The mechanism of graft transmission of sense and antisense gene silencing in tomato plants

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Abstract We investigated the effect of target mRNA level on grafting-transmitted gene silencing in tomato plants by using a strong ACC oxidase 1 (ACO1) silencer as the stock and transgenic ACO1 overexpressers as scions. Manifestation of graft transmission of sense gene silencing required a high initial level of target mRNA in the scion. A relatively high level of siRNA, similar to that in the strong ACO1 silencer, was also detected in the silencing-susceptible strong ACO1 overexpressers prior to grafting. After grafting the silencing signal from the stock enhanced the level of the siRNAs in the scion and the ACO1 mRNA level was reduced dramatically. Using stock and scions producing different siRNAs we provided evidence that the transmissible silencing signal does not correspond to the bulk siRNAs in the stock. We also showed, contrary to a previous report, that antisense silencing was graft-transmissible but it took longer to manifest itself. The delay in graft transmission from antisense-silenced plants could be attributed to the difference in the nature or strength of the signal or the mechanism of its amplification, but is further evidence of mechanistic similarities between sense and antisense silencing.

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1. Introduction

Post-transcriptional gene silencing (PTGS), also known as RNA silencing, is a sequence-specific RNA regulatory mechanism in higher plants. PTGS had been considered as a plant specific phenomenon [1–5] before RNA interference (RNAi) in animal systems was discovered in 1998 [6]. Inhibition of gene expression by antisense genes predates sense transgene PTGS in plants [7,8] and there has long been a discussion about their similarities [4]. RNA silencing is a defence mechanism common to plants and animals that causes sequence-specific RNA degradation of invading foreign DNA or RNA molecules, such as transgenes, viruses and exogenous double-stranded RNA (dsRNA). The core of the mechanism is the processing of dsRNA by a dsRNA-specific RNAse called Dicer into small interfering RNA (siRNA), which is then incorpo-

rated into an RNA-induced silencing complex (RISC) and guides the degradation of target mRNA by the RISC through sequence complementarity.

One fascinating characteristic of the RNA silencing signal in plants is that it is mobile and can be spread systemically to distal parts. Grafting has been used to demonstrate the spreading of RNA silencing in plants. By using this technique Palaqui et al. [9] showed unequivocally for the first time that the systemic silencing signal can move over long distances through plasmodesmata and phloem [10]. The movement of the signal is bidirectional, occurs more efficiently upward rather than downward and the direction can be manipulated by altering sink-source relationship [11,12]. Although no mobile silencing signal has been characterized so far, siRNAs have been favoured as the components of such signals, based on their characteristics, being long enough to convey sequence-specificity and small enough to move through plasmodesmata [13]. In support of the proposed role of siRNAs in systemic silencing, Klahre et al. [14] showed 21-nt siRNAs and high molecular weight single-stranded RNAs, delivered into plant tissues by a biolistic approach, could cause silencing that is able to spread from cell to cell and systemically. It was previously proposed, by the analysis of two size classes of green fluorescent protein (GFP) siRNAs associated with local and systemic silencing of GFP in N. benthamiana, that the shorter siRNAs (21-22 nt) were correlated with target mRNA degradation whereas the longer siRNAs (24–26 nt) correlated with the systemic silencing, hence suggesting the longer siRNAs as the possible systemic signal of silencing [15]. This finding, however, contrasts with some of the experimental evidences that imply a functional role of 21nt-secondary siRNAs, rather than the longer siRNAs, as a mobile silencing signal in transitive RNAi [16,17].

