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A novel link between tomato GRAS genes, plant disease resistance and mechanical stress response

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SUMMARY

Members of the GRAS family of transcriptional regulators have been implicated in the control of plant growth and development, and in the interaction of plants with symbiotic bacteria. Here we examine the complexity of the GRAS gene family in tomato (*Solanum lycopersicum*) and investigate its role in disease resistance and mechanical stress. A large number of tomato ESTs corresponding to GRAS transcripts were retrieved from the public database and assembled in 17 contigs of putative genes. Expression analysis of these genes by real-time RT-PCR revealed that six SlGRAS transcripts accumulate during the onset of disease resistance to *Pseudomonas syringae* pv. *tomato*. Further analysis of two selected family members showed that their transcripts preferentially accumulate in tomato plants in response to different avirulent bacteria or to the fungal elicitor EIX, and their expression kinetics correlate with the appearance of the hypersensitive response. In addition, transcript levels of eight SlGRAS genes, including all the *Pseudomonas*-inducible family members, increased in response to mechanical stress much earlier than upon pathogen attack. Accumulation of SlGRAS transcripts following mechanical stress was in part dependent on the signalling molecule jasmonic acid. Remarkably, suppression of *SlGRAS6* gene expression by virus-induced gene silencing impaired tomato resistance to *P. syringae* pv. *tomato*. These results support a function for GRAS transcriptional regulators in the plant response to biotic and abiotic stress.

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INTRODUCTION

The development of genomics techniques for the study of gene expression profiling allowed significant progress in the characterization of plant responses to pathogen attack. Microarray analyses of the Arabidopsis transcriptome revealed that hundreds of genes exhibit differential expression upon triggering the defence programme by a variety of pathogens, messenger molecules and elicitors (e.g. Marathe *et al.*, 2004; Navarro *et al.*, 2004; Schenk *et al.*, 2000). Similarly, over 400 genes were found to be differentially expressed during the resistance response of tomato plants to the bacterial pathogens *Pseudomonas syringae* pv. *tomato* (Mysore *et al.*, 2002) and *Xanthomonas campestris* pv. *vesicatoria* (Bonshtien *et al.*, 2005; Gibly *et al.*, 2004). A complex interplay between activating and repressing transcription factors from multiple families appears to regulate expression of the plant defence transcriptome (Eulgem, 2005). Representatives of the ERF, WRKY, Myb, TGA-bZIP and Whirly families of transcription factors have been shown to bind to promoters of defence-related genes and regulate their expression (Rushton and Somssich, 1998). Moreover, functional analysis of several transcription factors demonstrated their central role in disease resistance (Eulgem, 2005).

Members of the GRAS gene family encode transcriptional regulators that so far have not been directly linked to plant disease resistance. The family name derives from the first three functionally characterized GRAS genes: *GAI*, *RGA* and *SCR* (Di Laurenzio *et al.*, 1996; Peng *et al.*, 1997; Silverstone *et al.*, 1998). These genes are proposed to encode transcription factors based on nuclear localization and transcriptional activation capabilities that have been shown for several family members (Itoh *et al.*, 2002; Morohashi *et al.*, 2003; Silverstone *et al.*, 1998). GRAS proteins exhibit considerable sequence homology to each other in their C-terminus, where several distinguishing domains are located (Pysh *et al.*, 1999; Tian *et al.*, 2004). By contrast, their amino acid sequences are highly variable at the N-terminus, suggesting that this region is responsible for the specificity of their biological functions (Tian *et al.*, 2004). In this regard, a

subgroup of GRAS proteins, which function in several plant species as repressors of gibberellin signalling, share the amino acid sequence DELLA in their N-terminal region and are thus referred to as DELLA proteins (Silverstone *et al.*, 1998). Interestingly, substitutions or deletion of the DELLA motif in proteins of this GRAS subgroup result in a gibberellin-insensitive dwarfish phenotype (Itoh *et al.*, 2005).

Molecular genetic studies in several plant species have demonstrated that GRAS proteins play various roles in fundamental processes of plant growth and development, including gibberellin and phytochrome A signal transduction (Bolle *et al.*, 2000; Silverstone *et al.*, 1998), root radial patterning (Helariutta *et al.*, 2000) and gametogenesis (Morohashi *et al.*, 2003). Recently, two GRAS genes have been shown to be involved in the interaction of *Medicago truncatula* with symbiotic rhizobial bacteria (Kalo *et al.*, 2005; Smit *et al.*, 2005). In tomato, the only characterized GRAS family member is the *Lateral suppressor* (*Ls*) gene, which is required for the initiation of axillary meristems (Schumacher *et al.*, 1999). Recent expression profiling studies have provided first evidence for a possible function of GRAS transcriptional activators in the regulation of plant defence responses: transcripts corresponding to GRAS genes were differentially expressed in resistant tomato plants inoculated with avirulent phytopathogenic bacteria (Bonshtien *et al.*, 2005; Mysore *et al.*, 2002). In addition, tobacco GRAS homologues were reported to be induced upon treatment with hydrogen peroxide, which is well known for its involvement in plant defence responses (Vandenabeele *et al.*, 2003). Moreover, two GRAS genes from rice and a GRAS homologue from solanaceous wild species were found to be induced by the fungal elicitor *N*-acetylchitooligosaccharide and upon attack by the herbivore *Manduca sexta*, respectively (Day *et al.*, 2003; Schmidt *et al.*, 2005).

In this study we analysed the complexity of the GRAS gene family in tomato and investigated the involvement of family members in disease resistance. We identified groups of tomato GRAS genes that show coordinated expression during the onset of disease resistance, or as a result of mechanical stress. Finally, we provide evidence that one of these genes is required for full tomato disease resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato*.

RESULTS

Complexity of the GRAS gene family in tomato plants

Recent analysis of gene expression profiles revealed that members of the GRAS gene family are differentially expressed in tomato plants resistant to phytopathogenic bacteria: in an open-architecture expression profiling study, five expressed sequence tags (ESTs) showing similarities to GRAS genes were found to be specifically induced during the tomato resistance response to *P. syringae* pv. *tomato* (*Pst*) (Table 1) (Mysore *et al.*, 2002). Interestingly, microarray analyses have shown that expression of three of the *Pst*-induced ESTs and two additional GRAS ESTs were up-regulated upon inoculation of resistant tomato lines with *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) strains expressing either the avirulence gene *avrRxv* or *avrXv3* (Table 1) (Bonshtien *et al.*, 2005; A. Gibly and G. Sessa, unpublished data). By sequence analysis we found that the seven bacterial-induced GRAS ESTs correspond to six different genes that were named *SlGRAS1* to *SlGRAS6* (Table 1).

