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Phloem flow strongly influences the systemic spread of silencing in GFP *Nicotiana benthamiana* plants

Barthélémy Tournier[*,†], Martin Tabler[‡] and Kriton Kalantidis
Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology – Hellas, PO Box 1527, GR-71110 Heraklion/Crete, Greece

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[*]For correspondence (fax +30 2810 394 366; e-mail barthelemy.tournier.1@ulaval.ca or kriton@imbb.forth.gr).
[†]Present address: Hôpital St François d'Assise, CHUQ, URGHM-CRSFA local D0-730, 10 rue de l'Espinay, QC, Canada G1L 3L5.
[‡]Deceased 3 April 2005.

Summary

The term 'RNA silencing' describes a process that results in the specific degradation of an RNA target. In plants, silenced tissues can initiate the spreading of the process into non-silenced regions by a mobile signal that can be transmitted over long distances. In the present work, we made use of a modified grafting approach to elucidate the driving force behind long-distance transport of the silencing signal. We made reciprocal grafts of two GFP-transgenic *Nicotiana benthamiana* lines, the non-silenced line 16c (sensor) and the silenced line 6.4 (inducer). We show that the direction of systemic spread of silencing from inducer to sensor can be manipulated by altering sink/source relations in the plant. Using radioactive phosphate as a phloem tracer, we demonstrated that plants that transmitted silencing from silenced scion to non-silenced rootstock had developed a persisting phloem flow from scion to rootstock. These data provide experimental proof of what has been hypothesized so far, that the silencing signal travels via phloem from source to sink. We present here evidence that the appearance of systemic silencing is not an accidental stochastic process, but can be predicted on the basis of the direction of phloem flow.

Keywords: systemic silencing, mobile signal, GFP, graft, phloem transport, phloem tracer.

Introduction

Higher eukaryotes have developed a mechanism of sequence-specific RNA degradation called 'RNA silencing', an idiom combining the previous terms 'post-transcriptional gene silencing' (PTGS) and 'RNA interference' (RNAi). Despite common features of RNA silencing, there are differences between the animal and plant kingdoms and also amongst species (for review see for example Baulcombe, 2004; Meister and Tuschl, 2004; Mello and Conte, 2004; Sontheimer, 2005; Vance and Vaucheret, 2001; Voinnet, 2002). The core of the RNA degradation pathway is the generation of short interfering RNA (siRNA) from a double-stranded RNA by a double-strand-specific RNase called Dicer. The siRNAs are incorporated into the RNA-induced silencing complex (RISC), and, after strand separation, the remaining single-stranded RNA guides the sequence-specific cleavage of a target RNA.

The mechanism of RNA silencing in plants is more complex than in most animals. Different size classes of siRNAs ranging from 21 to 26 nt can be found (Hamilton *et al.*, 2002), as well as different forms of Dicer (Schauer *et al.*, 2002). Further, plant RNA silencing is non-cell-autonomous. It can spread from an initially silenced cell to a neighbouring cell, and silencing can spread over a long distance to different parts of the plant (Palauqui *et al.*, 1997; Voinnet and Baulcombe, 1997; Winston *et al.*, 2002). Local cell-to-cell spreading seems to be signalled by the shorter siRNA (21 or 22 nt), which are translocated trough plasmodesmata. The longer siRNA (24–26 nt) are believed to be involved in the long-distance spread of silencing (Hamilton *et al.*, 2002; Himber *et al.*, 2003), but other RNA forms seem to be required for that step as well, because the viral suppressor protein HC-Pro could inhibit siRNA accumulation without interfering with the spread of systemic silencing (Mallory *et al.*, 2003). Thus, despite the recent detection of siRNA in the phloem sap (Yoo *et al.*, 2004), the exact nature of the mobile silencing signal is still under investigation (Baulcombe, 2004; Fagard and Vaucheret, 2000). A candidate could be a plant-derived single-stranded RNA of about 85 nucleotides derived from GFP-silenced plants that could efficiently induce GFP-silencing in *Caenorhabditis elegans* (Boutla *et al.*, 2002).

The spread of a systemic virus infection in plants and the long-distance spread of silencing show a lot of similarities, suggesting that the mobile signal could be transported in the phloem sap in the same way as viral particles (Fagard and Vaucheret, 2000; Jorgensen *et al.*, 1998; Klahre *et al.*, 2002; Mallory *et al.*, 2003; Mlotshwa *et al.*, 2002; Palauqui *et al.*, 1997; Voinnet *et al.*, 1998). Processes that characterize the spread of silencing may be studied by grafting experiments that combine silenced and non-silenced plant tissues (reviewed in Kalantidis, 2004). Studies have shown that a silenced rootstock could induce gene-specific silencing in an originally non-silenced scion (Palauqui *et al.*, 1997; Voinnet *et al.*, 1998). While Palauqui *et al.* (1997) observed transmission of silencing of the endogenous nitrate reductase gene (*Nia*) to the scion in all tobacco grafts, Voinnet *et al.* (1998) found transmission of GFP silencing in *Nicotiana benthamiana* in 10 of 16 grafts. Thus, the transgene, the plant species and the size of the scion might influence the rate of successful transmission of silencing. Neither of the two pioneering groups observed spread of silencing from a silenced scion to a non-silenced rootstock. To our knowledge such a transmission has been reported only once, with a low efficiency, for *N. benthamiana* (Sonoda and Nishiguchi, 2000). The importance of the grafting method in the transmission of silencing was also demonstrated by Crete *et al.* (2001). It is an open question why RNA silencing cannot be efficiently transmitted to the rootstock unlike most viral infections, which will eventually pass to the rootstock (Hull, 2002).

Such stochastic rates and patterns of silencing spread questioned the model of a phloem-transported signalling molecule. In this study, we wished to unravel the transmission of the silencing signal, taking advantage of our *Nicotiana benthamiana* line 6.4 (Boutla *et al.*, 2002 and Kalantidis *et al.*, 2006). Line 6.4 contains a single GFP transgene copy expressing GFP at high levels before it is spontaneously post-transcriptionally silenced without the need for further induction of silencing by an ectopic expression of GFP. By grafting, we combined silenced tissue of line 6.4 with non-silenced GFP-expressing tissues of *N. benthamiana* line 16c, and found that transmission of silencing is also possible from scion to rootstock if a phloem flow is established in that direction. By using a phloem tracer, we provide experimental evidence for a strict correlation between the establishment of phloem sink–source relations and the spread of silencing.