A viral protein p19 of tombusvirus can suppress the spreading of the silencing signal, probably by binding the siRNAs [18]. However, two reports about viral suppressors of PTGS suggest a different mechanism. PVX p25 protein, a cell-to-cell movement protein of potato virus X (PVX), suppresses PTGS by interfering with the mobile silencing signal without affecting the accumulation of siRNAs [19]. Suppression of PTGS by a viral suppressor, a helper component proteinase (HC-Pro) of potyviruses, eliminates the accumulation of siRNAs but not the mobile signal [20,21], leaving unanswered the question about the exact nature of the mobile signal. Recently, however, a report from Yoo et al. [22] shed light on some aspects of the mobile silencing signal. They have characterized a SMALL RNA BINDING PROTEIN1 (CmPSRP1), extracted from cucumber (Cucurbita maxima) phloem sap. PSRP1 is

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implicated in small RNA trafficking through its capacity to bind and form stable complexes only with 25-nt single stranded RNA (ssRNA) but not with double stranded RNA (dsRNA). It has also been found that the PSRP1-ssRNAs complex could move through plasmodesmata whereas ssRNA alone could not.

From the grafting experiments to date, it is now evident that the efficient transmission of the silencing signal partly depends on the grafting methods used. Créte et al. [23] described three types of grafting methods but only top-grafting, where a wedge-shaped cut surface of the scion base is inserted into the vascular ring at the cut surface of the root stock, resulted in scions that were systemically silenced by a rootstock signal. They also reported that only sense silencing can be systemically transmitted to scions, whereas antisense silencing cannot, suggesting that the elaboration of a mobile signal is not an essential feature of antisense gene silencing. This finding gives rises to the questions: what prevents transmission in antisense graft-transmitted silencing and are sense and antisense silencing fundamentally different?

In an effort to understand more fully the nature of sense and antisense gene silencing, we assessed the ability of 1-amino-cyclopropane-1-carboxylate oxidase (ACC oxidase)-silenced tomato rootstocks to generate a mobile silencing signal that can cross a graft junction and induce silencing in grafted scions. In this paper, we report that a high level of target mRNA in the scion is required for the grafting transmitted sense silencing signal from the stock to be effective in causing silencing. We also show that antisense as well as silencing stocks can cause grafting-transmitted silencing, which contradicts earlier reports.

2. Materials and methods

2.1. Plant materials

Tomato plants were grown in a greenhouse. The transgenic tomato Lines T and V are homozygous lines and are progenies of the primary transformants as described by Hamilton et al. [24] and Han and Grierson [25]. Plants of the Line C series are the progenies of transgenic lines overexpressing the ACO1 gene. Line V plants are the progeny of a plant with an ACO1 transgene containing an inverted repeat (IR) showing PTGS of the endogenous ACO1 gene described by Hamilton et al. [24]. Line T is derived from the ACO1 co-suppression line described by Hamilton et al. [24] and Han and Grierson [25] but unlike Line V, its transgene does not contain an inverted repeat region. The antisense (AS) line is the progeny from a pTOM13 (ACO1) antisense line described by Han and Grierson [25] and Hamilton et al. [26]. To assay the mRNA level of the endogenous ACO1 gene in these various lines in response to mechanical wounding, young leaves were cut into small pieces and incubated for 1 h at 24 °C prior to RNA extraction [24].

2.2. Grafting procedures

We used a grafting method similar to top grafting described previously [23]. In all experiments, the transgenic ACO1-overexpressing Line C plants were used as a source of scions while Lines V, T and AS served as rootstocks. When the rootstock plants were about 30 cm in height (after forming about five compound leaves), the shoot was cut off at about 3 cm from the top. Then one vertical cut about 1–1.5 cm in length was made from the middle of the cut surface. The base of a scion shoot was cut to a wedge-shape and inserted into the vertical cut of the rootstock. The graft junction was wrapped with Parafilm and the scions covered with a transparent plastic bag for 5 days to maintain humidity and reduce water loss by transpiration. The lateral shoots that emerged in the rootstock after grafting were cut off to favour growth of the scion. Young leaves of the scions were sampled and RNA isolated approximately every 2 weeks at intervals from 2 to 10 weeks after grafting.

