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Origin, biogenesis and non-cell autonomous effect of small RNAs in *Arabidopsis thaliana*

Dissertation

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1 Introduction

All cellular processes depend on the correct expression of different genes. Cell growth, cell division and many other routine cellular processes are directly reliant on accurately timed gene expression. Likewise, in response to environmental signals, cellular organisms have to trigger, suppress or modulate gene expression to better adapt to the new changing conditions. In multi-cellular organisms, cellular differentiation is also dependent on the proper control of gene expression. Due to expression of different genes, in particular developmental stages, cells with the same genomic content can differentiate in the diverse cells types with specialized functions. Reflecting this important role for cellular organisms, the control of gene expression can be made at different level, spanning chromatin structure, initiation of transcription, processing and stability of the transcript, mRNA transport to the cytoplasm, translational and pos-translational control.

For a long time, RNA was considered to be mainly involved with the synthesis of proteins, either by transmitting the genetic information from genes to proteins (mRNA) or by being involved with the translation process (tRNA and rRNA). However, this view has now changed. The discovery of small RNA (sRNA) molecules ranging from 19-24 nt and their function has placed RNAs as one of the main regulators of the gene expression. These sRNAs are main part of a pathway that results in gene silencing, either by methylation of the target gene, which interferes with the gene transcription (also known as transcriptional gene silencing; TGS), or by affecting the transcript stability and/or mRNA translation. The last process is known by different names depending on which organism it occurs, such as pos-transcriptional gene silencing (PTGS) in plants, RNA interference (RNAi) in animals or quelling in fungi.

1.1 Small RNAs in plants

As in animals, plant sRNAs can be divided into two different classes: small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Chapman & Carrington, 2007; Ghildiyal & Zamore, 2009; Vazquez, 2006). Together, these classes of sRNA are involved in virtually all process of the plant life, including development, stress and nutritional responses, chromatin structure and defense (Chuck *et al*, 2009; Lu & Huang, 2008; Mallory & Vaucheret, 2006).

Long before the mechanisms of sRNAs were known, RNAi and PTGS were already used as a tool for gene silencing. While studying the requirements for RNAi in the model organism *Caenorhabditis elegans*, Fire and colleagues (1998) have shown that perfectly-paired double stranded RNA (dsRNA) was a potent trigger of this phenomenon. But, it was only after the work of Hamilton and Baulcombe (1999) with plants that sRNAs were finally associated with gene silencing. These authors showed that plants presenting transgene-induced or virus-induced gene silencing accumulate sense and antisense sRNAs of about 25 nt specific to the silenced locus. With the discovery that these 21-25 nt long sRNAs were directly derived from the trigger dsRNA molecule (Bernstein *et al*, 2001; Yang *et al*, 2000) and that, in addition, they are the molecules conferring the specificity to the cleavage of the target RNA in the RNAi/PTGS phenomenom (Hammond *et al*, 2000; Zamore *et al*, 2000), these sRNAs were referred to as small interfering RNAs, or siRNAs.

Initially, siRNAs were thought to be a defense mechanism against exogenous sequences (exo-siRNA), more specifically transgenes and virus derived RNA. Many plants virus genomes can be found, at least at some point of its life cycle, as dsRNA. These virus-derived dsRNA trigger the production of siRNAs that, in turn, target back the original viral sequence. In addition, these siRNAs can spread to uninfected cells,

where they can act avoiding the spread of the infection (Lindbo & Dougherty, 2005; Mlotshwa *et al*, 2008; Wang & Metzlaff, 2005). siRNAs are also often generated from transgenes. RNA-mediated silencing of transgenes was first described in plants (Linn *et al*, 1990; Matzke *et al*, 1989; Napoli *et al*, 1990; Smith *et al*, 1990; van der Krol *et al*, 1990). Perhaps the best known case is the one described by Napoli and colleagues (1990). While trying to manipulate anthocyanin biosynthesis in petunia petals, the authors generated plants over-expressing a copy of chalcone synthase (CHS), a key enzyme of this pathway. Surprisingly, almost half of the plants presented white flowers caused by the lack of anthocyanins, rather than deeper purplish flowers, as expected. Analysis of the plants showed that both, transgene and endogenous CHS copies, were silenced. This phenomenon was called co-suppression. It was not clear why some transgenes can trigger this process more efficiently than others; however, once it is triggered, there is the recruitment of RNA dependent polymerases (RDRs) that are responsible for the conversion of single strand RNA (ssRNA) to dsRNA, which is then processed into siRNA that promote methylation of the transgene and the endogenous copy. The fact that most transgenes are introduced with strong constitutive promoters could explain why silencing occurs. The high levels of expression could result in many imperfect mRNA copies (uncapped or missing poly A tail for example) to escape cell quality controls and became RDRs template (Baulcombe, 2004; Ghildiyal & Zamore, 2009; Mello & Conte, 2004).

Apart from protecting against virus and exogenous genes, it became later clear that plants produce a high number of siRNAs derived from endogenous sequences (endo-siRNA). One class of endo-siRNAs comprises *cis*-acting siRNAs (casiRNAs). As the name suggests, casiRNAs act in *cis* causing the silencing of the locus where they originate from, which in most cases regards transposons, repetitive elements and

tandem repeats (note that siRNAs involved in transgenes silencing can also be considered casiRNAs). These 24 nt long molecules cause transcriptional silencing by promoting methilation of the target locus. Therefore, casiRNAs are seen as guardians of the genome, controlling the multiplication and over-expression of such elements (Chapman & Carrington, 2007; Ghildiyal & Zamore, 2009; Vaucheret, 2006).

A second class of endo-siRNA in plants is constituted by natural antisense-derived siRNA (nat-siRNA). As the name suggests, nat-siRNA originates from natural antisense transcripts (NATs), i.e. genes that are under the control of opposing promoters and which transcripts are overlapping. Up to date there are two reports of nat-siRNAs in plants (Borsani *et al*, 2005; Katiyar-Agarwal *et al*, 2006). In both cases, one of the NAT genes is constitutively expressed, while the other gene is induced by abiotic or biotic stress. The expression of the complementary transcript result in dsRNA, which is then processed in 21-24 nt nat-siRNA. Nonetheless, the number of nat-siRNAs might be larger, as suggested by the 1340 potential NATs pair found in *A. thaliana* genome (Wang *et al*, 2005). However, it is important to mention that probably, not all these NATs pair will originate nat-siRNA. As shown by Henz and colleagues (2007), the majority of these potential NAts does not seem to produce more sRNAs than non-overlapping gene pairs, suggesting that further requirements (than overlapping regions) are necessary to drive NATs into sRNAs pathways.

Plants additionally possess a unique class of endo-siRNA, the so-called trans-acting siRNA (tasiRNA). This class of endo-siRNA originates from non-coding genes called *TAS*. (Allen *et al*, 2005; Peragine *et al*, 2004; Rajagopalan *et al*, 2006; Vazquez *et al*, 2004b; Williams *et al*, 2005). tasiRNA production is triggered by cleavage of the TAS transcript by a specific micro RNA (miRNA, see below) (Allen *et al*, 2005; Rajagopalan *et al*, 2006) and different from the other classes of siRNAs they act in

trans, driving the cleavage of transcripts not related to the *TAS* gene which they come from (Chapman & Carrington, 2007). *A. thaliana* has four different families of *TAS* genes (*TAS1-4*), but only *TAS3* seems to be conserved throughout the plant kingdom (Allen *et al*, 2005; Axtell *et al*, 2006; Axtell *et al*, 2007; Rajagopalan *et al*, 2006; Talmor-Neiman *et al*, 2006; Vazquez *et al*, 2004b). In agreement with being the only family evolutionary conserved, *TAS3*-derived tasiRNAs are the only ones with an important role in plants identified so far. *TAS3* tasiRNAs target two *AUXIN RESPONSE FACTORS* (*ARF3* and *ARF4*) mRNAs, which have a central role in proper patterning and developmental timing (Adenot *et al*, 2006; Allen *et al*, 2005; Fahlgren *et al*, 2006; Garcia *et al*, 2006; Hunter *et al*, 2006; Williams *et al*, 2005).

miRNAs are a class of endogenous sRNA ranging from 20-24 nt that act post-transcriptionally to regulate gene expression. miRNAs originate from transcripts displaying an imperfect foldback structure and differently from other siRNA classes, usually only one miRNA is processed out of its precursor (Voinnet, 2009). The discovery of miRNAs date back to the year of 1993, with the identification of *lin-4*, a sRNA involved with the regulation of development timing in *Caenorhabditis elegans* (Lee *et al*, 1993). At the time, it was thought *lin-4* regulation was an exotic mechanism restricted to *C. elegans*, since no evidences of *lin-4*-like genes were known from other species and no similar molecule was known in nematodes either. The report of a second miRNA came only seven years after, with the identification of *let-7*, another *C. elegans* sRNA involved in the control of the developing time (Reinhart *et al*, 2000; Slack *et al*, 2000). This time however, homologous of *let-7* were promptly identified in flies and humans and shortly after, tens of new miRNAs were identified in animals (Lagos-Quintana *et al*, 2001; Lau *et al*, 2001; Lee & Ambros, 2001). In plants, miRNAs were initially identified in *A. thaliana*, with many

of them being conserved in other plant species (Reinhart *et al*, 2002). Plants miRNAs play a central role in the regulation of many important developmental processes (Chuck *et al*, 2009). For instances, miR319 (also known as JAW), the first plant miRNA functionally characterized, has an critical impact in the definition of leaf morphology (Palatnik *et al*, 2003). Nonetheless, miRNA function in plants is not restricted to development regulation, on the contrary, many miRNAs seems to have a important role in the adaptive response of plants to abiotic and biotic stress (Lu & Huang, 2008; Mallory & Vaucheret, 2006; Voinnet, 2008).

1.2 Biogenesis and action of plant sRNAs

It is reasonable to expect that the presence of such different classes of sRNAs would result in an equally diverse variation in the way these molecules are produced. Indeed, one of the main parameter to classify sRNA classes is based on the precursor from which they derived, as well on the enzymes that are part of this pathway (Chapman & Carrington, 2007). Nonetheless, the biogenesis of the different classes of sRNAs shares a few common steps: in all cases, RNA silencing relies on the presence of a dsRNA molecule, which in turn is processed in 19-24 nt long sRNAs that have the 3' end 2' OH-methylated. These sRNAs are then either retained in the nucleus or transported to the cytoplasm, where they associate with different proteins to form a complex. This sRNA/protein complex cause gene downregulation by either driving cleavage or translation inhibition of the target gene (PTGS), or by leading to heterochromatin formation and blockage of transcription (TGS) (Carthew & Sontheimer, 2009; Chapman & Carrington, 2007; Ghildiyal & Zamore, 2009).

As already discussed, the hallmark for triggering RNA silencing is the presence of dsRNA. Double stranded RNA can be formed directly as a consequence of the transcript characteristic, virus and transposons replication process or be synthesized by RDRs. miRNAs originate from primary transcripts with self-complementarity, resulting in the formation of an imperfect dsRNA hairpin-like molecule (Voinnet, 2009). The origin of dsRNA-precursors that spawn siRNAs is more diverse. Like miRNAs, siRNAs can also be formed from stem-loop structures, however with a more perfect pairing than the one presented by miRNA precursors. This stem-loop can be part of a secondary structure of some transcripts or be result of inverted-repeated sequences (Kasschau *et al*, 2007; Lu *et al*, 2006). As part of the replication process, many virus and transposons can be found in some stage as dsRNA, which is promptly processed into siRNAs. However, in most cases formation of dsRNA derived from transgenes, virus, transposons and repetitive elements is dependent on the action of RDRs, a class of polymerase that uses ssRNA as substrate to produce dsRNA (Chapman & Carrington, 2007; Ghildiyal & Zamore, 2009). Plants have six RDRs (RDR1-6) identified, with RDR2 and RDR6 being the only members with direct roles described so far. RDR6 is involved in the production of secondary siRNA from virus and transgene-related siRNAs, as well as the amplification of endo-siRNAs (Chapman & Carrington, 2007; Xie & Qi, 2008). RDR6 has also a key role in the generation of tasiRNAs, process that will be discussed in more detailed further on. RDR2 in order hand is involved manly in the generation of casiRNAs that are involved in heterochromatin formation. This pathway requires transcription mediated by RNA polymerase IVa (PolIV), an enzyme only described in plants. The transcript is then converted to dsRNA by RDR2 (Chapman & Carrington, 2007; Ghildiyal & Zamore, 2009; Xie & Qi, 2008). Another member that was associated with RNA

silencing is RDR1 that seems to play a role in plants resistance against virus (Yu *et al*, 2003), however is not clear if this molecule is directly involved in the production of siRNAs. There is still no evidence for the involvement of other members of RDR family in RNA silencing.

Once present in the cell, dsRNA is processed into sRNAs by a class of RNase III enzymes called DICER-LIKE (DCL). Plants have four different DCLs (DCL1-4), which suggests a subdivision of function. Indeed, the different DCLs seem to be involved in different pathways. For instance, DCL1 is mainly involved in the processing of miRNA precursors (Voinnet, 2009). DCL2 is responsible for producing the 22 nt long siRNAs from exogenous elements and natsiRNAs, while DCL3 is the main enzyme in the generation of the 24 nt long heterochromatic casiRNAs. In turn, DCL4 is responsible for the production of 21 nt long siRNAs and tasiRNAs (Carthew & Sontheimer, 2009; Chapman & Carrington, 2007). However, there are many cases where DCLs function seems to overlap. Although there is a hierarchy for substrate preference, in some occasions (specially the ones involving the overexpression of the siRNA precursor or lack of one of the DCLs) a different DCL can have access to the precursor, which initially would preferentially diced by another member of the family (Deleris *et al*, 2006; Dunoyer *et al*, 2007; Gasciolli *et al*, 2005). For example, DCL4 and DCL2 can process dsRNA derived from RDR2 action, which normally would be a substrate for DCL3 (Gasciolli *et al*, 2005). The relative expression levels of a given DCL can also alter the preferential access to the substrate. Vazquez and colleagues (2008) demonstrated that in inflorescences, DCL3 (which is 10 times more expressed than in leaves) can produce miRNAs that are 24 nt in lengh. DCL slicing activity is assisted by co-factors, which include a group of five dsRNA-binding (DRB) enzymes. Like DCLs and RDRs, the different plants DRBs seem to have subdivision of

function (Chapman & Carrington, 2007; Xie & Qi, 2008). HYPONASTIC LEAVES1 (HYL1) is the founder member of the DRB family and has been linked to the production of miRNAs, together with DCL1 (Han *et al*, 2004; Vazquez *et al*, 2004a), while DRB4 interacts with DCL4 to produce tasiRNAs (Adenot *et al*, 2006; Nakazawa *et al*, 2007). It is not clear which is the function of the others DRB members, however it is reasonable to think they may also interact with specific DCLs. sRNA processing by DCLs also depends on other co-factors that are not specific to the sRNA pathway, such as SERRATE (SE) (Lobbes *et al*, 2006; Yang *et al*, 2006a). SE is a C2H2-zinc finger protein that likely act together with proteins of the cap-binding complex to promote proper miRNA processing and splicing (Gregory *et al*, 2008; Laubinger *et al*, 2008).