Results

RNA silencing is transmittable from scion to rootstock

Voinnet and Baulcombe (1997) showed for the stably GFP-expressing *N. benthamiana* line 16c that RNA silencing can be transmitted with high efficiency (10/16 plants) from a silenced rootstock to a non-silenced scion, but not in the reverse direction. We modified these grafting experiments with a slight variation: while we used the same transgenic line 16c as a non-silenced sensor, we changed the source of the GFP silencer. Instead of a 16c plant that had been silenced by an ectopically expressed GFP construct, we used as an inducer of RNA silencing our spontaneously silencing *N. benthamiana* line 6.4. Plants of this transgenic line express GFP at high levels at an early stage of development, but then – without a noticeable inducer – they develop silenced spots on their leaves, followed in some cases by subsequent systemic silencing (Kalantidis *et al.*, 2006). As silencing occurs in a spontaneous manner relatively early during development, young silenced 6.4 plants can be used as scions for grafting. At the molecular level, we compared the siRNA content of systemically silenced tissues from lines 6.4 and 16c. Northern blots of small RNAs and Southern analysis of transgene insertion showed that both lines have a single copy of the GFP transgene with similar accumulation of GFP-specific siRNAs (Figure S1).

When we grafted the non-silenced line 16c onto a fully silenced rootstock of a 6.4 plant (Figure 1a) we observed spread of silencing in more than 75% of the experiments (about 20 grafts done at different times), confirming an efficient transfer of the silencing signal from rootstock to scion as observed earlier (Palauqui *et al.*, 1997; Voinnet *et al.*, 1998). The transmission rate was age-dependent and the best results were obtained using 16c scions made of a 3–4 cm apex collected from a non-flowering branch, grafted onto a young fully developed and silenced 6.4 rootstock (plants 1.5 to 2 months old with several expanded leaves and flowers).

As a next step, we prepared the reverse graft using large shoots of young silenced 6.4 plants as a scion, on top of young 16c rootstocks (about eight leaves, of which the lower four were kept in the rootstock). The young scions had a strong growth potential and quickly developed large leaves. After 1 month, approximately one-third of the grafts (again about 20 grafts in different experiments) showed silencing in the rootstock (Figure 1b). The typical silencing pattern was observed initially for several leaves; however, only on some branches of the rootstock (Figure 1b,c). In a parallel grafting experiment, we first silenced 16c plants at a very young stage (three leaves) by ectopic agro-infiltration of GFP hairpin. The apical parts of the plants had been removed in order to allow the development of lateral young and silenced buds, which were then used as scions on top of non-silenced 16c rootstock. This experiment basically reproduced the experimental set-up used previously by Voinnet *et al.* (1998), with the important modification that great care was taken to use young scions with high growth potential in order to increase the likelihood that the scion would develop into a metabolic source tissue. By this modification, we obtained silencing transmission to the rootstock at a significant rate

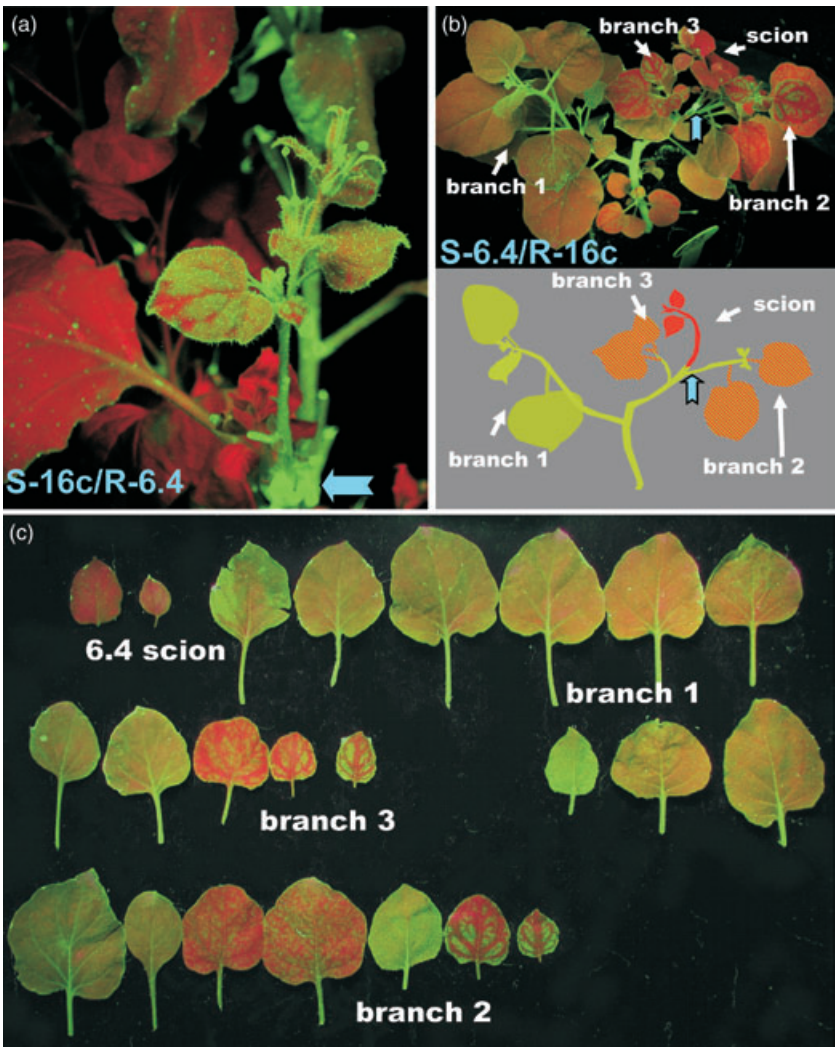

Figure 1. Reciprocal grafts between the silenced *N. benthamiana* GFP line 6.4 and non-silenced GFP line 16c.
All pictures were taken under UV light 1 month after grafting; the graft junction is indicated by a blue arrow.
(a) Graft of a non-silenced 16c scion (S-16c) made of a small apical stem segment on a silenced 6.4 rootstock (R-6.4). The scion is in the process of GFP silencing.
(b) Graft of a scion, consisting of a shoot with four leaves from a non-flowering silenced 6.4 plant (S-6.4) on a non-silenced 16c rootstock, derived from a plant with about eight leaves, of which four remained in the rootstock at the time of grafting. Three branches of the rootstock are marked; some leaves of branches 2 and 3 show silencing, but no leaves of branch 1 show silencing symptoms. A schematic diagram with only some leaves shows in red the silenced scion, in green the non-silenced rootstock branch, and in red–green stripes the partly silenced rootstock branches.
(c) Individual leaves of different parts of the graft in (b). Silencing can be seen in several leaves of branches 2 and 3.

(nine plants on 15 grafts) (data not shown), in contrast to the previous report (Voinnet *et al.*, 1998).

Removal of leaves from the rootstock enforces silencing transmission from silenced scion to rootstock by altering the sink–source relationship

For about two-thirds of the 6.4 silenced scions grafted on a 16c rootstock, there was no spread of silencing to the rootstock 1 month after grafting (Figure 2a), in accordance to what has been described for this type of graft. To test whether reversion of the sink–source relationship would induce transmission of silencing in these plants, we removed all developed leaves from the rootstock (Figure 2b). This made the rootstock a strong sink tissue, and 7–10 days later, we observed initiation of systemic silencing in the new leaves of the rootstock for about half of the defoliated rootstocks (Figure 2c–e). If we kept removing the newly grown developed leaves of the rootstock, we progressively obtained silencing transmission in almost all of the grafts. These results suggest that systemic transmission of silencing is directed from metabolic source to sink. In order to mimic the effect of leaf removal from the rootstock, we covered the entire non-silenced rootstock with aluminium foil, thus converting it into sink tissue, but left the silenced scion exposed to light. Two weeks later, systemic spread of silencing could be detected in some of the rootstock tissue as well (data not shown). However, long-term covering of the rootstock (>15 days) often led to leaves rotting or senescence, and thus is in a way similar to defoliation.