2.3. Isolation of total RNA and Northern analysis of ACO1 mRNA

Total RNA was extracted from mechanically wounded leaf by using the RNeasy Plant Mini Kit (Qiagen). The tissues were frozen in liquid nitrogen and ground to a fine powder in a mortar. Total RNA (10 μg per sample) was fractionated by electrophoresis in a 1% agarose gel containing 25 mM sodium phosphate (pH 6.5) and 3.7% formaldehyde. The RNA then was transferred to Genescreen hybridisation membranes (NEN) as described in [24]. The ACO1 sense-specific riboprobe used for Northern analysis was made from an in vitro transcription system (Promega Corporation, Madison, WI, USA) from a PCR product amplified from pGEM3 vector containing the ACO1 cDNA (1400 bp). The riboprobe was 32P-labelled and corresponded to the full-length ACO1 cDNA. The hybridization was carried out in a buffer containing 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulphate and 100 μg/ml denatured salmon sperm DNA at 42 °C overnight.

The membranes were washed in 2× SSC/0.1% SDS and 0.1× SSC/0.1% SDS at 42 °C for 15 min each and exposed to Kodak X-omat film between two intensifying screens. The hybridization signals of the ACO1 mRNA prior to grafting (Table 1) were measured and compared with the hybridization signals of the ACO1 mRNA following grafting (Table 1). The signals were quantified using a Phosphoimager and software (Cyclone Storage Phosphor System) from Packard Instrument Company.

2.4. Isolation and detection of small RNAs

The siRNAs were isolated and transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech) as described previously [27]. Sense-specific riboprophes corresponding to the ACO1 transgene were generated using an in vitro transcription system (Promega). Hybridization was carried out in 40% formamide, 7% SDS, 0.3 M NaCl, 0.05 M Na2HPO4–NaH2PO4 (pH 7), 1× Denhardt’s solution, 100 μg/ml denatured salmon sperm DNA at 30 °C for 16 h and the membranes filters were washed with 2× SSC/0.2% SDS at 50 °C for 3 × 10 min before being exposed to Kodak X-omat film between two intensifying screens.

3. Results

3.1. Silencing in scions by silencing stocks depends on the level of target mRNA in the scions

Grafting experiments were used to study transmission of a mobile silencing signal from silenced rootstocks across a graft junction and induction of silencing in scions. A diagram of the experimental setup for grafting scion onto a silenced rootstock is shown in Fig. 1. The ACO1-strong silencer Line V was used as the rootstock. In this line, the 5′ end of the ACO1 transgene includes two additional inverted and complementary copies (79 bp each) of the ACO1 5′ UTR. This construct has been shown previously to cause highly efficient silencing in tomato [24]. Line C plants used as scions contained a CaMV35s-driven sense ACO1 transgene without an inverted repeat and showed over expression of ACO1 mRNA transcripts. Line C plants (C75, C89, C81 and C88) showing different levels of ACO1 mRNA production upon wounding, the standard method for inducing endogenous ACO1 mRNA, were grafted onto the inverted repeat (IR)-associated gene silencing Line V (Fig. 2). We categorised the ACO1-overexpressing transgenic lines into two groups based on the phosphorimage densitometry signals of the ACO1 mRNA prior to grafting (Table 1). The phosphorimage results reveal that the ACO1 mRNA level in Lines C81 and C88 were double that in Lines C75 and C89 (Table 1a). Therefore, Lines C75 and C89 were referred to as ‘weak’ overexpressers and Lines C81 and C88 were called ‘strong’ overexpressers. Controls involved grafting wild type shoots onto Line V stocks (wt/V in Fig. 2). Approximately 2 weeks after grafting, RNA was extracted from leaves of the grafted and non-grafted scions and the ACO1 expression
was assayed by Northern blot analysis. Reduction of ACO1 expression only occurred in the grafted scions that showed strong ACO1 expression (C81 and C88, Fig. 2a), whereas scions that showed weak ACO1 expression (C75 and C89), were not silenced when grafted on the silencing rootstocks.