To analyse the complexity of the GRAS gene family in tomato plants, BLAST searches were performed in the tomato EST database of The Institute for Genomic Research (TIGR) by using

Table 1 Fold-change of GRAS gene expression during incompatible vs. compatible interactions of tomato plants with phytopathogenic bacteria.

		Treatment		
TIGR identifier	SlGRAS gene	*Pst* T1A*	*Xcv* T2(*avrXv3*)†	*Xcv* T3(*avrRxv*)‡
EST276979	*SlGRAS1*		3	
EST279139	*SlGRAS2*	2.7	3.2	
EST248297	*SlGRAS2*	2.6		
EST277417	*SlGRAS3*	4.4	2.6	
EST543162	*SlGRAS4*	1.7		5.7
EST299029	*SlGRAS5*			2.6
EST527461	*SlGRAS6*	2.2		

*Resistant and susceptible lines were inoculated with *Pseudomonas syringae* pv. *tomato* strain T1 expressing the *avrPto* avirulence gene (Mysore *et al.*, 2002).

†The tomato line Hawaii 7981 was inoculated with an avirulent strain of *Xanthomonas campestris* pv. *vesicatoria* race T2 (*Xcv* T2) expressing the *avrXv3* gene, or with the virulent strain *Xcv* T2 (A. Gibly and G. Sessa, unpublished).

‡The tomato line Hawaii 7998 was inoculated with an avirulent strain of *Xcv* race T3 expressing the *avrRxv* gene or with the virulent strain *Xcv* T3 (Bonshtien *et al.*, 2005).

coding regions of the previously characterized *Ls* GRAS gene from tomato and of GRAS genes from Arabidopsis and rice (Schumacher *et al.*, 1999; Tian *et al.*, 2004). A large pool of ESTs were retrieved, fully sequenced and assembled in 17 contigs: *SlGRAS1* to *SlGRAS6* included the bacterial-induced ESTs described above (Table 1), and *SlGRAS7* to *SlGRAS17* represented previously uncharacterized transcripts. Sequence analysis of full-length and partial SlGRAS cDNA clones confirmed that they encode proteins with characteristics of GRAS transcriptional activators (Bolle, 2004; Tian *et al.*, 2004). Similar to their counterparts in other plant species, SlGRAS proteins show a high degree of homology in their C-termini, where the majority of them contain five motifs highly conserved in GRAS proteins appearing in the following order: the leucine heptad repeat I (LHRI), the VHIID motif, the leucine heptad repeat II (LHRII), the PFYRE motif and the SAW motif (see supplementary Fig. S1). In addition, a large number of family members contain sequences related to the consensus motif LXXLL, which has been shown to mediate the binding of transcriptional co-activators to nuclear receptors (Heery *et al.*, 1997). As typically observed for other GRAS proteins, the N-terminal sequence of SlGRAS proteins is highly divergent. Interestingly, in several SlGRAS proteins we observed homopolymeric stretches of different amino acids, including Ser, Gln, Thr, Ala, Pro and Asn. Sequences corresponding to putative nuclear localization signals (NLS) were found in SlGRAS1 and 4. To determine the evolutionary relationship among the SlGRAS proteins, a phylogenetic tree was constructed by the neighbour-joining method using an alignment that included the conserved C-terminal amino acid sequences of the following proteins: 14 deduced SlGRAS proteins, for which the C-terminus was available (see 'Experimental procedures'), the tomato Ls, and nine GRAS proteins from other plant species representing previously defined GRAS subfamilies (Lim *et al.*, 2005) (Fig. 1).

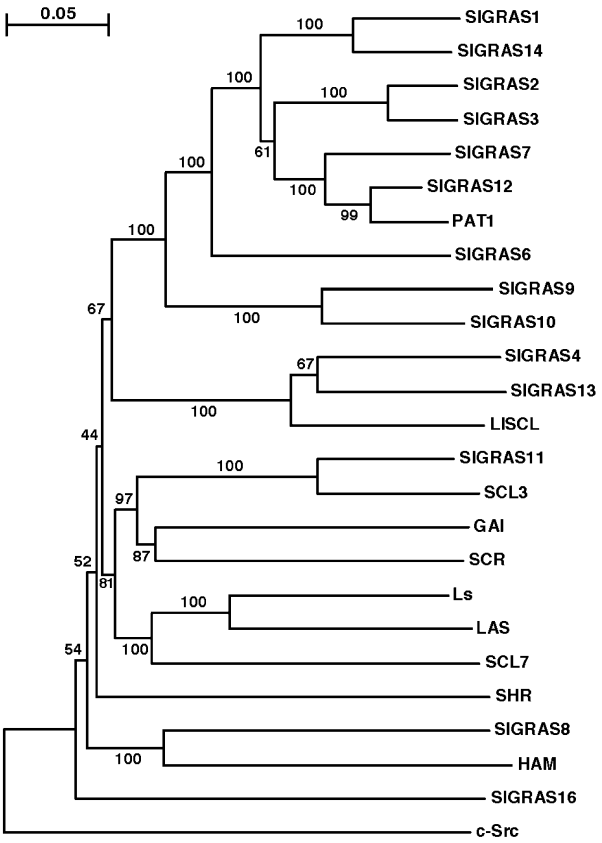

Fig. 1 Phylogenetic tree of GRAS proteins from tomato and other plant species. The neighbour-joining method was used to construct a phylogenetic tree based on an alignment that included the C-terminal amino acid sequences of SlGRAS proteins, of the tomato Ls protein and of nine GRAS proteins from other plant species representing previously defined GRAS subfamilies (Lim *et al.*, 2005) (see 'Experimental procedures' for GenBank accession numbers). The tree was rooted using the C-terminal region of the human STAT c-Src protein as outgroup, as previously reported (Bolle, 2004; Lim *et al.*, 2005). Scale above the tree represents branch length measured by the number of amino acid replacements per position. Bootstrap values are shown for each branch as a percentage of 1000 replicates.

