

RESEARCH PAPER

# A mobile signal transported over a long distance induces systemic transcriptional gene silencing in a grafted partner

Songling Bai<sup>1,2</sup>, Atsushi Kasai<sup>1</sup>, Kaori Yamada<sup>1</sup>, Tianzhong Li<sup>3</sup> and Takeo Harada<sup>1,2,\*</sup>

<sup>1</sup> Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki 036-8561, Japan.

<sup>2</sup> The United Graduate School of Agricultural Sciences, Iwate University, Morioka 020-8550, Japan

<sup>3</sup> College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, China

\* To whom correspondence should be addressed. E-mail: [tharada@cc.hirosaki-u.ac.jp](mailto:tharada@cc.hirosaki-u.ac.jp)

Received 10 February 2011; Revised 24 March 2011; Accepted 26 April 2011

## Abstract

**Transcriptional gene silencing (TGS) can be induced by promoter-targeted small interfering RNA (siRNA). Long-distance transmission of TGS by viral infection in plants has been reported. However, systemic TGS has not been observed in the case of using an inverted repeat transgene as the silencing trigger. Here it is reported that a mobile signal, presumably the siRNA, produced from a hairpin structure transgene controlled by a companion cell-specific promoter can also induce transmissible TGS in both a modified agroinfiltration and a grafting system. Although the transmissible TGS occurred only in cells located in the vicinity of a leaf vein in the scion, very strong silencing was observed in the root system, especially the lateral roots, including the root apical meristem. The transmissible TGS was maintained through tissue culture and subsequently inherited by the progeny. The results suggest the potential application of mobile promoter-targeting siRNA in horticulture for improvement of plant cultivars by grafting.**

**Key words:** Epigenetic change, grafting, RNA-directed DNA methylation, short interfering RNA, systemic silencing, transcriptional gene silencing.

## Introduction

Small interfering RNA (siRNA)-mediated gene silencing occurs in most eukaryotes across all kingdoms from fungi to mammals. In plants, for which the phenomenon has been relatively well studied, it plays a role in virus resistance, suppression of transgenes, and also inactivation of transposable elements (Waterhouse *et al.*, 2001). Double-stranded RNAs (dsRNAs), the key trigger derived from hairpin structure RNA or overexpressed gene mRNA, are processed into 19–25 nucleotide siRNA by Dicer-like proteins (DCL1–DCL4 in *Arabidopsis*) to silence the target gene. Such siRNA silencing can be classified into two major types: sequence-specific RNA degradation or translation arrest [post-transcriptional gene silencing (PTGS)] and repression of transcriptional activity [transcriptional gene silencing (TGS)] by RNA-directed DNA methylation (RdDM) (Baulcombe, 2004). PTGS is non-cell autonomous and can spread from the silenced parts to other tissues. In plants, the PTGS signal can be delivered over a long

distance through the phloem, usually from source to sink, following the direction of phloem flow (Tournier *et al.*, 2006). On the other hand, short-distance spreading of PTGS signals via plasmodesmata is limited to within 10–15 cells (Himber *et al.*, 2003), unless the target transcript works as a template for RNA-dependent RNA polymerase 6 (RDR6), in which case the PTGS can spread through the entire plant by transitivity (Brosnan *et al.*, 2007).

Transmissible TGS has been achieved successfully by virus-induced gene silencing (VIGS). Inoculation by virus vectors carrying the DNA fragment of the cauliflower mosaic virus (CaMV) 35S promoter sequence led to TGS of the transgenes 35S:GFP (green fluorescence protein) (Jones *et al.*, 1999) and 35S:GUS ( $\beta$ -glucuronidase) (Jones *et al.*, 2001) in systemic leaves. In the case of VIGS, RDR6 is required to produce secondary siRNAs that drive a more effective antiviral response (Vaistij and Jones, 2009). With regard to the endogenous gene, systemic gene silencing and

inheritable RdRM have been successfully induced by the cucumber mosaic virus (CMV)-based virus vector targeting the endogenous chalcone synthase gene (CHS-A) promoter in petunia and the colourless non-ripening gene (LeSPL-CNR) promoter in tomato (Kanazawa *et al.*, 2011). As the virus-induced TGS, but not PTGS, is inheritable independently without the virus trigger (Jones *et al.*, 2001), the practical application of VIGS to plant breeding is attracting attention (Kanazawa *et al.*, 2011). However, VIGS has certain disadvantages. First, most viruses infect a specific host, thus limiting their extensive application to non-host-range plants. Secondly, although the virus can be eliminated in the progeny, because the virus itself is not transmitted to seeds (Kanazawa *et al.*, 2011), the technique is not applicable to plants that propagate vegetatively, such as most fruit trees and some flowers and vegetables. Thirdly, there is a risk of potential generation of new infectious viruses through recombination and mutation generated by errors during the replication of genomes (Allison *et al.*, 1990). Therefore, there is a need to derive some method other than VIGS for TGS in the breeding of horticultural crops.

The production of dsRNA corresponding to a target gene promoter by a transgene system would be an ideal trigger of systemic TGS. However, it appears that the TGS triggered by transgene-derived siRNA is not graft transmissible (Mlotshwa *et al.*, 2002; Mourrain *et al.*, 2007). Furthermore, a study of a transgenic locus containing multiple copies of a plasmid showed that the PTGS was graft transmissible, whereas the TGS from the same locus was not (Mourrain *et al.*, 2007). Recently, using an *Arabidopsis* mutant in which siRNA biogenesis was blocked, Molnar *et al.* (2010) demonstrated that transgene-derived siRNA moved across the graft union. Although the mobile siRNA in the recipient tissue was three orders of magnitude less than in the source tissue, it functioned efficiently, initiating PTGS of the transgene in the grafted root. Furthermore, the authors provided evidence that a 24 nucleotide mobile siRNA from an endogenous gene was able to direct epigenetic DNA methylation in the genome of the recipient cells (Molnar *et al.*, 2010). Since the mobile signal is the siRNA itself, the grafting transmission of PTGS and TGS can be unified as the transmission of the siRNA with a different target (the coding region for PTGS and the promoter for TGS). For PTGS, the amplification system (Brosnan *et al.*, 2007) in which the target transcript plays the key role could be involved in the systemic transmission. On the other hand, in the case of transgene TGS, such an amplification system is not feasible, because no transcript of the transgene promoter exists in the ordinary state.

Therefore a system was tested for generation of epigenetically modified plants using siRNA transmission from the graft partner, which was given competency for the production of hairpin RNA derived from the transgene. It was shown that dsRNA-derived mobile siRNA from the rootstock successfully triggered TGS in the scion, through *de novo* DNA methylation in the promoter target region and not degradation of the mRNA. The systemic gene silencing was manifested only in the tissue located around the leaf

vein. On the other hand, when the scion was used as the siRNA donor, strong systemic silencing was observed in the root, with almost total silencing in the lateral roots. Regenerated plants with the silenced phenotype were also obtained from the tissue showing the TGS, which was further inherited by the next generation. The results suggest that this approach using mobile promoter-targeting siRNA would be applicable to horticulture for improving plants by grafting.