The pattern of silencing spread can be predicted by tracing the phloem flow

The above-described experiments showed that, unlike earlier reports, transmission of silencing is possible from scion to rootstock. The enhancement of transmission of silencing by defoliation or covering the rootstock with aluminium foil

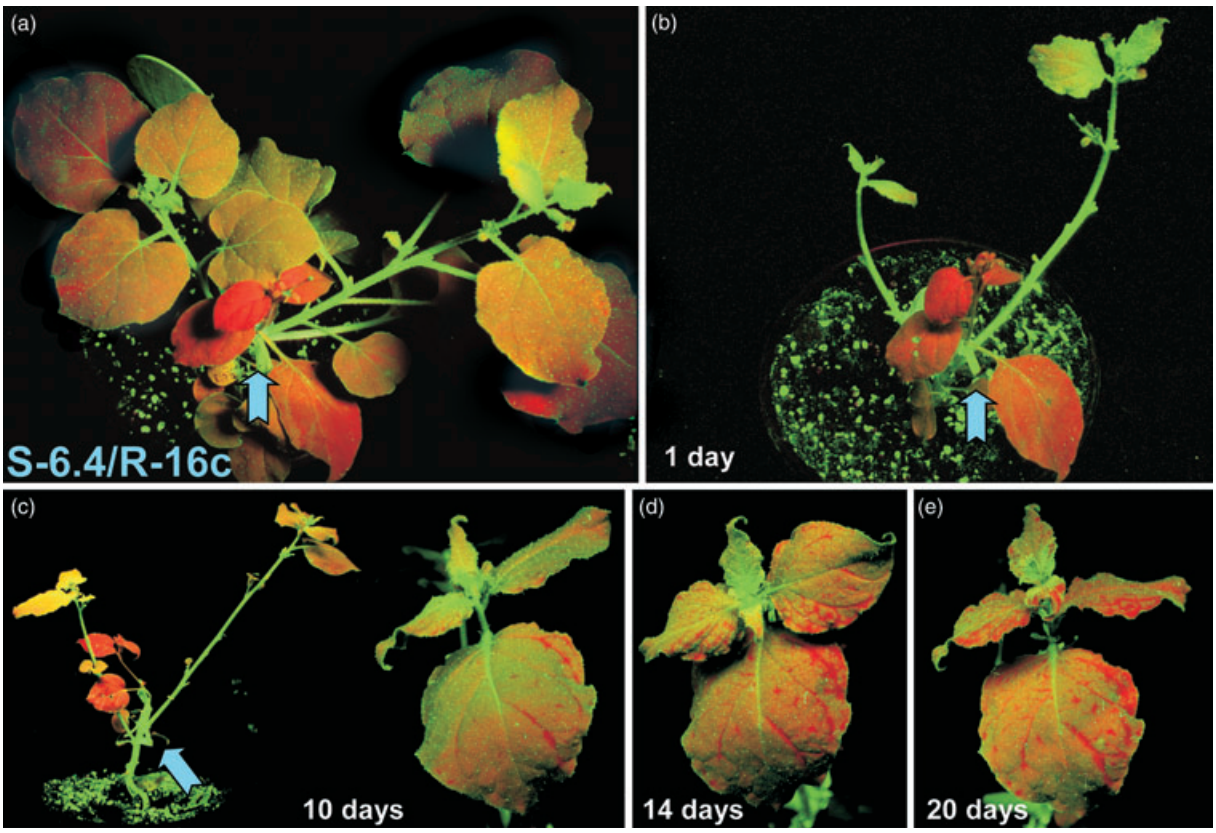

Figure 2. Removal of rootstock leaves induces transmission of silencing from scion to rootstock.
(a) Graft of a silenced 6.4 scion on a 16c rootstock as in Figure 1(b) but without transmission of silencing 1 month after grafting. This phenotype was seen in about two-thirds of the plants.
(b) The grafted plant in (a) 1 day after cutting off leaves from the rootstock.
(c) Defoliated plant in (a) and (b) 10 days after pruning; first signs of silencing can be observed in one branch of the rootstock.
(d,e) Silencing spreads in the rootstock branch, 14 and 20 days after defoliating.

suggested that we had converted our scions into source tissue. We intended to verify this by tracing the flow of phloem. The classical tracer carboxyfluoresceine could not be used here, because it interfered with the emission spectrum of GFP. Instead, we used radioactively labelled inorganic phosphate. Early work with turnip had shown that leaf-injected ^{32}P-labelled phosphate (Pi) will travel in the phloem sap to sink organs, predominantly in the form of pyrophosphate and sugar phosphates (Bieleski, 1969). We infiltrated in one or two of the fully developed source leaves about 1000 nCi of ^{32}Pi in 50–100 μl of a 7% sucrose solution. After 2–3 h, we exposed either the entire plant or selected leaves to an X-ray film. In *N. benthamiana*, similarly to turnip, labelling appeared to be highly phloem-specific, revealing signals in strong sink organs, such as roots, the apical meristem and the growing fruits (Figure 3a–c).

Next, we analysed the pattern of phloem flow in our grafted plants. We always injected radiolabelled Pi into the silenced tissue to monitor movement of the phloem sap. First, we assayed a classical graft of a 16c scion on a silenced 6.4 rootstock, at the stage where the scion showed the first detectable signs of silencing (Figure 3d). Figure 3(d–f) shows that the radioactive tracer was transmitted from the infiltrated leaf in the rootstock directly to the scion (leaves 2 and 3), and only very low amounts of radioactivity could be detected in the developed upper leaf 1 (a source leaf) of the rootstock, although it is much closer to the ^{32}P-treated leaf. It should be noted that the first indications of GFP silencing could be seen along the main vein in leaf 3, but not in leaf 2 at that time. The influx of radioactively labelled phosphate correlated with this pattern, as leaf 3 had a stronger influx of radioactivity. Leaf 2 also received some ^{32}P, and it would possibly have shown systemic silencing at a later stage if not destroyed for the purposes of the experiment.