Identification of GRAS genes differentially expressed during incompatible and compatible interactions of tomato with *Pst* bacteria

To identify GRAS genes that may play a role in tomato disease resistance, we examined by real-time RT-PCR RNA levels of tomato GRAS genes following *Pst* infection. These experiments were performed with the tomato line Rio Grande PtoR (RG-PtoR), which carries the *Pto* resistance gene and is resistant to *Pst* strain T1 expressing the *avrPto* gene (*Pst* T1A) (Pedley and Martin, 2003). RG-PtoR plants were inoculated with *Pst* T1A or mock-inoculated, and leaf samples were harvested 3 h after treatment, based on a preliminary analysis of expression kinetics showing high transcript accumulation of *SlGRAS4* and *SlGRAS6* at this time point (data not shown). Total RNA was extracted from samples and used to perform real-time RT-PCR reactions with gene-specific primers for each tomato GRAS gene (see supplementary Table S1). Gene products of the expected size were successfully amplified for 16 of the 18 genes analysed. Efforts to amplify *SlGRAS5* and *Ls* with different primer sets failed, probably due to low abundance of their transcripts in leaf tissues. As shown in Fig. 2A, transcripts of a group of SlGRAS genes, consisting of *SlGRAS1/2/3/4/6* and *13*, accumulated with statistical significance ($P < 0.05$) in *Pst*-infected resistant RG-PtoR plants at higher levels as compared with mock-inoculated plants. This analysis confirmed that in resistant plants *SlGRAS2/3/4* and *6* are responsive to *Pst* T1A (Mysore *et al.*, 2002), and extended this observation to *SlGRAS1* and *13*. The involvement of these genes in basal defence was then examined in the RG-prf3 tomato line, which bears a mutation in the *Prf* gene and is susceptible to *Pst* T1A (Salmeron *et al.*, 1996). Plants were inoculated with *Pst* T1A

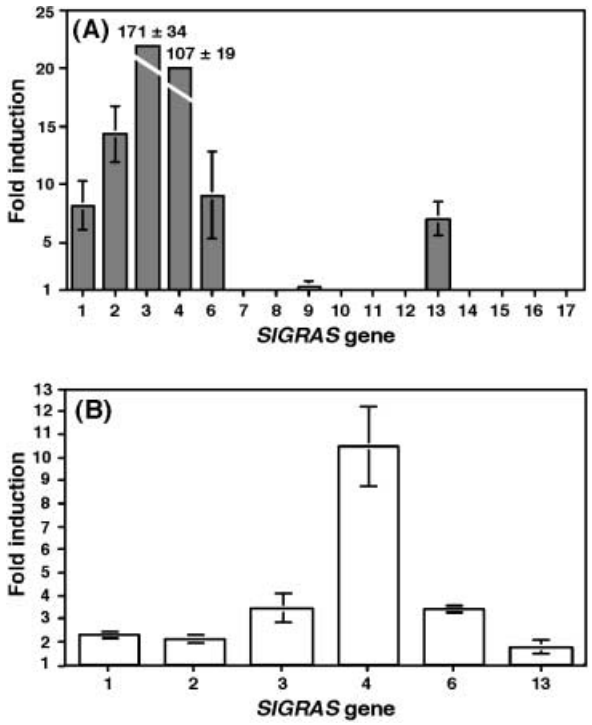

Fig. 2 SlGRAS gene expression in resistant and susceptible tomato plants infected with *Pst* bacteria. Plants of the tomato lines Rio Grande-PtoR (RG-PtoR) (A), or Rio Grande-prf3 (RG-prf3) (B) were infected with a suspension of *Pst* T1A (10^8 cfu/mL), or with a 10 mM $MgCl_2$ control solution. Total RNA was extracted from leaf samples collected 3 h post-inoculation and used to perform real-time RT-PCR analysis for the indicated SlGRAS genes. Expression levels were normalized based on internal standards and used to calculate fold-change in treated vs. mock-inoculated plants. In (A) and (B), values are the average of three and two independent experiments ± SE, respectively. Each experiment included three replicates for each gene.

and gene expression levels were examined, as described above. Interestingly, the *SlGRAS2/3/4/6* and *13* genes accumulated significantly ($P < 0.05$) and with a fold-change of at least 1.5 during the compatible interaction of susceptible RG-prf3 plants with *Pst* T1A (Fig. 2B). However, it should be noted that the fold-change of these genes was much lower in the compatible than in the incompatible interaction. These results define a group of GRAS genes that accumulate with different amplitudes during tomato gene-for-gene disease resistance and basal defense.

Transcripts of tomato GRAS genes accumulate during multiple race-specific resistance responses and following treatment with the EIX elicitor

To test inducibility of SlGRAS genes during incompatible and compatible interactions of tomato plants with different phytopathogenic bacteria, we monitored transcript accumulation of two representative SlGRAS genes in response to three different combinations of virulent and avirulent isogenic bacteria differing only by expression of an avirulence gene. The SlGRAS genes selected for this analysis were *SlGRAS4* and *SlGRAS6*, which in real-time RT-PCR experiments were found to accumulate in response to *Pst* T1A at high and intermediate levels, respectively. The tomato lines used in these experiments were: RG-PtoR, resistant to *Pst* T1A and susceptible to *Pst* T1; Hawaii 7998, resistant to *Xcv* race T3 expressing *avrRxv* [T3(*avrRxv*)] and susceptible to *Xcv* T3 (Bonshtien *et al.*, 2005); and Hawaii 7981, resistant to *Xcv* T2 expressing *avrXv3* (T2[*avrXv3*]) and susceptible to *Xcv* T2 (Gibly *et al.*, 2004). Plants were inoculated with suspensions of avirulent or virulent isogenic strains. A mock-inoculation treatment was also included to control for changes in gene expression resulting from vacuum-infiltration. As expected, the appearance of a typical hypersensitive response (HR) in plants treated with avirulent strains was earlier than that of disease symptoms in plants treated with corresponding isogenic virulent strains. Northern blot analysis of RNA extracted from leaf tissues sampled at different times after treatment showed that *SlGRAS4* and *SlGRAS6* accumulated significantly in resistant plants during incompatible interactions, but at much lower levels during isogenic compatible interactions (Fig. 3A–C). *SlGRAS4* and *SlGRAS6* RNA levels peaked at 4 h after treatment in RG-PtoR plants infected with *Pst* T1A (Fig. 3A). The two transcripts reached maximal levels at 10 h post-inoculation in Hawaii 7981 treated with *Xcv* T2(*avrXv3*) (Fig. 3B), whereas their accumulation peaked at 12 h in Hawaii 7998 infected with *Xcv* T3(*avrRxv*) (Fig. 3C). These gene expression kinetics correlated well with appearance of the HR in the different lines: as reflected by the earlier accumulation of *SlGRAS4* and *SlGRAS6* in these plants, RG-PtoR showed the fastest response with an HR appearing at 6 h after treatment, whereas in Hawaii 7981 and Hawaii 7998 the HR developed later, at 16 and 18 h post-infection, respectively.