## Materials and methods

### *Plant material and growth condition*

GFP transgenic *Nicotiana benthamiana* line 16c has been described previously (Ruiz *et al.*, 1998a). Transgenic *N. benthamiana* was obtained by *Agrobacterium*-mediated transformation. The plants were grown in a cultivate room at 24 °C under a 16 h light/8 h dark photoperiod with cool fluorescent light at  $\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

### *Agroinfiltration*

Agroinfiltration was performed as described before (Voinnet *et al.*, 2003) with some modifications. The first to the ninth leaves (except the seventh leaf) were cut off 4-week-old *N. benthamiana* plants, leaving only the seventh leaf and apical bud. A 20  $\mu\text{l}$  aliquot of the *Agrobacterium* suspension was infiltrated into four sites of the seventh leaf via a needle-less 1 ml syringe (Supplementary Fig. S2 available at *JXB* online).

### *GFP imaging*

GFP fluorescence was photographed using a digital camera (Panasonic DMC-FZ50, Osaka, Japan) with a UV-Cut filter (Marumi MC-Y2, Tokyo, Japan) under UV light from a hand-held 1000 W long-wavelength UV lamp (B1000AP; Ultraviolet Products, Upland, CA, USA).

### *Micrografting*

Micrografting was modified from a method described previously (Turnbull *et al.*, 2002). Seven-day-old seedlings of *N. benthamiana* germinated on Murashige and Skoog (MS) agar (0.7%) were used for micrografting. The rootstock donor hypocotyl at  $\sim 5$  mm below the cotyledon was cut horizontally and the cut side of the root part was inserted into a silicone tube (2 mm length, 0.5 mm external  $\times$  0.4 mm internal diameter; TechJam, Osaka, Japan). The cut surface of the scion partner prepared in the same way was adhered against that of the root part midway in the tube. All grafting procedures were performed under a stereomicroscope on a clean bench. The grafts were grown on MS agar in a Petri dish by setting up using an agarose block (3 mm cube). After 7 d, the tube was cut off from the graft interface and then the grafted plant was transferred to Rockwool (Nitto Boseki Co., Tokyo, Japan) in a standard nutrient solution (Otsuka House Nos. 1 and 2, Otsuka Chemical Co., Osaka, Japan). For the grafting that used CoYMV:35SIR as the scion, the silencing phenotypes were observed in the root system 1 week later. For the grafting that used CoYMV:35SIR as the root stock, all leaves were cut off 2 weeks after transplant. The silencing phenotypes were monitored in the newly developing leaves after another 1 week (Supplementary Fig. S2 at *JXB* online).

### *Total RNA extraction and qRT-PCR analysis*

Total RNA was extracted from leaves with TRIzol (Invitrogen). Genomic DNA was eliminated with a TURBO DNA-free Kit (Ambion). The cDNAs used for qRT-PCR of GFP were synthesized from 500 ng of total RNA with a SuperScript VILO cDNA Synthesis Kit (Bio-Rad). The qRT-PCR was performed with

a Chromo4 real-time PCR detector (Bio-Rad). cDNA corresponding to 10 ng of total RNA was used in 20  $\mu$ l reactions with iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. Changes in the target genes were determined by normalizing to NbUbi (AY912494). The primers used for qRT-PCR are described in Supplementary Table S1 at *JXB* online.

#### Extraction of small RNAs and detection of siRNA

Small RNA was enriched using methods described previously (Senda *et al.*, 2004). For siRNA detection, probes were synthesized by an *in vitro* transcription system from pBluescript II SK+ carrying the corresponding PCR fragments. The detection by northern blot hybridization was performed as described before (Senda *et al.*, 2004).

#### Methylation assays

Genomic DNA was isolated from leaves using a DNeasy Plant mini kit (Qiagen). Bisulphite treatment of DNA was conducted using an EpiTect Bisulphite kit (Qiagen) according to the manufacturer's instructions. For bisulphite sequencing, at least 10 clones were sequenced by an ABI 3500 sequencer from each sample. Sequencing data were analysed with the online software Kismeth (<http://katahdin.mssm.edu/kismeth>) (Gruntman *et al.*, 2008).

The methylation-sensitive real-time PCR was conducted as described previously (Jones *et al.*, 2001) with some modification. A 1  $\mu$ g aliquot of genomic DNA was digested by *Ava*II overnight and each sample were adjusted to 10 ng  $\mu$ l<sup>-1</sup>. The undigested control was treated in the same way without the enzyme in the digestion system. A 10 ng aliquot of each sample was used in a 20  $\mu$ l reaction system. Each sample was quantitatively normalized by a fragment of the GFP gene without the *Ava*II site. The level of digestion was evaluated by the digestion rate of the *Ava*II site within the GFP coding region. The *Ava*II site distribution map within the 35S:GFP locus is shown in Supplementary Fig. S4 at *JXB* online. Bisulphite PCR was performed as described by Khraiwesh *et al.* (2010). Primer sequences can be found in Supplementary Table S1.

#### Microscopic observation

The samples were embedded in 7% low melting-point agarose and sectioned (100  $\mu$ m thick) transversally using a vibratome (Series 1500 Leica, St Louis, MO, USA). Each sample was monitored with a biological fluorescent microscope (BX61, Olympus, Tokyo, Japan), and their digital images were captured with a digital camera (DP71, Olympus) connected to the microscope. For the fluorescence imaging, a confocal laser scanning microscopy system FluoView 1000 (Olympus, Tokyo, Japan) was used for detection of the fluorescence of mGFP and chlorophyll.

