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Development of a virus-induced gene-silencing system for functional analysis of the *RPS2*-dependent resistance signalling pathways in *Arabidopsis*

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Abstract Virus-induced gene silencing (VIGS) offers a rapid and high throughput technique platform for the analysis of gene function in plants. Although routinely used in some Solanaceous species, VIGS system has not been well established in *Arabidopsis thaliana* (L.) Heynh. We have recently reported some factors that potentially influence tobacco rattle virus (TRV)-mediated VIGS of phytoene desaturase (PDS) and actin gene expression in *Arabidopsis*. In this study, we have further established that the *Agrobacterium* strain used for agro-inoculation significantly affects the VIGS efficiency. Strain GV3101 was highly effective; C58C1 and LBA4404 were invalid, while EHA105 was plant growth stage-dependent for TRV-induced gene silencing. Furthermore, the VIGS procedure optimised for the *PDS* gene was applied for the functional analysis of the disease resistance gene *RPS2*-mediated resistance pathway. Silencing of *RPS2* led to loss of resistance to the otherwise avirulence strain of *Pseudomonas syringae* pv. *tomato* DC3000 carrying the avirulence gene *AvrRpt2*. Silencing of *RIN4*, a *RPS2* repressor gene, gave rise to conversion of compatible interaction to incompatible. Silencing of *NDR1*, *RAR1* and *HSP90*, known to be required for the *RPS2*-mediated resistance, resulted in loss of the resistance, while silencing of *EDS1* and *SGT1b*, which are not required for the *RPS2*-mediated resistance, caused no change of the resistance. These results indicate that the optimised procedure for the TRV-based VIGS is a potentially powerful tool for dissecting the signal transduction pathways of disease resistance in *Arabidopsis*.

Keywords *Arabidopsis thaliana* · Disease resistance · RPS2 · Signal transduction · Tobacco rattle virus (TRV) · Virus-induced gene silencing (VIGS)

Abbreviations

Agro-inoculation	*Agrobacterium* inoculation
cfu	colony-forming units
dpa	days post agro-inoculation
dpi	days post inoculation
EST	expressed sequence tag
OD	optical density
PDS	phytoene desaturase gene
Pst	*Pseudomonas syringae* pv. *tomato*
TRV	tobacco rattle virus
VIGS	virus-induced gene silencing

Introduction

Arabidopsis thaliana (L.) Heynh. is a model plant species to study gene functions. Its whole genome has been sequenced (*Arabidopsis* Genome Initiative 2000). Collections of genome-wide mutant populations generated through mutation approaches have provided a

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robust platform for *Arabidopsis* gene function analysis (Alonso et al. 2003). However, these mutant collections have limitations such as lack of genome-wide coverage, gene target bias, lethality, and functional redundancy (Alonso et al. 2003). A recently developed technique, virus-induced gene silencing (VIGS) can overcome these shortcomings, offering an attractive alternative to elucidate *Arabidopsis* gene functions.

VIGS is a technology that employs recombinant viruses to knock down expression of plant endogenous genes involving a homology-based degradation mechanism triggered by double stranded RNA (Baulcombe 1999). VIGS has obvious advantages over other known approaches for gene function analysis. For instance, it does not require development of stable genetic transformants, and thus is rapid and less laborious. It is applicable for the functional analysis of genes for which only partial sequences are available, such as expressed sequence tags (ESTs). Additionally, it allows characterisation of phenotypes that are lethal in stable lines (Lu et al. 2003b; Burch-Smith et al. 2004).

An increasing number of VIGS vectors for plant gene function analysis have been exploited during the recent years (Burch-Smith et al. 2004; Constantin et al. 2004; Fofana et al. 2004; Tao and Zhou 2004). Most of these vectors function only in Solanaceous species (Burch-Smith et al. 2004; Brigneti et al. 2004; Faivre-Rampant et al. 2004; Valentine et al. 2004), while barley stripe mosaic virus vector works in monocots; barley and wheat (Hein et al. 2005; Holzberg et al. 2002; Lacomme et al. 2003; Scofield et al. 2005). VIGS in *Arabidopsis* is much less studied than in Solanaceous species. So far, only tobacco rattle virus (TRV)- and cabbage leaf curl virus (CbLCV)-based vectors have been examined for VIGS in *Arabidopsis* (Dalmay et al. 2000; Ratcliff et al. 2001; Turnage et al. 2002; Valentine et al. 2004). Delivery of CbLCV-based vector requires expensive and laborious bombardments (Turnage et al. 2002). Regarding TRV-based VIGS vector, there are three versions, constructed by groups of Drs. Baulcombe (Ratcliff et al. 2001), Dinesh-Kumar (Liu et al. 2002) and Lacomme (Valentine et al. 2004), and thus abbreviated here as TRV-B, TRV-DK and TRV-L, respectively. Compared with TRV-B, the earliest version of TRV-based VIGS vector, TRV-L retains the 2b gene (Valentine et al. 2004), while TRV-DK has a duplicated 35S promoter and a ribozyme at the C-terminus, as well as some amino acid changes in the viral sequence (Liu et al. 2002). Efforts to obtain stable and satisfactory TRV-induced gene silencing were not successful when the vector was agro-infiltrated directly into *Arabidopsis* plants (Ratcliff et al. 2001), and instead a two-step strategy, involving agro-infiltration followed by viral extraction from another host plant *N. benthamiana*, and subsequent inoculation of *Arabidopsis* plants, is suggested and currently used (Lu et al. 2003b; Valentine et al. 2004). However, this procedure is obviously time-consuming and laborious.