After maturation, sRNAs are protected against degradation by the action of HUA ENHANCER1 (HEN1), a protein responsible for methylation of the 2′-hydroxyl group on the ribose of 3′ terminal nucleotide (Li *et al*, 2005; Yang *et al*, 2006b; Yu *et al*, 2005). sRNAs that act in the cytoplasm are exported from the nucleus through the action of HASTY, a plant homologous of exportin-5 (Park *et al*, 2005). However, it is likely that another transport mechanism exists in plants, since some miRNAs seem to be HASTY-independent (Voinnet, 2009).

In order to promote RNA silencing, sRNAs need to be associated with a protein complex known as RNA-induced silencing complex (RISC), for which the sRNAs act as a guiding molecule. The main protein component of RISC is ARGONAUTE (AGO), which contain an RNA-binding domain (PAZ) and the slicer activity (PIWI domain) responsible for the sRNA-mediated cleavage of transcripts (Carthew & Sontheimer, 2009; Ghildiyal & Zamore, 2009; Xie & Qi, 2008). The *A. thaliana* genome encodes 10 different AGOs. AGO1 is the main effector protein

associated with sRNAs in PTGS. AGO4 and AGO6 interact with the 24 nt long siRNA involved in DNA and histone methylation, while AGO7 is required in one of the pathways leading to tasiRNA production (Chapman & Carrington, 2007; Ghildiyal & Zamore, 2009; Xie & Qi, 2008). But this subdivision of function is not only restricted to the different pathways. Characterization of the sRNA population associated to the different AGOs shows a preference regarding the loading of sRNAs, which is dependent on the identity of the first nucleotide in the 5' end of the sRNA (Mi *et al*, 2008; Montgomery *et al*, 2008). AGO1 for instance, associates manly with molecules that the first nucleotide is an uridine. This is in accordance with AGO1 function as the main slicer for miRNAs activity, which are mostly starting with this nucleotide. AGO2 and AGO4 seem to prefer adenosine as the first nucleotide, while AGO5 are enriched for sRNAs that have a cystidine in the first position.

As mentioned before, RNA silencing can act at the level of transcription (TGS) or post-transcriptionally through effects on transcript stability (PTGS). In plants, TGS involves both DNA-directed and H3K9 methylation (methylation of lysine 9 residue of the histone 3). This epigenetic effect is driven by DCL3-generated 24 nt siRNAs; it is dependent on NRPD1a and NRPD1b (two isoforms of the PolIV), RDR2, AGO4 and requires the action of DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) (Chapman & Carrington, 2007; Henderson & Jacobsen, 2007; Moazed, 2009). RNA silencing can also occur post-transcriptionally. Indeed, cleavage of the target transcript was promptly recognized as a consequence of sRNA-directed silencing (Hammond *et al*, 2000; Zamore *et al*, 2000). Guided by the sRNA, the RISC complex, by action of the slicing activity of the associated AGO, cause the cut of the complementary transcript leading to its degradation (Carthew & Sontheimer, 2009).

In plants, PTGS triggered by miRNAs usually result in cleavage of the target mRNA, in a similar way as described above. However a second mode of action is often recognized. Analysis of the *C. elegans* miRNA *lin-4* and its target LIN-14 showed that this sRNA does not affect the levels of the target mRNA (a consequence of RISC cleavage). Instead, it affects protein synthesis by interfering with translation. Therefore, this mode of action of miRNAs is referred as translation inhibition. In plants, translation inhibition was first observed for miR172, being described as the main mechanism in the silencing of AP2 (Aukerman & Sakai, 2003). In general, translation inhibition has long been considered to be the main mode of operation of animal miRNAs, while in plants, it might be a secondary activity observed in a few miRNAs. The main reason for this difference in mode of actions is believed to be due to the extent of miRNA-target pairing (Carthew & Sontheimer, 2009). Recent findings however, suggest that the picture might be different. The characterization of *miRNA-action deficient* (mad) mutants in *A. thaliana* shows that miRNA degradation and protein translation inhibition probably occur at the same time and have the same sequence requirements (Brodersen *et al*, 2008). Nonetheless, the mechanism how translation inhibition occurs is still controversial. Initially, it was suggested that the RISC complex either avoid the initiation of translation or act by repressing the elongation of the peptide chain, which in both cases would prevent protein accumulation without affecting the mRNA levels (Ghildiyal & Zamore, 2009). A second model has been suggested to explain miRNA action not dependent on target cleavage. This model is based on the destabilization of the target mRNA caused by miRNA interaction, routing of the target mRNA to degradation and consequently decrease in mRNA levels (Carthew & Sontheimer, 2009). This scenario is supported

by recent findings on ribosome profiling of mammalians cells, which shows that translation is not affected by miRNA targeting (Guo *et al*, 2010).

1.3 Biogenesis of tasiRNAs

Most transcripts when cleaved by miRNA are driven for degradation. *TAS* transcripts on the contrary, are not destroyed, but used as template by RDR6 to generate dsRNA that will be processed by DCL4, forming 21 nt tasiRNAs that are in phase regarding the miRNA-guided cleavaged site (Allen *et al*, 2005; Gasciolli *et al*, 2005; Peragine *et al*, 2004; Vazquez *et al*, 2004b; Williams *et al*, 2005; Xie *et al*, 2005; Yoshikawa *et al*, 2005). miR173 triggers tasiRNA synthesis from *TAS1* and *TAS2*, while *TAS4* is targeted by miR828 (Allen *et al*, 2005; Rajagopalan *et al*, 2006). In all three cases, miRNA cleavage leads to tasiRNA production 3' of the initial cut. *TAS3* on the other hand, is targeted by miR390 and spawns tasiRNAs from the region located upstream of the cleavage site (Allen *et al*, 2005).

Why are *TAS* transcripts not targeted to degradation, but instead, directed to a pathway that results in secondary sRNA production? Axtell and colleagues (2006), when studying sRNAs in the moss *Physcomitrella patens*, identified in *TAS3* transcripts the existence of a second functional cleavage site for the miR390. Interesting, this new site was located upstream to the original described site, with most of the tasiRNAs being localized in between these two cleavage motifs. They could also recognize the same pattern in the gymnosperm *Pinus taeda* and *A. thaliana*, with the difference that in the latter species the 5' sites are not cleavable, but still necessary for efficient tasiRNA production. In addition, they have also described loci that seem to produce secondary sRNAs from regions that are in between two

sRNA target sites. All these observations led to the suggestion that tasiRNAs are often spawned when transcripts are targeted at two positions by one or more sRNAs, idea that is known as the "two-hit" trigger hypothesis for tasiRNA generation. However, an alternative or complementary mechanism is still necessary to explain the generation of tasiRNAs, based on the fact that no evidence for a secondary miRNA cleavage site was found in the other families of *TAS* genes. This idea was further reinforced after deep analysis of *A. thaliana TAS3* locus. By replacing both miR390 recognition sites in *TAS3* for alternative miRNAs sites and/or not functional cleavage motifs, Montgomery and colleagues (2008) have shown that tasiRNA production in this locus depend on the specific interaction of miR390 in the 5', but not in the 3' recognition site. Nonetheless, miRNA cleavage of the 3' site was still necessary to start the process, although this could be replaced by another miRNA-mediated cleavage. These results suggest that not only the double targeting is important for tasiRNA production, but also the nature of the miRNA/*TAS3* interaction. Indeed, the authors have also shown that AGO7 interacts specifically with miR390 and that such interaction is necessary for proper tasiRNA production.

1.4 Origin and evolution of new miRNAs

With exception of miR319, the first plant miRNA to be identified based on a forward genetic screen and the first shown to be important for plant development (Palatnik *et al*, 2003), the first plant miRNAs described were found by high-throughput cloning (Llave *et al*, 2002; Mette *et al*, 2002; Park *et al*, 2002; Reinhart *et al*, 2002). Many of these miRNAs were later shown to have essential roles in key developmental pathways and to be conserved in other plant species (Voinnet, 2009).

The employment of new deep-sequencing technologies have allowed the identification of miRNAs with low abundance that otherwise would be masked by miRNAs with higher expression (Fahlgren *et al*, 2007; Rajagopalan *et al*, 2006). The majority of these more recently identified miRNAs are species specific, suggesting a high level of birth and death of new miRNAs. This scenario was confirmed in flies, with only 4% of the new miRNAs being retained in the genome (Lu *et al*, 2008).

Where do all this new miRNA loci come from? Allen and colleagues (2004) identified two miRNAs which the targets present extensive similarity with their precursors. Close analysis showed that these miRNAs likely originated from an inverted repeated duplication of what then became the target loci. In this scenario, the inverted repeat would probably generate heterogeneous siRNAs resembling those originating from perfect dsRNA, which are usually processed by DCL4 and DCL3. Corroborating this view, some evolutionarily young miRNAs are dependent on DCL4, instead of DCL1 processing (Rajagopalan *et al*, 2006). In some cases, positive selection would lead to accumulation of mutations and consequently to fold-back mis-pairing and eventually release of specific mature miRNAs. Continuous accumulation of mutation in the new miRNA genes would cause further drift of the mature miRNA surrounding arms resulting finally in an old miRNA gene unrelated to the parental locus. This model for the evolution of miRNA genes seems to be true for over 30% of the *A. thaliana* recently evolved miRNAs identified by deep-sequencing (Fahlgren *et al*, 2007; Rajagopalan *et al*, 2006). miRNAs can also evolve from inverted duplications of non-target sequences in a similar way described above (Fahlgren *et al*, 2007).

Not all miRNAs seems to originate by duplication events. This observation is based on the fact the many recently evolved miRNAs do not resemble any of the

properties related to such process (Fahlgren *et al*, 2007; Rajagopalan *et al*, 2006). Transposable elements can be an alternative source of miRNAs. DNA-type nonautonomous elements known as miniature inverted-repeat transposable elements (MITEs) have been shown to fold in hairpin-like structure typical of miRNAs. In addition, some putative miRNAs as well siRNAs have been mapped back to MITEs locus (Piriyapongsa & Jordan, 2008). Nonetheless, alternative models are necessary to explain the whole spectrum of new miRNA genes (*MIRNAs*) observed in plants.

1.5 Non-autonomous effect of sRNAs

Even before the identification of sRNAs and RNA silencing, PTGS and co-suppression in plants have been described to be non-cell-autonomous, i.e. the silencing occurs not only in the cells where it is produced, but also can spread to the surrounding cells and eventually to the whole organism (Kalantidis *et al*, 2008; Voinnet, 2005). Movement of silencing was first described in tobacco plants overexpressing nitrate reductase (Nia) and nitrite reductase (Nii) genes (Palauqui *et al*, 1996). In this system, some plants developed a spontaneous co-suppression leading to localized chlorosis that could then spread to the rest of the leaf and even to other leaves. In the same system, it was shown that the co-suppression-triggered silencing could spread to naive scions after they were grafted on stocks that had the silencing trigger (Palauqui *et al*, 1997). About the same time, it was shown that silencing of a constitutively expressed GFP initiated locally by infection of *Agrobacterium* expressing GFP could spread systemically (Voinnet & Baulcombe, 1997). The fact that systemic silencing can be initiated by inoculation of *Agrobacterium* that introduces exogenous transgenes into plant cells suggests that

siRNAs are responsible to trigger the process (Palauqui & Balzergue, 1999; Voinnet *et al*, 1998). In agreement, systemic silencing can be induced by bombardment of dsRNA and most notably, by synthetic siRNA duplex, with the same efficiency (Klahre *et al*, 2002). The observation that RNA silencing follows the same direction of the phloem, suggests that this is the main channel for the spreading (Palauqui & Balzergue, 1999; Sonoda & Nishiguchi, 2000; Voinnet *et al*, 1998). Indeed, many sRNAs have been show to exist in plants phloem sap (Buhtz *et al*, 2008; Yoo *et al*, 2004).

siRNA-triggered silencing can also spread cell-to-cell (Himber *et al*, 2003; Palauqui *et al*, 1996; Voinnet & Baulcombe, 1997). Initially, silencing spread for 10-15 cells, however, in some cases silencing can spread further in a mechanism that is dependent on RDR6 and SILENCING DEFECTIVE 3 (SDE3) (Himber *et al*, 2003). Nonetheless, this amplification mechanism seems to be restricted to silencing initiated by exogenous sequences, like virus and transgenes (Himber *et al*, 2003; Vaistij *et al*, 2002). Cell-to-cell silencing depends on the 21 nt produced by DCL4, however it was not sure if it is the siRNA that actually moves, the precursor or some downstream factor (Dunoyer *et al*, 2005; Himber *et al*, 2003). Recently, it has been shown that the 21 nt long sRNA duplex works as the mobile silencing signal in between plant cells and that 24 nt long sRNAs are transported through the phloem and are responsible for the systemic silencing (Dunoyer *et al*, 2010; Molnar *et al*, 2010). In addition, RDR2, NRPD1a and CLASSY1 are necessary for proper siRNA-triggered spreading (Dunoyer *et al*, 2007; Smith *et al*, 2007). tasiRNAs are another class of sRNAs that seems to be non-cell-autonomous. Recent evidences suggest that *TAS3*-derived tasiRNA could act at long distances to confer proper leaf patterning (Chitwood *et al*,

2009; Schwab *et al*, 2009), however this putative tasiRNA movement need to be better characterized.

Silencing triggered by miRNAs seems to be more controversial, with evidence supporting both cell-autonomous and non-cell-autonomous effects. Many experiments using natural and artificial constructs expressed under different promoter suggest that miRNAs would have a cell-limited area of action (Alvarez *et al*, 2006; Parizotto *et al*, 2004; Schwab *et al*, 2006; Tretter *et al*, 2008; Válóczi *et al*, 2006). On the other hand, miRNAs have been reported to act in areas different from where they are produced, as a long distance molecule in phosphate homeostasis (Pant *et al*, 2007) or involved in the leaf development (Nogueira *et al*, 2009). In addion, miRNAs are also part of the sRNA population found in the phloem sap of some plants (Buhtz *et al*, 2008; Yoo *et al*, 2004).