In the reciprocal graft, we labelled the silenced 6.4 scion on top of a 16c stock (Figure 4). We observed a strong phloem flow within the scion, from the infiltrated source leaf to the apical meristem (Figure 4c,d). In about two-thirds of the grafts, those that did not show silencing transmission from a silenced 6.4 scion to a 16c rootstock, very little (if any) radioactivity could be detected in the rootstock (data not shown). However, when we labelled a 6.4 scion that had

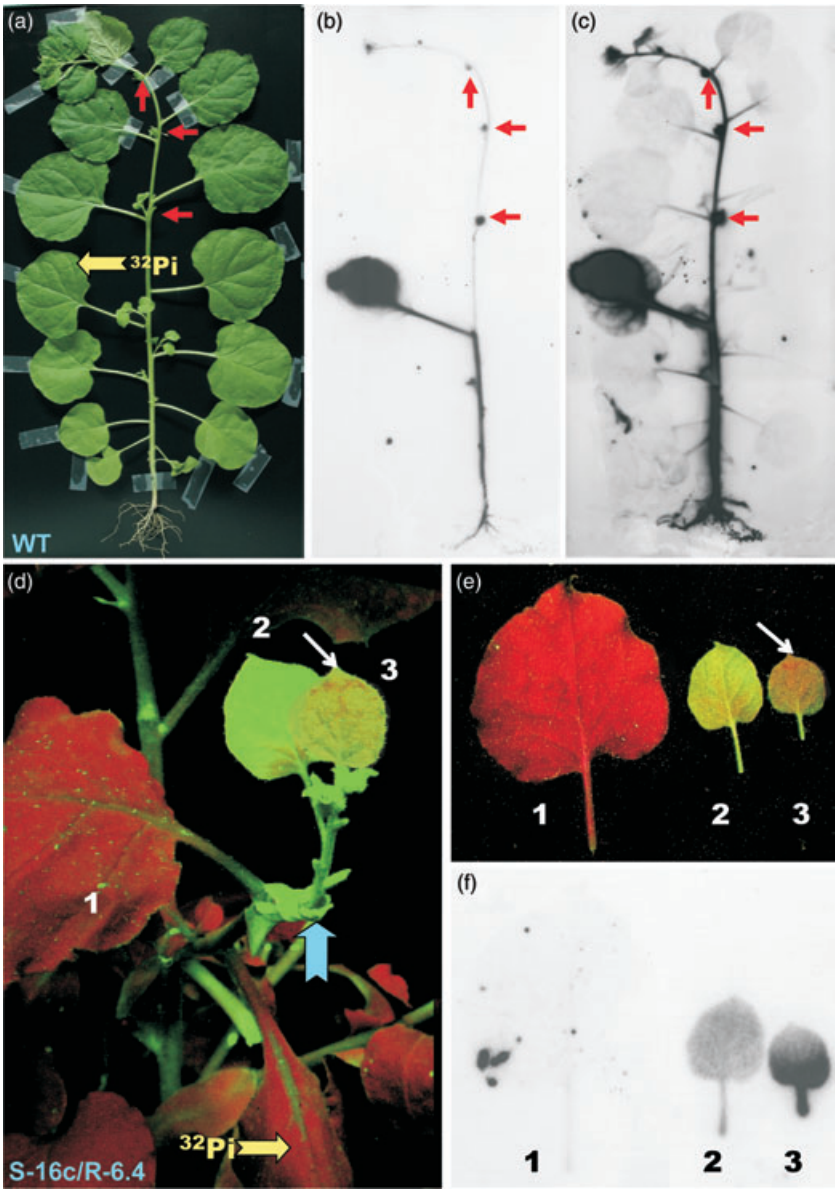

Figure 3. The use of radioactively labelled phosphate to trace specific phloem flow in *Nicotiana benthamiana*.
(a) Non-grafted *Nicotiana benthamiana* plant used to study the distribution of ^{32}P-labelled phosphate 2.5 h after infiltration; the infiltrated source leaf is marked (yellow arrow) and the plant is ready for exposure to X-ray film.
(b) Distribution of the radioactive tracer after 1 day exposure to autoradiographic film.
(c) The same plant as in (b) after 7 days exposure. (b) and (c) demonstrate the transmission of the tracer to root and other sink tissues, including fruit tissues (red arrows).
(d) ^{32}P-phloem tracing in a plant 1 month after grafting a non-silenced scion 16c on a silenced 6.4 rootstock. The graft junction and the infiltrated leaf are indicated. Leaf 1 is a source leaf of the rootstock, just above the infiltrated leaf and leaves 2 and 3 are derived from the scion. Leaf 3 shows first indications of GFP silencing.
(e) Harvested leaves 2 h after infiltration; the arrow indicates regions of GFP silencing in leaf 3.
(f) Autoradiograph obtained from leaves 1–3 (3 days exposure). Leaf 3 is strongly labelled, leaf 2 less strongly, while leaf 1 contains no tracer – the spot on leaf 1 is most likely an artefact on the film.

transmitted silencing to the 16c rootstock, we always observed a strong translocation of the Pi signal to the rootstock (Figure 4c,d). Apparently, those plants had established a phloem flow from the scion to the rootstock, through which the silencing signal had been transmitted. The phosphorus labelling verified that this route of transmission was still active at a stage when the first symptoms of GFP silencing were already detectable in the rootstock. The silenced 6.4 scion could transmit silencing only to some, but not all branches of the rootstock (Figures 1b,c and 4). In accordance with the silencing pattern, the non-silenced branches did not show any ^{32}P signal (branch 2 in Figure 4), while all leaves that showed systemic silencing symptoms were labelled (leaves 4, 5, 6 and 8 in Figure 4). Moreover, the intensity of silencing was directly correlated with the quantity of phosphorus influx (Figure 4d, compare leaves 4, 5, 6 and 8). Two different silencing patterns could be discriminated: leaves with strong silencing along the veins showed the strongest labelling, while leaves with a spotted silencing phenotype were weakly labelled (Figure 4d, leaf 4). We also observed leaves that displayed a GFP-silencing pattern in one half only, and, in accordance with this, the ^{32}P loading was stronger in this area (Figure 4d, leaves 4, 5). Moreover, we detected some labelled leaves that did not yet show any silencing phenotype (Figure 4d, leaves 1 and 2). Most likely these represent leaves with a newly established phloem flow, where silencing had not yet been initiated. It should be noted that such old leaves could also become phloem sink leaves under certain conditions (see Figure S2).