In an effort to establish an efficient and direct TRV-mediated VIGS procedure for gene function analysis in *Arabidopsis*, we examined the factors that potentially influence the VIGS efficiency, which include the concentration and pre-incubation of *Agrobacterium* inocula; the concentration of acetosyringone in the pre-incubation medium; the *Agrobacterium* inoculation (agro-inoculation) method; the growth stage of the plants for agro-inoculation; and the growth temperature of the agro-inoculated plants. We optimised the procedure for TRV-DK-induced silencing of *Arabidopsis* PDS and actin genes through direct delivery of the VIGS vector into the plants by agro-inoculation (Wang et al. 2006). In this paper we report further observation on the effect of the *Agrobacterium* strains on VIGS efficiency, and application of the procedure for the functional analysis of the *RPS2*-mediated disease resistance pathways. VIGS of seven genes; *RPS2*, a resistance gene, *RIN4*, a repressor of the RPS2 (Axtell and Staskawicz 2003; Mackey et al. 2003), *RAR1, HSP90* and *NDR1*, genes required for the *RPS2*-dependent disease resistance, and *SGT1* and *EDS1*, genes required for other resistance genes but not the *RPS2*-dependent disease resistance (Dodds and Schwechheimer 2002; Tornero et al. 2002; Holt et al. 2003; Takahashi et al. 2003), was analysed. Our results demonstrate that the optimised procedure for the TRV-based VIGS is a potentially powerful reverse genetics tool for functional analysis of genes involved in signal transduction pathways of disease resistance in *Arabidopsis*.

Materials and methods

Construction of the gene silencing constructs

Fragments of the *RPS2* gene and six genes differentially involved in the *RPS2*-mediated resistance, *EDS1*, *NDR1*, *RAR1*, *SGT1b*, *HSP90* and *RIN4*, were obtained by reverse transcriptase-PCR (RT-PCR) from the template of *Arabidopsis* complementary DNA, and the primers listed in supplementary table 1. They were cloned into pYL156, an improved TRV VIGS vector (Liu et al. 2002) through the sites *Eco*RI and *Bam*HI. The pYL156 recombinant constructs obtained were electroporated into cells of *Agrobacterium tumefaciens* by a Gene Pulser Xcell (Bio-Rad, USA). *Arabidopsis PDS* gene silencing construct were made previously (Wang et al. 2006).

Agrobacterium strains

Four commonly used laboratory strains of *A. tumefaciens*, GV3101, EHA105, C58C1 and LBA4404, were used for transformation of the cloned silencing constructs in the experiments of evaluating the effect of the *Agrobacterium* strains on VIGS efficiency.

VIGS manipulation procedure

TRV-based VIGS was conducted following the procedure optimised previously for the *PDS* gene silencing analysis (Wang et al. 2006). To prepare the agro-inocula, *Agrobacterium* carrying pTRV1 and pYL156 recombinant constructs was cultured in YEBi medium (per 100 ml containing 0.5 g beef extract, 0.1 g yeast extract, 0.5 g bacto-peptone, 0.2 ml of 1 M MgSO4 [pH 7.0], 10 μl of 200 mM acetosyringone, 1 ml of 1 M MES, and 100 μg ml^{-1} kanamycin), and grown till the OD_{600} reached a value between 0.8 and 1.2. After centrifugation, the collected pellets were suspended with MMAi solution (per 100 ml containing 0.5 g MS salts, 0.195 g MES, 2 g sucrose, 100 μl of 200 mM acetosyringone, pH 5.6) into suspensions with an OD_{600} of 4.0, followed by shaking at a speed of 50 rpm at 25°C for 2 h. The suspensions of *Agrobacterium* carrying pTRV1 and pYL156 recombinant constructs were then mixed in a 1:1 ratio so that the final OD_{600} value of each type in the mixture prior to inoculation was 2.0. The prepared agro-inocula were delivered into *Arabidopsis* plants by vacuum infiltration. Whole plants were submerged into agro-inocula in a beaker within a desiccator, pulled by a vacuum pump till the pressure reached 0.07 kPa, maintained for 5 s, and then quickly released to connect the vacuum container to the air, allowing the agro-inocula to rapidly enter the plant tissues. The agro-inoculated plants were grown in a plant growth chamber at 22°C with a 12/12 h light/dark regime.

Plant materials and growth conditions

Seeds of *Arabidopsis* (ecotype Col-0) were sown in small pots, and grown in a plant growth chamber at 22°C with a 12/12 h light/dark regime.

Plant inoculation and *in planta* pathogen growth analysis

The pathogen *Pseudomonas syringae* pv. *tomato* DC3000 with or without the avirulence gene *AvrRpt2* were grown at 28°C in low salt LB medium with kanamycin (25 μg ml^{-1}) and rifampcin (100 μg ml^{-1}) till the OD_{600} reached 0.6–1.0. After centrifugation, the collected bacteria were re-suspended with sterilised 10 mM M_gCl_2 solution containing 0.05% Tween-20. The plants in trays were sprayed with the bacterial inocula with a density of 5×10^8 colony-forming units (cfu) ml^{-1}. The trays were then wrapped with a sterilised plastic membrane to keep a high humidity for 24 h. The plants were grown in a plant growth chamber at 25°C with a 12/12 h light/dark regime. *In planta* pathogen growth analysis was conducted as described (Katagiri et al. 2002).