1.6 Aim of this work

This PhD thesis focused on three main areas of sRNA evolution and function in plants, as follow:

a) Identification of new recently evolved miRNAs in *A. thaliana* and possible scenarios for the origin and evolution of those sRNAs.

b) Elucidation of the biogenesis process that result in tasiRNA production, more specifically, the role of miR173 in triggering tasiRNAs generation in *TAS1* and *TAS2* genes.

c) Characterization of the putative non-cell-autonomous effects of miRNAs and tasiRNAs in plants.

2 Results

2.1 "Evolution of Arabidopsis thaliana microRNAs from random sequences"

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Contributions

FFF, KS, TD, DHH and DW planned the experiments. TD developed the pipeline for identification of *A. thaliana* specific sRNAs. FFF performed the isolation and validation of all new miRNA candidates. Genome and transcriptome alignments were done by KS. FFF and KS compared the *A. thaliana* MIRNA loci to *A. lyrata* genome. FFF, KS and DW analyzed the data. FFF and DW wrote the manuscript with contribution from KS and the other authors.

Synopsis

In silico analysis has shown that a typical plant genome contains hundreds of thousands of potential partially self-complementary foldback sequences (Jones-Rhoades & Bartel, 2004). We hypothesized that these sequences, once expressed (as a consequence of a promoter trapping or strong expression of adjacent genes, for example) could be the source of new *MIRNAs*. If this is the case, one could expect that the *MIRNA* genes in question would have no similarity to other regions of the plant genome, opposite to what has been described in cases where the new *MIRNA* evolve through duplication events (Allen *et al*, 2004; Fahlgren *et al*, 2007; Rajagopalan *et al*, 2006).

Conserved miRNAs likely arose before species speciation; therefore they are often referred as "old" miRNAs. Because "old" miRNAs tend to accumulate more mutations, evolutionary history can be hard to be assigned due to sequence drift. Therefore, evolutionary studies on *MIRNA* genes require the availability of recently evolved ("young") miRNAs, which are usually over-represented among species-specific miRNAs. To this end, I developed a new functional assay to identify and validate *A. thaliana* specific *MIRNAs*, which, at the beginning of this project were under-represented. Using this assay I was able to validate five new *A. thaliana* specific miRNAs. Those, together with a set of "young" miRNAs identified by several independent large-scale small RNA sequencing projects (Fahlgren *et al*, 2007; Lu *et al*, 2006; Rajagopalan *et al*, 2006), were analyzed for their similarity to the rest of the genome and transcriptome. Based on this, we were able to divide these *MIRNAs* into two groups, according to similarity to some other region of the genome/transcriptome. *MIRNAs* belonging to the group sharing similarity to other genome regions probably evolved through processes relying on duplication events. Indeed, many of these *MIRNAs* were identified as being the consequence of a target inverted duplication event (Fahlgren *et al*, 2007; Rajagopalan *et al*, 2006). To test if the alignment result from the second group (*MIRNAs* without obvious similarity) was statically significant, we performed a second analysis where we randomly shuffled the miRNA arms 1000 times and aligned these again against the genome/transcriptome of *A. thaliana*. These allowed us to identify *MIRNAs* that seem to have originated from a unique region. Finally, for each of the *A. thaliana MIRNA* genes without significant alignment scores, we examined their orthologous regions in the genome of *A. lyrata*, a close relative of *A. thaliana*. In none of the cases the *MIRNA* gene was substantially conserved. However, in some cases it was possible to identify a putative foldback

structure, but without the mature miRNA present, or a relative conserved mature miRNA could be detect, but the secondary structure of the possible precursor was unlike to be used as a template for DCLs. Together, these observations led us to suggest that some *MIRNAs* could indeed originate from random sequences.

2.2 *"Triggering the formation of tasiRNAs in Arabidopsis thaliana: the role of microRNA miR173"*

Felipe Fenselau de Felippes and Detlef Weigel

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Contributions

FFF and DW planned and analyzed the experiments and wrote the manuscript. All experiments were carried out by FFF.

Synopsis

One of the main questions concerning tasiRNA biogenesis regards the mechanism that results in *TAS* transcripts being directed to the SGS3/RDR6 pathway instead of being degraded, which is the normal fate of transcripts targeted by miRNAs. It has been recently suggested that transcripts that are targeted twice by sRNAs are more prone to generating tasiRNAs, a concept known as the "two-hit" model for tasiRNA production. However, this hypothesis cannot explain tasiRNA generation from *TAS1*, *TAS2* and *TAS4* families, since those transcripts do not seem to be targeted twice by sRNAs (Axtell *et al*, 2006). In addition, miR390/AGO7 interaction with the *TAS3* transcript has been shown to be essential for proper tasiRNA production, suggesting a main role for the miRNA in tasiRNA biogenesis (Montgomery *et al*, 2008).

We have studied the role of miR173 in the production of tasiRNAs from *TAS1*. For this purpose, I developed an artificial tasiRNA (atasiRNA) based on the *CH42* gene (atasi-SUL), which is required for chloroplast function (Koncz *et al*, 1990). tasiRNA production from the atasi-SUL construct results in pale plants, due to downregulation of *CH42* (Himber *et al*, 2003). We first tested whether miR173-

mediated cleavage was essential for TAS1 tasiRNA production. While expression of the original atasi-SUL resulted in most plants having a bleached phenotype, replacing the miR173 target site with the one recognized by the strongly expressed miR159 did not have such an effect. This result suggested that miR173 cleavage is required for proper tasiRNA generation. We have then created deleted versions of the atasi-SUL construct to assay whether *TAS1* transcript relies on an extra site for triggering tasiRNAs. Our results suggested that miR173 in sufficient to start the tasiRNA production process. If this assumption is correct, miR173-cleavage alone should be sufficient to trigger secondary sRNA production even in non-*TAS* transcripts. To test this hypothesis, we used a second system also based on the silencing of *CH42*. We showed that placing the site recognized by miR173 in front of a fragment of the *CH42* gene results in secondary sRNA production, while the same fragment cleaved by miR159 is not affected. Finally, we have shown that flanking the *CH42* fragment with the miR390 recognition site found in *TAS3* (but not miR159) also leads to tasiRNA production, corroborating the idea that the miRNA that mediates the cleavage has a main role in directing TAS transcript to SGS3/RDR6 pathway.

Addendum

The reason why miR173 is so unique was partially solved by two recently papers published by Chen *et al* (2010) and Cuperus *et al* (2010). Both groups have found, independently, that most miRNAs (including miR173) and tasiRNAs triggering secondary sRNAs are usually 22nt in length (most miRNAs and tasiRNAs are 21nt long). In accordance, the two groups showed that changing the precursor of miR173 to produce a 21nt long miRNA abolishes its ability to trigger tasiRNA production. Also, engineering a 21nt long miRNA, which normally does not trigger

transitivity (such as miR319), to release 22nt long miRNA is sufficient to support secondary sRNA production. In addition, they have shown that asymmetric pairing between miRNA and miRNA* is the reason why 22nt long miRNAs are processed by DCL1.

2.3 *"Comparative analysis of non-autonomous effects of tasiRNAs and miRNAs in* Arabidopsis thaliana"

Felipe Fenselau de Felippes, Felix Ott and Detlef Weigel

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Contributions

FFF and DW designed the experiments and analyzed the data. FFF performed all experiments, and FO was responsible for the analysis of the sRNA sequencing libraries. FFF and DW wrote the manuscript with contributions from FO.

Synopsis

RNA silencing triggered by siRNAs has been shown to be mobile, spreading from one cell to another and also systemically. On the other hand, non-autonomous effects of other sRNA classes are still a matter of discussion. I have focused in the characterization of the possible non-autonomous effects of miRNAs and tasiRNAs. To do so, I initially designed an artificial miRNA (amiRNA), amiR-SUL, targeting the *CH42* gene. Expression of the amiR-SUL form the *SUC2* promoter allowed us to follow the movement of the silencing signal from its production site, i.e. the phloem companion cells where the *SUC2* promoter is strongly active, to neighboring cells. As a control, I generated an inverted repeat using a fragment of *CH42* (siR-SUL), which in turn spawns siRNAs targeting *CH42*. SUC2:*siR-SUL* plants showed the typical bleaching around veins, caused by spreading of the silencing signal over 10 to 15 cells (Himber *et al*, 2003). Similarly, lines carrying the SUC2:*amiR-SUL* presented similar phenotypes to the one described for SUC2:*siR-SUL* plants, suggesting that miRNA-triggered silencing could spread the same distance as siRNA silencing.

I ruled out the possibility that the silencing movement observed in the amiR-SUL lines would be caused by siRNAs by crossing these plants to *rdr6* and *dcl*2-3-4 triple mutant, which cannot undergo secondary and primary sRNA production, respectively. Next, I tested the possible effects of the miRNA precursor on the spreading of the RNA silencing. For this purpose, I constructed amiR-SUL variants that produce the same mature miRNA but from alternative precursors. All the variants resulted in the same bleaching pattern seem in the original amiR-SUL line, with the exception of the amiR-SUL based on the miR164b. However, careful analysis showed that the miRNA was not properly processed out of the miR164 precursor, suggesting that the expression level of the miRNA is an important parameter influencing the extent of spreading of RNA silencing. In addition, I could also conclude that the miRNA precursor plays at most a minor role in defining the range of sRNA movement.

Using the same approach described above, I designed an atasi-SUL construct to study the non-autonomous effect of tasiRNAs. The advantage of our system is that both constructs, the amiR-SUL and the atasi-SUL, spawn the same mature sRNA, allowing us to compare and test the effects of different pathways on the mobility of the silencing signal. Surprisingly, plants carrying the SUC2:*atasi-SUL* constructs showed spread of bleaching throughout the whole leaf, instead of the limited 10 to 15 cells observed for miRNAs and siRNAs. This long-range cell-to-cell movement has also been described for siRNAs. In this case, amplification of the signal by means of RDR6 has been suggested as the mechanism allowing such extended movement. Unfortunately, because RDR6 is also necessary for the production of the original tasiRNA, direct analysis of the dependency on transitivity is difficult to be accessed. Nonetheless, priming-dependent 5'-to-3' amplification does not appear to be

necessary for the long-range movement of tasiRNA-triggered silencing. However, analysis of the sRNAs associated with the *CH42* locus, which I carried out with help from Felix Ott, showed that atasi-SUL targeting seems to trigger the production of small amounts of secondary siRNAs. Whether these secondary sRNAs play a role in the long-range movement of tasiRNAs is currently still unclear.

At last, I tested the genetic requirements for spreading of miRNA and tasiRNA-triggered silencing; my results suggested that there are alternative mechanisms for spreading of miRNAs and tasiRNAs.

2.4 *"MIGS: an efficient gene silencing approach for plant functional genomics"*

Felipe Fenselau de Felippes, Jia-Wei Wang and Detlef Weigel.

Manuscript in preparation for submission to Nature Methods

Contributions

FFF, JW and DW designed the experiments. FFF has done all the experiments with contribution of JW in the construction of a plasmid collection for the use of MIGS. FF and DW analyzed the data and wrote the manuscript with contribution of JW.

Synopsis

It was only with the discovery of sRNAs that gene silencing became a frequently used and reliably applicable technology, not only for research, but also for medicine and agriculture. The first techniques to trigger RNAi were based on the production of siRNAs from perfectly complementary dsRNA. In plants, Virus Induced Gene Silencing (VIGS) and hairpin RNAi are today the two most successful of these techniques (Ossowski *et al*, 2008; Watson *et al*, 2005). Another method widely used is the artificial miRNA (amiRNA) approach. As its name suggests, amiRNAs are based on production of specific miRNAs, instead of a collection of siRNAs, designed to target the gene(s) of interest (Ossowski *et al*, 2008; Schwab *et al*, 2006). All these methods present advantages and disadvantages; for example, while amiRNAs are very specific, this requires full background knowledge of a genome, and the stringent sequence requirements do not allow amiRNA design for every gene.

We have previously shown that flanking a fragment of *CH42* with the target site for a specific miRNA, miR173, which is a trigger of transitivity (Allen *et al*,

2005),was sufficient to trigger secondary sRNA production and consequently silencing of the endogenous gene (Felippes & Weigel, 2009). I then hypothesized that this approach could be generalized, and that it could be broadly used as a new gene-silencing tool. First, I have shown that miR173-triggered targeting of *AGAMOUS* (*AG*), *EARLY FLOWERING 3* (*ELF3*), *FLOWERING LOCUS-T* (*FT*) and *LEAFY* (*LFY*) results in plants with phenotypes similar to the respective loss-of-function mutants. This provides for an alternative method to downregulate gene expression in *A. thaliana*, which we named MiRNA Induced Gene Silencing (MIGS).

Next, we tested whether MIGS could be used to silence more than one gene. To this end, I generated constructs where an *AG* fragment was both linked to *FT* or *ELF3* fragments, and flanked by a single miR173 target site. To address possible positional effects, I tested *AG* both in the miR173 proximal and distal position (*35S:173ts_AG_FT* and *35S:173ts_FT_AG*; *35S:173ts_AG_ELF3* and *35S:173ts_ELF3_AG*). Silencing could be detected in all cases; however, *AG* and *FT* were only partially downregulated when the respective fragment was located in the distal position, suggesting loss of silencing efficiency with increasing distance from the miR173 target site. I then assessed the effect of a second miR173 target site in front of the second gene fragment. With this approach, both genes were silenced to a similar extent as in single-gene MIGS.

By using transient assays in *Nicotiana benthamiana* plants, I have also shown that MIGS can be readily extended to plants other than *A. thaliana*. Because outside the Brassicaceae, co-expression of the miR173 is necessary due to the family-specific character of miR173, I have developed a collection of plasmids to facilitate the usage of MIGS. These vectors are all based on the pGreen plasmid and are Gateway compatible.

In summary, we have developed an alternative technique for efficient gene silencing in plants, which we called MIGS. MIGS differentiated itself from other gene silencing methods due to its design simplicity and efficacy in multi-gene silencing. In addition, we generate a collection of plasmids for convenient use of MIGS.

3 Conclusions

This PhD thesis was focused on different aspects of sRNA silencing in plants, namely: the origin and evolution of miRNAs; the biogenesis of tasiRNAs; and the non-cell autonomous effect of miRNAs and tasiRNAs.

