 1), derived from six genes (ACO1, ACO5, CTR1, EIN2, ERF3 and TSRF1) known or predicted to be involved in ET biosynthesis and signaling pathways, were amplified from H7996 and used to develop TRV constructs for VIGS tests in H7996. As revealed in Fig. 1A, the transcript accumulation of these targetedgenes in stembases of silenced plants was reduced at various levels. Except for ACO1 and TSRF1, cross-silencing of other genes trigged by the same fragments is unlikely to occur, as no significant sequence homology (21 continuous identical nucleotides, Thomas et al. 2001) was found between these fragments and ESTs or genes currently available in tomato sequence databases (Table 1). The transcript accumulation of ACO1 and ACO3 was significantly reduced in stembases of ACO1-silenced plants, but not that of ACO4, which shares less sequence homology with ACO1 (Fig. 1A). Additionally, no apparent effects on growth and development of the silenced plants were observed 15 days after agroinfiltration, except that silencing of CTR1 led to typical epinastic phenotypes of constitutive ET response (data not shown).

To determine whether ET biosynthesis and signaling pathways are involved in the resistance of tomato to BW, gene-silenced plants were inoculated with *R. solanacearum* by root drenching 10 days after agroinfiltration. At this time point, VIGS efficacy had already started in roots, stembase and even in young leaves (our unpublished data). Resistance to *R. solanacerum* in tomato is usually correlated with

**Table 1.** Tomato genes tested in this study and comparison of the most closely related paralogs of the test genes. Unigene numbers for tomato were taken from SGN website. Nucleotide identity indicates the overall sequence identity between the two genes. Identical length indicates the length of longest continuous stretch where the sequences are identical between the two genes. '—' indicates no paralog was identified in current databases. N/A, not applicable.

CorrespondinTargetedgenes in tobatomato genesUnigene numberor Arabidopsi		Corresponding genes in tobacco or <i>Arabidopsis</i>	Description	Paralogs [accession no., nucleotide identity %, identical length (bp)]		
ACO1	SGN-U314592		1-aminocyclopropane-1-carboxylate	ACO3 (SGN-U314591, 98%, 194)		
			UNITED STORES	ACO4 (SGN-U314593, 84%, 47)		
ACO5	SGN-U323861		1-aminocyclopropane-1-carboxylate oxidase	—		
CTR1	SGN-U316111		A MAPK kinase kinase (MAPKKK) involved in tomato ET signaling pathway	—		
EIN2	SGN-U319128	AtEIN2 (AT5G03280)	A downstream target ER-membrane protein of CTR1 in <i>Arabidopsis</i> ET signaling pathway	—		
ERF3	SGN-U315194	NtERF3, NbCD1	A member of class II EFF, a transcriptional repressor	_		
TSRF1	SGN-U331411	NtTSRF1	A member of ERF sub-group B-3	ERF1 (SGN-U320682, 88%, 23)		
NPR1	SGN-U320977	AtNPR1 (At1G64280)	A key regulator of the SA-mediated systemic acquired resistance pathway	_		
TGA2.2	SGN-U318626	AtTGA2.2 (At5G06950)	Putative NPR1-interaction transcription factor of the b-ZIP family	<i>TGA-2.1</i> (SGN-U316694, 84%, 23)		
TGA1a	SGN-U321979	AtTGA1a (AT5G65210)	Putative transcription factor with DNA-binding and calmodulin-binding domain	_		
ΜΡ3Κα	SGN-U323157		MEK kinase (MAP3K $\alpha$ )			
МКК2	SGN-U319074	NtMEK2	A member of MAPKK involved in Pto-mediated resistance in tomato	_		
МККЗ	SGN-U325519	NtMEK1	A member of MAPKK	_		
MKK4	SGN-U315916	NtSIPKK	A member of MAPKK involved in Pto-mediated resistance in tomato	_		
MPK1	SGN-U316697	NtSIPK	A member of MAPK involved in pathogen resistance, wounding, ET biosynthesis, oxidative stress and osmotic stress	MPK2 (NtNTF4) (SGN-U316695, 90%, 77		
				<i>МРКЗ</i> (SGN-U313928, 81%, 21)		
				MPK16 (SGN-U216717, 96%, 23)		
МРКЗ	SGN-U313928	NtWIPK	A member of MAPK involved in pathogens resistance, wounding, cold, salinity stress.	<i>MPK1</i> (SGN-U316697, 81%, 21)		
				CDPK (SGN-U332911, 100%; 21)		
MPK4	SGN-U323634	NtNTF6	A member of MAPK	<i>MPK5</i> (SGN-U313996, 83%, 25) <i>MPK16</i> (SGN-U318101, 100%, 24)		