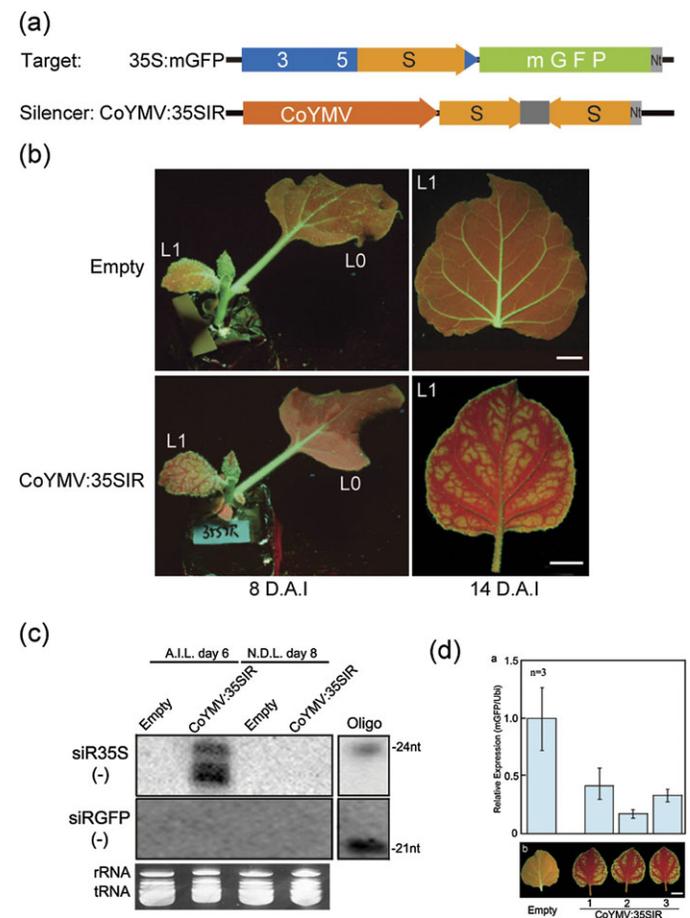
## Results

### Gene silencing in remote sink tissue by transient siRNA production

In this study, the promoter of the 35S:GFP locus in *N. benthamiana* transgenic line 16c was targeted using promoter region siRNA derived from hairpin RNA (35SIR) to induce translational TGS by which loss of the GFP fluorescence was expected. The TGS initiator consisted of an inverted repeat structure of part of the CaMV 35S promoter (-32 to -343) (Okano *et al.*, 2008). The hairpin mRNA was controlled by a companion cell-specific promoter, CoYMV (*Commelina* yellow mottle virus) (CoYMV:35SIR, Fig. 1a), to increase the potential siRNA level in the phloem.

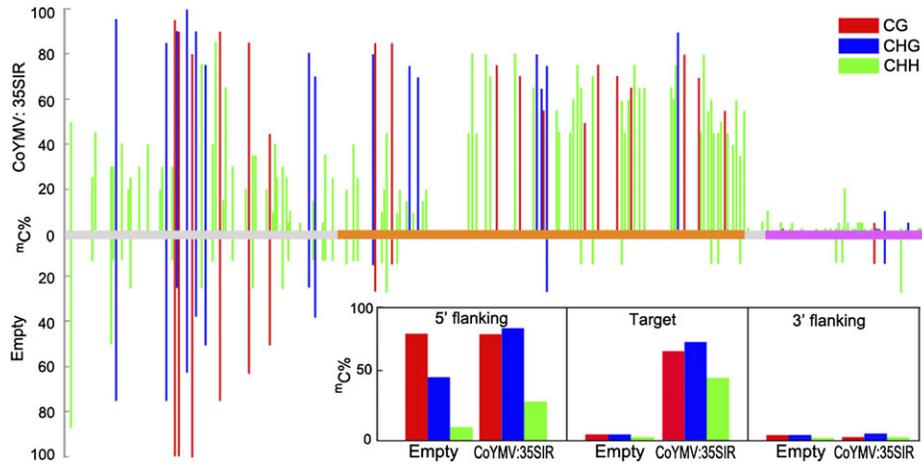
Agroinfiltration of CoYMV:35SIR using the methods described (English *et al.*, 1997; Voinnet *et al.*, 1998) induced GFP silencing in the upper developed leaves, but the degree of evident silencing was fairly low (Supplementary Fig. S1 at *JXB* online). As siRNA is usually transported from the source organs to the sink organs (Crete *et al.*, 2001; Tournier *et al.*, 2006), the agroinfiltration procedure was slightly modified, using plants from which the leaves had been removed, leaving only the first fully expanded leaf, in order to force siRNA transmission (Supplementary Fig. S2). As a control, the infiltration was performed with *Agrobacterium* harboring a construct without the CoYMV:35SIR structure.

One week after infiltration, GFP fluorescence began to disappear in the region infiltrated by CoYMV:35SIR, but



**Fig. 1.** Transmission of GFP silencing by agroinfiltration.

(a) Schematic representation of the constructs used for the target and the silencer. (b) 16c plants infiltrated by an empty control vector ('Empty') and CoYMV:35SIR. The plants at 8 dpi (days post-infiltration) injected with empty vector and CoYMV:35SIR. L0, injected leaf; L1, the first newly developing leaf. The L1 of both the empty vector and CoYMV:35SIR at 14 dpi is also shown. (c) RNA gel blot analysis of siRNA targeting the 35S promoter (upper panel) and mGFP (lower panel) in agroinfiltrated leaves (A.I.L. at 6 dpi) and newly developing leaves (N.D.L. at 8 dpi). (d) qRT-PCR analysis of GFP mRNA at 12 dpi. One empty control and three silenced leaves (lower panel) were analysed using three technical replicates. Scale bars=1 cm.

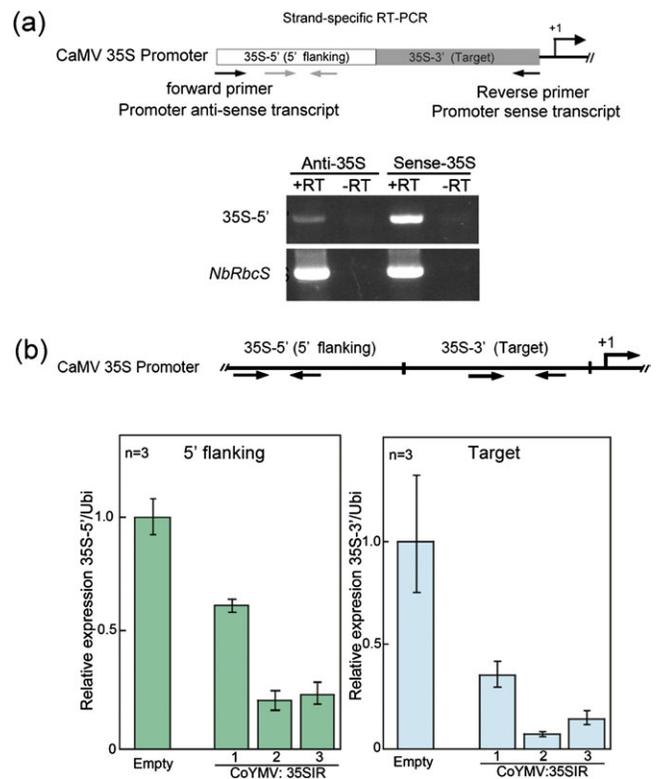


**Fig. 2.** DNA methylation status in the target and flanking regions of the transgenic 35S promoter. The leaf tissue showing GFP silencing was harvested under UV light and subjected to bisulphite sequencing. The percentages of methylation at individual cytosines in CG (red), CHG (blue), and CHH (green) located in the target (−343 to −32, orange), 5′-flanking (−344 to −594, grey), and 3′-flanking (−32 to +161, violet) regions in the silenced leaves after injection of CoYMV:35SIR (upper) and empty vector (lower). The methylation rates of cytosines with different sequence backgrounds are shown in the inset box.

not in plants infiltrated by the empty control (Fig. 1b, L0). The silenced region contained 21, 22, and 24 nucleotide siRNA (Fig. 1b) from the dsRNA 35S promoter sequence, which had presumably resulted from multiple DCL enzymes acting on abundant hairpin RNAs in the plants (Fusaro *et al.*, 2006). Meanwhile, no GFP-targeting siRNA was detected (Fig. 1c), thus excluding the possibility that the GFP expression had been suppressed by the PTGS pathway.