Next, we assessed whether expression of SlGRAS genes is affected exclusively by bacterial avirulence proteins, or also by other elicitors of plant defence responses. To this aim, *SlGRAS4* and *SlGRAS6* transcript accumulation in response to the ethylene-inducing xylanase (EIX) elicitor from *Trichoderma viride* was tested in the near isogenic lines M82 and IL7-5, which are sensitive and insensitive to EIX, respectively (Ron and Avni, 2004). The response to the EIX elicitor is mediated by the membrane-bound LeEIX2 receptor and is typified by enhanced ethylene biosynthesis and localized cell death. M82 and IL7-5 plants were vacuum-infiltrated with a solution of EIX or mock-inoculated, and a typical HR was observed 36 h after treatment only in M82 plants treated with EIX. The appearance of the HR in M82 was preceded by the accumulation of increasing amounts of *SlGRAS4* and *SlGRAS6* transcripts (Fig. 3D), while very low accumulation was observed in the EIX-insensitive line IL7-5. These observations strengthen and widen results of high-throughput expression profiling studies (Table 1), and suggest that SlGRAS genes may participate in

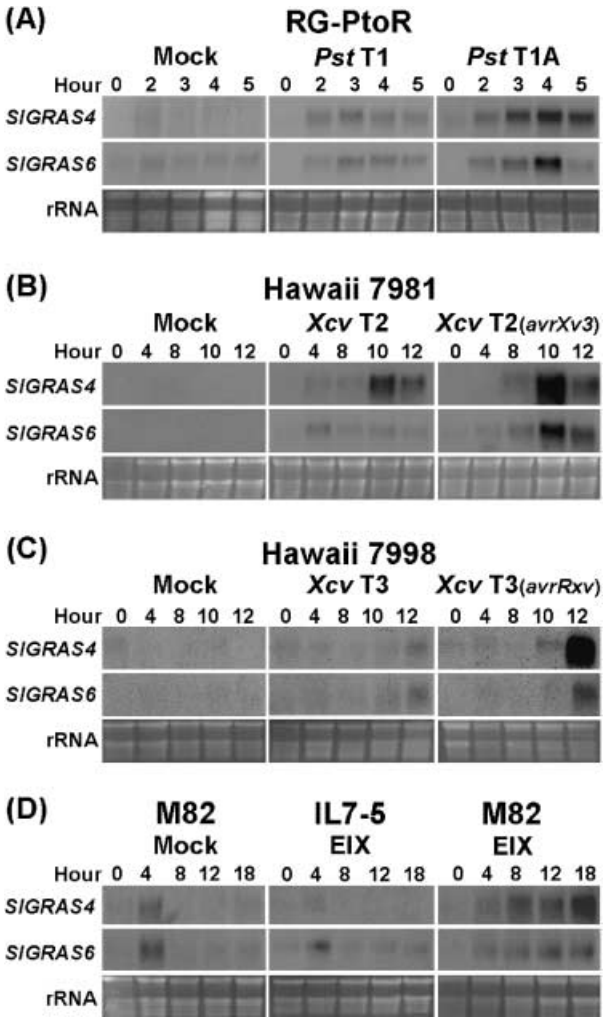

Fig. 3 *SlGRAS4* and *SlGRAS6* transcript accumulation in response to *Pst* and *Xcv* infection and to the fungal elicitor EIX. Tomato plants were infiltrated with a 10 mM $MgCl_2$ control solution (Mock) or treated as follows: (A) Rio Grande-PtoR (RG-PtoR) plants inoculated with *Pst* T1 or T1A (10^8 cfu/mL); (B) Hawaii 7981 plants inoculated with *Xcv* T2 or *Xcv* T2(*avrXv3*) (10^8 cfu/mL); (C) Hawaii 7998 plants inoculated with *Xcv* T3 or *Xcv* T3(*avrRxv*) (10^8 cfu/mL); (D) M82 and IL7-5 plants infiltrated with a solution of 0.5 mg/L EIX. Total RNA was extracted from leaf samples collected at the indicated time points and subjected to Northern blot analysis using *SlGRAS4* and *SlGRAS6* gene-specific probes. Equal loading of RNA was confirmed by ethidium bromide staining of ribosomal RNA (rRNA).

defence responses triggered by specific recognition events between bacterial effectors and their corresponding resistance proteins, and between a cultivar-specific elicitor and its receptor.

Transcripts of tomato GRAS genes accumulate in response to mechanical stress and wounding

While investigating gene expression kinetics of *SlGRAS4* and *SlGRAS6* during the tomato resistance response, we often noticed a significant accumulation of these transcripts at early time points after treatment in bacterial-infected as well as in mock-inoculated plants (Fig. 3). This observation raised the possibility that these genes respond to the mechanical stress caused by vacuum-infiltration and are involved in the plant response to abiotic stress. To test this hypothesis, we monitored changes in *SlGRAS4* and *SlGRAS6* gene expression in response to wounding caused by rubbing the leaf surface with carborundum powder or to mechanical stress caused by vacuum-infiltration. A rapid and transient accumulation of *SlGRAS4* and *SlGRAS6* transcripts was observed in response to both treatments (Fig. 4A). The two transcripts reached maximal level between 30 min and 1 h after treatment and slowly decreased therafter. It should be noted that the peak of *SlGRAS4* and *SlGRAS6* accumulation triggered by mechanical stress is significantly earlier than that observed during the response to avirulent bacteria, which were infiltrated in the plants by vacuum.

To identify additional SlGRAS genes whose expression is modulated by mechanical stress, we tested the inducibility of SlGRAS family members in response to vacuum-infiltration. RNA was extracted from samples collected 30 min after treatment or from untreated plants, and used in real-time RT-PCR experiments, as described above. Analysis of the data indicated that RNA levels of eight family members, *SlGRAS1/2/3/4/6/9/12* and *13*, significantly ($P < 0.05$) increased in response to mechanical stress (Fig. 4B). Interestingly, with the exception of *SlGRAS9* and *SlGRAS12*, transcripts of all other SlGRAS genes were previously found to accumulate also in response to *Pst* but with different timing (Fig. 3A). These findings indicate that a large group of SlGRAS genes may be involved in stress responses that are not necessarily associated with host cell death. In addition, transcripts of a subset of these genes may accumulate with distinct kinetics in response to both biotic and abiotic stimuli.

The signalling molecule jasmonic acid (JA) plays a central role in wound-induced gene expression (Wasternack *et al.*, 2006). To test whether accumulation of SlGRAS genes triggered by mechanical stress is mediated by JA, we used plants of the tomato line Castlemart carrying a mutation in the *jai1-1* gene that are impaired in JA signalling (Li *et al.*, 2004). Wild-type and *jai1-1* mutant plants were subjected to mechanical stress and leaf samples were harvested 30 min after treatment. Real-time RT-PCR analysis was then performed for SlGRAS genes whose RNA levels were found to increase following mechanical stress (*SlGRAS1/2/3/4/6/9/12* and *13*; Fig. 4B). In Castlemart plants, transcripts of all tested SlGRAS genes accumulated in response to mechanical stress with statistical significance ($P < 0.05$) (Fig. 4C). However, although RNA of most of them accumulated also in the *jai1-1* mutant, their transcript levels in these plants were reduced as compared with wild-type plants. These results indicate that the transcript accumulation observed for a group of SlGRAS genes in response to mechanical stress is mediated in part by JA signalling.