The sequencing of very large populations of sRNAs in *A. thaliana* has made it possible to identify many new, low expressed miRNAs that have not been isolated before (Fahlgren *et al*, 2007; Rajagopalan *et al*, 2006). Many of these are specific to *A. thaliana* and likely recently evolved *MIRNA* genes. This assumption was confirmed by two recent studies comparing the miRNA populations of *A. thaliana* and its close relative *A. lyrata*. Despite their recent speciation (about 10 million years ago), 18% and 22% of the miRNA loci in *A. lyrata* and *A. thaliana*, respectively, are either unique or substantially diverged (Fahlgren *et al*, 2010; Ma *et al*, 2010). But how do new *MIRNAs* arise? It seems that some *MIRNAs* are the result of inverted duplication events (Allen *et al*, 2004; Fahlgren *et al*, 2007; Rajagopalan *et al*, 2006). However, duplication events do not seem to be responsible for the rise of all new *MIRNA* genes. Most of the recently evolved *MIRNAs* in *Drosophila* do not originated by inverted duplication, but more likely from non-miRNA related sequences of random origin (Lu *et al*, 2008). In plants, it has been speculated that transcription of random foldbacks could be the source of new miRNAs (Axtell, 2008).

Based on the fact that the *A. thaliana* genome contains hundreds of thousands of hairpin-like structures (Jones-Rhoades & Bartel, 2004), I hypothesized that some of these structure could be the source of new *MIRNAs*. In accordance, we have identified a set of recently evolved miRNAs that seem to be unique in the genome and therefore, unlikely to have evolved through duplication events. In addition, comparison of those

MIRNA genes with their homologous regions in *A. lyrata* resulted in partial conservancy, with some aspects of the miRNA missing, suggesting that those could be some sorts of pre-*MIRNA* genes. Finally, we suggest that random sequences that present some features of miRNAs, such as a foldback, could be the origin of new *MIRNAs*. In this case, if a newly evolved miRNA fortuitously guides cleavage of an mRNA, this interaction could become the subject of either negative selection (if the interaction is deleterious for the organism) or positive selection (if the interaction is advantageous) in a similar way as observed for transcription factors (Dermitzakis & Clark, 2002).

I have also studied the role of miR173 in starting tasiRNA production from *TAS1*. My results suggested that miR173 plays a central role in this process, being necessary for triggering *TAS1*-tasiRNAs. In addition, miR173 seems to be sufficient by itself, with other regions of *TAS1* gene having little or no effect in the generation of tasiRNAs. It is quite likely that my findings can also be applied to *TAS2*, which shares the same miR173-trigger (Allen *et al*, 2005). This uniqueness of miR173 cleavage seems to be a property of miRNAs involved in tasiRNA production. Indeed, analysis of miRNAs and tasiRNAs leading to the production of secondary sRNAs shows an over-representation of molecules that are 22 nt in length, including miR173. In addition, genetic engineering of miR173 to produce a mature miRNA of 21 nt instead of 22 nt results in the loss of its capability to produce secondary sRNAs. Conversely, increasing the length of miRNAs that are originally 21 nt to 22 nt long (such as miR319) converts them to siRNA triggers (Chen *et al*, 2010; Cuperus *et al*, 2010). How the difference in size affect the capacity of an sRNA to trigger secondary sRNA production is still unclear. It is possible that size differences affect AGO1

conformation, resulting in the recruitment of RDR6 and SGS3 and consequently production of secondary sRNAs.

Interestingly, *TAS3*-derived tasiRNAs are triggered by miR390, which is 21 nt long. Clearly the 22 nt rule does not apply in this case. *TAS3* differs from the other *TAS* families in being targeted twice by the miRNA (Axtell *et al*, 2006). Montgomery and colleagues (2008) have shown that, at least for the miRNA target site located at the 5' region of *TAS3*, miR390 is necessary for *TAS3*-tasiRNA generation. Most importantly, the authors also described the specific interaction of miR390 with AGO7, and how this interaction is important for tasiRNA production. An interesting speculation is that AGO7 differs from AGO1 (the AGO presented in the RISC associated with the majority of miRNAs, including miR173) in its ability to recruit RDR6 and SGS3. While AGO1 would require association with 22 nt long sRNAs to be able to initiate transitivity, AGO7 would naturally trigger this process, eliminating the need of a 22 nt size for miR390.

Finally, I have shown that miR173-cleavage can initiate transitivity in non-*TAS* loci. Based on the unique activity of this miRNA, I have developed a new method for efficient gene silencing in plants, called MIGS. As other methods, MIGS has pros and cons. Perhaps, the greatest advantage of MIGS is the ease of use. With a single step PCR it is possible to generate MIGS constructs and clone them into a binary vector of choice. Other methods usually rely on more time consuming cloning procedures, multiple step PCR and/or prior *in silico* screens (Ossowski *et al*, 2008; Watson *et al*, 2005). Another beneficial feature of MIGS is its ability to silence multiple genes. With a few additional steps, it is possible to generate MIGS constructions to silence two or more genes, without the necessity of any relationship degree between them. Similar approaches can be used for VIGS; however, application

of this technique tends to be associated with phenotypes resembling virus infection, what can complicate the results interpretation (Watson *et al*, 2005). One of the main concerns when using hpRNAi or VIGS refers to the possibility of off-targeting. If the fragment used for these techniques has any sequence homology to other regions of the genome, silencing of unwanted targets might occur. The same concern applies for MIGS, since it also relies on the use of gene fragments. To reduce the chances of off targeting it is advisable to select regions of the gene with low sequence similarity to the rest of the genome (where known).

Building on my experience with artificial small RNAs directed against *CH42*, I developed a system based on the downregulation of *CH42* to compare the non-autonomous effect of miRNAs and tasiRNAs expressed in phloem companion cells. Interesting, the same sRNA produced by two distinct pathways presented completely different behaviors. This suggested that the pathway through which the sRNA is generated is very important for determining the extent of non-autonomous RNA silencing. Similarly, systemic movement of siRNA-triggered silencing also seems to be dependent on the pathway generating the signal. In tobacco, silencing generated by siRNA produced from inverted repeats was able to move systemically, while amplicon-derived siRNAs were unable to start systemic silencing (Mallory *et al*, 2003). The insensitivity of miRNA and tasiRNA silencing movement to the loss of RDR2 and NRPD1a (which are necessary to siRNA spread) reinforces the role that the pathway has an impact to the spread of silencing.

Which would be the factors responsible for the long-range cell-to-cell movement of tasiRNA-triggered silencing? Studies of the silencing started by siRNAs have shown that in some cases, silencing can spread longer than the usual 10-15 cells, and eventually reach the whole leaf lamina (Himber *et al*, 2003; Palauqui *et al*, 1996;

Voinnet & Baulcombe, 1997). In these cases, which are usually related to foreign sequences (GFP, virus-derived sequences for example) the long-range spreading relies on an amplification mechanism that is dependent on RDR6 and SDE3 (Himber *et al*, 2003). We could show that tasiRNA-triggered silencing does not depend on 5'to 3' primed transitivity, however our data is not sufficient to conclude whether 3' to 5' or priming-independent transitivity is necessary for the amplification of the tasiRNA signal and consequently long range movement of the silencing. Deep sequencing revealed that tasi-SUL cleavage seems to trigger production of some secondary sRNAs. Although the levels of secondary sRNAs are low, we can unfortunately not exclude with confidence that these molecules do not contribute to the observed phenotype.

The pathway that is responsible for production of the sRNA is likely not the sole factor affecting the spreading of the silencing signal. Among the different classes of sRNAs, miRNAs seems to be the ones more affected by these factors, with the silencing triggered by miRNAs ranging from complete cell-autonomy to systemic spreading (Alvarez *et al*, 2006; Nogueira *et al*, 2009; Pant *et al*, 2007; Parizotto *et al*, 2004; Schwab *et al*, 2006; Tretter *et al*, 2008; Válóczi *et al*, 2006). The level of expression is clearly one of these factors. I have found that the poor accumulation of amiR-SUL caused by insufficient processing of the miR164 precursor results in no spreading phenotype. In accordance, the same positive correlation has been detected for siRNA silencing cell-to-cell (Dunoyer *et al*, 2005) and systemic movement (Palauqui & Balzergue, 1999). Cells from different tissues and developmental stages have different exclusion limits of the plasmodesmata (Kim *et al*, 2005). Since the silencing signal is believed to spread through these channels (Voinnet *et al*, 1998), one could expect that the tissue where the miRNA is produce could influence the

silencing movement. In support to this idea, siRNA silencing triggered in epidermal cells cannot spread systemically, while the same construct expressed in the whole leaf can (Ryabov *et al*, 2004).

In summary, I would like to suggest that the spreading of silencing triggered by sRNAs, especially miRNAs, is dependent on many aspects. The circumstances controlling cell-autonomy versus non-cell-autonomy would range from factors related to time, local and intensity of the miRNA expression, which together with different pathways involved in the sRNA production and translocation would lead to a range of mobility, where in some cases miRNAs would either act cell-autonomously, forming expressions gradients or even act as long distance messengers.

Although siRNA movement is known for some time now, the mechanism by which silencing spreads is still unclear. For instance, does it depend on a carrier or does it just diffuse through the plasmodesmata? How is the range of the movement controlled and which molecules are actually mobiles? These and other questions still need to be answered. Genetic screens are an excellent starting point to address such problems. However, screens performed so far have failed to answer most of these questions. A reporter line based on the silencing of *PHYTOENE DESATURASE (PDS)*, silencing of which results in bleaching similar to silencing of *CH42*, has been used for this purpose by two independent groups. Interestingly, both genetic screens produced a similar collection of mutants, with mutations in genes that affect siRNA spreading, namely *RDR2*, *NRPD1a* and *CLASSY1* (Dunoyer *et al*, 2007; Smith *et al*, 2007). CLASSY1 is an SNF2-contaning domain protein, and although its function is not known, it probably function in DNA methylation. RDR2 and NRPD1a are known members of the pathway that generates the 24 nt long siRNAs involved in TGS. Together with CLASSY1, it is thought that these proteins affect the accumulation,

rather than the movement of the siRNA itself, and that the effect on spreading of the silencing is an indirect one. In addition, the fact that two independent mutant hunts resulted in very similar outcomes, suggests that more specific designs for spreading assays are necessary. In this regard, two reporter lines described in this work, SUC2:*atasi-SUL* and the SUC2:*amiR-SUL*, might be good candidates for new genetic screens aimed to find factors directly involved with sRNA trafficking. One of their advantages compared to the siRNA line targeting *PDS* used before is that neither requires RDR2 and NRPD1a for its phenotypic effects; therefore they might escape the 24 nt siRNA pathway, and consequently increase the chances of finding alternative factors. In addition, SUC2:*atasi-SUL* appears to be a very interesting system to study long-range *versus* short-range spreading of gene silencing.

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5 Appendix

5.1 Publications originating from this work

5.1.1 "Evolution of *Arabidopsis thaliana* microRNAs from random sequences"

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Evolution of *Arabidopsis thaliana* microRNAs from random sequences

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ABSTRACT

One mechanism for the origin of new plant microRNAs (miRNAs) is from inverted duplications of transcribed genes. However, even though many young *MIRNA* genes have recently been identified in *Arabidopsis thaliana*, only a subset shows evidence for having evolved by this route. We propose that the hundreds of thousands of partially self-complementary foldback sequences found in a typical plant genome provide an alternative path for miRNA evolution. Our genome-wide analyses of young *MIRNA* genes suggest that some arose from DNA that either has self-complementarity by chance or that represents a highly eroded inverted duplication. These observations are compatible with the idea that, following capture of transcriptional regulatory sequences, random foldbacks can occasionally spawn new miRNAs. Subsequent stabilization through coevolution with initially fortuitous targets may lead to fixation of a small subset of these proto-miRNA genes.

Keywords: *Arabidopsis thaliana*; microRNAs; evolution

INTRODUCTION

Similar to their animal counterparts, plant miRNAs are produced from endogenous transcripts that contain self-complementary foldbacks. These precursors are processed by DICER-LIKE1 (DCL1), generating the mature miRNAs that are incorporated into RISC, a protein complex that uses miRNAs as specificity components to regulate target genes (for reviews, see Jones-Rhoades et al. 2006; Chapman and Carrington 2007).

While the biogenesis and the mechanisms of action of miRNAs are increasingly well understood, less is known about the evolutionary origins of individual *MIRNA* genes. Allen and colleagues (2004) showed that in plants, miRNAs genes could arise from inverted duplication of what will then become a target of the miRNA. More elaborate scenarios for an inverted duplication origin have been described (Rajagopalan et al. 2006; Fahlgren et al. 2007), but common to all of them is that the origin of the new *MIRNA* is dependent on duplication and inversion events.

However, these scenarios do not seem to account for the appearance of all new miRNAs. Recently, ultradeep sequencing of *Arabidopsis thaliana* small RNA (sRNA) populations (Rajagopalan et al. 2006; Fahlgren et al. 2007) showed that several recently evolved miRNAs could not be explained by the inverted duplication hypothesis. Searching for *MIRNA* gene candidates, Jones-Rhoades and Bartel (2004) had previously found 138,864 imperfect inverted repeats in the genome of *A. thaliana*. We speculated that such genomic regions with the potential to generate hairpin-like RNAs could be the source of new miRNAs, as proposed recently also by Axtell (2008). We report that analysis of miRNAs that are unique to *A. thaliana* (i.e., not found in *A. lyrata*, poplar, or rice) suggests that some of these miRNAs arose from sequences that either have self-complementarity by chance or that represent highly degenerate inverted duplications. We propose that miRNAs can evolve spontaneously from foldback sequences after these have come under the control of transcriptional regulatory sequences.

RECENTLY EVOLVED *MIRNA* GENES IN *A. THALIANA*

One of the premises for studying the evolutionary origin of individual miRNAs is the identification of young *MIRNA* genes, i.e., ones that are species specific, and hence more

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likely to have evolved recently. These young *MIRNA* genes are expected to retain some sequence similarity to the region from which they have originated, making it possible to track their evolutionary history. On the other hand, miRNAs deeply conserved across species must have originated a long time ago, and the accumulated mutations will obscure their origin. In *A. thaliana*, several recently evolved *MIRNA* genes have high similarity to their locus of origin, indicating that *MIRNAs* can arise by inverted duplication of such sequences (Allen et al. 2004; Rajagopalan et al. 2006; Fahlgren et al. 2007).

Recently, the results for several exhaustive small RNA sequencing efforts have been reported for *A. thaliana* (Lu et al. 2006; Rajagopalan et al. 2006; Fahlgren et al. 2007). Among the miRNAs newly discovered in these studies, several were not found in the monocot species rice, *Oryza sativa*, or even in the more closely related poplar, *Populus trichocarpa*. These miRNAs include four new miRNA candidates that we had identified before the results of deep sequencing efforts had been published, using a newly developed functional assay (see Supplemental Figs. 1,2; Supplemental Tables 1–4). We used this set of miRNAs with limited conservation in subsequent analyses.