In the first emergent leaf (L1) of CoYMV:35SIR-injected plants, loss of GFP fluorescence was observed in the cells around the veins (Fig. 1b, L1). However, 1 week later, the distinct silencing manifestation had not expanded to the whole leaf, unlike the case for PTGS (Fig. 1b) (Ruiz *et al.*, 1998b). The lower mRNA level of GFP paralleled the loss of GFP fluorescence (Fig. 1d), indicating that the 35S:GFP transgene was silenced in the cells coloured in red under UV light. However, the siRNAs corresponding to the 35S target region or the GFP coding region could not be detected by northern blotting (Fig. 1c), presumably due to a very low amount of the mobile siRNA from the infiltrated leaves having induced the GFP silencing as reported (Molnar *et al.*, 2010).

To clarify the *de novo* DNA methylation status in the target and its flanking regions, bisulphite sequencing was carried out. The tissue showing the GFP fluorescence vanishing in newly developing leaves was harvested carefully under UV light and used for bisulphite sequencing. Most of the cytosine residues in the target promoter region were methylated in the silenced parts of the CoYMV:35SIR-infiltrated plants, ~70% of CG, 80% of CHG, and 50% of CHH sites being methylated (Figs 2, S3). In contrast, the cytosine residues in the 3′-flanking region, which encodes GFP protein, showed a negligible methylation level, almost equivalent to that obtained with the



**Fig. 3.** 35S promoter RNA transcript expression. (a) Total RNA was prepared from the young leaves of 16c plants. Both the sense and the antisense transcript were detected with the strand-specific reverse transcription primers. NbRbcS mRNA was applied as the internal control for the reverse transcription reaction. (b) Down-regulation of the transcript level in silenced leaves shown in Fig. 1d was determined by qRT-PCR separately. qRT-PCRs were carried out with the target region-specific primers (left) and the 5′-flanking region-specific primers (right).

empty control, again excluding the possibility that the loss of GFP in these leaves had been due to degradation of the mRNA (PTGS), which is accompanied by strong DNA methylation in the target region (Jones *et al.*, 1999). Therefore, the siRNA triggered the TGS in the remote developing leaves in 16c plants.

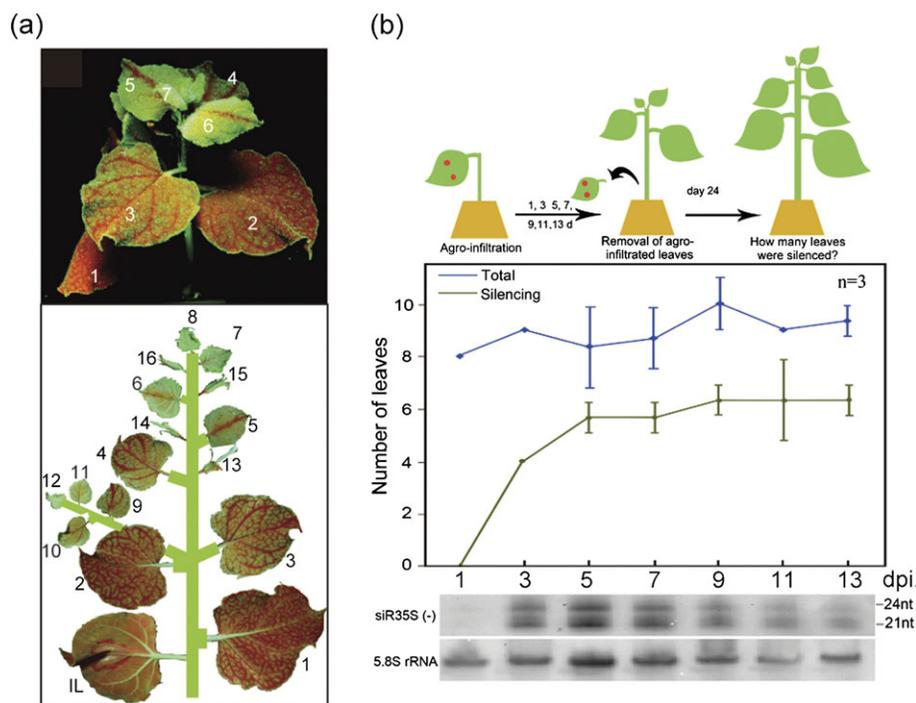
The bisulphite sequencing results also showed that the 5'-flanking region in the empty control plants exhibited a high level of symmetric DNA methylation (~80% of CG and 50% of CHG sites) background, and the methylation level of the CHG and CHH sites was additionally increased in the silenced leaves in CoYMV:35SIR-infiltrated plants (Fig. 2), suggesting possible spreading of the RdDM, in which the promoter transcripts could be involved (Daxinger *et al.*, 2009). Indeed, RT-PCR analysis revealed the presence of bidirectional transcripts of the 35S promoter region in 16c plants (Fig. 3a). Both the transcripts were down-regulated in the same portions of the newly developing leaves as those showing the decrease of GFP mRNA detected with the primer sets located in the target region and the 5'-flanking region (Fig. 3b), revealing the degradation of RNA after infiltration. However, using northern blotting, it was not possible to detect the transitive siRNA targeting the 5'-flanking region, probably due to insufficient sensitivity of the digoxigenin (DIG)-labelled probe. However, it seems that the putative siRNA transitivity only

occurred in the 3' to 5' direction but not the opposite direction, because the DNA methylation did not increase in the 3'-flanking region. The DNA methylation status in and around the target region was also confirmed by methylation-sensitive real-time PCR (Supplementary Fig. S4 at *JXB* online).

#### Limited TGS in newly developed leaves

As the new leaves developed in sequence, the strength of GFP silencing declined (Fig. 4a). Initially, a few newly developed leaves exhibited unequivocal silencing in the major and minor veins. In the fourth to fifth leaf, stronger silencing was seen only in the tips, and the leaves that developed subsequently only showed silencing in the main vein or even the petiole. Finally, leaves above the seventh leaf were completely devoid of silencing. GFP silencing was also observed in leaves on secondary branches, peduncle, and collateral flowers above the infiltrated leaf (IL; Fig. 4a).