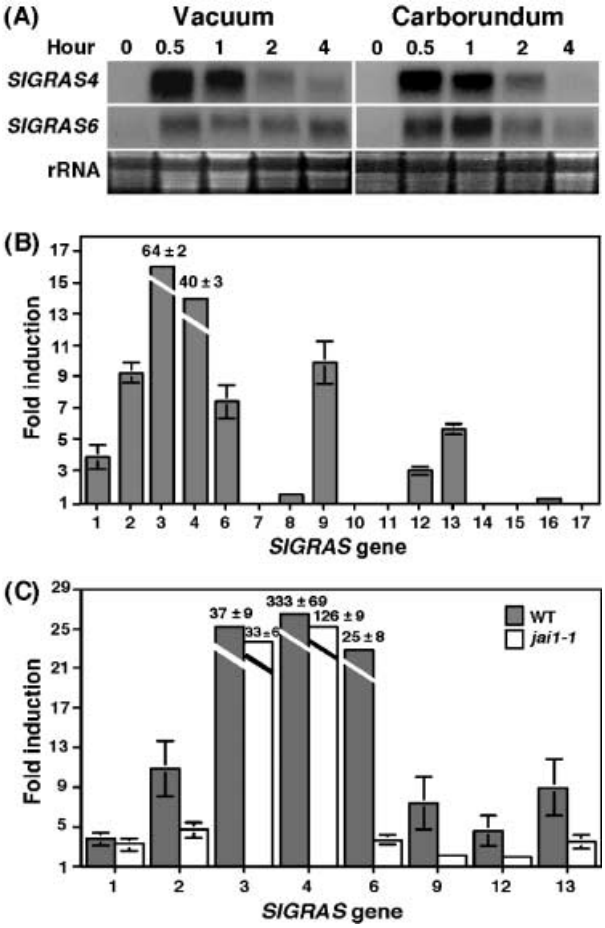

Fig. 4 Accumulation of SlGRAS transcripts in response to wounding and mechanical stress. (A) Total RNA was extracted from leaf samples collected at the indicated time points from Rio Grande (RG-PtoR) plants that were rubbed with wet carborundum or vacuum-infiltrated with a 10 mM $MgCl_2$ solution. Northern blot analysis was performed with *SlGRAS4* and *SlGRAS6* gene-specific probes. Equal loading was confirmed by ethidium bromide staining of ribosomal RNA (rRNA). (B) RG-PtoR plants, (C) Castlemart wild-type or *jai1-1* mutant plants were vacuum-infiltrated with a 10 mM $MgCl_2$ solution. Total RNA was extracted from leaf samples collected 30 min after treatment and used for real-time RT-PCR analysis. Expression levels were normalized, and the fold-change in treated vs. untreated plants was calculated. Values are average of three independent experiments ± SE. Each experiment included three replicates for each gene.

Reduction of *SlGRAS6* gene expression compromises tomato disease resistance to *Pst* bacteria

To investigate the role of SlGRAS genes in disease resistance, we reduced the expression of selected *Pst*-inducible GRAS genes in RG-PtoR tomato plants by virus-induced gene silencing (VIGS). VIGS techniques have been successfully used in several plant species to assess the requirement of certain genes in plant disease resistance (e.g. Cai *et al.*, 2006; Ekengren *et al.*, 2003; Liu *et al.*, 2004). For these experiments, cDNA fragments corresponding to *SlGRAS2/3/4* and *6* were cloned into the tobacco rattle virus (TRV) vector pTRV2 (Liu *et al.*, 2002). Constructs were transformed into *Agrobacterium* and syringe-infiltrated into 1-week-old RG-PtoR seedlings in a mixture with *Agrobacterium* carrying the pTRV1 vector. As controls, seedlings were infected with empty TRV, with TRV carrying the phytoene desaturase (*PDS*) gene or a fragment of the *Prf* gene, which plays a central role in *Pto*-mediated disease resistance (Salmeron *et al.*, 1996). Silencing of *PDS* causes photobleaching of leaves and was used to ensure that conditions were conducive for silencing. To assess the effect of silencing GRAS genes on disease resistance, 3 weeks after TRV infection, plants were inoculated with a bacterial suspension of the *Pst* strain DC3000, which expresses the avirulence genes *avrPto* and *avrPtoB*, and is avirulent to RG-PtoR tomato plants (Kim *et al.*, 2002; Ronald *et al.*, 1992). Five days later, plants were scored for the appearance of bacterial speck symptoms. Among the four tested GRAS genes, silencing of only *SlGRAS6* caused a breakdown of disease resistance (Fig. 5A). Symptoms of disease appeared in five out of nine *SlGRAS6*-silenced plants, and in ten out of 12 *Prf*-silenced plants. However, silencing of *SlGRAS6* determined the appearance of fewer and smaller lesions, as compared with those caused by silencing of the *Prf* gene. This difference probably reflects the downstream position of SlGRAS6 in the resistance pathway relative to Prf, or may result from gene redundancy. Requirement of *SlGRAS6* for full disease resistance was also supported by measurements of bacterial growth in *SlGRAS6*-silenced leaves. As shown in Fig. 5B, silencing of *SlGRAS6* caused a statistically significant ($P < 0.05$), although moderate, increase of bacterial populations in *SlGRAS6*-silenced leaves, as compared with leaves infected with empty TRV. In agreement with the observed degree of lesioning, bacterial counts in *Prf*-silenced leaves were considerably higher than in *SlGRAS6*-silenced plants (Fig. 5B). Semiquantitative RT-PCR analysis showed that *SlGRAS6* transcript levels in silenced plants were reduced by approximately 85% (Fig. 5C). Because Pto recognition of either AvrPto or AvrPtoB, which are expressed in the *Pst* DC3000 strain used in these experiments, is sufficient to mediate tomato disease resistance (Kim *et al.*, 2002; Ronald *et al.*, 1992), it is likely that SlGRAS6 is an important component of signalling pathways downstream of both the Pto/AvrPto and the Pto/AvrPtoB recognition events. Taken together, these results provide the first functional evidence for the involvement of a GRAS gene in plant disease resistance to phytopathogenic bacteria.