RT-PCR analysis

Transcript abundance of the genes targeted for silencing analysis in plants was examined by RT-PCR analysis. Total RNA was extracted from leaves of silenced and non-silenced plants with TRIZOL reagents (Invitrogen). The first-strand cDNA was synthesised from 0.1 mg of total RNA with oligo (dT) primer and superscript reverse transcriptase (Promega, USA). Semiquantitative RT-PCR was performed as described (Wang et al. 2006). Primers used for RT-PCR for the silencing genes are listed in supplementary table 1. They anneal outside the region targeted for silencing so that only the endogenous genes would be probed. The ubiquitin gene served as an internal constitutively expressed control. For a negative control, RT reaction mix without reverse transcriptase was used as a template in the reaction. The intensities of PCR-generated fragments were analysed and quantified using Gel Doc 2000 and Quantity One Version 4.3 (Bio-Rad, USA).

Statistical analysis and illustration of the data

Each experiment on evaluation of the effect of *Agrobacterium* strains on VIGS efficiency and silencing analysis of genes involved in the RPS2-dependent resistance was conducted in triplicate with 20 plants per strain or gene. The *PDS* gene-silencing efficiency was calculated as the percentage of plants showing photo-bleaching phenotype in at least three leaves, among which at least one nearly complete white, 4 weeks after agro-inoculation out of the total number of plants tested. The final result was indicated as the mean value and the standard deviation. The data of the VIGS efficiency and the *in planta* pathogen growth assay were analysed and illustrated with the software Origin 6.0 (Microcal Software Inc.).

Results

Effect of the *Agrobacterium* strains on VIGS in *Arabidopsis*

Phytoene desaturase (PDS) is a key enzyme regulating carotenoid biosynthesis. Silencing of the *PDS* gene results in loss of carotenoids, causing the *Arabidopsis* plants to exhibit a photo-bleaching phenotype (Dalmay et al. 2000; Turnage et al. 2002). A 975-bp fragment of the *Arabidopsis PDS* gene was cloned into the improved TRV VIGS vector pYL156 (Liu et al. 2002) to silence the *Arabidopsis PDS* gene and evaluate the effect of *Agrobacterium* strains used for agro-infiltration on gene silencing in *Arabidopsis*.

Four *Agrobacterium* strains that are most commonly used worldwide, GV3101, EHA105, C58C1 and LBA4404, were compared for their efficiency to induce VIGS. Agro-inoculation with strains C58C1 and LBA4404 did not give rise to any *PDS* gene silencing regardless of plant growth stage when agro-inoculated, while agro-infiltration with the strain GV3101 resulted in consistent and highly efficient VIGS, which was slightly affected by the plant growth stage at which it was used for agro-inoculation. When the plants were inoculated at before-flowering stages, the VIGS efficiency reached nearly 100%, while it reduced slightly to 91% when the plants were inoculated at the flowering stage (Fig. 1A). The photo-bleaching phenotype developed in the plants in all cases initially in the newly emerged leaves at 11–12 days post agro-inoculation (dpa), later in branches, stems and inflorescence, and constantly occurred in the newly emerged tissues for at least 6 weeks (Fig. 1B). However, VIGS resulting from agro-infiltration of the strain EHA105 was dependent on the plant growth stage when used for agro-inoculation. When the plants were inoculated at 5–6-leaf stage, it reached 98%, which was nearly as high as the strain GV3101. When the plants were inoculated at 8–9-leaf stage, it reduced greatly to 23%, whereas when the plants were inoculated at flowering stage, not a single plant showed any photo-bleaching phenotype (Fig. 1A). Additionally, development of the photo-bleaching phenotype in the plants that were agro-inoculated at 8–9-leaf stage was delayed for 5–8 days, and was less consistent and profound than in those were agro-inoculated at 5–6-leaf stage (data not shown).

To confirm the VIGS at the molecular level, RT-PCR analysis was undertaken to investigate accumulation of the *PDS* gene transcript in the leaves showing photo-bleaching phenotype. Compared with non-silenced leaves of wild type TRV-infected control plants, abundance of the *PDS* gene transcript in leaves, seen in nearly white, of plants inoculated with *Agrobacterium* GV3101 carrying TRV-PDS reduced by 92% (Fig. 1C). The *PDS* gene transcript in non-photo-bleached leaves of plants inoculated with *Agrobacterium* strains EHA105, C58C1 and LBA4404 carrying TRV-PDS accumulated to a level similar to the counterparts of wild type TRV-infected control plants (data not shown).

Taken together, these results demonstrate that the strain of *Agrobacterium* used for agro-infiltration significantly affects VIGS in *Arabidopsis*. Among the four strains tested GV3101 has the best results for TRV-based gene silencing study in *Arabidopsis*.