the pathogen distribution and bacterial density in the stem (Grimault et al. 1994, Wang and Lin 2005). Therefore, both visual symptom development and internal bacterial density in stem parts were determined in this experiment. 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 not their response to *R. solanacearum* (Fig. 2). L390 plants first showed wilting symptoms 3 days post-inoculation (DPI) and completely wilted 5 DPI, when the internal

*R. solanacearum* density reached at a mean greater than  $10^9$  CFU g<sup>-1</sup> plant tissue at the mid-stems. None of silenced H7996 plants with reduced transcript level of ET-related genes developed apparent wilting symptoms 12 days after *R. solanacearum* inoculation. Large variations were observed in the bacterial density of assayed H7996 plants. This is because of several reasons, including the high detection limit of the direct plating method ( $\geq 10^3$  CFU g<sup>-1</sup> plant tissue), the effect of plant physiological status and environment on the progress of the disease and the uneven gene silencing degree over tested plants even within the same plant.



**Fig. 1.** VIGS-mediated reduction of specific transcript levels in tomato. BW-resistant cultivar H7996 (A, C, D) and BW-susceptible cultivar L390 (B) were agroinfiltrated with *Agrobacterium tumefaciens* strain GV3101 containing pTRV1 plus pTRV2 containing the targeted genes (silenced plants) or plus pTRV2 empty vector (TRV-infected plants). The targeted genes are known or predicted to be involved in ET biosynthesis and signaling pathways (A, B), SA defense signaling pathway (C) and MAPK cascades (D). Silencing of genes known or predicted to be involved in ET biosynthesis and signaling pathways and genes for which we also examined a closely related paralog are shown in brackets. Ethidium bromide stained 1% agarose gels with RT-PCR products. 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 using gene-specific primers (see Supporting information). Amplification of the internal control *Ubi3* was performed for every gene-silenced plant with similar results (data not shown). 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.

Nevertheless, pair-wise *t*-test indicated that *ACO1/3-*, *EIN2-* or *ERF3-*silenced plants had significantly higher bacterial density in stembases and mid-stems when compared with H7996 plants inoculated with the empty vector (Table 2). These results indicated that silencing the ET signaling pathway decreased the resistance of tomato to BW.

Because *CTR1* is known to be a negative regulator of the ET signaling pathway (Ouaked et al. 2003), the effect of silencing of this gene in response to *R. solanacearum* infection was further assessed in L390. The level of *CTR1* transcript accumulation in stembases of the silenced L390 plants was reduced (Fig. 1B), leading to obvious typical phenotypes of constitutive ET response



**Fig. 2.** Wilting symptoms caused by *Ralstonia solanacearum* on silenced tomato plants. (A) BW-susceptible cultivar L390 plants were agroinfiltrated with *Agrobacterium tumefaciens* strain GV3101 containing pTRV1 plus pTRV2-*CTR1* (*CTR1*) or the pTRV2 empty vector (TRV2); (B) BW-resistant cultivar H7996 plants were agroinfiltrated with *A. tumefaciens* strain GV3101 containing pTRV1 plus the pTRV2 empty vector (TRV2); pTRV2-*TGA2.2* (*TGA2.2*), TRV2-*TGA1a* (*TGA1a*), TRV2-*MPK1* (*MPK1*) or TRV2-*MKK2* (*MKK2*). Ten days after agroinfiltration, the silenced plants and the control plants (those infiltrated with pTRV2 empty vector) were inoculated with *R. solanacearum* by soil drench as described in the Materials and methods. Photos were taken (A) 7 days or (B) 5 days after inoculation with *R. solanacearum*. The BW-susceptible cultivar L390 in (B) was used as a positive control for pathogen inoculation. Arrows indicate wilted leaves.