DISCUSSION

Transcriptional regulators of the GRAS family play essential roles in a variety of growth and developmental processes that are unique to plants (Achard *et al.*, 2006; Bolle, 2004; Kalo *et al.*,

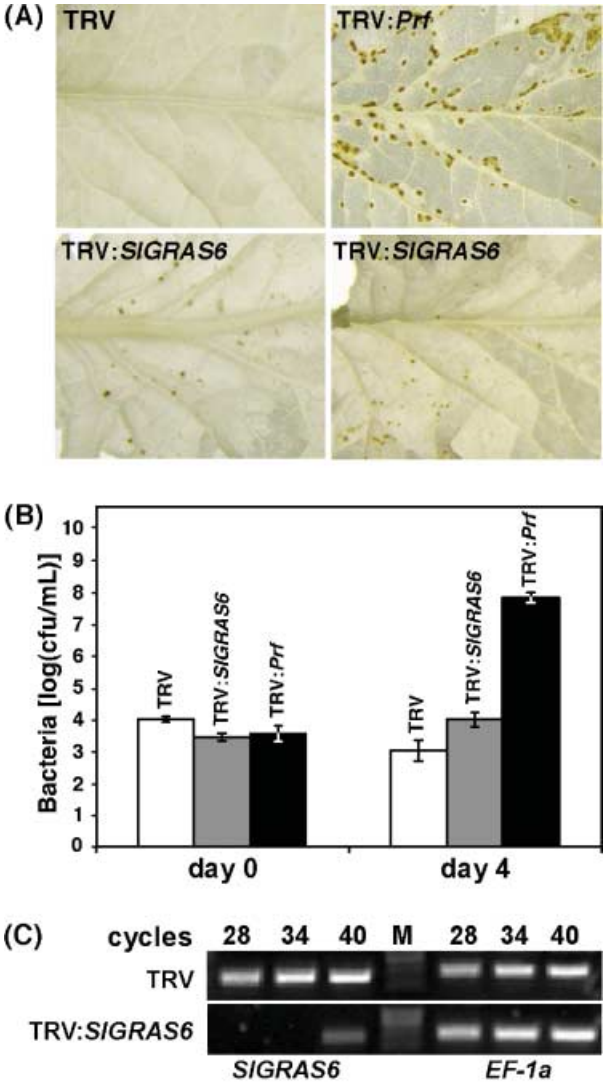

Fig. 5 Effect of SlGRAS6 virus-induced gene silencing in tomato leaves on resistance to *Pst* bacteria. (A) Symptoms of bacterial speck disease on Rio Grande-PtoR (RG-PtoR) tomato leaves silenced for *SlGRAS6* or *Prf*. RG-PtoR plants resistant to *Pst* DC3000 were infected with TRV, TRV:*Prf* or TRV:*SlGRAS6* as indicated. Three weeks later, plants were sprayed with a suspension of *Pst* DC3000 and monitored for the appearance of bacterial speck symptoms. Leaves were cleared in 10% acetic acid, 30% chloroform, 60% ethanol and photographed. Experiments included at least three plants for each treatment and were repeated three times with similar results. (B) Growth of *Pst* in leaf tissue of *SlGRAS6*-silenced plants. The number of colony-forming units (cfu) per millilitre of plant extract was determined 1 h (day 0) and 4 days (day 4) after infection in leaves of RG-PtoR plants treated as in A. Data represent the average and standard deviation of three leaf discs for each of three independently silenced plants. (C) RT-PCR analysis of *SlGRAS6* (left) and *EF-1a* (right) transcripts in plants inoculated with TRV:*SlGRAS6* (lower panel) and with the empty TRV vector (upper panel). PCR products were sampled at the indicated cycles, separated on agarose gel and stained with ethidium bromide. Lane M denotes DNA marker.

2005; Smit *et al.*, 2005). In this study, by database searches we identified a large number of GRAS genes that are expressed in tomato plants. In addition, by gene expression analysis and functional studies we established a link between a group of SlGRAS family members, plant disease resistance and the response to mechanical stress.

Analysis of EST sequences retrieved from public databases, along with that of the previously characterized *Ls* gene (Schumacher *et al.*, 1999), revealed that at least 18 members of the GRAS family are expressed in tomato. Based on similar searches of EST databases, groups of 19, 31 and 20 GRAS genes were reported to be expressed in Arabidopsis, rice and maize, respectively (Bolle, 2004; Lim *et al.*, 2005; Tian *et al.*, 2004). However, it should be noted that in the genomes of Arabidopsis, rice and maize GRAS gene families are significantly larger and consist of 33, 57 and 50 genes, respectively. This apparent discrepancy has been explained by the presence in the plant genome of a number of GRAS pseudogenes and possibly by low expression levels of certain family members (Tian *et al.*, 2004). By aligning encoded amino acid sequences of tomato GRAS genes, we found that all five GRAS signature motifs (Bolle, 2004) are conserved in the majority of SlGRAS family members. As previously observed for GRAS proteins of other plant species and in line with their proposed function, many of them harbour typical motifs of transcriptional regulators. However, a rigorous analysis of transcriptional activation capabilities, DNA binding and subcellular localization will be required to demonstrate definitely a function for SlGRAS proteins as transcription factors or co-activators.

Detailed expression kinetics examined for two family members, *SlGRAS4* and *SlGRAS6*, revealed that they accumulate during the incompatible interactions of tomato with *Xcv* and *Pst* bacteria with kinetics correlating well with the different timing of HR appearance in the infected plants. Enhanced transcript accumulation of both genes was also detected in response to the fungal elicitor EIX, indicating that SlGRAS genes can be induced by a wide variety of race-specific avirulence factors and elicitors. Consistent with this observation, two GRAS genes from rice were recently shown to be induced by the *N*-acetylchitooligosaccharide elicitor of defence responses (Day *et al.*, 2003). The co-ordinated kinetics observed for *SlGRAS4* and *SlGRAS6* gene expression may reflect a common regulatory mechanism responsible for transcript accumulation of several SlGRAS family members in response to multiple elicitors of defence responses.

Expression analysis of the whole SlGRAS gene family by real-time RT-PCR identified a group of six SlGRAS genes that accumulate in resistant tomato plants infected with an avirulent *Pst* strain. This analysis confirmed our previous expression profiling study, which revealed the inducibility of *SlGRAS2/3/4* and *6* during the incompatible interaction of tomato plants with *Pst* bacteria (Mysore *et al.*, 2002), and extended this observation to two additional SlGRAS genes, *SlGRAS1* and *SlGRAS13*.

Interestingly, *SlGRAS1/2/3* and *4* were recently found in microarray experiments to accumulate also in at least one of two incompatible interactions of tomato plants with *Xcv* bacteria (Table 1), whereas *SlGRAS13* was not previously associated with disease resistance. Fold-change values were considerably higher in the present analysis as compared with those determined in microarray experiments, probably reflecting the higher sensitivity and accuracy of real-time RT-PCR techniques. A slight but significant increase in RNA levels of a similar group of SlGRAS genes was observed also during the compatible interaction of susceptible tomato plants with a *Pst* virulent strain. This is consistent with the notion that susceptible and resistant plants respond to pathogen attack by activating similar molecular mechanisms but with different timing and amplitude (Tao *et al.*, 2003). Transcript accumulation observed for a large group of GRAS genes during the interaction of tomato plants with phytopathogenic bacteria suggests that these putative transcriptional regulators play a role in the reprogramming of gene expression that is required for the activation of defence responses. Interestingly, the involvement of GRAS proteins in plant–microbe interactions is not unprecedented: the NSP1 and NSP2 GRAS proteins from the legume plant *M. truncatula* were recently reported to regulate gene expression changes that lead to the establishment of a symbiotic interaction between the plant and rhizobial bacteria (Kalo *et al.*, 2005; Smit *et al.*, 2005). In addition, a root-knot nematode secretory peptide involved in parasitism was recently shown to interact physically with two Arabidopsis GRAS proteins (Huang *et al.*, 2006).