VIGS analysis of the *Arabidopsis* disease resistance gene *RPS2*

We are interested in the exploitation of VIGS as a robust tool for functional analysis of disease resistance pathways in *Arabidopsis*. To achieve the goal, VIGS technique was employed to dissect the signalling pathway leading to the *RPS2*-mediated resistance. As the first step, VIGS analysis of the *Arabidopsis RPS2* gene was conducted. A 547-bp fragment of the *RPS2* gene was obtained by RT-PCR, and cloned into pYL156. The resulting construct pYL156::*RPS2* was used to silence the *Arabidopsis RPS2* gene in the plants at 4–6-leaf stage. Using the *Agrobacterium* strain GV3101 and following the VIGS procedure optimised previously for analysis of the PDS and actin genes (Wang et al. 2006), we successfully silenced the *Arabidopsis RPS2* gene. Transcripts of the *RPS2* gene in leaves of the recombinant TRV-infected plants at four weeks post agro-inoculation decreased by 90%, compared to the wild-type TRV-infected control, while the transcript of the ubiquitin gene, a constitutively expressed gene used as inner standard, accumulated to a similar level in both plant types (Fig. 2A). The *RPS2* gene confers resistance to *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 carrying the avirulence gene *AvrRpt2*. The *RPS2/AvrRpt2*-dependent resistance in the *RPS2*-silenced and non-silenced plants was compared. As expected, the wild-type TRV-infected, and thus non-silenced control plants (genotype $RPS2^{+}$) did not show any disease symptom (Fig. 2B), and strictly limited the growth of the pathogen after inoculation with the avirulent strain Pst DC3000 ($AvrRpt2^{+}$) (Fig. 2C). However, the recombinant TRV-infected, and thus the *RPS2*-silenced plants (genotype $RPS2^{+}$) allowed a rapid growth of the bacterium within 4 dpi (Fig. 2C), and showed chlorosis and necrosis, the disease