3.2. Accumulation of small interfering RNA (siRNAs) in grafted and non-grafted plants

siRNAs are associated with silencing and their production is believed to be triggered by the formation or introduction of dsRNA in cells. In general, siRNAs can be derived from all regions of perfect duplex RNAs and, at least in plants, they accumulate with both sense and antisense polarities [28]. For both polygalacturonase (PG)-silenced and ACO1-silenced tomato plants, siRNAs were produced from the transgene, preferentially from the 3′ region [25,29], although it was shown that the site of preferential production of siRNAs was shifted to the 5′ region for an ACO1-transgene containing an inverted repeat in that region [25]. This makes it possible to distinguish siRNAs made in the scion from those made in the stock. We probed for the presence of siRNAs in grafted and non-grafted plants by using an ACO1 full length antisense-specific riboprobe. As expected, the reduction in ACO1 mRNA levels in grafted plants (C81 and C88) was accompanied by the presence of siRNAs in the scions (Fig. 2b). Surprisingly, however, siRNAs as abundant as those in the Line V were also detected in these strong overexpressers prior to grafting, whereas no detectable siRNA level was found in the weak overexpressers before and after grafting (Fig. 2b). These siRNAs must be generated from the ACO1 transgene as they are accumulated in leaves before the endogenous ACO1 gene is induced by wounding (data not shown). Furthermore, the level of the siRNAs from the strong overexpresser lines increased significantly after the grafting (C81/V and C88/V). Additional transgenic ACO1 strong overexpressers, Line C331 and C332 containing the same ACO1 transgene as in the other Line C plants were grafted onto Line V plants and showed a similar pattern of mRNA reduction after grafting (data not shown). siRNA analysis using strand-specific riboprobes showed that grafting increased the accumulation of siRNA in C331/V and C332/V compared to C331 and C332 (Fig. 2d), and furthermore, that in the scions the siRNAs were derived from the 3′ region of ACO1 gene, even though most of the siRNAs in the silencing stock (Line V) were generated from the 5′ region.

3.3. Antisense silencing can be transmitted to scions but at a slower rate

Crété et al. [23] concluded, using chitinase sense and antisense genes in tobacco, that only sense silencing can be transmitted to scions and not antisense silencing. We repeated the experiments in tomato using a similar type of grafting setup described above, with three ACO1 strong overexpressers grafted onto sense- and antisense ACO1 silenced rootstocks. We discovered that, 10 weeks after grafting, the ACO1 mRNA level was reduced not only in scions grafted to the sense silencing stock but also those to the antisense silencing stock (data}

Fig. 1. (A) Diagram of the experimental setup for grafting an ACO1-overexpressing scion onto a silenced rootstock by using a wedge-grafting procedure. The plant used for rootstock was decapitated and a vertical notch was cut in the top of the stem, while the base of the scion stem was trimmed at the bottom into a wedge and inserted into the notch. The graft junction was secured with Parafilm and the grafted plant was kept in a humid environment for 5 days. (B) Representative grafted tomato plant. Photographs taken 5 days after grafting showing an overexpresser Line C scion grafted onto a sense-silenced Line V rootstock (inset).
Based on these early results, we repeated the experiment using more strong overexpressing lines and a more detailed time-course of shoot sampling. In total, strong ACO1-overexpresser scions from 14 different Line C plants with similar ACO1 expression levels as in C81 and C88 were grafted in triplicate onto sense-silenced rootstocks (Line V and Line T) and antisense-silenced rootstocks (Line AS). Controls were performed by grafting ACO1 overexpresser scions onto wild-type shoots (Line V) as control.

Table 1
The ratio of hybridization signals of the ACO1 and NPTII mRNAs were analyzed and quantified using a phosphor imaging system as described in Section 2.

(a) Ratios of ACO1 mRNA level in Lines C81 and C88 were double those in Lines C75 and C89

<table>
<thead>
<tr>
<th>Lines</th>
<th>C81:C75</th>
<th>C81:C89</th>
<th>C88:C75</th>
<th>C88:C89</th>
</tr>
</thead>
<tbody>
<tr>
<td>C81</td>
<td>2.19</td>
<td>1.99</td>
<td>2.13</td>
<td>1.94</td>
</tr>
</tbody>
</table>

(b) Ratios of ACO1 mRNA levels before and after grafting

<table>
<thead>
<tr>
<th>Lines</th>
<th>C75:C75/V</th>
<th>C89:C89/V</th>
<th>C81:C81/V</th>
<th>C88:C88/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>C81</td>
<td>1.02</td>
<td>2.8</td>
<td>2.57</td>
<td></td>
</tr>
</tbody>
</table>