Transcripts of the six SlGRAS genes whose RNA levels increase during the response of resistant plants to *Pst* bacteria and of two yet uncharacterized family members, *SlGRAS9* and *12*, accumulate in concert and at early time points following mechanical stress. This finding suggests a role for this group of SlGRAS genes in the plant defence response to insect attack. In support of this hypothesis, gene expression of a GRAS family member was recently found to be induced in the solanaceous species *Nicotiana attenuata* and *Solanum nigrum* upon attack of the herbivore *Manduca sexta* (Schmidt *et al.*, 2005). Many defence-related genes are activated in plants by wounding, largely through a signal transduction pathway mediated by the lipid-derived molecule JA (Wasternack *et al.*, 2006). However, not all wound-induced genes require the signalling molecule JA for their expression: expression profiling of JA-insensitive Arabidopsis mutants identified large groups of genes activated by wounding in a JA-dependent or JA-independent manner (Devoto *et al.*, 2005). The finding that transcript accumulation of a number of SlGRAS genes in response to mechanical stress is only partially compromised in the JA-insensitive mutant *jai1-1* strongly suggests that their activation is subject to control of both JA-dependent and JA-independent signalling pathways.

Functional analysis of pathogen-induced SlGRAS genes by virus-induced gene silencing revealed that *SlGRAS6* is required for full tomato disease resistance to *Pst* bacteria mediated by the resistance gene *Pto*. As previously observed by silencing the expression of other genes involved in resistance to *Pst* (Ekengren *et al.*, 2003), symptoms of bacterial speck disease in *SlGRAS6*-silenced plants were less severe than those seen in control plants silenced for *Prf*. In addition, *Pst* bacterial populations increased significantly in *SlGRAS6*-silenced plants but at a lower extent than in *Prf*-silenced plants. These differences probably reflect the downstream position of SlGRAS6 in the Pto signal transduction pathway relative to Prf, which is thought to participate in very early signalling events of the pathway (Pedley and Martin, 2003). SlGRAS6 thus appears to act in one of several parallel Pto/Prf-activated pathways that contribute additively to overall resistance. Alternatively, it is possible that residual gene expression levels in silenced tissues or functional redundancy between pathogen-induced SlGRAS genes may account for the relatively low impact of *SlGRAS6* silencing on disease resistance, as well as for the lack of a phenotype in plants silenced for other *Pst*-induced SlGRAS genes. A concern related to gene silencing is that genes closely related to the target gene can also be silenced. Effective VIGS has been reported to require a region of perfect nucleotide identity over at least 23 nucleotides (Thomas *et al.*, 2001). A comparison between the *SlGRAS6* fragment used for silencing and available sequences of other SlGRAS genes did not reveal any stretch of perfect sequence identity longer than 23 nucleotides. Moreover, we and others have reported that in several instances of VIGS experiments designed as for the SlGRAS gene family, transcript levels of genes homologous to the target gene were not reduced despite their nucleotide identity over a $>$ 23-bp region (Ekengren *et al.*, 2003; Liu *et al.*, 2004). Although we cannot exclude the possibility that in our experiments we have silenced other GRAS genes whose sequences are highly homologous to *SlGRAS6* and not yet present in the tomato EST database, these observations support a substantial degree of specificity in our VIGS approach.

In conclusion, our findings related to the requirement of *SlGRAS6* for full disease resistance to *Pst*, and to the RNA accumulation of groups of SlGRAS genes in response to different biotic stimuli and mechanical stress, provide support for a novel defence function for the multifaceted GRAS family of transcriptional regulators.

EXPERIMENTAL PROCEDURES

Bacterial strains and plant material

Bacterial strains used are: *Pseudomonas syringae* pv. *tomato* strain DC3000, T1 and T1 expressing *avrPto* (*Pst* T1A) (Ronald *et al.*, 1992); *Xanthomonas campestris* pv. *vesicatoria* race T2 strain 110C (*Xcv* T2), and *Xcv* T2 expressing *avrRxv* (Bonshtien *et al.*, 2005); *X. campestris* pv. *vesicatoria* race T3 strain 97-2

(*Xcv* T3) and *Xcv* T3 expressing *avrXv3* (Gibly *et al.*, 2004); *Agrobacterium tumefaciens* strain GV3101.

Tomato (*Solanum lycopersicum*) cultivars used are: Rio Grande PtoR (*Pto/Pto*, *Prf/Prf*), Rio Grande prf3 (*Pto/Pto*, *prf/prf*) (Pedley and Martin, 2003), Hawaii 7981 (Scott *et al.*, 1995), Hawaii 7998 (Yu *et al.*, 1995), near-isogenic lines M82 and IL7-5 (Ron and Avni, 2004), Castlemart and *jai-1* mutant in Castlemart background (Li *et al.*, 2004).

Plant treatments and measurement of bacterial growth *in planta*

Cultures of *Xcv* or *Pst* bacteria were prepared as described by Mayrose *et al.* (2004), and diluted to a concentration of 10^8 cfu/mL in 10 mM $MgCl_2$ and 0.5% (v/v) Silwett-L77. Six-week-old plants were inoculated with bacterial suspensions by vacuum-infiltration or by spraying in gene expression and VIGS experiments, respectively. Bacterial populations were measured in leaf tissue collected 1 h and 4 days after inoculation as described by Ekengren *et al.* (2003), and differences among treatments were statistically analysed by Student's *t*-tests for independent samples with the level of significance set at $P < 0.05$. EIX and wounding treatments were carried out as described by Mayrose *et al.* (2004).

DNA sequence analysis

SlGRAS ESTs were retrieved from the database of The Institute for Genomics Research (TIGR), obtained from the Sol Genomics Network stock centre (SGN; Ithaca, NY), and sequenced by using an ABI Prism 3100 Genetic analyser (PerkinElmer, Wellesley, MA). A full-length *SlGRAS1* cDNA clone was obtained combining TIGR EST276979 and a cDNA fragment amplified by 5′-RACE with the BD SMART™ RACE cDNA Amplification Kit (BD Biosciences, Clontech, San Jose, CA). A full-length *SlGRAS6* cDNA clone was isolated by screening a tomato cDNA library constructed from Rio Grande-PtoR plants inoculated with *Pst* T1A bacteria. GenBank accession numbers of *SlGRAS* genes are: *SlGRAS1*, DQ399824; *SlGRAS2*, DQ399825; *SlGRAS3*, AW033846; *SlGRAS4*, DQ399826; *SlGRAS5*, DQ399827; *SlGRAS6*, DQ399828; *SlGRAS7*, DQ399829; *SlGRAS8*, AW931618; *SlGRAS9*, DQ399830; *SlGRAS10*, DQ399831; *SlGRAS11*, AI772452; *SlGRAS12*, BI211172; *SlGRAS13*, BI922397; *SlGRAS14*, AI485697; *SlGRAS15*, AW220126; *SlGRAS16*, DQ399832; *SlGRAS17*, BG132364.