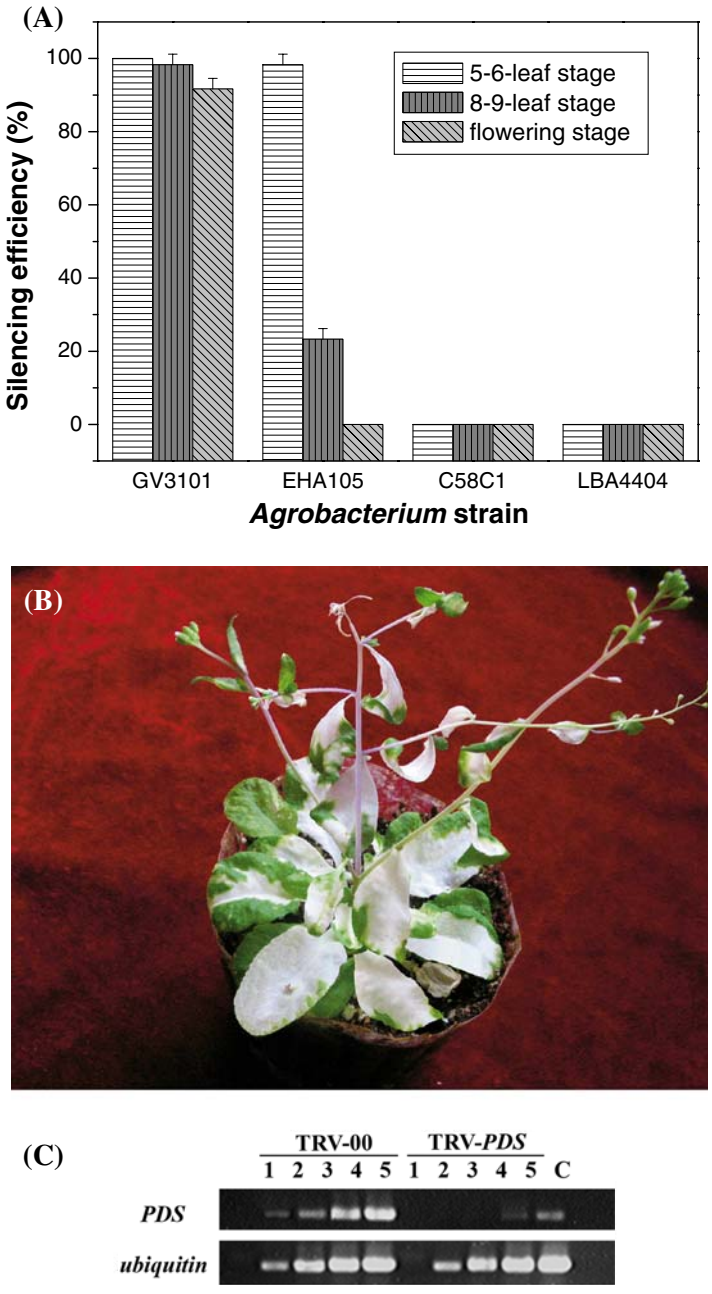

Fig. 1 Effect of the *Agrobacterium* strain on VIGS efficiency in *Arabidopsis*. (**A**) comparison of VIGS efficiency mediated by four *Agrobacterium* strains, GV3101, EHA105, C58C1 and LBA4404. The four strains were used for the *PDS* gene silencing analysis in *Arabidopsis* plants at 5–6-leaf, 8–9-leaf and flowering stages, respectively. The photo-bleaching phenotype resulting from the *PDS* gene silencing was investigated 4 weeks after agro-infiltration. The VIGS efficiency was calculated as the percentage of plants showing photo-bleaching phenotype in at least three leaves, among which at least one was nearly completely white, out of the total number of plants tested. (**B**) A typical plant showing photo-bleaching phenotype subjected to GV3101-mediated *PDS* VIGS analysis. The plant was agro-infiltrated at eight-leaf stage. The photograph was taken 6 weeks after agro-inoculation. (**C**) Confirmation of the *PDS* gene silencing in leaves showing photo-bleaching phenotype. RT-PCR analysis was conducted using total RNA extracted from leaves, seen in nearly white, of plants inoculated with *Agrobacterium* GV3101 carrying TRV-PDS and the corresponding non-silenced leaves of the wild type TRV (TRV-00)-infected control plants. Typical PCR products are also shown for ubiquitin, used as an internal standard to correct the quantity, from the same tissues. Lanes 1-5 correspond to products from PCR of cycles 20, 24, 28, 32 and 36, respectively. Lane C represents the negative control, in which the RT reaction mix without reverse transcriptase was used as a template in the reaction

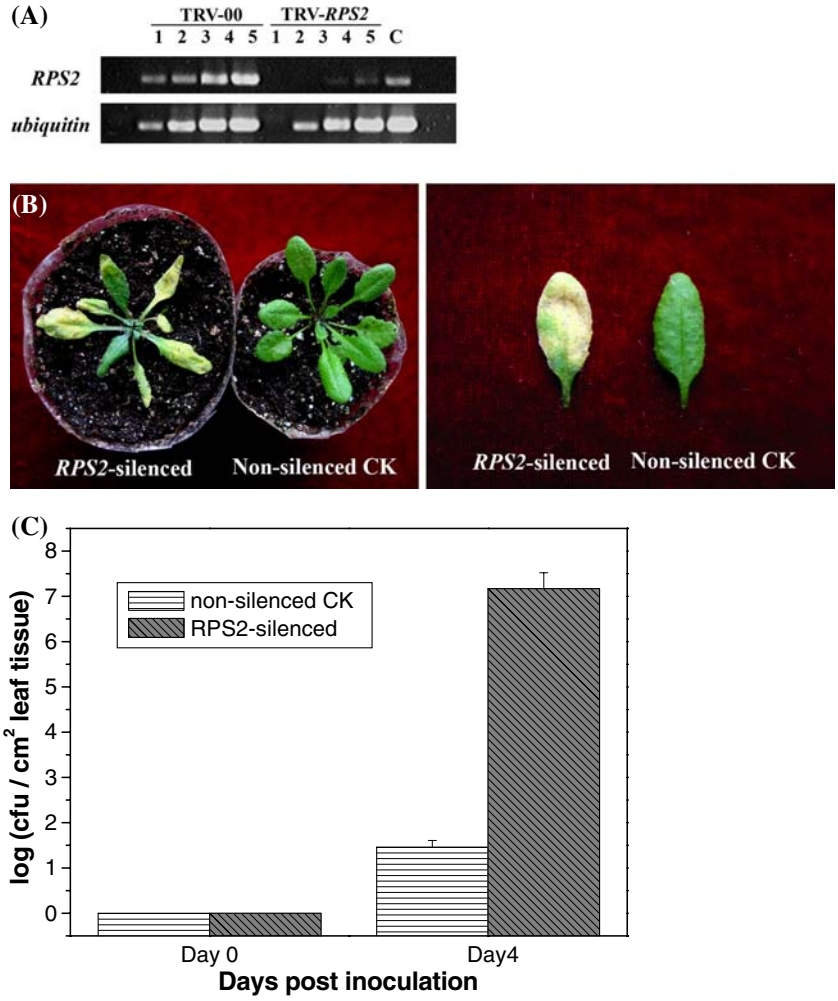

Fig. 2 VIGS analysis of the *Arabidopsis* disease resistance gene *RPS2*. The *RPS2*$^+$ plants at 4–6-leaf stage were agro-infiltrated with either the wild-type TRV (TRV-00, non-silenced control) or the recombinant TRV containing an insert of a 547-bp fragment of the *RPS2* gene (TRV-*RPS2*). These plants were spray-inoculated with bacterial suspensions of Pst DC3000 (*AvrRpt2*$^+$) four weeks post agro-infiltration. (**A**) Confirmation of the *RPS2* gene silencing in the TRV-*RPS2*-infected plants before resistance test. Total RNA was extracted from the leaves of TRV-*RPS2*-infected plants whose counterpart showed nearly complete photo-bleached phenotype in reporting control plants subjected to *PDS* silencing analysis. RT-PCR analysis was conducted as described in Fig. 1C; (**B**) A typical plant and leaf of the two plant types at 5 dpi with Pst; (**C**) *in planta* bacterial density in leaves of the two plant types at 4 dpi

symptom of the otherwise avirulent pathogen Pst DC3000 (*AvrRpt2*$^+$) at 5–6 dpi (Fig. 2B). These results reveal that silencing of the *RPS2* gene results in loss of resistance to the otherwise avirulent pathogen Pst DC3000 (*AvrRpt2*$^+$).