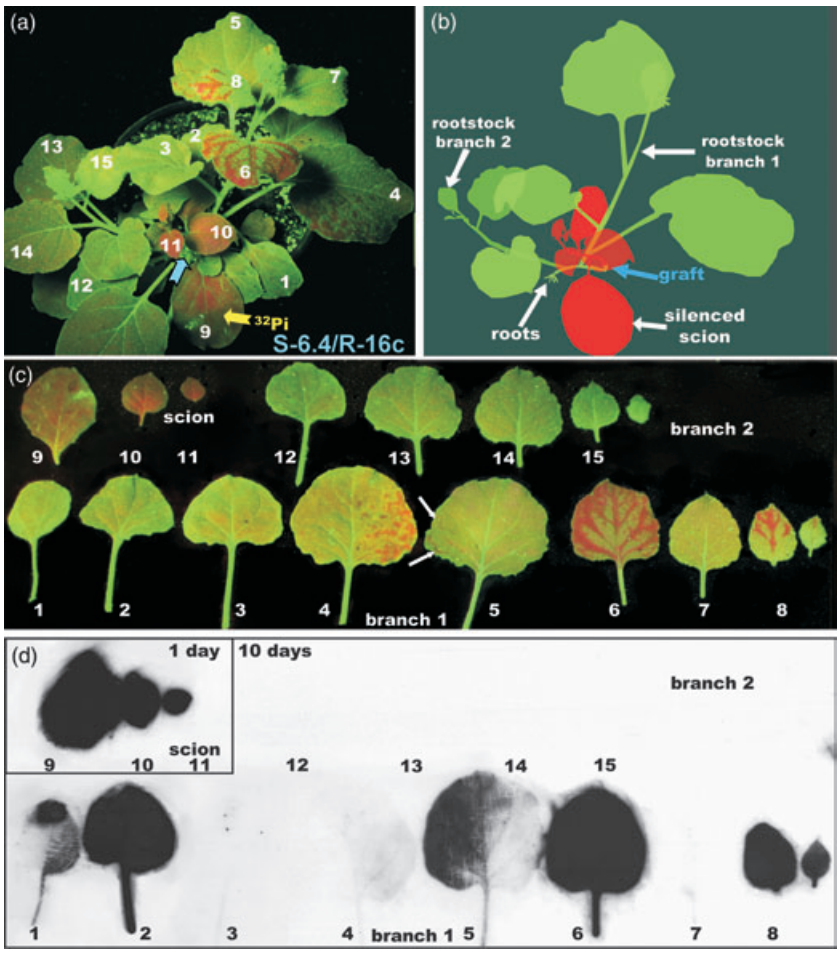

Figure 4. Spread of silencing correlates with the direction of phloem flow.
(a) Phloem tracing in a plant, where transmission of silencing to the rootstock is detectable 1 month after grafting a silenced 6.4 scion on a non-silenced 16c rootstock, similar to the plant shown in Figure 1(b). The graft junction is indicated; leaves are numbered, and leaf 9, a source leaf of the scion, was infiltrated with ^{32}P-phosphate.
(b) Schematic representation of the plant shown in (a).
(c) Detached leaves infiltrated with the phloem tracer; white arrows indicate small veins showing systemic silencing.
(d) Autoradiograph taken from the leaves of (c); scion leaves were exposed for 1 day and the residual leaves for 10 days. The pattern and intensity of GFP silencing correlates with the radiolabelling, except for leaves 1 and 2 which were labelled but not silenced. This suggests that they may have become sink tissues very recently and will only show silencing later. This hypothesis is tested in the experiment presented in Figure 6. See also Figure S1 for some analysis with another plant.

A similar correlation between silencing and phloem spread was observed in plants whose leaves had been removed from the rootstock (Figure 5). Figure 5(c,d) shows that 10 days after the removal of leaves from the original nonsilenced rootstock, the scion became an effective phloem source for leaves 1 and 2. Leaf 1 was already silenced, but not yet the very young leaf 2. The more developed leaves 3 and 4, in another branch, were neither silenced nor labelled, suggesting that they were not a phloem sink for the scion. The branches not silenced in this graft were fully capable of receiving and responding the silencing signal however, since they were systemically silenced when re-grafted to a silenced 6.4 rootstock (data not shown).

These results show that silencing spread through grafts seems to rely only on the establishment of phloem flow, with a very good correlation between phloem labelling and silencing spread. However, we identified a few cases of leaves that were labelled but not silenced (such as leaves 1 and 2 in Figure 4). We hypothesized that silencing may have appeared later if the leaves were not destroyed by exposure to film. Therefore, to test this hypothesis, we had to replace our signal detection method by a non-destructive, although less accurate, technique. In Figure 6, we used a 16c plant induced for systemic silencing by agro-infiltrating two leaves with a 35S–GFP hairpin construct (Koscianska *et al.*, 2005). As soon as silencing appeared in systemic leaves (10 days later), we labelled the phloem flow by infiltrating ^{32}Pi in the previously agro-infiltrated leaves. All leaves were subsequently removed from the plant, but instead of exposing the leaves to autoradiographic film, we measured the ^{32}Pi exported to other leaves using a scintillation counter and a handheld Geiger radiation counter, in a non-destructive manner. Although the scintillation measurements of whole leaves (Hülsen and Prenzel, 1968) are unlikely to be very accurate, this has allowed further analysis of the development of silencing after labelling and detachment from the plant by maintaining the leaves *in vitro*. Figure 6 shows a plant with a simple phloem circuit as it is not branched. Silencing spread from the two agro-infiltrated leaves (leaves 2–4) to the upper half of the plant (leaves 6, 7, 9 and 10). The phloem labelling and the silencing spread were similar in manner, 'avoiding' the old leaf 5 (labelling received is only 1.6 nCi g^{-1}) as well as the very young leaf 8 (labelling received is 0.3 nCi g^{-1}). Leaves 6 and 7 were