Alignment and phylogenetic analysis

A multiple sequence alignment for the conserved C-terminus of deduced SlGRAS proteins and GRAS proteins from other plant species was performed by ClustalW (Thompson *et al.*, 1994). Proteins included in the alignment are: SlGRAS1/2/3/4/6/7/8/9/10/11/12/13/14/16 and Ls (GenBank accession no. AAD05242), HAM (AAM90848), SHR (NP_195480.1), SCL7 (NP_190634), LAS (NP_175954), SCR (NP_190990.1), GAI (NP_172945.1), SCL3 (NP_175459), PAT1 (NP_974903.1) and LlSCL (BAC77269). Because full-length clones were not available for the *SlGRAS12/13* and *14* genes, only partial sequences of their deduced proteins were aligned. SlGRAS5 and 17 were not included in the alignment because predicted translation frames of the *SlGRAS5* and *SlGRAS17* genes were interrupted by stop codons, whereas SlGRAS15 was not included because sequences of the 3′ end of the *SlGRAS15* gene were not available. The C terminal region of a human (*Homo sapiens*) STAT protein (c-Src, NP_004374) was aligned and used as outgroup in the phylogenetic analysis as previously reported (Bolle, 2004; Lim *et al.*, 2005). A phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) as implemented in ClustalW, and bootstrap analyses were conducted using 1000 replicates to measure node robustness.

Northern blot analysis

Leaf samples were used to extract RNA with the SV total RNA isolation kit (Promega Co., Madison, WI). Total RNA was fractionated, blotted and hybridized as described by Mayrose *et al.* (2004). The gene-specific probes used are: for *SlGRAS4* (GenBank accession no. DQ399826), a fragment from position 209–588 in the *SlGRAS4* nucleotide sequence; for *SlGRAS6* (DQ399828), a fragment from position 1808–2293 in the *SlGRAS6* nucleotide sequence.

Real-time RT-PCR analysis

Total RNA was extracted from 100 mg leaf tissue using the Plant RNA Isolation Reagent (Invitrogen, Carlsbad, CA). To remove residual genomic DNA, RNA was treated with RNase-free DNase I (Promega) and purified with the RNeasy Plant Mini kit (Qiagen, Valencia, CA). cDNA was synthesized from RNA samples using the SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen). Gene-specific primers for SlGRAS genes used in the reactions are given in Table S1. PCR reactions were performed in triplicate and contained template cDNA, 200 nM gene-specific primers, and SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) in a volume of 15 μL. Reactions were carried out using an ABI Prism 7700 and Sequence Detection System (Applied Biosystems) with the following cycling programme: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 54 °C and 30 s at 72 °C. Fluorescence was monitored at the end of each cycle. The absence of non-specific products and primer dimers was confirmed by analysis of melting curves and agarose gel electrophoresis. For data analysis, threshold cycle values of three independent biological replicates were averaged,

normalized and used to calculate relative transcript levels as described by Pfaffl (2001). The tomato genes used as internal standards for normalization were: *actin* (GenBank accession no. AB199316), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; U97257) and *ATPase* (CD002473). Data were compared using a Student's *t*-test for independent samples (mock vs. treated), with the level of significance set at $P < 0.05$.

Semiquantitative RT-PCR analysis

Three leaf discs per plant (50 mg tissue) were collected from the upper leaves of TRV-silenced plants, total RNA was extracted and RT-PCR was performed as described (Ekengren *et al*., 2003) using the following gene-specific primers: for *SlGRAS6*, 5′-AAACCAAAGAGACCAGCTTCTGCG-3′ and 5′-AATAAAGTGCACCTGCCTCCTCCT-3′ for *EF-1a*, 5′-GGTGGTTTTGAAGCTGGTATCTCC-3′ and 5′-CCAGTAGGGCCAAAGGTCACA-3′. A 20-μL aliquot was removed from each reaction after 28, 34 and 40 cycles. PCR products were separated on a 1.2% agarose gel and abundance of the product after 28 cycles of PCR was determined by densitometry. The abundance of *SlGRAS6* transcripts in *SlGRAS6*-silenced and TRV-only infected plants was normalized against the amount of *EF-1a* transcript. The RT-PCR analysis was repeated for two independently silenced plants with similar results.

Virus-induced gene silencing plasmids and procedures

Plasmids pTRV1, pTRV2, and the pTRV2 derivatives pTRV2:*Prf* and pTRV2:*PDS* were previously described (Ekengren *et al*., 2003; Liu *et al*., 2002). For the generation of pTRV2:*SlGRAS2/3/4* and *6* the following TIGR ESTs were PCR-amplified using Gateway-compatible primers: for *SlGRAS2*, EST248297 (GenBank accession no. AI489958); for *SlGRAS3*, EST298835 (AW222024); for *SlGRAS4*, EST463889 (BG130997); and for *SlGRAS6*, EST527461 (BI209421). Sequences of the Gateway-compatible primers used were: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATCCCCCGGGCTGCAGGAATTC-3′, and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTGGTACCGGGCCCCCCCTCGAG-3′. PCR products were recombined into pYL279 (pTRV2) with the Gateway system (Invitrogen), confirmed by sequencing and plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. Plants were inoculated with *Agrobacterium* cultures as described by Ekengren *et al*. (2003), and incubated in growth chambers at 20 °C for 3 weeks.

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SUPPLEMENTARY MATERIAL

The author has provided the following supplementary material, which can be viewed alongside the article at http://www.blackwell-synergy.com:

Table S1 Genes and oligonucleotide sets used in real-time RT-PCR experiments.

Fig. S1 Sequence alignment of the C-terminal conserved region of tomato GRAS proteins. Alignment of the C-terminal deduced amino acid sequences of SlGRAS proteins was performed using the ClustalW program. Included in the alignment are tomato GRAS proteins encoded by the genes listed in Table S1, except for *SlGRAS5* and *SlGRAS17*, whose predicted translation frames were interrupted by stop codons, and *SlGRAS15*, for which sequences of the 3′ end were not available. Gaps were introduced to maximize alignment. Amino acid sequences of SlGRAS12/13 and 14 are partial. Residues conserved in at least three of the 15 aligned SlGRAS proteins are highlighted in black. Residues defining the conserved domains and typical motifs of GRAS proteins are indicated above the sequence alignment.