The kinetics of GFP loss in the present system were assessed by periodic removal of the injected leaf (Fig. 4b). The amount of siRNA in the removed infiltrated leaf was analysed, and the position in the leaf that the silencing had reached was inspected at 24 days post-infiltration (dpi). SiRNA from CoYMV:35SIR had accumulated at 3 dpi, and reached a maximum at 5 dpi, followed by a gradual decrease



**Fig. 4.** The systemic spreading of TGS in the plant. (a) A typical 16c plant exhibiting TGS manifestation of the 35S:mGFP transgene by CoYMV:35SIR agroinfiltration. The plant (upper), the detached leaves (abaxial side), and the flowers were photographed under UV light and digitally arranged (lower). Note that the GFP silencing was limited to the tissue around the vein and that the silencing strength declined with the plant development. (b) Kinetics of transmission of the silencing signal. The infiltrated leaf was periodically removed (upper), and the number of silenced newly developing leaves was counted at 24 dpi (middle). Three plants were monitored for each time point. The siRNA accumulation in the removed leaves was also detected with northern blotting (lower). Note that the number of silenced leaves remained the same in all the plants whose infiltrated leaf had been removed after 5 dpi although the siRNA was continuously produced in infiltrated leaves.

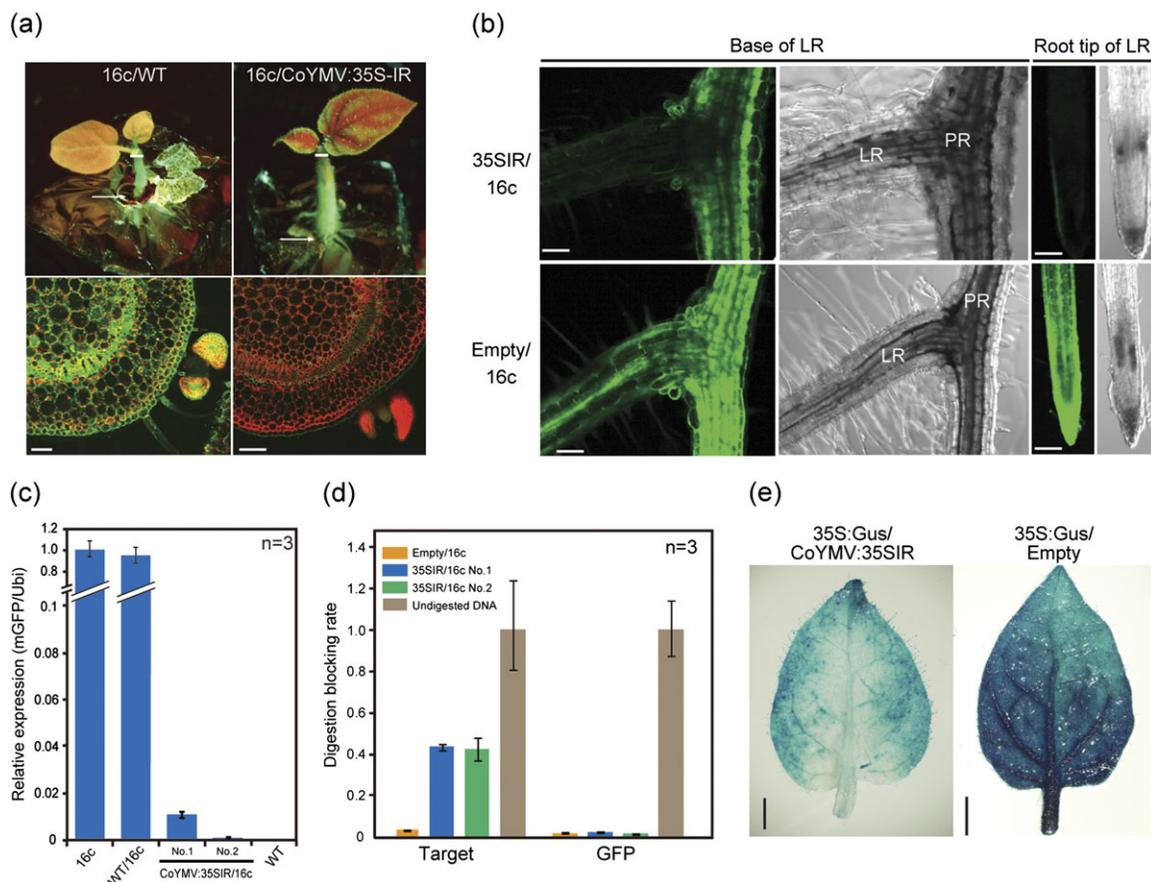
in the infiltrated leaf. The leaf position that the silencing signal reached was almost the same in all the plants whose infiltrated leaf had been removed after 5 dpi (Fig. 4b). These results confirmed that the siRNA produced in infiltrated leaf and transmitted within 5 dpi was responsible for induction of silencing in the newly developed leaves, whereas siRNA transmitted beyond 5 dpi was ineffective. These findings suggested that the amount of siRNA derived from the injected leaf became insufficient for induction of silencing as the volume of shoots increased.

#### TGS induction by grafting

To investigate whether the siRNA that induces TGS in an agroinfiltration system is graft transmissible, transgenic plants harboring the CoYMV:35SIR were prepared. In the initial micrografting experiment, the 16c shoot was grafted

onto a CoYMV:35SIR root. Only one out of a total of 30 grafted plants showed the silenced phenotype in a limited area of tissue around the leaf vein (Supplementary Fig. S1 at *JXB* online). The experimental procedure was then modified by removing all the developed leaves 3 weeks after grafting to promote the root-to-scion phloem flow (Supplementary Fig. S2). In this experiment, the newly developed leaves in all 80 grafted plants exhibited GFP loss around the veins (Fig. 5A). Cross-sections of the stem taken at various points along the route of siRNA signal transport demonstrated suppression of the GFP phenotype (Fig. 5a).