(Fig. 2A; Liu et al. 2002). When the response of L390 to *R. solanacearum* inoculation was evaluated 5 DPI, all of the TRV-infected control plants displayed severe wilting symptoms. Nevertheless, when *CTR1* was silenced in L390, only 50% of the plants developed partial wilting symptoms and the rest did not show any obvious wilting symptoms (Fig. 2A). These data suggested that silencing of *CTR1* in BW-susceptible tomato L390, which led to constitutive ET response, resulted in reduced severity and incidence of wilting symptoms.

Silencing of genes required for the SA signaling transduction pathway reduced the resistance of tomato to BW.

To determine whether SA-related signaling pathway is involved in the resistance of tomato to BW, fragments derived from tomato homologs of *Arabidopsis NPR1*, *TGA1a* and *TGA2.2* were prepared from and used for VIGS tests in H7996 (Table 1). As shown in Fig. 1C, the level of transcript accumulation of these genes in stembases of the silenced plants was reduced at various levels. Additionally, the silenced plants did not display apparent growth and development defects 15 days after agroinfiltration.

Upon the infection of R. solanacearum, silencing of TGA1a or TGA2.2 in H7996 resulted in a partial wilting phenotype in some, but not all, of the silenced plants 5 DPI (Fig. 2B). Additionally, much higher proportions of TGA1a- or TGA2.2-silenced plants were detected with the presence of the pathogen in stembase and mid-stems compared with the controls (Table 2). Pair-wise t-test also revealed that the silenced plants had significantly higher bacterial density in stembases and mid-stems compared with H7996 plants inoculated with the empty vector (Table 2). Although silencing of NPR1 did not cause wilting symptoms in the silenced plants, a higher proportion of colonized plants and significantly higher density of planta R. solanacearum were detected in midstems and stembases of the silenced plants (Table 2). Although NPR1-silenced plants harbored increased mean bacterial population comparable with that in

**Table 2.** Assessment of *Ralstonia solanacearum* density in silenced tomato H7996 plants. Assessment was carried out 5 DPI by a method described in Materials and methods, and data were collected from at least three independent experiments. Numbers of plants with positive detection of the pathogen in stembases or mid-stems were shown by symbol '+'. Pair-wise mean comparisons were made between TRV-infected and silenced plants with Student's *t*-test method. *P*-values are indicated in parentheses.

			Stembases (silenced/TRV infected)			Mid-stems (silenced/TRV infected)		
Signaling pathways	Silenced genes	Sample no. (silenced/TRV infected)	+	log (CFU g <sup>-1</sup> plant tissue) ( <i>P</i> -value)		+	log (CFU g <sup>-1</sup> plant tissue) ( <i>P</i> -value)	
ET	AC01/3	30/30	20/6	4.91/1.40	(0.0001)	10/4	2.77/0.93	(0.0304)
	ACO5	36/36	12/11	2.27/2.30	(0.9750)	4/3	0.84/0.60	(0.7768)
	CTR1	24/24	14/9	4.35/2.70	(0.1316)	7/2	2.10/0.75	(0.1537)
	EIN2	36/36	17/4	4.16/0.76	(<.0001)	12/3	2.55/0.50	(0.0039)
	ERF3	54/54	28/14	3.92/1.81	(0.0024)	15/6	2.34/0.75	(0.0078)
	TSRF1	30/30	12/13	3.15/3.10	(0.9641)	5/2	1.52/0.50	(0.1313)
SA/JA	NPR1	42/42	22/11	4.45/1.84	(0.0053)	15/4	2.63/0.62	(0.0028)
	TGA2.2	30/30	23/15	6.56/3.73	(0.0040)	21/11	4.35/1.24	(0.0017)
	TGA1a	30/22	15/7	3.84/2.27	(0.1400)	9/0	2.52/0.00	(0.0009)
МАРК	МРЗК-а	30 /30	12/13	2.99/3.13	(0.8861)	2/7	0.47/1.56	(0.0890)
	MKK2	36/36	22/14	5.15/2.75	(0.0095)	15/7	3.53/1.29	(0.0102)
	МККЗ	36/36	13/11	2.73/2.20	(0.5323)	5/5	1.16/1.09	(0.9184)
	MKK4	42/42	23/12	3.93/1.99	(0.0120)	11/6	0.87/0.22	(0.1733)
	MPK1/2	30/30	19/8	4.85/1.82	(0.0013)	10/3	2.84/0.75	(0.0189)
	МРКЗ	42/35	25/12	4.52/2.26	(0.0068)	14/1	2.25/0.21	(0.0004)
	MPK4	48/48	21/16	2.11/1.39	(0.2836)	8/6	1.20/1.03	(0.7600)