VIGS analysis of the *RPS2* repressor gene *RIN4*

To further test whether the VIGS procedure can be efficiently applied to functional analysis of the *RPS2*-dependent disease resistance signalling pathways, VIGS of the *RIN4* gene was analysed. The RIN4 is a negative regulator of the *RPS2*-dependent disease resistance. AvrRpt2-dependent elimination of RIN4 activates *RPS2* (Axtell and Staskawicz 2003; Mackey et al. 2003). Therefore, it is conceivable that silencing of the *RIN4* gene should constitutively activate the *RPS2*-dependent disease resistance, mimicking the role of the avirulence elicitor AvrRpt2 upon inoculation with the avirulent pathogen Pst DC3000 (*AvrRpt2*$^+$).

VIGS analysis of the *Arabidopsis RIN4* gene was conducted by inoculating plants at 4–6-leaf stage with the construct pYL156::*RIN4*, containing a 437-bp fragment insert of the *RIN4* gene. Following the described VIGS procedure, we efficiently silenced the *Arabidopsis RIN4* gene. Transcripts of the *RIN4* gene in leaves of the recombinant TRV-infected plants at 4 weeks post agro-inoculation reduced by 92%, compared to the wild-type TRV-infected control (Fig. 3A). Furthermore, TRV-*RIN4*-infected plants showed retarded growth with less and smaller leaf exhibiting some necrosis (Fig. 3B), which fits the fact that elimination of RIN4 results in block of plant development due to activation of *RPS2*-mediated defence (Mackey et al. 2003), confirming that the *RIN4* gene in these plants has been efficiently silenced. Comparative assay on the *RPS2/AvrRpt2*-dependent resistance in the two plant types was conducted. The non-silenced control plants (genotype *RPS2*$^+$) allowed a rapid growth of the bacterium within 4 dpi (Fig. 3C), and displayed typical disease symptom at 5–6 dpi with the virulent strain Pst

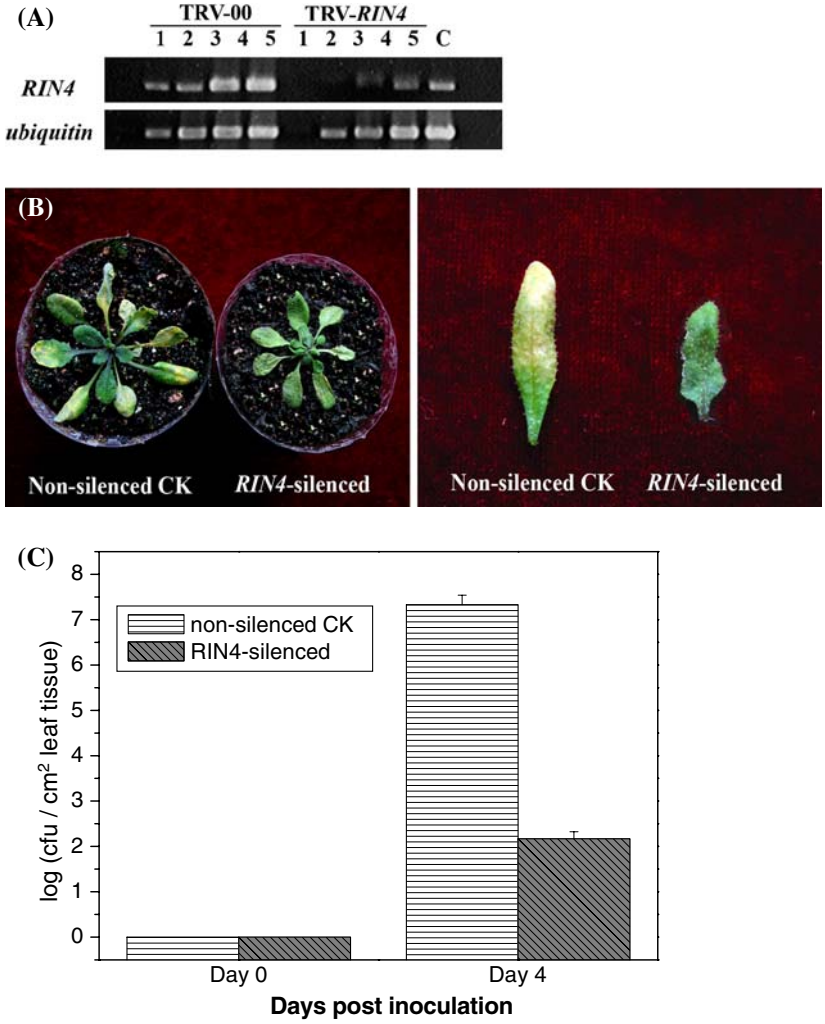

Fig. 3 VIGS analysis of the RPS2 repressor gene *RIN4*. The *RPS2*$^+$ plants at 4–6-leaf stage were agro-infiltrated with either the wide-type TRV or the recombinant TRV containing an insert of a 437-bp fragment of the *RIN4* gene (TRV-*RIN4*). These plants were spray-inoculated with bacterial suspensions of Pst DC3000 (*AvrRpt2*$^-$) four weeks post agro-infiltration. (**A**) Confirmation of the *RIN4* gene silencing in the TRV-*RIN4*-infected plants before resistance test. RT-PCR analysis was conducted as described in Fig. 1C; (**B**) a typical plant and leaf of the two plant types at 5 dpi; (**C**) *in planta* bacterial density in leaves of the two plant types at 4 dpi

DC3000 (*AvrRpt2*$^-$) (Fig. 3B). However, the *RIN4*-silenced plants (genotype *RPS2*$^+$) did not show the disease symptom (Fig. 3B), and greatly repressed the growth of the otherwise virulent pathogen Pst DC3000 (*AvrRpt2*$^-$) (Fig. 3C). These results indicate that silencing of the *RIN4* gene enables the plants to develop resistance to the otherwise virulent pathogen Pst DC3000 (*AvrRpt2*$^+$).