Since it is known that siRNA transport from shoot to root is more efficient than vice versa (Molnar *et al.*, 2010), reverse grafting of a CoYMV:35SIR scion onto a 16c rootstock was performed. Two weeks after grafting, GFP silencing was clearly evident in the root. It was noteworthy that lateral



**Fig. 5.** Systemic TGS in the grafting system. (a) Manifestation of TGS in a 16c plant grafted onto CoYMV:35SIR rootstock. GFP fluorescence in tissue surrounding the vein in newly developed leaves was lost (upper). GFP in a cross-section of the stem of a 16c scion (white bars in upper) was also silenced (lower). Photographs were taken a week after pruning. Arrowheads indicate the graft union. Bar=50  $\mu$ m. (b) TGS induction in lateral roots. GFP expression in 16c root stock was suppressed completely in the lateral roots, including the root apical meristem. The roots were observed 14 d after grafting. The GFP fluorescence of the entire root system is shown in Supplementary Fig. S5 at *JXB* online. LR, lateral root; PR, primary root. Bar=100  $\mu$ m. (c) qRT-PCR analysis of mGFP mRNA in the 16c root grafted under the CoYMV:35SIR shoot. Roots from two individual grafts (no. 1 and no. 2) were harvested 3 weeks after grafting. (d) The DNA methylation status of the 35S target region analysed by methylation-sensitive RT-PCR in the 16c rootstocks. The *Av*11 site map at the 35S:GFP locus is shown in Supplementary Fig S4 at *JXB* online. (e) GUS staining of the newly developed leaf for the 35S:GUS/CoYMV:35SIR grafting combination. GUS expression was suppressed in the tissue around the vein. Photographs were taken 1 week after pruning, in accordance with Supplementary Fig. S2. Bar=4 mm. The graft combination is marked as scion/rootstock.

roots, including the root apical meristem, showed a dramatic loss of GFP fluorescence (Fig. 5b). The quantity of GFP mRNA in the 16c root grafted under the CoYMV:35SIR shoot was as low as <1% of that in the root grafted under the empty control (Fig. 5c), and a similar DNA methylation level in the 35S promoter region to that in silenced leaf tissue was detected in the silenced root by methylation-sensitive real-time PCR (Fig. 5d, Supplementary Fig. S4 at *JXB* online), again illustrating that the TGS was induced by the CoYMV:35SIR scion.

Moreover, a 35S:GUS-transgenic plant which had been prepared, in which the 35S promoter transcript was not detected by RT-PCR unlike in the case of 16c, was also used to induce TGS by the mobile siRNA with a similar method. As a result, GUS expression in tissue around the main vein was strongly inhibited in the newly developed leaves of the 35S:GUS scion grafted onto the CoYMV:35SIR rootstock, but not the empty control (Fig. 5e). These results confirmed that the TGS signals were transmissible through the graft union, and could effectively induce TGS in the root system, especially the lateral roots. Moreover, the promoter transcript in the recipient tissue is not necessary to induce systemic TGS because of the absence of 35S promoter transcript in 35S:GUS.

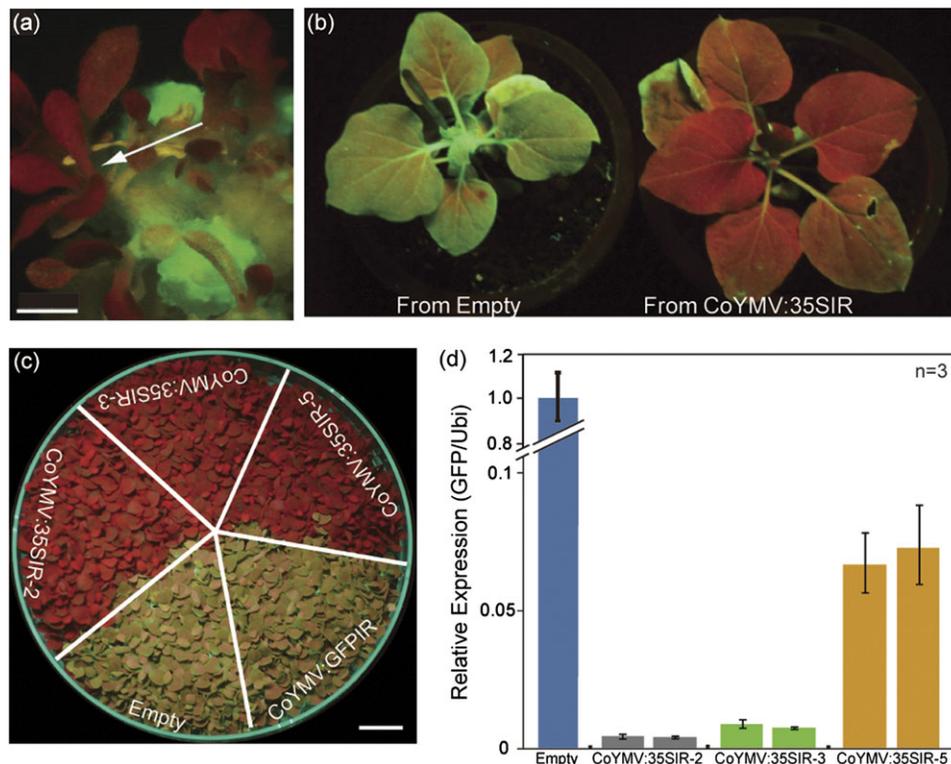
### Maintenance of TGS through *in vitro* regeneration

To obtain plants with epigenetic modification of 35S:GFP, *in vitro* regeneration was performed with silenced leaf tissue in newly developing leaves from 16c infiltrated by CoYMV:35SIR. Among the many regenerated shoots, several exhibited no GFP fluorescence (Fig. 6a), whereas no shoots showed loss of GFP in the regeneration experiment using the empty control (Fig. 6b). All of the selfed progeny of three independent silenced shoots showed the GFP-silenced phenotype (Fig. 6c), although their GFP transcription was not completely arrested in these selfed progeny, but suppressed to <10% of the control level (Fig. 6d).

Kanazawa *et al.* (2011) reported that both the symmetric and the asymmetric DNA methylation can be meiotically maintained in the absence of an RNA trigger for RdDM in virus-induced TGS. The present results also showed that the systemic TGS could be maintained through *in vitro* regeneration and further passed to the selfed progeny.

## Discussion

It is well known that PTGS is non-cell autonomous, and can spread to other parts of a plant through a process known as



**Fig. 6.** Regenerated plants from the silenced tissue and their progeny. (a) The silenced shoot (white arrow) regenerated from the callus. The silenced tissue around the vein in newly developing leaves 14 dpi was used for tissue culture as described in the Materials and methods. Bar=1 cm. (b) The plants regenerated from the empty control (left) and the silenced tissue (right). (c) Selfed progeny from three independent regenerated plants showed GFP silencing. As a control, the progeny corresponding to 'Empty' plants are shown. To illustrate the difference compared with post-transcriptional gene silencing (PTGS), the progeny of the regenerated plants from the systemic silenced leaf tissue of the plants injected with CoYMV:GFPIR are also shown. Bar=1 cm. (d) The GFP expression in the progeny of the regenerated lines shown in (c) was detected by qRT-PCR. Two individual plants were selected for each line as biological replicates.