EVOLUTIONARY ORIGIN OF *MIRNA* GENES

According to the inverted duplication hypothesis (Allen et al. 2004), a recently evolved *MIRNA* gene should have long stretches of sequence similarity to the gene that gave origin to it, allowing the identification of the founder gene. The same is true for new *MIRNA* genes that originated by related mechanisms involving duplication (Rajagopalan et al. 2006).

To test the additional hypothesis that random foldbacks could lead to new miRNAs, we selected 29 *A. thaliana* specific miRNAs, which were not detectable in a preliminary assembly of the *A. lyrata* genome using microHARVESTER (Supplemental Table 5; Dezulian et al. 2006). We first divided the *MIRNA* foldbacks into miRNA and miRNA* containing arms and aligned the arms to the set of all annotated cDNAs (from now on called "transcriptome") and the reference genome sequence of *A. thaliana*. Based on these results, two groups of *MIRNA genes* were distinguished (Fig. 1).

The first group contains *MIRNA* foldbacks with at least one arm that has significant similarity to some other genomic region (E VALUE $\leq$ 0.05). This group includes *MIRNA* genes that apparently arose through an inverted duplication (miR163, miR447, miR778, miR824, miR842, miR843, miR856, and miR866) (Fahlgren et al. 2007), and one of our candidates that has not yet been confirmed by other studies, mpss05 (see Supplemental Materials). Among these, the best alignment of miR842 was between the miRNA* arm and At1g52130, a gene encoding a jacalin lectin and belonging to the same family as two validated targets (Supplemental Fig. 2, At5g38550 and At1g60130). These results suggest that the origin of miR842 is likely through duplication from a gene related to its target. Both arms of the mpss05 candidate had high similarity to two separate regions of the *A. thaliana* genome (chromosome 3: 16,815,951–16,816,018, and chromosome 4: 6009,736–6,009,804). In silico folding of the chromosome 3 region indicates a self-complementary structure that is related to the *MIRNA* foldback (Supplemental Fig. 3). Thus, mpss05 could have originated by direct duplication/transposition of a genomic region that contained a foldback structure by chance.

The second group of *MIRNA* genes included those for which no statistically significant alignment with another region of the genome could be found. To evaluate alignments with scores above the significance threshold, we randomly shuffled the sequence of both arms 1000 times and again aligned against the transcriptome and genome. We define *rank* as the number of alignments of permuted sequences that had higher alignment scores than the original sequence. Scores with low rank indicate that the original alignment, while highly degenerate, was statistically

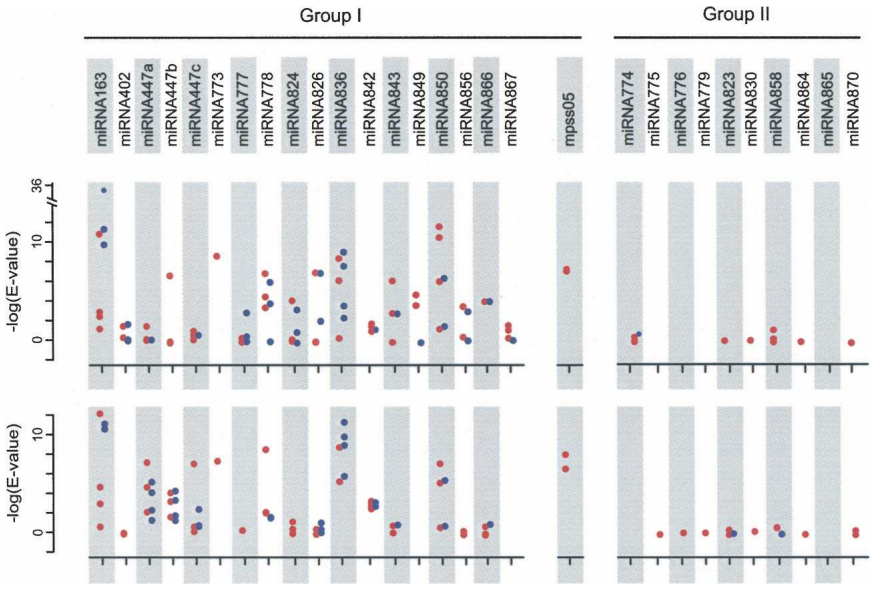

FIGURE 1. Detection of *MIRNA* related sequences in the *A. thaliana* transcriptome (blue) and genome (red). *MIRNA* foldbacks of *A. thaliana* specific miRNAs were divided into miRNA containing arm (*top*), and miRNA* containing arm (*bottom*). Each arm was aligned using FASTA, and the best four hits are reported. Group I contains *MIRNAs* with significant similarity to some other genomic/transcriptomic region (E value $\leq$ 0.05). *MIRNA* genes for which no significant similarity could be found are indicated in Group II.

significant (Table 1). This exercise showed that the similarity between *MIR858* and a genomic region on chromosome 4 (10,406,453–10,406,508), as well as between *MIR774a* and At3g19890, a validated target (Supplemental Fig. 2; Lu et al. 2006), is significant. For the other *MIRNA* genes, any similarity to other regions of the genome is apparently fortuitous.

Finally, for each of the *A. thaliana MIRNA* genes without significant alignment scores, we examined their orthologous regions in the genome of *A. lyrata*, which diverged from *A. thaliana* about 5 million years ago (Koch et al. 2000). First, we identified orthologs for the protein-coding genes flanking each of the new *MIRNA* genes. In seven cases the syntenic relationships of the orthologous genes were conserved in *A. lyrata*, allowing the comparison of the *MIRNA*-containing regions between the protein coding genes with their respective counterparts in *A. lyrata*. In none of the cases was the entire foldback including the miRNA substantially conserved, confirming the microHARVESTER results, which had indicated that no homologs were present in *A. lyrata* (Fig. 2). The exception is miR823, which seems to be conserved in *A. lyrata*. Both, miRNA and foldback can be easily recognized in the homologous region of *A. lyrata*, but the fragment that can be aligned to the foldback contains two insertions. This causes a drastic change of the predicted secondary structure, although this alternative structure could still be subject to DCL1-dependent processing (Fig. 3). In four other cases, there was partial sequence conservation with the possibility of a foldback (Fig. 3), but the miRNA and miRNA* sequences themselves were not conserved. In the remaining three cases, the flanking genes were on different contigs in the *A. lyrata* genome sequence or the *MIRNA* foldback could not be meaningfully aligned to the *A. lyrata* intergenic region.

TABLE 1. Rank values for *MIRNA* arms aligned to the *A. thaliana* genome/transcriptome, with respect to alignments of 1000 permuted sequences

	miRNA arm rank		miRNA* arm rank	
	Genome	Transcriptome	Genome	Transcriptome
miRNA774	356	17†	678	NA
miRNA775	NA	NA	537	NA
miRNA776	NA	NA	380	NA
miRNA779	NA	NA	355	NA
miRNA823	481	NA	211	201
miRNA830	474	NA	372	NA
miRNA858	30†	NA	123	248
miRNA864	474	NA	575	NA
miRNA865	NA	NA	NA	NA
miRNA870	675	NA	286	NA

Rank value 1 refers to the alignment with the highest score. Only the top 5% (indicated by "†") were considered to be significant. NA indicates sequences without sensible alignments.

In addition, we examined in detail the genomes of *Carica papaya* and *P. trichocarpa*, the two closest *Arabidopsis* relatives for which advanced drafts of genome sequences are available (Tuskan et al. 2006; Ming et al. 2008). The synteny-based strategy applied to *A. lyrata* failed, because we could not detect homologs of the *MIRNA* flanking genes in these two species. However, this does not exclude the possibility that *MIRNA* homologous sequences are located in different regions of the genome. For this reason, we also performed a whole-genome search against *P. trichocarpa* and *C. papaya* using Blast and blat (Altschul et al. 1990; Kent 2002). None of the *MIRNAs* had significant conserved counterparts in the other two genomes. These observations corroborate the idea of new miRNAs being spawned by random sequences that have appeared only recently in evolution.

CONCLUSIONS

The only hypotheses that have so far explicitly been advanced for the origin of *A. thaliana* miRNAs rely on the duplication of genic regions that subsequently will become the target of the new miRNA (Allen et al. 2004; Rajagopalan et al. 2006; Fahlgren et al. 2007). In some cases, such a newly evolved miRNA could also target another gene that is unrelated to the founder locus (Fahlgren et al. 2007). Alternatively, as suggested by Rajagopalan and colleagues (2006), a new *MIRNA* gene could arise from the duplication/transposition of a gene that has been the subject of a prior duplication event. Finally, Axtell (2008) has speculated that spurious transcription of random foldbacks could be a first step in the evolution of new miRNAs in plants.

In support of the hypothesis of a random origin of some *A. thaliana MIRNA* genes, we have found that some evolutionarily young *A. thaliana MIRNA* genes have no similarity to other regions of the *A. thaliana* genome, which suggests that they have evolved directly from a sequence that fortuitously contained certain features of *MIRNA* genes, such as the ability to produce an RNA with a hairpin-like structure. Indeed, in silico folding of the *A. thaliana* reference genome has shown that it has the potential to form hundreds of thousands of imperfect foldbacks (Jones-Rhoades and Bartel 2004). It is conceivable that acquisition of promoters could lead to transcription of such foldbacks, which in turn could become substrates for *DCL1* processing. Svoboda and Di Cara (2006) had speculated that animal miRNAs could originate from random sequences, emphasizing that a random match between miRNA and target would be much more likely in animals, because of the much lower sequence complementarity required for animal miRNA targeting. Based on a comparison of three *Drosophila* species, a random origin, accompanied by high birth and death rates, has been proposed for the majority of miRNAs in this genus

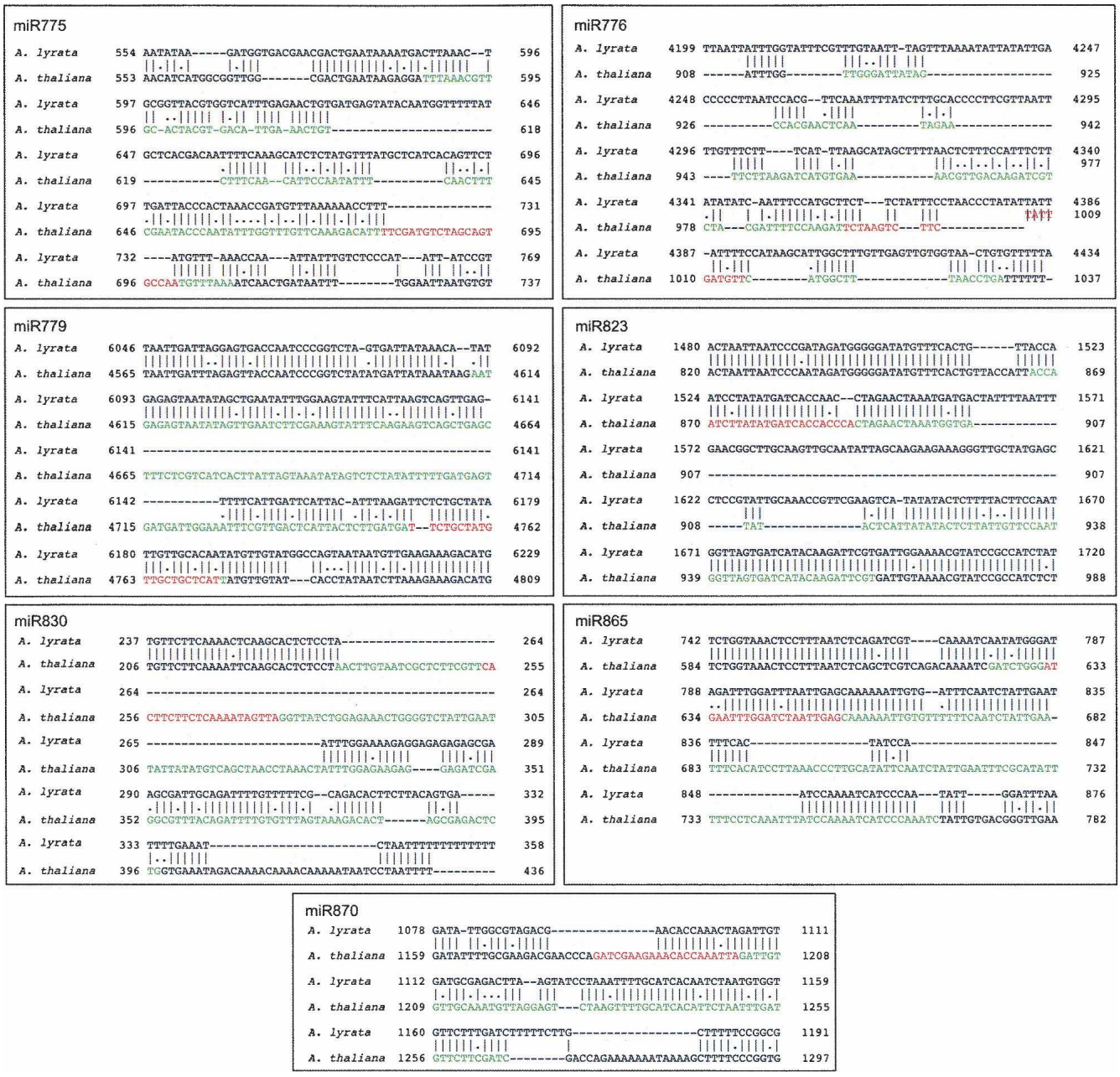

FIGURE 2. Alignments of *MIRNA*-foldback regions and surrounding sequences from *A. thaliana* with their orthologous counterparts in *A. lyrata*. Nucleotides involved in the *MIRNA* foldback are represented in green and the mature miRNA in red. Numbers *next* to the alignments indicate the position within the respective intergenic region.

(Lu et al. 2008). Among the evolutionarily young *MIRNA* genes, none appeared to have formed by inverted duplication, and only a few shared a common origin with other *MIRNA* loci. Therefore, Lu and colleagues (2008) suggested that such *MIRNAs* originated from non-miRNA sequences after accumulation of mutations.