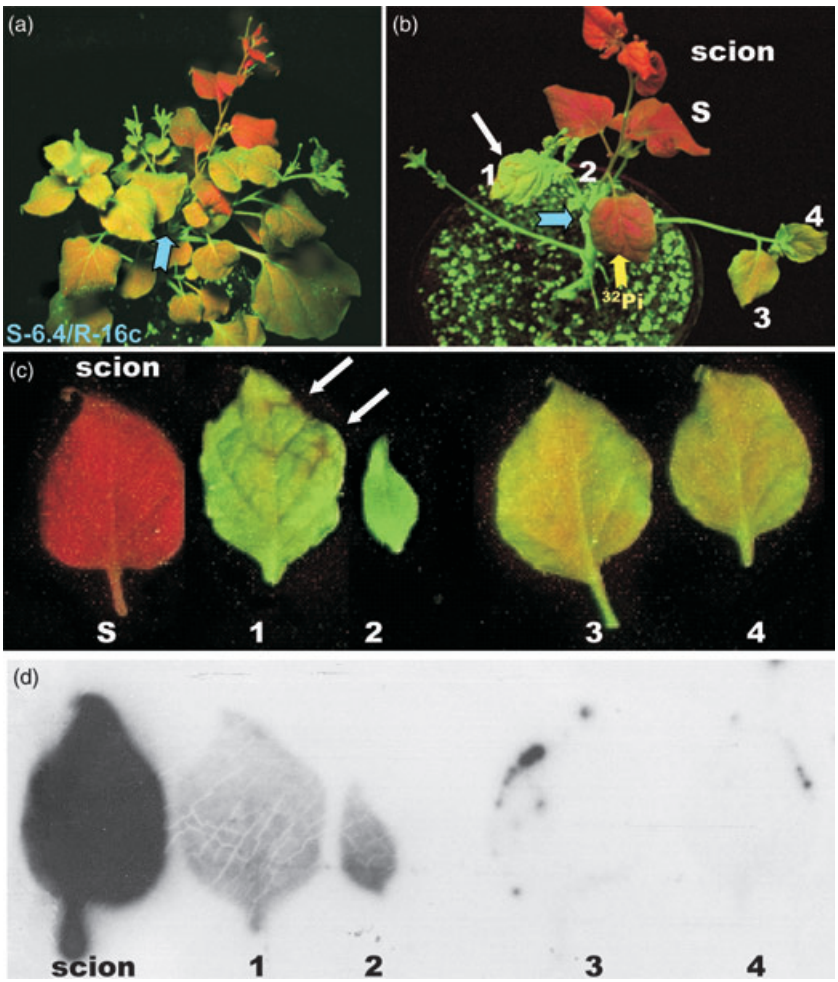

Figure 5. Silencing in defoliated rootstock is accompanied by re-establishment of phloem flow from scion to rootstock.
(a) Similar graft as in Figure 3, i.e. silenced 6.4 scion on a non-silenced 16c rootstock, without transmission of silencing 1 month after the graft.
(b) Plant in (a) 7 days after defoliating the rootstock, with first indication of silencing on leaf 1 (white arrow). This plant was infiltrated on the largest scion leaf with the radioactive phloem tracer (yellow arrow).
(c) Harvested leaves as in (b) 2 h after labelling.
(d) Autoradiography of the leaves in (c) (4 days exposure). The transfer of radioactive tracer is strongest in leaf 1, which is starting to silence.
A similar correlation between silencing and phloem spread was observed in plants whose leaves had been removed from the rootstock. (c) and (d) shows that 10 days after the removal of leaves from the original non-silenced rootstock, the scion became an effective phloem source for leaves 1 and 2. Leaf 1 was already silenced, but not yet the very young leaf 2. The more developed leaves 3 and 4, in another branch, were neither silenced nor labelled, suggesting that they were not a phloem sink for the scion. However, the branches not silenced in this graft were fully capable of receiving and responding to the silencing signal, as they were systemically silenced when re-grafted to a silenced 6.4 rootstock (data not shown).

partially silenced and labelled although they were fully expanded. Leaf 9 was highly labelled and silenced (770 nCi g^{-1}). Finally the apical shoot was divided into two parts: (i) leaf 10, which was well labelled (12.5 nCi g^{-1}) and showed silencing initiation, and (ii) bud 11, which was labelled but not yet silenced at all. We kept all leaves *in vitro* on tissue culture media for 2 weeks and followed the development of silencing. In accordance with phloem labelling, leaves 1, 3, 5 and 8, which were not found to have received a substantial amount of ^{32}Pi, never showed silencing. On the contrary, the silencing pattern in the young leaf 10, which was strongly labelled but slightly silenced, evolved quickly to full silencing. The fully GFP-expressing apical bud 11, which had received a significant amount of ^{32}Pi, quickly developed silencing even though it had been detached from the plant.

Restricted GFP silencing of branches is possibly due to an independent phloem circuit

GFP line 6.4 shows spontaneous occurrence of systemic silencing in a stochastic manner. Normally, the first systemic silencing appears early in plant development, so that it will spread systemically all over the plant. Occasionally, however, silencing will appear later, when the plant has already developed several branches. We have sporadically observed plants in which silencing is confined to a single branch (Figure 7a), while the other parts of the plant continue to express GFP, some with spots of local silencing which does not spread systemically. In order to understand why silencing does not spread throughout the plant, we infiltrated one of the oldest leaves of the silenced branch with ^{32}Pi. After 3 h, we exposed the plant to an X-ray film. As expected, we detected a strong flow within the silenced branch from the older leaves to the apical part in correlation to the spread of silencing within the branch (Figure 7b). However, almost no phloem tracer could be detected that was flowing out of this branch and entering other parts of the plant, suggesting that at this stage the GFP-silenced branch represents a relatively independent phloem circuit, which correlates with the restriction of GFP silencing to this branch.

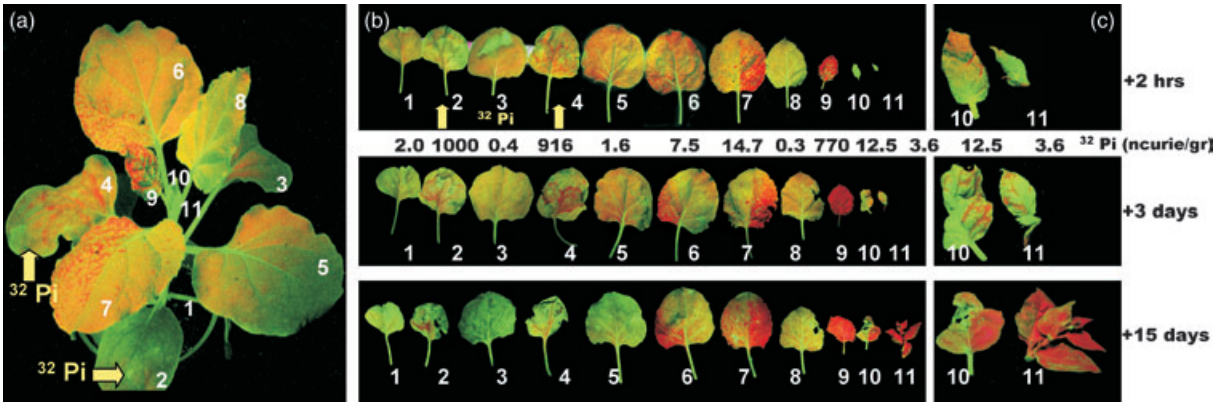

Figure 6. Phloem labelling can predict the evolution of silencing in distant leaves.
(a) A 16c plant was agro-infiltrated in two leaves with a construct producing a GFP hairpin. Ten days after agro-infiltration, silencing had spread only to some leaves. The phloem spread from these two leaves was then labelled by injecting ^{32}Pi (yellow arrows).
(b) Leaves were collected 1.5 h after labelling, photographed under UV light, and rolled carefully in scintillation pots to count in a non-destructive manner the ^{32}Pi received. Indicative counts correlated with fresh leaf weight are shown below individual leaves. After a short sterilization, leaves were grown *in vitro* and photographed again after 3 and 15 days. Silenced leaves (6, 7, 9) were labelled, while non-labelled leaves (1, 3, 5, 8) were not silenced initially and never showed silencing. The initially non-silenced apical tip (11) received radioactive label and developed silencing later, after being detached from the plant.
(c) Close-up of the youngest leaf and the apical tip.