(c) Ratios of ACO1 and NPTII mRNAs before and after grafting

<table>
<thead>
<tr>
<th>Lines</th>
<th>C75</th>
<th>C75/V</th>
<th>C89</th>
<th>C89/V</th>
<th>C81</th>
<th>C81/V</th>
<th>C88</th>
<th>C88/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACO1:NPTII</td>
<td>0.86</td>
<td>0.90</td>
<td>1.00</td>
<td>0.95</td>
<td>1.07</td>
<td>0.44</td>
<td>1.14</td>
<td>0.43</td>
</tr>
</tbody>
</table>
type rootstocks. The time-course of manifestation of systemic silencing of ACO1 in grafted and non-grafted scions was investigated by collecting the shoot samples at 2, 4, 6, 8 and 10 weeks after grafting. At week 2, systemic silencing was detected in the Line V-grafted scions (Fig. 3a) and ACO1 mRNA accumulation was dramatically reduced in sense silenced Line V-grafted scions (C/V) as compared to nongrafted plants (C), which were used as controls. In the scions grafted to the AS rootstocks (C/AS), no silencing was observed at 2 weeks and the scions continued to express the transgene mRNA at a high level as was the case for the controls. However, at 4 weeks after grafting, scions grafted onto AS rootstocks began to

Fig. 3. Antisense silencing can be transmitted from stock to scion but at a slower speed than for sense silencing. Line C31 and C811 plants with similar strong ACO1 over expression levels as in the C81 and C88 lines in Fig. 2 were grafted onto sense- and antisense-silenced rootstocks. (a) The ACO1-overexpressing transgenic Line C31 was used as a source of scion while Line V (containing sense ACO1 transgene with two copies of inverted-repeat 5'UTR) and Line AS served as sense and antisense silencing rootstocks, respectively. The expression of ACO1 mRNA in scions was analysed at 2, 4, 6, 8 and 10 weeks after grafting by gel blot analysis of RNA isolated from grafted scions (C/AS, C/V and C/wt) and scions of the source plants (C). Transmission of sense silencing (C/V) was observed as early as 2 weeks after grafting, but antisense silencing (C/AS) was only apparent 8 weeks after grafting. No silencing was observed in wild-type grafted scions (C/wt). (b) The ACO1-overexpressing transgenic Line C811 was used as a source of scion, together with Line V, Line AS and Line T as rootstocks. Line T shows sense silencing and produces a mobile signal but the gene construct lacks the inverted-repeat region present in the Line V. All the grafted plants showed transmission of a silencing effect (C/V, C/T and C/AS) except for the control (C/wt). Antisense silencing of Line C811 was manifested 6 weeks after grafting. The photograph of ethidium bromide staining of rRNA indicates the loading control for total RNA. (c) The frequency of successful sense and antisense silencing transmission at different times after grafting. Fourteen different strong ACO1-overexpressing Line C plants were used in this experiment and 71% of them showed induced sense silencing in the scion 2 weeks after grafting to the Line V, as compared to only 35% of those grafted over antisense silencing plant stocks. Ten weeks after grafting however, the percentage have increased to 93% and 71% for sense silencing and antisense silencing stocks, respectively.
demonstrate systemic silencing. ACO1 mRNA levels in each scion grafted onto AS rootstocks, as well as in scions grafted onto sense-silencing rootstocks, were reduced considerably as compared to the controls (C and C/wt in Fig. 3a), suggesting that silencing had been transmitted from both sense and antisense silenced rootstocks to the scions. The same pattern of silencing effect has also been observed in Line C811 grafted onto silenced Line T, together with Line V and Line A5 rootstocks (Fig. 3b). Unlike Line V, the Line T transgene does not contain an inverted repeat region (IR) at the 5' end of the transgene [24]. The reduction of ACO1 mRNA expression in the AS-grafted scions of C811 was again detected at a much later stage (6 weeks) than for V- and T-grafted scions (2 weeks). Similar results were obtained with 12 other transgenic ACO1 strong overexpresser scions grafted onto sense and antisense silenced rootstocks (data not shown). Of the 14 sets of grafted plants tested, 71% of the scions were silenced by a sense (PTGS) silencing signal as early as 2 weeks after grafting, as compared to only 35% by an antisense silencing signal (Fig. 3c). Ten weeks after grafting however, the percentage increased to 93% and 71% for the sense silencing and antisense silencing signal, respectively. The silenced state for most of the scions from sense- and antisense-rootstocks was stable for up to at least 10 weeks after grafting (data not shown).