VIGS analysis of the *RPS2* downstream signalling genes

To further elucidate whether the VIGS procedure is valid for functional analysis of the *RPS2*-dependent disease resistance signalling pathways, VIGS of a set of well-known signalling genes, including *RAR1*, *HSP90* and *NDR1*, genes required for the *RPS2*-dependent disease resistance, and *SGT1* and *EDS1*, genes required for other resistance genes but not the *RPS2*-dependent disease resistance (Dodds and Schwechheimer 2002; Tornero et al. 2002; Holt et al. 2003; Takahashi et al. 2003), was analysed. It is expected that silencing of the genes *RAR1*, *HSP90* and *NDR1*, but not the genes *SGT1* and *EDS1*, should compromise the *RPS2*-dependent disease resistance.

VIGS analysis of these genes was conducted by inoculating plants at 4–6-leaf stage with the recombinant pYL156 constructs containing an insert of a 520-bp-, 910-bp-, 471-bp-, 752-bp- and 769-bp fragment of the *Arabidopsis* genes *RAR1*, *HSP90.1*, *NDR1*, *SGT1b* and *EDS1*, respectively. Transcripts of the five genes in leaves of the recombinant TRV-infected plants at 4 weeks post agro-inoculation reduced by 88%, 90%, 91%, 86% and 87%, respectively, compared to the wild-type TRV-infected control (Fig. 4A). Resistance test revealed that, as expected, similar to the non-silenced control plants (genotype *RPS2*$^+$), the *SGT1b*- and *EDS1*-silenced plants did not show any