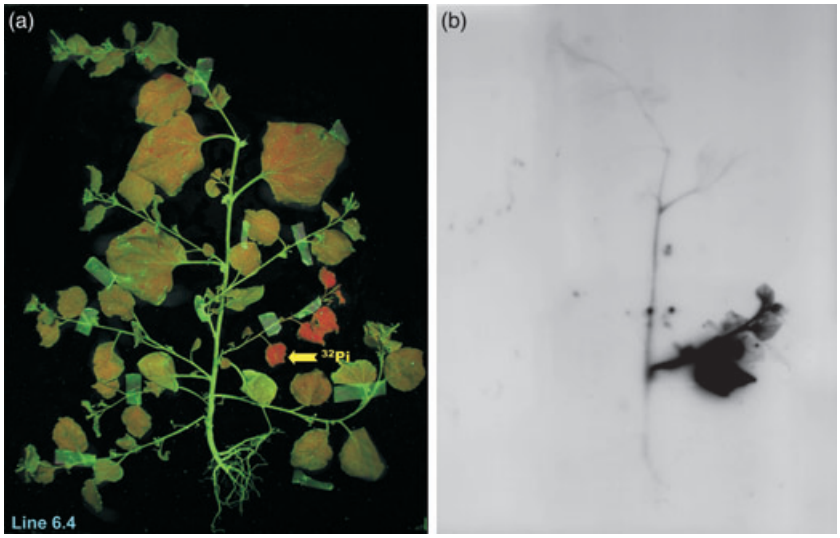

Figure 7. A line 6.4 plant showing a chimeric silencing pattern displays an independent phloem flow circuit.
(a) Example of a line 6.4 plant with a spontaneously GFP-silenced branch. Silencing has spread restrictively within this single branch. Some spots of local silencing that did not spread may be identified in other leaves. The indicated oldest leaf of the silenced branch was infiltrated with the phloem tracer.
(b) Autoradiograph taken from the plant in (a) 3 h after infiltration (7 days exposure). Radioactive labelling is largely confined to the silenced branch.

Discussion

Transmission of silencing can be bi-directional

The spread of RNA silencing follows a similar pattern to the spread of a systemic virus (Cruz *et al.*, 1998; Palauqui *et al.*, 1997; Roberts *et al.*, 1997; Voinnet *et al.*, 1998). It is not clear which RNA species is actually translocated over long distances and is responsible for the spread of silencing. Although siRNAs have been cloned recently from phloem sap (Yoo *et al.*, 2004), short RNAs appear not to be the only signalling molecules (Boutla *et al.*, 2002; Mallory *et al.*, 2001; Mlotshwa *et al.*, 2002). Regardless of the chemical nature of the mobile signal, one would expect that a silenced scion could direct phloem flow to the sink roots and lower meristems, thus initiating silencing in the rootstock. However, transmission in this direction had not been reported until now (Mallory *et al.*, 2003; Palauqui *et al.*, 1997; Voinnet *et al.*, 1998), or only with low efficiency (Sonoda and Nishiguchi, 2000).

We reasoned that the genetic background of the lines used can influence the 'strength' of the silencing source and accordingly the transmission rate of silencing in grafting experiments (Garcia-Perez *et al.*, 2004; Crete *et al.*, 2001). Thus we repeated reciprocal grafts using our GFP-expressing *Nicotiana benthamiana* line 6.4 as a source of silencing (Kalantidis *et al.*, 2006). All young plants of this line show strong GFP expression (excluding any form of transcrip-

tional silencing), but some plants spontaneously silence and generate high levels of siRNA, so that no ectopic expression of GFP is necessary to obtain silenced scions or rootstocks. Using 6.4 silenced scions, we obtained a high rate of silencing spread to a 16c rootstock, in contrast to previous reports (Palauqui *et al.*, 1997; Sonoda and Nishiguchi, 2000; Voinnet *et al.*, 1998). Surprisingly, when grafting was performed taking care to produce a metabolic-source scion, silencing transmission to rootstock could also be obtained equally well from a 16c silenced scion. Both lines are sense transgene lines, express GFP mRNA at similar level, and accumulate a similar pattern of siRNA when silenced. Once silencing is established it is perfectly stable in both lines, and scions of identical metabolic strength seem to behave similarly as far as spread of silencing is concerned. Thus silencing transmission as realized here depends on the grafting procedure rather than on the genetic background of the lines used. The importance of the grafting method used on the transmission efficiency of silencing has been shown previously (Crete *et al.*, 2001). It cannot be excluded that other transgenic lines with different transgene structures (antisense, inverted repeat, direct repeats etc.) may be found to have modified patterns of silencing spread.

The quality of the phloem flow influences the degree of transmission of silencing

It is established that phosphate is taken up from the soil, and transported via xylem sap to the leaves, where it is either incorporated into biomolecules or stocked (Bieleski, 1973; Schachtman *et al.*, 1998). Plants are able to mobilize the phosphates from old leaves for re-allocation in growing tissues, using a second flow travelling through the phloem back to the roots, where phosphates are pumped again into the xylem (Jeschke *et al.*, 1997; Mimura, 1995). Other phosphorus-containing compounds, such as sugar phosphates, ATP and ADP, are transported directly in the phloem. Taking advantage of these processes, we used radioactively labelled phosphate to demonstrate that, in all plants showing successful transmission of silencing, a phloem source to sink flow had been established between the silenced (6.4) tissue and the parts of the (16c) plants that were undergoing silencing. This included flow from silenced scions to rootstocks that were in the process of silencing. The amount of phosphor tracer transmitted from the scion to the rootstock correlated with the degree of silencing: strongly silenced tissues showed a higher influx of radioactivity than weakly silenced tissues. This could even be observed within a single leaf when silencing and phloem labelling are similarly restricted to the same half of the leaf (Figure 4). We never identified silenced leaves that escaped labelling, but we did identify some labelled leaves that were not yet silenced. To test the possibility that these tissues would have developed silencing later, we set up a similar experiment in which label translocation from the agro-infiltrated leaves to the rest of the plant was traced in a non-destructive manner. The leaves eventually had to be removed from the plant, but the evolution of silencing could be further monitored in tissue culture (Figure 6). It was not obvious that tissue-cultured leaves would behave as *in planta* regarding silencing spread. However, we found that all silenced tissues maintained silencing. Furthermore, leaves that were strong receivers of phloem sap from the agro-infiltrated silenced leaves, but which were not yet silenced at the time of determination of labelling, were eventually silenced later during *in vitro* culture. This indicates that after a certain signalling threshold has been exceeded, silencing can develop independently of the original signalling source.

The congruent patterns of RNA silencing and phosphate tracer are remarkable, as transmission of silencing is a process that requires several days, while the transmission of the phosphate is a matter of a few hours. These data suggest that once a phloem flow has been established it will be maintained, at least until the metabolic sink–source relations in the plant are seriously altered. In the experiment presented here, care was taken to agro-infiltrate and label leaves in a relatively stable sink–source relation state by choosing mature but not old leaves.

Our experiments indicate that transmission of silencing requires a steady influx of the silencing signal for several days. Most likely the amount of signalling molecule travelling is rather low, and the receiving cells might require a critical dose before responding to the signal. In this context, the spotted pattern of silencing is of interest (Figure 4, leaf 4); this is a tissue that is receiving signal from the scion, but in a rather inefficient manner so that only localized silencing can be observed. It is not possible to predict with certainty how this kind of silencing will evolve. From our experience, when such silencing phenotype appears, silencing does not spread further or spreads very weakly. In contrast, highly labelled influx of phloem sap along the veins (Figure 5, leaf 5) signals efficient spread of silencing.