4. Discussion

There have been a number of reports dealing with the transmission of the PTGS signal in plants involving grafting experiments [9,23,30,31]. In most cases, grafting of a non-silenced transgenic plant onto a transgenic rootstock with the same silenced transgene or homologous endogenous gene causes the scion to become silenced. Silencing can spread systematically within the entire plant in a sequence-specific manner. Although the nature of the transmitted signal is unclear, this sequence-specificity, as with all silencing, strongly suggests that it must incorporate a nucleic acid, probably an RNA molecule [13,32,33].

A high level of target mRNA in the scion source is associated with graft-transmitted silencing. This was proposed by Palauqui et al. [9] based on the fact that 100% graft transmitted silencing was achieved with all scions tested (either transgenic lines or a nitrate reductase mutant) and the target mRNA levels in these lines prior to grafting were higher than in wild type. However, as we demonstrated in this paper, the weak ACO1 overexpressers (Line C75 and C89, Fig. 2a), even though the target mRNA in these lines was higher than that in wild type, did not respond to the silencing signal from the stock. Strong ACO1 overexpressers (Line C81 and C88), on the other hand, with much higher ACO1 mRNA than the weak overexpressers, responded to the signal and exhibited silencing. This suggests that systemic silencing was only manifested in the scions that showed strong ACO1 expression over a certain level or threshold prior to grafting. A similar suggestion was made by García-Pérez et al. [17]. Interestingly, siRNAs as abundant as those in the ACO1 strong silencers V [29] and T lines (Fig. 2b) were detected in the strong overexpressers (C81 and C88) prior to grafting but not in the weak overexpressers (C75 and C89).

In all other cases reported to date, the existence of siRNAs has been linked with activation of PTGS and yet, from our results, these two lines were still expressing a high level of ACO1 mRNA yet contained siRNAs (Fig. 2b). This raises the questions: (1) Is silencing going on in these strong overexpressers? (2) How did the grafting trigger the strong silencing in the scions? Since these lines contain the NPTII selection marker gene and were generated through selection on kanamycin-containing media, we presume that the NPTII gene(s) in these lines is not silenced. Based on this, we would expect that the ratio of ACO1 and NPTII mRNA levels in C81 and C88 should be similar to those in C75 and C89 (no siRNA detected and presumably no silencing, Fig. 2b) if no detectable silencing occurs to C81 and C88. Indeed, phosphorimage densitometry analysis of ACO1/NPTII mRNA levels confirmed that the ratios were similar among these lines, approximately 1.0 (Table 1C). In contrast, after grafting the ratio dropped to 0.44 and 0.43 for C81 and C88 after grafting, respectively, whereas it did not change much for C75 and C89 (0.90 and 0.95). This suggests that the ACO1 genes in the strong overexpressers (Lines C81 and C88) are not silenced or that only very weak silencing occurs at an undetectable level or in a few cells only. Transient expression of viral silencing suppressors using agro-infiltration in the strong overexpressers may reveal whether silencing happens at all in these lines, but it could be difficult to tell if it occurs at a very low level or in specific cell types only.