Our analysis of orthologous regions between *A. lyrata* and *A. thaliana* revealed limited sequence conservation for several *A. thaliana MIRNA* genes. Although we cannot exclude that the *MIRNA* genes have degenerated in *A. lyrata*, the fact that these *MIRNA* genes are also not conserved in *C. papaya* and *P. trichocarpa* (nor in the more distantly related *O. sativa*) indicates that they all arose after the split between *A. thaliana* and its nearest relative 5 million years ago. This observation suggests that these regions were not under strong selective pressure and therefore available for mutations that eventually led to the origin of new *MIRNA* genes. If in any of these cases a newly evolved miRNA fortuitously guides cleavage of an mRNA, this interaction could become the subject of either

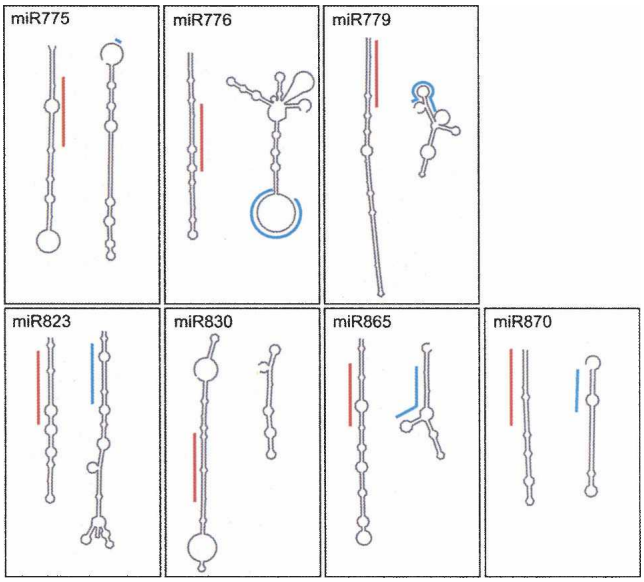

FIGURE 3. Secondary structure of *A. thaliana* miRNA foldbacks (*left*) compared to predicted secondary structure of the orthologous sequences from *A. lyrata* (*right*). The red line indicates the mature *A. thaliana* miRNA sequence, while the blue line refers to the corresponding *A. lyrata* sequence.

negative selection (if the interaction is deleterious for the organism) or positive selection (if the interaction is advantageous). This potential route of miRNA/target coevolution would be similar to what has been suggested for transcription factor binding sites, which are often surprisingly transient, with considerable turnover rates (Dermitzakis and Clark 2002).

SUPPLEMENTAL DATA

Supplemental material can be found at http://www.rnajournal.org.

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Supplementary Material

For

Felippes et al.: Evolution of plant miRNAs from random sequences

Supplementary Results

Prediction of new miRNA candidates

Meyers, Green and colleagues (Lu et al., 2005) were the first to provide a deep account of the small RNA component of the transcriptome, using an adaptation of MPSS technology. They described more than 2 million MPSS tags from three different libraries. Among the non-redundant signatures, tags for known miRNAs and related sequences were the most abundant. Since many high-abundance MPSS signatures were miRNA associated, we speculated that some signatures at the lower end of known miRNA abundance correspond to miRNAs that are not evolutionarily conserved. We therefore developed a set of filters for MPSS tags, as outlined in Supplementary Figure 1. This resulted in 13 candidate sequences, which were consecutively labeled mpss01 to mpss13 (Supplementary Table 1).

While this work was in progress, results from several large-scale small RNA sequencing projects were reported (Lu et al., 2006; Rajagopalan et al., 2006; Fahlgren et al., 2007). We compared our candidates to small RNAs derived from exact sequencing methods in the recently updated Arabidopsis Small RNA Project (ASRP, http://asrp.cgrb.oregonstate.edu/) database, and found small RNAs for most of the mpss candidates (Supplementary Table 1). mpss01 was identified as miR774a (Lu et al., 2006), mpss02, mpss08 and mpss11 as miR842, miR839 and miR822, respectively (Rajagopalan et al., 2006). Finally, in addition to mpss13, a new miRNA (miRNA869.1) was identified as being derived from the putative precursor molecule of mpss13 (Fahlgren et al., 2007).

Characterization of miRNA candidates

Because the abundance of MPSS tags indicated that the corresponding small RNAs are rare, we decided to test directly whether genomic regions that gave rise to the MPSS tags was sufficient to generate small RNAs (as opposed to small RNAs being generated from independently transcribed sense and antisense RNAs). To this end, we transiently overexpressed the predicted precursors for 11 candidates under the control of a constitutive promoter in *Nicotiana benthamiana* leaves (Llave et al., 2002). Five candidates, miR774a (mpss01), miR842 (mpss02), mpss05, mpss07 and miR822 (mpss11), were processed as expected, producing a small RNA in the size range typical for miRNAs (Supplementary Figure 2A). Generation of small RNAs was confirmed with stable *A. thaliana* transformants (Supplementary Figure 3), all of which presented normal development and morphology. Overexpression of many conserved miRNAs induces strong gain-of-function phenotypes, indicating that their targets are central regulators of plant physiology and development (Jones-Rhoades et al., 2006). Overexpression of the new miRNA candidates did not cause any obvious defects. Recently evolved protein-coding genes are underrepresented among genes with genetically defined functions (Domazet-Loso & Tautz, 2003), and it appears that the same applies to miRNA genes.

DCL1 dependency of the miRNA candidates was tested by introducing the transgenes for overexpression into *dcl1*-11 mutants (Supplementary Figure 2B). As shown before for the endogenous locus, production of miR774a (mpss01) was decreased in *dcl1* (Lu et al., 2006). The *dcl1* mutation also greatly reduced abundance of miR842 (mpss02) and mpss05 in the overexpressers. No strong effect was seen for mpss07 or miR822 (mpss11). miR839 (which derives from the same foldback as mpss07) and miR822 have been shown before to be DCL4-, rather than DCL1-dependent, apparently because they derive from a foldback that shows much higher than average self-complementarity (Allen et al., 2004; Xie et al., 2004; Rajagopalan et al., 2006).

To determine whether miR774a (mpss01), miR842 (mpss02) and mpss05 had the expected property of miRNAs, namely ability to cause cleavage of partially complementary mRNAs, we searched the *A. thaliana* genome for potential targets (http://wmd2.weigelworld.org; Supplementary Table 2). Predicted targets included two related F-box protein-encoding genes (miR774a/mpss01) (Lu et al., 2006), a gene encoding an expressed protein of unknown biochemical function (mpss05), and several genes encoding jacalin related proteins (miR842/mpss02) (Rajagopalan et al., 2006). We used a modified 5' RACE protocol for cleavage site mapping of miRNA targets (Llave et al., 2002). We detected RACE products that ended at the expected cleavage site opposite of nucleotides 10 and 11 of the miRNA for miR842/mpss02 targets At5g38550 and At1g60130, but only in plants overexpressing this miRNA, which also had strongly reduced expression of the targets (Supplementary Figure 2C). Similarly, all RACE products of miR774a/mpss01 target At3g19890 terminated at the expected position (Supplementary Figure 2C). The same RACE product could also be found in wild-type plants, confirming a previous report (Lu et al., 2006). Finally, although we could find RACE products for At1g43130, predicted to be targeted by mpss05, none of them terminated at the expected position. Interestingly, several products terminated about 50 bases upstream of the expected region, in a region with some complementarity to the predicted miRNA (Supplementary Figure 2C).

Supplementary Experimental Procedures

Plant material

N. benthamiana, wild type, *dcl1*-11 (Sascha Laubinger, pers. communication) and transgenic *A. thaliana* plants (Col-0 ecotype) were grown in continuous light or long days (16 hrs light) at 23°C.

Identification of miRNA candidates from MPSS data

All 100,452 MPSS sequence tags from the small RNA database (Lu et al., 2005) were initially

used. As a first step, all signatures with abundance less than 15 TPQ were removed, resulting in 7,582 tags. Then each of these tags was mapped onto the genome and the number of possible originating loci was counted. We removed a sequence if we could not map it onto the genome or if we found more than 9 possible originating loci — 4,166 sequences passed this step. Next, we determined for each sequence its similarity to any published miRNA precursor or repetitive sequence using BLAST. We removed all sequences that were similar to a sequence in one of these databases with a cutoff E-value of 0.1; 2,982 sequences passed this test.

For each possible originating locus of each remaining sequence we extracted two preliminary miRNA precursor candidates from the genome: one with the potential mature miRNA (derived from the tag) located in the 5' arm of the precursor and the other with the potential mature miRNA on the 3' arm of the precursor. For this, we extended the putative miRNA matching locus 20 nucleotides to one side of the miRNA candidate and 650 nucleotides on the other side. This procedure resulted in 5,263 miRNA precursor candidate pairs.

In the next step, we used each miRNA precursor candidate together with the 21 nucleotide putative mature miRNA segment as input to the microHARVESTER2 server (Dezulian et al., 2006) using default settings except that we allowed up to 6 mismatches between mature miRNA and miRNA segment and thus increased sensitivity at the price of additional false positives. This procedure essentially imposed the structural constraints observed in published miRNAs onto our candidates. 1,433 precursor candidates passed the test applied using the microHARVESTER. Subsequently, overviews of the putative RNA folding structure were generated for each of these. We manually inspected each document and selected 13 precursor candidates for further analysis, which we labeled consecutively from mpss01 to mpss13. Our primary selection criteria were: strength of expression (TPQ), a preference for a uridine at the first position, as few originating loci in the genome as possible, and the foldback quality of the predicted precursor structure.

Transgenic plants

The genomic regions containing predicted miRNA precursors were amplified by PCR (Supplementary Table 3). PCR products were cloned into pSK+ (Stratagene) or pGemT-easy (Amersham) vectors, and shuttled into pHB (Sang et al., 2005) or pMS37 (R. Schwab, pers. communication), and then into pMLBart (Gleave, 1992). *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* (strain ASE or GV3101) carrying the binary vectors (Llave et al., 2002), and leaves were collected after four days. *A. thaliana* was transformed by floral dip (Weigel & Glazebrook, 2002).

RNA analyses

Total RNA and polyA+ RNA were isolated with Trizol reagent (Invitrogen) and Oligotex kit (Qiagen), respectively. For small RNA blots, total RNA was resolved on a 17% PAGE under denaturing conditions (7 M urea) and hybridized with probes given in Supplementary Table 3 (Llave et al., 2002). Cleavage sites were mapped with the GeneRacer kit (Invitrogen), using specific primers (Supplementary Table 4).

Analysis of sequence similarity

MiRNA arms were aligned against the genome and transcriptome with FASTA using default parameters (version 3.4) (Pearson, 1990). Permuted sequences were obtained using shuffleseq from the EMBOSS package (http://emboss.sourceforge.net).

Analysis of orthologous sequences in the *A. lyrata* genome

Using the coding sequence of genes flanking new *MIRNA* loci for which no homolog had been identified by microHARVESTER, we determined the location of orthologous *A. lyrata* (MN47 accession) genes in the draft assembly of the *A. lyrata* genome using blat (Kent, 2002). Intergenic regions were then aligned using needle from the EMBOSS package with default parameters. The corresponding *A. thaliana MIRNA* region in the *A. lyrata* genome was folded

using the Vienna RNA Secondary Structure Prediction, with default settings (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

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Supplementary Table 1. mpss candidates.

Candidates	miRNA sequence	Location in genome	ASRP[a]	Abundance[b]
mpss01	UUGGUUACCCAUAUGGCCAUC	between At1g60070 and At1g60075	(miR774[c])	106
mpss02	UCAUGGUCAGAUCCGUCAUCC	between At1g61215 and At1g61230	(miR842)	97
mpss03	GGUGAACGACCUGUGUCCCC	between At1g29960 and At1g29965, and between At5g40100 and At5g40110	13209[d]	50
mpss04	UUCACUACCGAACGAUUCU	between At2g14860 and At2g14870	12445d	75
mpss05	UGGCCUUGUCAUCUCAACCGU	intron of At1g44100	62267	67
mpss06	UGGUCGUGAUCUACUGGUUUC	between At1g55010 and At1g55020	211760 d	62
mpss07	UCGGCUCAGGACCAUUGCGGU	between At1g67480 and At1g67490	148334 d	82
mpss08	UACCAACCUUUCAUCGUUCCC	between At1g67480 and At1g67490	(miR839)	168
mpss09	UUGGCUUCUACCGCAAGAGUU	between At3g06433 and At3g06435	87800	154
mpss10	UUGACGGAAUUGUGGCGGGAU	exon of At3g30110	71626	120
mpss11	UGCGGGAAGCAUUUGCACAUG	between At5g03550 and At5g03555	(miR822)	589
mpss12	CUUCAUCGCAAUGGCUAUGGA	between At5g11660 and At5g11670	177532	58
mpss13	UCAACUCCAGGAUUGGACCAG	between At5g39690 and At5g39700		114

[a]http://asrp.cgrb.oregonstate.edu/db/

[b]transcripts per quarter (TPQ) million molecules in MPSS library

[c]mpss sequence completed with additional nucleotides based on the precursor structure

[d]overlap, in the case of mpss04 on opposite strand

Supplementary Table 2. Potential mRNA targets for mpss miRNA candidates.

miRNA candidate	Target IDs	Target description/function
miR774/mpss01	At3G19890.1	F-box family protein
	At3G17490.1	F-box family protein
miR842/mpss02	At3G43610.1	Tubulin family protein
	At1G60130.1	Jacalin lectin family protein
	At1G57570.1	Jacalin lectin family protein
	At3G63400.1	Peptidyl-prolyl cis-trans isomerase cyclophilin-type family
	At5G38550.1	Jacalin lectin family protein
	At1G62750.1	Elongation factor Tu family protein
	At2G37340.2	Splicing factor RSZ33 (RSZ33)
	At1G19570.1	Dehydroascorbate reductase, putative,
mpss03	At4G11890.1	Protein kinase family protein
	At3G59000.1	F-box family protein
	At3G58820.1	F-box family protein
	At5G50250.1	31 kDa ribonucleoprotein, chloroplast, putative
	At4G24770.1	31 kDa ribonucleoprotein, chloroplast, putative
mpss04	NONE	
mpss05	At1G43130.1	Expressed protein
mpss06	At3G15510.1	No apical meristem (NAM) family protein (NAC2)
mpss07	NONE	
miR839/mpss08	At1G63430.1	Leucine-rich repeat transmembrane protein kinase, putative
mpss09	At1G60800.1	Leucine-rich repeat family/protein kinase family protein
mpss10	NONE	
miR822/mpss11	At2G13900.1	DC1 domain-containing protein
	At5G02350.1	DC1 domain-containing protein
	At5G02330.1	DC1 domain-containing protein
	At2G02620.1	DC1 domain-containing protein / PHD finger protein-related
mpss12	At3G51280.1	Male sterility MS5, putative
mpss13	NONE	

Supplementary Table 3. Oligonucleotides used for PCR amplification of candidate precursors and for small RNA blot.