*TGA1a*-silenced plants, no visual wilting symptoms were observed. This is because the internal bacterial density in all plant parts of *NPR1*-silenced plants did not reach the level required for visual wilting. There was a large variation in internal bacterial density over and within *TGA1a*-silenced plants, where the bacterial density reached a sufficient level to cause wilting on a few leaves of a few plants. Together, these results indicated that silencing of the SA-related signaling pathway compromised the resistance of tomato to BW.

Silencing of genes involved in MAPK cascades caused reduction of the resistance of tomato to BW.

To examine roles of MAPK cascades in the resistance of tomato to BW, defined sequence fragments, derived from seven genes (MP3Ka, MKK2, MKK3, MKK4, MPK1, MPK3 and MPK4) known or predicted to be involved in MAPK pathways, were used for VIGS tests in H7996 (Table 1). Except for the case in MPK1, cross-silencing of other genes trigged by the same fragments is unlikely to occur (Table 1). Additionally, although a calciumdependent protein kinase gene (CDPK, SGN-U332911) sharing 21 continuous identical nucleotides with MPK3 was identified, its transcripts were not detected in roots, collars, stems and leaves (data not shown). As illustrated in Fig. 1D, the transcript accumulation of all targeted genes, including MPK2, which shares sufficient sequence homology with MPK1, in stembases of silenced plants was reduced at various levels. Furthermore, no

apparent altered phenotypes were observed on the silenced plants 15 days after agroinfiltration.

Upon the inoculation of *R. solanacearum*, silencing of *MKK2* or *MPK1/2* in H7996 resulted in a partial wilting phenotype in some of the assayed plants (Fig. 2B), while silencing of other targeted genes did not cause apparent wilting 12 DPI. However, in most cases, higher proportions of the silenced plants were detected with the presence of the bacterium in stembases and/or mid-stems compared with the controls (Table 2). Consistently, pairwise *t*-test further illustrated that silencing of five (*MKK2, MKK4, MPK1, MPK2* and *MPK3*) of the test genes resulted in significantly increased bacterial proliferation in stembases and/or mid-stems compared with the empty vector (Table 2). These results indicated that silencing of MAPK cascades led to reduced the resistance of tomato to BW.

### Discussion

In this study, we used TRV-VIGS approach to systematically uncover whether certain defense signaling pathways play roles in the resistance of tomato to BW. When VIGS is used for functional studies of genes, concerns about the possible suppression of non-targeted genes are often raised as a shortcoming (Baulcombe 1999; Burch-Smith et al. 2004). However, our results (Table 1, Fig. 1, *ACO4*) along with previous reports (Bhattarai et al. 2007, Ekengren et al. 2003, Senthil-Kumar et al. 2007, Thomas et al. 2001) suggested that,

while a stretch of 21-23 nucleotides with 100% identity to a targeted mRNA is sufficient to trigger gene silencing, it is frequently insufficient to elicit noteworthy co-silencing of paralogs; longer stretches of identical sequences are required. Therefore, although the possibility that we have co-silenced some other related genes cannot be completely ruled out because of the unavailability of the complete tomato genome sequence, we projected that there would be a considerable level of specificity in our VIGS experiments. Another limitation of VIGS is that it often does not lead to uniform and complete repression of the expression of a targeted gene throughout an infected plant (Burch-Smith et al. 2004, Ekengren et al. 2003). A threshold for effective silencing of each gene to exhibit significant and detectable effects on a specific functional context could be different from others, particularly at the translational and the functional activity levels. Therefore, this study is not aimed at evaluating the importance of individual genes that have been silenced, but at evaluating the involvement of certain pathways in the resistance to R. solanacearum, judging from the silencing effect of several genes in the same pathway.