In the scions C81 and C88, the silencing signal from the stock after grafting appeared to stimulate production of more siRNA. As found for lines C81 and C88, two more strong overexpressers (Lines C331 and C332; Fig. 2d) showed siRNA accumulation before grafting and grafting induced silencing. Differences in the structure of the ACO1 PTGS transgenes in the V and T lines means that siRNAs generated from each gene can be distinguished one from the other [25]. Due to the introduction of an IR to the 5' region of the transgene, the silencing stock (Line V) makes siRNAs mainly from the 5' end [25, Fig. 2d], but the source plant of the scions contain an ACO1 transgene without an inverted repeat and generate siRNAs from the 3' end [25]. On grafting, more siRNAs were generated in the scions, but these were only from the 3' end, even though the siRNAs in the stock (Line V) were preferentially generated from the 5' end (Fig. 2d). This indicates clearly that the siRNAs are the products of transmission to the scions of a silencing signal system which amplifies siRNAs, rather than transmission of specific siRNAs themselves. The accumulation of siRNAs in the scion of the source plants before grafting is consistent with this, since if siRNAs were the signal for systemic silencing, the scions would be expected to show strong silencing before grafting. Together with the finding that the elimination of siRNA production by a silencing suppressor does not prevent grafting-transmitted silencing [31,34], these observations point to the possibility of another specific nucleic acid species that acts as a systemic silencing signal.

Aberrant RNAs (abRNAs), probably derived from the transgene or its mRNA, have long been considered as the silencing initiation factor and the template for RNA-dependent RNA Polymerase (RdRP) [35]. Its level may be closely monitored by plant cells, leading to silencing initiation once it is over a threshold. It could be that such RNAs in the silencing stock move as the systemic silencing signal into the scions, adding to the level of abRNAs in the scion and stimulating the enhanced generation of siRNAs. The abRNAs may be in a decapped form as demonstrated by Gazzani et al. [36].
RNA hairpin structure formed due to the presence of the IR in the 5’ end of the ACO1 transgene in Line V [25] could lead to the generation of a pool of ACO1 mRNAs similar to the decapped mRNA. Such decapped ACO1 RNA could then be used as the templates by RdRP, leading to the production of siRNAs mainly from the 3’ region [25,29].

The graft transmission of a silencing signal from antisense plants was a noteworthy feature of these experiments, since antisense-induced silencing has been proposed to be ineffective in graft transmission of silencing [23,37]. We demonstrated with a total of 17 ACO1 strong overexpressers as scions that antisense-silenced rootstocks are capable of transmitting the signal to induce systemic silencing in these scions, albeit at a much slower pace than in sense-silenced rootstocks (Fig. 3). It is unclear whether this is related to the fact that in our experiments siRNAs were present in the scion source plant before grafting. It is possible that the delayed manifestation of transmission of antisense silencing that we found could make it difficult to observe. The variation in the time scale for the induction of silencing within different grafting experiments probably depends on how well the vascular tissues meet at the grafting junction. It is evident that the systemic spread of the silencing signal is strongly influenced by the phloem flow [12]. Perfect joining of the vascular systems of the scion and stock would guarantee a more effective flow and detection in the scions. This may also explain why graft-transmitted silencing is achieved more successfully using relatively young plants [23,37], as the vascular systems fuse more efficiently than for older plants.

Differences between sense and antisense silencing were reported by Jorgensen et al. [38] when they comparing the effects of sense and antisense Chalcone synthase (Chs) constructs in petunia populations. Recently, a nuclear model for sense and antisense transgene-mediated silencing by Wang and Metzlaflf [39] proposed that siRNAs arising from the sense transcript will have partial complementarity with the target mRNA in the cytoplasm. In their model, these siRNAs are then used as primers by RdRP to synthesize secondary dsRNA, resulting in propagation of more dsRNAs. On the other hand, the antisense-derived siRNAs will have perfect complementarity with the target mRNA and hence can be involved in direct cleavage of the mRNA as well as acting as primers for RdRP. We suggest that it is possible that if these RNAs are less accessible to act as primers for RdRP that might explain the reduced effectiveness of antisense silencing transmission. Although the ACO1 antisense line AS does have much lower levels of siRNAs than the V and T sense silencing lines [25], it is not clear if this is related to silencing transmission. It has been suggested that there is insufficient systemic signal for silencing to be transmitted in antisense lines [23] and it is possible that the delayed response we found indicates transmission of a weak silencing signal, although its precise molecular identity and origin remain unclear.

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