Candidate	Primer A	Primer B	Probe
miR774/mpss01	AAAGCCTCTGTCGGATTCAG	TCCAAGCAATCTACGAGCAA	GATGGCCATATGGGTAACCAA
miR842/mpss02	CGTTCAGGGTGACAGAAACA	AACCATTCAGCTTCCAATCG	GGATGACGGATCTGACCATGA
mpss03	CATCCAGTCATGGGTTAATGA	TTGTAGCAGCATTTTTCACACA	GGGGACACAGGTCGTTCACC
mpss05	GGAACCGATATGGAGAACCA	TTGGGTCAGGAGTGTTGTCA	ACGGTTGAGATGACAAGGCCA
mpss06	TGTTTCCTTGTTGTGCGAGA	AAAGTCGGTTTGGGGTATTT	GAAACCAGTAGATCACGACCA
mpss07	TGCACAGGTTGGGATATTCA	TAAAGGCAAAGCTGGTTGGT	ACCGCAATGGTCCTGAGCCGA
miR839/mpss08	TGCACAGGTTGGGATATTCA	TAAAGGCAAAGCTGGTTGGT	GGGAACGATGAAAGGTTGGTA
mpss09	GGACTATGAATGGGGTTTTCC	GGATTTTGTTTTCGGGGTTT	AACTCTTGCGGTAGAAGCCAA
mpss10	TGACGAAGACGACGAAGAGA	ACTCGCAACAACCCGAACTA	ATCCCGCCACAATTCCGTCAA
miR822/mpss11	TCGGAAGTGACAATCCTTTTT	TTGCTTGATGGGCTGTGA	CATGTGCAAATGCTTCCCGCA
mpss12	AAACACATCCTCGGAAGCAT	AGCGTTATCCCCTTTTACCC	TCCATAGCCATTGCGATGAAG

Supplementary Table 4. Primers used for cleavage site mapping by 5' RACE.

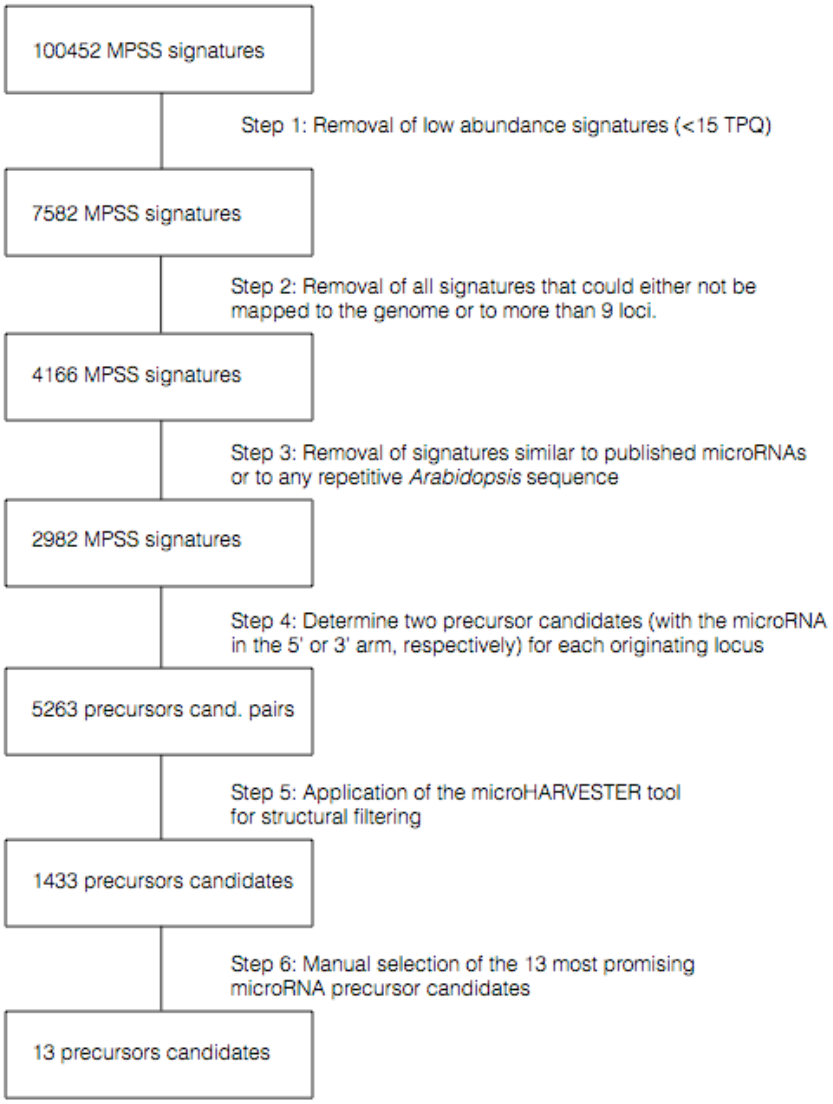
Candidate	Target	Primer	Nested Primer
miR774/mpss01	At3g19890	CGGCGTATCCTTAAATGGAA	AACCACCGCGAGTTTCTTCTCTTCG
miR842/mpss02	At1g60130	GGCATAAACTGCATCCGATT	CCTGCCCACGAGGGACCATAGAATG
	At5g38550	AATCCATCGTCCCACTTCTTGCTTCC	TTTCCTTGGACGGCTAGCGTAAACA
mpss05	At1g43130	TTGGTGAATACGCATTTGGA	TCACGATTGAGCACGACGCGTAAAC

Supplementary Table 5. MiRNA genes that had only been reported for *A. thaliana* and that were examined for conservation in other species.

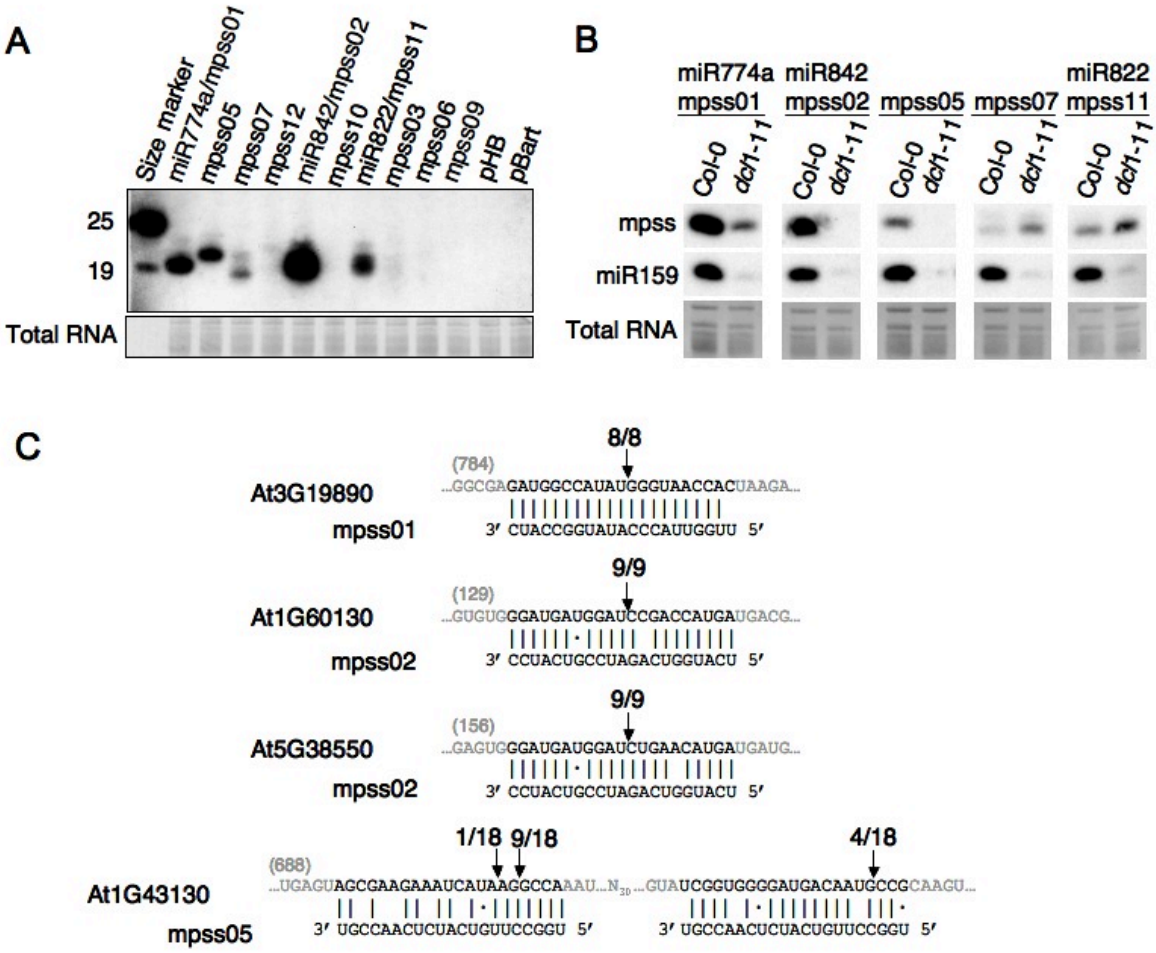
miRNAs
ath-miR163
ath-miR402
ath-miR447a
ath-miR447b
ath-miR447c
ath-miR773
ath-miR774a/mpss01
ath-miR775
ath-miR776
ath-miR777
ath-miR778
ath-miR779
ath-miR823
ath-miR824
ath-miR826
ath-miR830
ath-miR836
ath-miR842/mpss02
ath-miR843
ath-miR849
ath-miR850
ath-miR856
ath-miR858
ath-miR864-3p
ath-miR865-3p
ath-miR866-3p
ath-miR867
ath-miR870
mpss05

Supplementary Table 6. Location of the *A. thaliana* genes flanking the MIRNA genes for which no genomic region could be aligned.

miRNA	miRNA locus	Flanking genes ID	Intergenic region
775	1:29427345..29427467	AT1G78200	1:29426760..29427610
		AT1G78210	
776	1:22799283..22799400	AT1G61730	1:22798370..22801730
		AT1G61740	
779	2:9567841..9568003	AT2G22490	2:9563230..9570300
		AT2G22500	
823	3:4496829..4496925	AT3G13720	3:4495964..4497982
		AT3G13730	
830	1:4820402..4820496	AT1G14070	1:4820150..4822520
		AT1G14080	
864	1:6740491..6740582	AT1G19460	1:6739730..6741290
		AT1G19470	
865	5:5169993..5170134	AT5G15830	5:5169370..5171180
		AT5G15840	
870	5:21412771..21412855	AT5G52790	5:21413170..21411590
		AT5G52800	

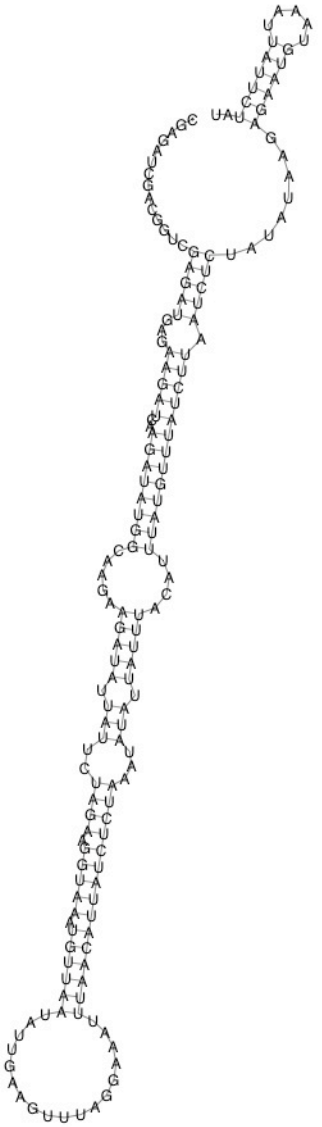


Supplementary Figure 1. Flowchart for prediction of new *A. thaliana* miRNAs.



Supplementary Figure 2. Characterization of miRNA candidates.

(A) Expression of small RNAs after transient transformation of *N. benthamiana* leaves. Empty vectors (pHB and pMLBart) were used as control. Total RNA is shown below is loading control. (B) Small RNA blot analysis of transgenic *DCL1*$^{+}$ (Col-0) and *dcl1*-11 *A. thaliana* plants. miR159 was used as a control. (C) Cleavage site mapping; fraction of clones with corresponding 5' end is indicated above sequence.



Supplementary Figure 3. RNA secondary structure of *A. thaliana* region in chromosome 3 (3:16815951..16816018).

5.1.2 "Triggering tasiRNA formation in *Arabidopsis thaliana*: the role of microRNA miR173"

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Triggering the formation of tasiRNAs in *Arabidopsis thaliana*: the role of microRNA miR173

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Trans-acting small interfering RNAs (tasiRNAs) comprise a class of endogenous small RNAs that are generated from *TAS* gene-derived transcripts after these are cleaved at a microRNA (miRNA) target site. *Arabidopsis thaliana* has four families of *TAS* genes: miR173 triggers tasiRNA production from *TAS1* and *TAS2*, miR390 from *TAS3* and miR828 from *TAS4*. The two-hit trigger model postulates that dual target sites in the same transcript are often sufficient to initiate tasiRNA production, but two hits are not always required for tasiRNA formation. Here, we characterize the function of miR173 in the formation of tasiRNAs from *TAS1* transcripts, as well as the importance of the *TAS1* and *TAS3* transcript sequences outside the miRNA-targeting sites for tasiRNA production. We show that tasiRNAs can be produced from heterologous transcripts containing miR173 or miR390 target sites, indicating that these trigger sequences are the only *cis* sequences essential for tasiRNA formation.

Keywords: microRNA; trans-acting small RNA; tasiRNA; transitivity; *Arabidopsis*

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INTRODUCTION

Trans-acting small interfering RNAs (tasiRNAs) are a specialized class of small RNAs (sRNAs) that originate from *TAS* gene transcripts and, similar to microRNAs (miRNAs), they act in *trans* to regulate messenger RNAs (mRNAs) at the post-transcriptional level (Vazquez *et al*, 2004). The generation of tasiRNAs itself is triggered by an miRNA that targets the *TAS* transcript, resulting in the production of 21 nucleotide sRNAs that are phased with respect to the miRNA cleavage site. This process depends on several proteins, including SUPPRESSOR OF GENE SILENCING 3 (SGS3), RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and DICER-LIKE 4 (DCL4; Peragine *et al*, 2004; Vazquez *et al*, 2004; Allen *et al*, 2005; Gasciolli *et al*, 2005; Xie *et al*, 2005; Yoshikawa *et al*, 2005).