Our study is the first report revealing that the tomato's defense mechanism against BW involves multiple signaling pathways. First, our data together suggested a positive role for the ET response pathway in the resistance of tomato to BW. The ET signaling pathway in plants has been well studied. ACO genes regulate ET biosynthesis, which then inactivates CTR1 (Gao et al. 2003). Inactivation of CTR1 results in de-repression of EIN2 and then activation of ERF1, finally resulting in defense response (Ouaked et al. 2003). Additionally, a Nicotiana benthamiana homolog of ERF3 was shown to induce expression of ERF1 (Nasir et al. 2005). It is known that the ET signaling and response pathway could play a positive or negative role in plant disease defense in different host-pathogen interactions (Lin et al. 2008b, van Loon et al. 2006). Previously, it has been shown that ein2 mutant of the susceptible Arabidopsis ecotype Col0 displayed delayed wilting symptom development compared with the wild-type plant in response to R. solanacearum infection (Hernandez-Blanco et al. 2007), suggesting that the ET signaling pathway may play a negative role in Arabidopsis tolerance to R. solanacearum. However, in this study, we showed that silencing of ACO1/3, EIN2 and ERF3 in BWresistant tomato H7996 caused a significant increase in *R. solanacearum* proliferation, and consistently silencing CTR1 in BW-susceptible tomato L390 led to enhanced tolerance to this bacterium. These results suggested that the ET pathway plays a positive role in the resistance of tomato to BW. Notably, our study on silencing of *COl1* in H7996 suggests that the JA signaling pathway is positively involved in the defense mechanism of tomato against BW (our submitted data). This result also is not in agreement with the report in *Arabidopsis* (Hernandez-Blanco et al. 2007), in which *coi1* mutant of ecotype Col0 displayed delayed development of BW symptom. Therefore, our findings provide additional support for differences in *R. solanacearum* interactions with tomato and *Arabidopsis* (Lin et al. 2008a). Additionally and most significantly, our study depicts the nature of resistance mechanisms of solanaceous plants to *R. solanacearum*, although knocking down of the target genes by taking a transgenic RNAi approach would further help fortify these results.

SA-related defense pathway has been well studied in Arabidopsis where NPR1 is required for positively regulating TGA2.2 and TGA1a to activate the expression of downstream defense genes, resulting in a final defense response. These defense genes, however, are also involved in the cross-communication between SA- and JA-dependent defense signaling pathways (Ndamukong et al. 2007, Thurow et al. 2005). Based on our data, it is suggested that the SA- and JA-related defense pathways are involved in the resistance of tomato against the attack of R. solanacearum. In Arabidopsis, previous reports indicated that inactivation of JAand ET-related signaling pathways, but not SA-related pathways, resulted in increased disease development and bacterial proliferation in the susceptible Col ecotype upon R. solanacearum infection (Hernandez-Blanco et al. 2007, Hirsch et al. 2002), while the resistance of ecotype Nd-1 was demonstrated to be partially SA dependent (Deslandes et al. 2002). Although the R gene (RRS1) has been isolated from Nd-1 and shown to confer enhanced resistance when over-expressed in Col plants (Deslandes et al. 2002), no direct evidence has been obtained for the association between the SA signaling pathway and the RRS1-mediated resistance. Therefore, it is important to further assess whether the studied tomato genes function in the Solanaceae SA-related signaling pathway similarly as in Arabidopsis by looking into the defense mechanism of tomato. Our study reveals a crucial scope for Solanaceae defense response against this bacterium. Further study on the definite function of these putative SA-related genes in tomato disease response is in progress.