Removal of rootstock leaves promotes transmission of silencing from scion to rootstock

In about two-thirds of the grafts, no transmission of silencing from the 6.4 scion to the 16c rootstock could be observed. Tracing the phloem flow revealed that no flow from scion to rootstock had been established in those plants. However, conversion of the source–sink relationship by either defoliation of the rootstock – or less efficiently by covering the source leaves with aluminium foil – made the rootstock a sink tissue and changed the direction of the flow, followed by transmission of silencing in almost all rootstocks tested.

'Independent' phloem circuits can control some aspects of plant phenotype by affecting long-distance RNA signal translocation

Collectively, the above data show that some apparently stochastic patterns of silencing spread (restricted to half of a leaf, to a single branch or only some grafts capable of transmission) can be understood in the light of phloem flow. Our labelling showed that, from a signalling point of view, phloem flow is more complicated than the simple movement from the oldest leaves to roots and meristems. Especially in the case of grafts, the nature of the phloem flow determines the spread of silencing between rootstock and scion. However, the regulation of RNA signal movement within the phloem might be of more general importance, beyond these grafting experiments. Our non-grafted 6.4 plants sometimes show silencing that is confined to a single branch (Figure 7). Using phosphor tracing, we showed that the silenced branch of the chimeric plant had an independent phloem circuit that was not connected in an efficient manner to the residual non-silenced parts of the plant. The occurrence of confined phloem environments, as we could visualize here, is of interest, as in the recent years many developmental signals have been identified that travel in the phloem, including mRNA (Ayre and Turgeon, 2004; Kim *et al.*, 2001; Ruiz-Medrano *et al.*, 1999, 2001; Xoconostle-Cazares *et al.*, 1999), proteins (Golecki *et al.*, 1999; Gomez and Pallas, 2004; Gomez *et al.*, 2005) and microRNA (miRNA) (Yoo *et al.*, 2004). Spatially restricted phloem streams might influence the translocation of signals and thus play an important role in plant development. On a more general note, it now seems possible that an RNA signalling mechanism functions in plants, as it was recently shown that local induction of the *flowering locus* gene (*FT*) in a single Arabidopsis leaf is sufficient to trigger flowering by transportation of the actual *FT* mRNA to the shoot apex, presumably via the phloem, where downstream genes are activated (Huang *et al.*, 2005). Determining the rules that govern the translocation of such signals will be critical in unravelling such regulatory mechanisms.

Experimental procedures

Plant material

The *pBIN 35S–mGFP4* construct (Haseloff *et al.*, 1997) was kindly provided by Jim Haseloff (Cambridge University, UK) and was used for the generation of transgenic line GFP 6.4. *Agrobacterium tumefaciens* strain LBA4404 was transformed with the *pBIN 35S–mGFP4* plasmid by tri-parental mating (Ditta *et al.*, 1980). *N. benthamiana* transformations were done as described previously (Kalantidis et al., 2002). Line 6.4 plants were regularly checked for fluorescence with a handheld 1000 W long-wavelength UV lamp (B100AP; Ultraviolet Products, Cambridge, UK), and plants undergoing systemic silencing were separated from the others. All photographs showing GFP silencing were also take under UV light. Line 16c is a highly GFP-expressing line that never shows spontaneous silencing. It was kindly provided to us by D. Baulcombe's laboratory.

Plant growth conditions

Explants and plants were grown at 25°C during the day and 18°C at night in a growth chamber with a 16 h photoperiod provided by cool white fluorescent tube lights to give 90 μmol m^{-2} sec^{-1} PPF (photosynthetic photon flux). Plantlets were transferred to the greenhouse at a controlled temperature of 23°C.

Agro-infiltration

Agro-infiltration of a 35S–GFP hairpin (hpGFP/s-As) was performed as described previously (Koscianska *et al.*, 2005). Briefly, *A. tumefaciens* LBA 4404 were grown overnight in LB medium with the appropriate antibiotics and 20 μM acetosyringon; then they were briefly spun down and re-suspended in MMA medium (MS salts, 10 mM MES, pH 5.6, 200 μM acetosyringon) and incubated for at least 1 h at 28°C. Subsequently, the bacteria were washed twice with 10 mM $MgCl_2$ and re-suspended in $MgCl_2$ to an OD_{600} of about 0.1. After agro-infiltration, the plants were kept in the growth chamber at 23°C during the day and 18°C at night with a 16 h photoperiod.

Grafting procedure

Line 6.4 plants were used as rootstocks when they were at least 1.5–2 months old (approximately 10 fully developed leaves), and always before old leaves had started yellowing. When 6.4 plants were used as a scion, an apical shoot made up of three or four young leaves was placed on a non-flowering stem after being cut with at least 2 cm long bevelled edges on two sides of the stem making a V-shaped edge. When 16c was used as a scion, a very small apical stem segment made up of one or two leaves was placed on a non-flowering branch and bevelled along 1 cm. As rootstock, 16c plants were used at the 6–8 leaves stage, by cutting three or four upper leaves. All scions were placed in a rootstock stem cleaved through the middle to a similar length. The graft junction was always made at a leaf level so that the uncut petiole made an ergot maintaining the scion. All plants were then covered with a transparent plastic bag to avoid drying, and these were progressively opened after 7–10 days.

Phloem labelling

Instead of purchasing labelled inorganic phosphate (^{32}Pi), we obtained it for reasons of convenience by treating γ^{32}P-ATP, with radioactivity of 1 μCi, with 2 units of CIP (New England Biolabs, Ipswich, MA, USA) for 1 h at 37°C in 50 μl of 1× CIP buffer. The solution was diluted with 7% sucrose to an activity of 10 nCi μl^{-1}. Fully developed leaves were injected with 50–100 μl of radioactive solution using a 1 ml syringe, and in no case was more than one-third of the leaf infiltrated. The apical end of the leaf was preferentially injected, avoiding major veins. After 2–3 h, the plant was photographed under UV light, transferred on to aluminium foil, covered with Saran wrap and exposed (wet) to X-ray film. Non-destructive measurements of leaf radioactivity were conducted using a handheld GM radiation counter (Mini 900 rate meter,

Thermo Electron Corporation, Reading, Berkshire, UK) placed directly on top of parafilm-covered leaves. For an approximate estimation of the radioactivity transferred to leaves, they were rolled carefully into a pot and radioactivity was counted for 2 min a Beckman LS 1701 scintillator counter without scintillating liquid (Beckman Coulter, Fullerton, CA, USA; Hülsen and Prenzel, 1968). ^{32}Pi loading is expressed per weight of fresh tissue. Leaves were then washed with 10% bleach (NaOCl) for 5 min, washed three times with water and grown in magenta pots with MS media.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Northern of siRNA production and Southern analysis of transgene integration for the different silenced plant lines used.

Figure S2. Spread of silencing correlates with the direction of phloem flow, similar to Figure 4 with another plant.

This material is available as part of the online article from http://www.blackwell-synergy.com

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