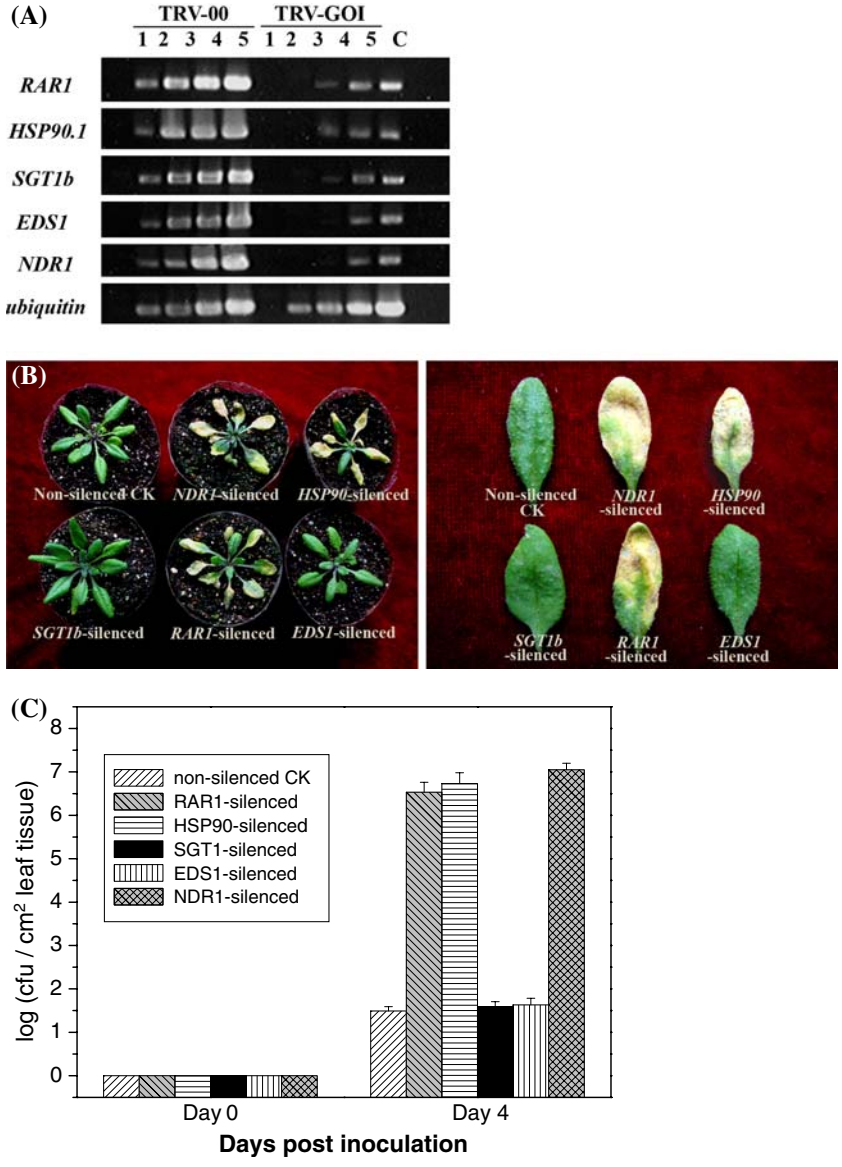

Fig. 4 VIGS analysis of the resistance signalling genes *RAR1*, *SGT1*, *HSP90*, *EDS1* and *NDR1*. The *RPS2*$^+$ plants at 4–6-leaf stage were agro-infiltrated with either the wild-type TRV or the recombinant TRV containing an insert of a 520-bp-, 910-bp-, 471-bp-, 752-bp- and 769-bp fragment of the *Arabidopsis* genes *RAR1*, *HSP90.1*, *NDR1*, *SGT1b* and *EDS1*, respectively (TRV-GOI). These plants were spray-inoculated with bacterial suspensions of Pst DC3000 (*AvrRpt2*$^+$) four weeks post agro-infiltration. (**A**) Confirmation of silencing of the genes *RAR1*, *HSP90.1*, *NDR1*, *SGT1b* and *EDS1* in the recombinant TRV-infected plants before resistance test. RT-PCR analysis was conducted as described in Fig. 1C; (**B**), a typical plant and leaf of the two plant types at 5 dpi; (**C**) *in planta* bacterial density in leaves of the two plant types at 4 dpi

disease symptom after inoculation with the avirulent strain Pst DC3000 (*AvrRpt2*$^+$) (Fig. 4B). *In planta* pathogen growth analysis revealed that growth of the pathogen was suppressed in these plants (Fig. 4C). However, the *RAR1-*, *HSP90.1-* and *NDR1*-silenced plants (genotype *RPS2*$^+$) allowed a rapid growth of the bacterium within 4 dpi (Fig. 4C), and exhibited disease symptoms at 5–6 dpi with the otherwise avirulent pathogen Pst DC3000 (*AvrRpt2*$^+$) (Fig. 4B). These results demonstrate that silencing of the *RAR1*, *HSP90* and *NDR1* genes, but not *SGT1* and *EDS1* genes, results in loss of *RPS2*-dependent disease resistance to the otherwise avirulent pathogen Pst DC3000 (*AvrRpt2*$^+$).

Taken together, these results prove that the TRV-mediated VIGS procedure using the GV3101 *Agrobacterium* strain is applicable for the functional analysis of the *RPS2*-dependent disease resistance signalling pathways.

Discussion

We found in this study that the most commonly used laboratory strains of *Agrobacterium*, GV3101, EHA105, C58C1 and LBA4404, are distinct in their ability to mediate the TRV-induced gene silencing in *Arabidopsis*. GV3101 is highly valid; C58C1 and LBA4404 are invalid, while EHA105 is dependent on the plant growth stage when used for TRV-induced gene silencing analysis (Fig. 1). This could be due to the different capability of the four strains in invading

the *Arabidopsis* cells and propagating to a sufficient level, which influenced the speed and level of the TRV virus accumulation in targeted tissues, and thus affecting the initiation and efficiency of VIGS. Our results suggest that attention should be paid to selection of the *Agrobacterium* strain for VIGS analysis.

Arabidopsis is a model plant species for the study of gene functions including disease resistance. Although collection of genome-wide mutant populations generated through T-DNA insertion has provided a robust platform for gene function analysis (Alonso et al. 2003), these mutant collections have obvious limitations, such as lack of genome-wide coverage, gene target bias, lethality, and functional redundancy. In fact, so far mutants are only available for less than 75% of the total predicted *Arabidopsis* genes (Alonso et al. 2003). VIGS offers an attractive alternative to mutagenesis approach. Significantly, VIGS does not require development of stable genetic transformants. It is rapid and easily manipulated. Additionally, it can be applied for functional analysis of genes for which only partial sequences are available, such as ESTs (Lu et al. 2003b; Burch-Smith et al. 2004).

Disease resistance is an important aspect of plant biology researches. Employment of *Arabidopsis* as plant material has greatly pushed forward for the study of disease resistance including those conferred by *R* genes such as *RPS2*. We have established an efficient and direct TRV-mediated VIGS procedure for gene function analysis in *Arabidopsis* through examining of the factors that potentially influence the VIGS efficiency, which involve preparation and subsequent inoculation of the *Agrobacterium* inocula and growth of the agro-inoculated plants (Wang et al. 2006). In this study we have further optimised the procedure for TRV-induced gene silencing in *Arabidopsis* by investigation of the effect of the *Agrobacterium* strains on VIGS efficiency. Furthermore, the optimised VIGS procedure has been validated for functional characterisation of the *RPS2*-dependent resistance pathways through successful VIGS analysis of the disease resistance gene *RPS2*, its repressor gene *RIN4*, and a set of *RPS2* downstream signalling genes. This procedure should therefore be applicable as a routine protocol for VIGS analysis of genes involved in *RPS2*-dependent resistance. Additionally, it can also be used for isolation of novel genes involved in *RPS2*-dependent resistance. One approach to achieve this goal is to construct an *Arabidopsis* cDNA library using TRV silencing vector as cloning vector, which is then subjected to VIGS analysis following the optimised procedure, followed by resistance examination, to screen in a high throughput manner for plants with alteration-of-resistance phenotypes, and finally obtain the genes involved in *RPS2*-dependent resistance. Similar strategy has been successfully applied for isolation of resistance-related genes using PVX silencing vector (Lu et al. 2003a).

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