systemic silencing (Palauqui *et al.*, 1997; Voinnet and Baulcombe, 1997; Voinnet *et al.*, 1998). The molecule responsible for long-distance spreading of the signal was recently defined as siRNA, which can be transmitted through the sieve element over long distances via phloem flow, and is able to induce DNA methylation in recipient tissue (Molnar *et al.*, 2010). It has been predicted theoretically that not only PTGS but also TGS can be induced by siRNA transmitted over a long distance. However, some reports have shown that TGS is not graft transmissible (Mlotshwa *et al.*, 2002; Mourrain *et al.*, 2007). In the present study, the agroinfiltration system was slightly modified by simplifying the source–sink relationship to promote transmission of the siRNA to a simple sink organ. Furthermore, visible marker genes (GFP and GUS) were applied to allow easy monitoring of the silenced phenotype. This approach allowed observation of the TGS in the initially developed leaves, and to prove the graft transmissibility of the TGS. However, it proved impossible to detect the expected siRNA in newly developed leaves, even though they manifested TGS, perhaps because the amount of transmitted siRNA was too low to be detected by the northern blot system used here, so the possibility that siRNA could be the mobile signal could not be excluded. Recently, by using a *dcl2,3,4* triple mutant of *Arabidopsis* that is unable to produce siRNA and deep sequencing technology, Molnar *et al.* (2010) demonstrated the transportation of siRNA and that the transferred siRNA can induce *de novo* DNA methylation in the recipient tissue. However, there are no reports that the manifestation of TGS was induced by the transported siRNA from the grafted partner.

As well as the systemic induction of TGS by transmission of an siRNA molecule from an inverted repeat transgene, like that demonstrated here, it has been shown that systemic TGS can also be induced by VIGS (Jones *et al.*, 1999, 2001). However, there are differences in characteristics between these processes. In the case of VIGS, not only the siRNA but also the virus moves over a long distance, whereas in the case of the hairpin RNA silencer used in the present system, the mobile molecule is exclusively siRNA. Thus, the absence of a system for amplification of siRNA within the cells to which it is transmitted would limit the degree of TGS spreading, and, as a result, TGS would be manifested in cells close to the veins in initially developing leaves. A similar phenomenon has been observed in VIGS using a virus lacking the ability to move from cell to cell (Voinnet *et al.*, 2000; Bayne *et al.*, 2005). The resulting TGS was also restricted to cells in the vicinity of leaf veins where the virus replicated (Himber *et al.*, 2003; Vaistij and Jones, 2009). Compared with these results, the present TGS method affected a much wider area along the veins than was the case for VIGS mentioned above. In the TGS induction system used here, the siRNA may be imported to newly developing leaves in the early stage of development, and thus subsequent propagation of the cells showing silencing would contribute to expansion of the silenced area because the TGS would be maintained through cell division.

The siRNA transmitted through the sieve element did not affect the shoot apical meristem (Voinnet *et al.*, 1998;

Vaistij and Jones, 2009). Unlike the shoot, almost total TGS induction was observed throughout the whole of the lateral roots, including the root apical meristems. Lateral roots in angiosperms are initiated from the cells of the pericycle, which is bound to phloem cells. Therefore, lateral roots originate endogenously from tissues lying inside the parent root (Lloret and Casero, 2002). The founder cells that undergo TGS become the lateral root primordium, which eventually forms a TGS lateral root. Therefore, it was demonstrated that siRNA transmitted over a long distance can induce epigenetic modification in lateral roots. In many plants the lateral roots constitute almost the entire root system. They influence many aspects that are important for environmental adaptation and absorption capacity, thus affecting plant size, plant production, vitality, and other characteristics (Lloret and Casero, 2002). The TGS induction system used here may allow the development of a new approach for improvement of the plant root system.

VIGS has been widely used to silence target genes in studies of gene functions in plants within different families (Burch-Smith *et al.*, 2004; Lu *et al.*, 2007; Zhou *et al.*, 2007; Renner *et al.*, 2009; Tang *et al.*, 2010). Some reports have also proposed the potential application of VIGS to plant breeding, because the TGS is heritable and normally the virus is not transmitted to seed (Kanazawa *et al.*, 2011). However, as most horticultural crops, such as fruit trees and some vegetables, are propagated vegetatively, it would be difficult to eliminate the virus from the plants. The almost complete silencing in lateral roots induced by the present system is of considerable interest, as it would enable regenerated TGS plants to be obtained from the root through tissue culture. Furthermore, some fruit trees or shrubs can readily form adventitious shoots (root suckers) from the root (Kormanik and Brown, 1967). Moreover, a plant that has acquired the ability to transmit siRNA could be grafted onto appropriate cultivars as a stock or scion as long as there is mutual grafting compatibility, and consequently the siRNA transmission would induce TGS in the graft partners, suggesting the possibility that several cultivars could be improved using only one transgenic plant.

The micrografting and pruning method used in this study is similar to the grafting technique usually employed for fruit tree production. Usually, in grafting for propagation, a small part of the shoot with a dormant bud is grafted onto the rootstock, creating a strong sink power in the scion during the period of initial growth. Therefore, in actual fruit tree grafting, siRNA transmission from the rootstock would be expected to induce TGS effectively in the scion. Future research goals will focus on the development of a breeding system by which endogenous genes can be transcriptionally silenced by mobile siRNA from a grafted transgenic partner.

#### Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1.** Weaker silencing manifestation by using the common agroinfiltration and grafting methods.

**Figure S2.** The process of agroinfiltration (A) and micro-grafting (B) methods.

**Figure S3.** DNA methylation status in the 35S:GFP locus in the silenced tissue of agroinfiltrated 16c.

**Figure S4.** DNA methylation status of the 35S promoter target region and the 5'-flanking region corresponding to Fig. 2A.

**Figure S5.** GFP expression and GFP silencing in the 16c root stocks.

**Table S1.** Sequences of the primers used.

## Acknowledgements

We are grateful to Professor David Baulcombe and Professor N. Olszewski for the *N. benthamiana* 16c line and the CoYMV promoter, respectively. We thank Ms S. Ohzeki and Ms S. Kida for technical assistance. Part of this work was done at the Gene Research Center of Hirosaki University. This work was supported by the Program for Promotion of Basic Activities for Innovative Bioscience (PROBRAIN) in Japan, a Grant-in-Aid for Scientific Research in Japan (22380003), and a Grant for Hirosaki University Institutional Research.