Our study suggested that tomato's defense mechanism against BW involves MAPK cascades. This is the first study suggesting the involvement of MAPK cascades in a plant defense mechanism against a xyleminfecting pathogen. Previous studies have demonstrated that MPK1, MPK2, MPK3 and their orthologs are convergent points in various disease-defense signaling networks of solanaceous species after the perception of viral, bacterial, fungal and nematode pathogens by resistance proteins or microbe-associated molecular pattern receptors (Ekengren et al. 2003, Jin et al. 2003, Li et al. 2006, Stulemeijer et al. 2007). In N. benthamiana, these MAPKs are activated by MAPK kinases (MAPKK), such as MEK1 (corresponding to tomato MKK3), MEK2 (corresponding to tomato MKK2) and SIPKK (corresponding to tomato MKK4); MAPKKKa  $(MP3K\alpha)$  acts as an upstream activator above the MAPKK(del Pozo et al. 2004). In tomato, both MKK2 and MKK4 can phosphorylate MPK2 and MPK3 (Pedley and Martin 2004). In tobacco, NTF6 (corresponding to tomato MPK4) is regulated by MEK1 (corresponding to tomato MKK3) (Calderini et al. 2001, Soyano et al. 2003) and SIPKK (corresponding to tomato MKK4) (Gomi et al. 2005). In this study, however, our data did not support the involvement of MP3Kα, MKK3 or MPK4 in tomato's defense mechanism against R. solanacearum. In this case, an unknown MP3K may be involved in the defense mechanism. Nevertheless, because it is possible that the decreased transcript level of these two genes by VIGS was still sufficient to produce enough functional protein in the silenced plant, we cannot definitively exclude their possible role in tomato's BW defense at this point. Future studies will lead to the identification and confirmation of the components of the MAPK cascades in the BW defense mechanism of tomato.

In nature, plants orchestrate multiple defense signaling pathways to achieve proper response to various pathogen attacks (Pieterse and Dicke 2007). On the basis of our results, we proposed that the resistance of tomato to BW involves, at least, ET-, SA- and MAPK-related signaling pathways. In addition, silencing of COI1 attenuated H7996 resistance to BW (our submitted data), suggesting that the JA signaling pathway is positively involved in tomato's defense mechanism against R. solanacearum as well. Hence, JA, ET and SA defense signaling pathways may interact synergistically, rather than antagonistically, in the resistance of tomato against BW. Our study adds to conclusions that JA/ET- and SA-related defense pathways do not always antagonize each other in different combinations of plant and pathogen/pest interactions (Block et al. 2005, Guo and Stotz 2007, Kanneganti et al. 2006, Mur et al. 2006, O'Donnell et al. 2003, Spoel et al. 2007, Thaler et al. 2002). Furthermore, as no case where the level of increased susceptibility to R. solanacearum observed in this study was as severe as seen in a susceptible tomato cultivar (Fig. 2B, Table 2), these defense signaling pathways are suggested to interact in an additive mode. Our previous study showed that over-expression of Arabidopsis NPR1, a central regulator of the SA signaling pathway, in a susceptible tomato cultivar did not lead to the full spectrum of H7996 resistance to R. solanacearum (Lin et al. 2004), further supporting the suggested additive interplays between these pathways in the overall resistance in tomato. Additionally, silencing of SIPK and/or WIPK resulted in the reduction of JA and thus led to SA accumulation in tobacco (Seo et al. 2007), and co-silencing of tomato MPK1 and MPK2 reduced JA biosynthesis and JAdependent signaling pathway (Kandoth et al., 2007). Moreover, MAPK cascades are suggested to be involved in ET signaling transduction pathway in Arabidopsis (Yoo et al. 2008). Thus, certain interplays between MAPK cascades, JA/ET and SA pathways in the tomato's BW defense network may also exist. How a BW-resistant tomato plant tailors the quantity, composition and timing of a defense signal signature in response to the systemic attack of R. solanacearum, which involves orchestration of multiple defense signaling pathways, remains to be investigated further.

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### **Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Appendix S1.** Primers used to amplify candidate genes from tomato H7996 and in RT-PCR analyses to evaluate transcript level of silenced genes. N/A, not applicable.

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