## References

- Allison R, Thompson C, Ahlquist P.** 1990. Regeneration of a functional RNA virus genome by recombination between deletion mutants and requirement for cowpea chlorotic mottle virus 3a and coat genes for systemic infection. *Proceedings of the National Academy of Sciences, USA* **87**, 1820–1824.
- Baulcombe D.** 2004. RNA silencing in plants. *Nature* **431**, 356–363.
- Bayne EH, Rakitina DV, Morozov SY, Baulcombe DC.** 2005. Cell-to-cell movement of Potato Potexvirus X is dependent on suppression of RNA silencing. *The Plant Journal* **44**, 471–482.
- Brosnan CA, Mitter N, Christie M, Smith NA, Waterhouse PM, Carroll BJ.** 2007. Nuclear gene silencing directs reception of long-distance mRNA silencing in Arabidopsis. *Proceedings of the National Academy of Sciences, USA* **104**, 14741–14746.
- Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP.** 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *The Plant Journal* **39**, 734–746.
- Crete P, Leuenberger S, Iglesias VA, Suarez V, Schob H, Holtorf H, van Eeden S, Meins F.** 2001. Graft transmission of induced and spontaneous post-transcriptional silencing of chitinase genes. *The Plant Journal* **28**, 493–501.
- Daxinger L, Kanno T, Bucher E, van der Winden J, Naumann U, Matzke AJM, Matzke M.** 2009. A stepwise pathway for biogenesis of 24-nt secondary siRNAs and spreading of DNA methylation. *EMBO Journal* **28**, 48–57.
- English J, Davenport G, Elmayan T, Vaucheret H, Baulcombe D.** 1997. Requirement of sense transcription for homology-dependent virus resistance and trans-inactivation. *The Plant Journal* **12**, 597–603.
- Fusaro AF, Matthew L, Smith NA, et al.** 2006. RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. *EMBO Reports* **7**, 1168–1175.
- Gruntman E, Qi YJ, Slotkin RK, Roeder T, Martienssen RA, Sachidanandam R.** 2008. Kismeth: analyzer of plant methylation states through bisulfite sequencing. *BMC Bioinformatics* **9**, 371.
- Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, Voinnet O.** 2003. Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO Journal* **22**, 4523–4533.
- Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe DC.** 1999. RNA–DNA interactions and DNA methylation in post-transcriptional gene silencing. *The Plant Cell* **11**, 2291–2301.
- Jones L, Ratcliff F, Baulcombe DC.** 2001. RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. *Current Biology* **11**, 747–757.
- Kanazawa A, Inaba J, Shimura H, Otagaki S, Tsukahara S, Matsuzawa A, Kim BM, Goto K, Masuta C.** 2011. Virus-mediated efficient induction of epigenetic modifications of endogenous genes with phenotypic changes in plants. *The Plant Journal* **65**, 156–168.
- Khraiweh B, Arif MA, Seumel GI, Ossowski S, Weigel D, Reski R, Frank W.** 2010. Transcriptional control of gene expression by MicroRNAs. *Cell* **140**, 111–122.
- Kormanik PP, Brown CL.** 1967. Root buds and the development of root suckers in sweetgum. *Forest Science* **13**, 338–345.
- Lloret PG, Casero PJ.** 2002. Lateral root initiation. In: Waisel Y, Eshel A, Kafkafi U, eds. *Plant roots—the hidden half*. New York: Marcel Dekker, 127–155.
- Lu HC, Chen HH, Tsai WC, Chen WH, Su HJ, Chang DCN, Yeh HH.** 2007. Strategies for functional validation of genes involved in reproductive stages of orchids. *Plant Physiology* **143**, 558–569.
- Mlotshwa S, Voinnet O, Mette MF, Matzke M, Vaucheret H, Ding SW, Pruss G, Vance VB.** 2002. RNA silencing and the mobile silencing signal. *The Plant Cell* **14 Suppl**, S289–S301.
- Molnar A, Melnyk CW, Bassett A, Hardcastle TJ, Dunn R, Baulcombe DC.** 2010. Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* **328**, 872–875.
- Mourrain P, van Blokland R, Kooter JM, Vaucheret H.** 2007. A single transgene locus triggers both transcriptional and post-transcriptional silencing through double-stranded RNA production. *Planta* **225**, 365–379.
- Okano Y, Miki D, Shimamoto K.** 2008. Small interfering RNA (siRNA) targeting of endogenous promoters induces DNA methylation, but not necessarily gene silencing, in rice. *The Plant Journal* **53**, 65–77.
- Palauqui JC, Elmayan T, Pollien JM, Vaucheret H.** 1997. Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO Journal* **16**, 4738–4745.
- Renner T, Bragg J, Driscoll HE, Cho J, Jackson AO, Specht CD.** 2009. Virus-induced gene silencing in the culinary ginger (*Zingiber officinale*): an effective mechanism for down-regulating gene expression in tropical monocots. *Molecular Plant* **2**, 1084–1094.
- Ruiz MT, Voinnet O, Baulcombe DC.** 1998a. Initiation and maintenance of virus-induced gene silencing. *The Plant Cell* **10**, 937–946.
- Ruiz MT, Voinnet O, Baulcombe DC.** 1998b. Initiation and maintenance of virus-induced gene silencing. *The Plant Cell* **10**, 937–946.

- Senda M, Masuta C, Ohnishi S, Goto K, Kasai A, Sano T, Hong JS, MacFarlane S.** 2004. Patterning of virus-infected *Glycine max* seed coat is associated with suppression of endogenous silencing of chalcone synthase genes. *The Plant Cell* **16**, 807–818.
- Tang Y, Wang F, Zhao JP, Xie K, Hong YG, Liu YL.** 2010. Virus-based microRNA expression for gene functional analysis in plants. *Plant Physiology* **153**, 632–641.
- Tournier B, Tabler M, Kalantidis K.** 2006. Phloem flow strongly influences the systemic spread of silencing in GFP *Nicotiana benthamiana* plants. *The Plant Journal* **47**, 383–394.
- Turnbull CGN, Booker JP, Leyser HMO.** 2002. Micrografting techniques for testing long-distance signalling in *Arabidopsis*. *The Plant Journal* **32**, 255–262.
- Vaistij FE, Jones L.** 2009. Compromised virus-induced gene silencing in RDR6-deficient plants. *Plant Physiology* **149**, 1399–1407.
- Voinnet O, Baulcombe DC.** 1997. Systemic signalling in gene silencing. *Nature* **389**, 553–553.
- Voinnet O, Lederer C, Baulcombe DC.** 2000. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* **103**, 157–167.
- Voinnet O, Rivas S, Mestre P, Baulcombe D.** 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *The Plant Journal* **33**, 949–956.
- Voinnet O, Vain P, Angell S, Baulcombe DC.** 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* **95**, 177–187.
- Waterhouse PM, Wang MB, Lough T.** 2001. Gene silencing as an adaptive defence against viruses. *Nature* **411**, 834–842.
- Zhou HB, Li SF, Deng ZY, et al.** 2007. Molecular analysis of three new receptor-like kinase genes from hexaploid wheat and evidence for their participation in the wheat hypersensitive response to stripe rust fungus infection. *The Plant Journal* **52**, 420–434.