Four families of *TAS* genes have been identified in *Arabidopsis thaliana*. *TAS1* and *TAS2* transcripts are recognized by miR173, which triggers the production of phased tasiRNAs downstream from the cleavage site (Allen *et al*, 2005). A similar pattern is seen for *TAS4*, which is targeted by miR828 (Rajagopalan *et al*, 2006). By contrast, miR390 triggers the production of tasiRNAs from *TAS3* transcripts upstream from the miR390-guided cleavage site (Allen *et al*, 2005).

One of the main questions about tasiRNA generation is why *TAS* transcripts, but not the vast majority of other miRNA-targeted transcripts, form siRNAs. Axtell *et al* (2006) proposed a two-hit trigger model in which tasiRNAs are often spawned when transcripts are targeted at two positions by one or more sRNAs. This model was based on the observation that *TAS3* transcripts in *Physcomitrella patens* and *Pinus taeda* have a second, cleavable miR390 target site, with most tasiRNAs being formed between the two miR390 target motifs. A second, upstream miR390 complementary motif is also present in *A. thaliana TAS3*, but owing to additional mismatches, it is not cleaved. Nonetheless, the production of tasiRNAs from *A. thaliana TAS3* is dependent on the presence of both sites (Axtell *et al*, 2006).

Replacing the downstream, cleavable miR390 target site with another miRNA complementary motif does not affect the generation of tasiRNAs, as long as the new site is recognized and cleaved through the activity of the alternative miRNA. By contrast, the upstream, non-cleavable miR390 target site in *TAS3a* is essential for the production of tasiRNAs. When this site is replaced with another miRNA-targeting motif, tasiRNAs are no longer formed, even if the mismatches in the alternative site are engineered to resemble the original site. Notably, miR390 is unique compared with other miRNAs and is preferentially loaded into ARGONAUTE 7 (AGO7; Montgomery *et al*, 2008).

In the case of *TAS1*, *TAS2* and *TAS4*, which seem to have only single miRNA target motifs, the specific functions of miR173 and miR828 in tasiRNA production are not yet clear. Here, we show that the miR173 target site in *TAS1* transcripts is not only necessary but also sufficient to trigger the formation of tasiRNAs. Similarly, the two miR390 target sites from *TAS3* transcripts are shown to be sufficient for tasiRNA production.

RESULTS

miR173 is necessary for tasiRNA formation from *TAS1*

The formation of *TAS1*- and *TAS2*-derived tasiRNAs is initiated by miR173. To investigate in more detail the function of miR173 in

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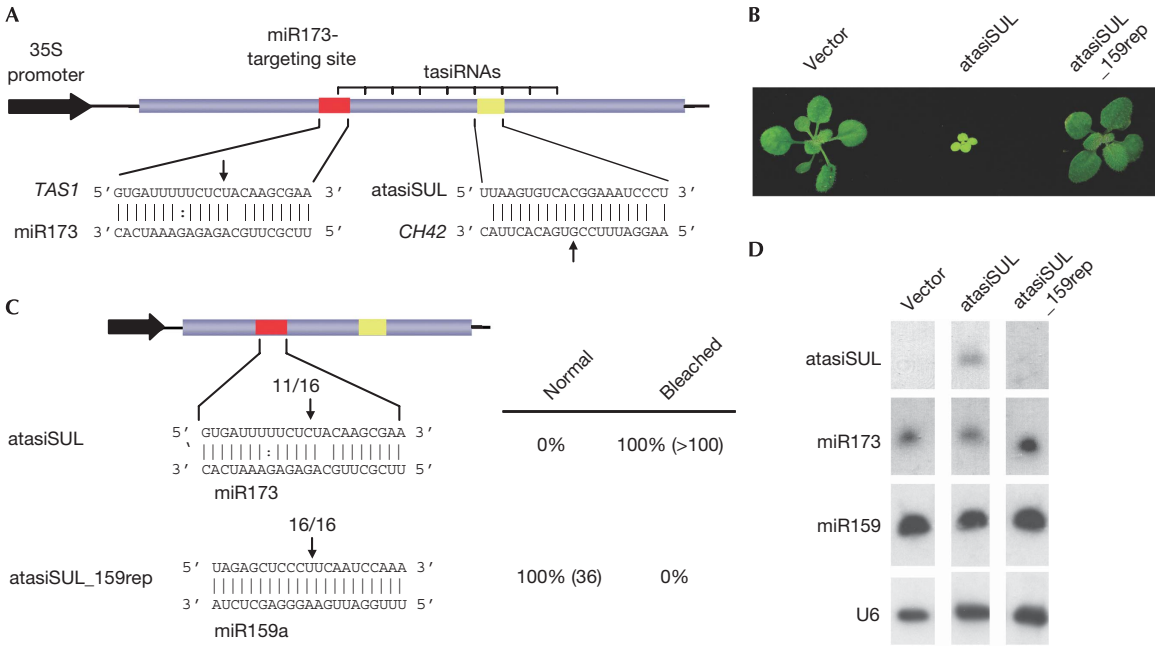

Fig 1 | A target site for miR173, but not miR159, triggers the generation of trans-acting small interfering RNAs. (**A**) Diagram of *TAS1a*-derived TAS1-atasiSUL construct. The tasiRNA-spawning region is indicated by brackets, the miR173 target site is shown in red and the atasiSUL sequence in yellow. Arrows indicate expected miRNA-guided cleavage in the TAS1-atasiSUL transcript or atasiSUL-guided cleavage in its *CH42* target. (**B**) Phenotype of atasiSUL-expressing plants. (**C**) Constructs testing miR173 and miR159 target site functionality. The numbers above arrows refer to the fraction of 5′ RACE products terminating at the canonical miRNA target site; the numbers of analysed plants are given in parentheses in the table. (**D**) Small RNA blot analysis; U6 was used as a loading control. atasiSUL, artificial (synthetic) tasiRNA-SULFUR; CH42, CHLORINA 42; miRNA, microRNA; RACE, rapid amplification of cloned ends; tasiRNA, trans-acting small interfering RNA.

this process, we took advantage of one of the properties of tasiRNAs—that the phase of production is determined by the miRNA-guided cleavage site. The phasing allows the prediction of the sRNAs that will be spawned from a *TAS* gene, which can be exploited to generate artificial (synthetic) tasiRNAs (atasiRNAs/syn-tasiRNAs; Montgomery *et al*, 2008). Rules developed for artificial miRNAs (Schwab *et al*, 2006) were used to design a sRNA, artificial tasiRNA-SULFUR (atasiSUL), that should specifically cause cleavage of the mRNA of *CHLORINA 42* (*CH42*), the *A. thaliana* homologue of tobacco *SULFUR* (Koncz *et al*, 1990; Ossowski *et al*, 2008). *CH42* encodes a magnesium chelatase involved in the biosynthesis of chlorophyll, and its inactivation causes bleaching of green tissue. The siR255 sequence in *TAS1a* was replaced with the atasiSUL sequence (Fig 1A; supplementary Fig 1 online). Plants expressing the TAS1-atasiSUL chimaera under the control of the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter were very pale and much smaller than wild type (Fig 1B).

To test the importance of miR173-guided cleavage for the production of tasiRNAs from the *TAS1a* transcript, we replaced the miR173 complementary motif in TAS1-atasiSUL with an miR159 target. miR159 is among the most abundant miRNAs in *A. thaliana*, it is broadly expressed, it is very effective in causing target cleavage in seedlings (Fig 1D; http://asrp.cgrb.oregonstate.edu/db; Allen *et al*, 2007; Palatnik *et al*, 2007) and has been used for studying the generation of *TAS* previously (Montgomery *et al*, 2008). Although the miR159 target site in the RNA transcribed from this construct, TAS1-atasiSUL_159rep, was cleaved at the expected position (Fig 1C), TAS1-atasiSUL_159rep did not seem to produce any atasiRNA against *CH42* (Fig 1D) and the plants were not bleached (Fig 1B). We conclude that the miR173 target site in *TAS1* transcripts is essential and that the normal function of miR173-containing effector complexes extends beyond transcript cleavage.

miR173 target site sufficient for tasiRNA production

The fact that two miR390 complementary motifs are necessary for tasiRNA production from *TAS3*, and that several other *TAS* transcripts spawn secondary sRNAs only from a portion of the transcripts, led Axtell *et al* (2006) to propose a two-hit trigger mechanism for tasiRNA production. As *TAS1* and *TAS2* lack obvious second target sites for known *A. thaliana* miRNAs, we aligned *TAS1* and *TAS2* family member sequences to identify conserved regions that might participate in the generation of tasiRNAs. In *TAS1* and *TAS2*, the miR173-targeting motif is upstream from the region that gives rise to tasiRNAs. According to the two-hit trigger model, one might therefore expect sequences downstream from the tasiRNA-spawning region to be involved in tasiRNA biogenesis; however, there was little sequence conservation downstream from the tasiRNA-generating region (Fig 2A; supplementary Figs 2,3 online). By contrast, the *TAS1* and *TAS2* loci had considerable sequence similarity in the region upstream from the miR173 target site.

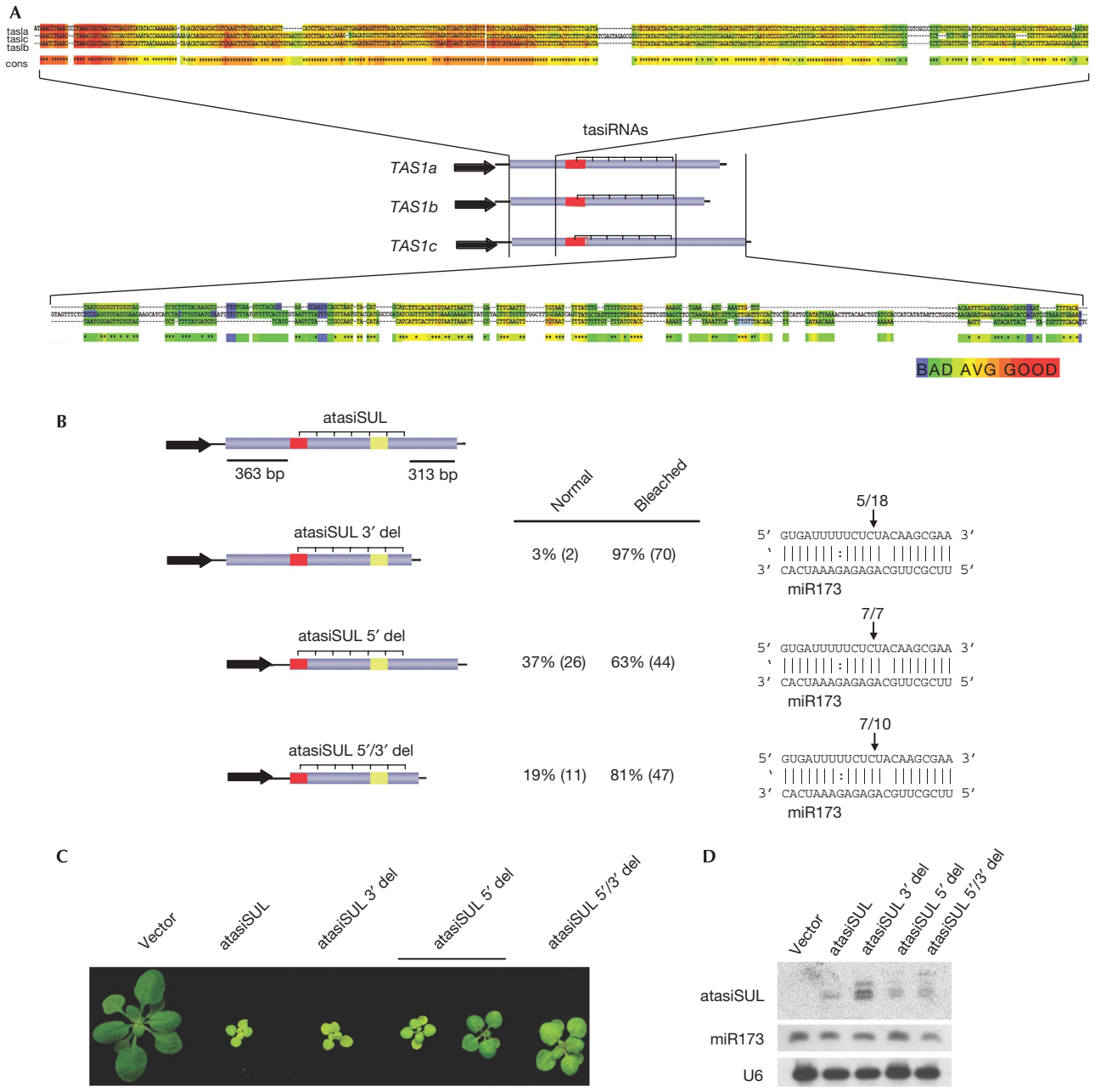

Fig 2 | Formation of artificial (synthetic) trans-acting small interfering RNAs from a *TAS1* derivative. (**A**) Alignment of *TAS1* family transcripts. The alignment of regions flanking the tasiRNA-producing region is shown; colours are based on the alignment score generated by the CORE function of T-Coffee (Llave *et al*, 2002). (**B**) TAS1-atasiSUL constructs with 5′ and 3′ deletions still generate tasiRNAs. The full-length construct is shown at the top (see Fig 1A). Numbers in parentheses indicate the number of plants analysed. (**C**) Plants expressing the various *TAS1* derivatives. (**D**) Small RNA blots of the various *TAS1* derivatives. atasiRNA, artificial (synthetic) trans-acting small interfering RNA.

To test whether this upstream conserved sequence or the sequences downstream from the tasiRNA-producing region are important for the generation of tasiRNAs, we deleted these sequences individually and in combination in the TAS1-atasiSUL construct. For the upstream region, we removed all sequences 5′ to the miR173 target site, whereas the downstream deletion started a few nucleotides after the last tasiRNA with a predicted target (Axtell *et al*, 2006; supplementary Fig 1 online). The deleted downstream region does spawn a few sRNAs without known targets (http://asrp.cgrb.oregonstate.edu/db/). On the basis of the production of sRNAs and the characteristic bleaching phenotype, we concluded that neither of these sequences has an essential role in the biogenesis of tasiRNAs (Fig 2B–D). Taken together, our observations suggest that the only sequence that is essential for the production of tasiRNAs in *TAS1a* is the miR173 target site.

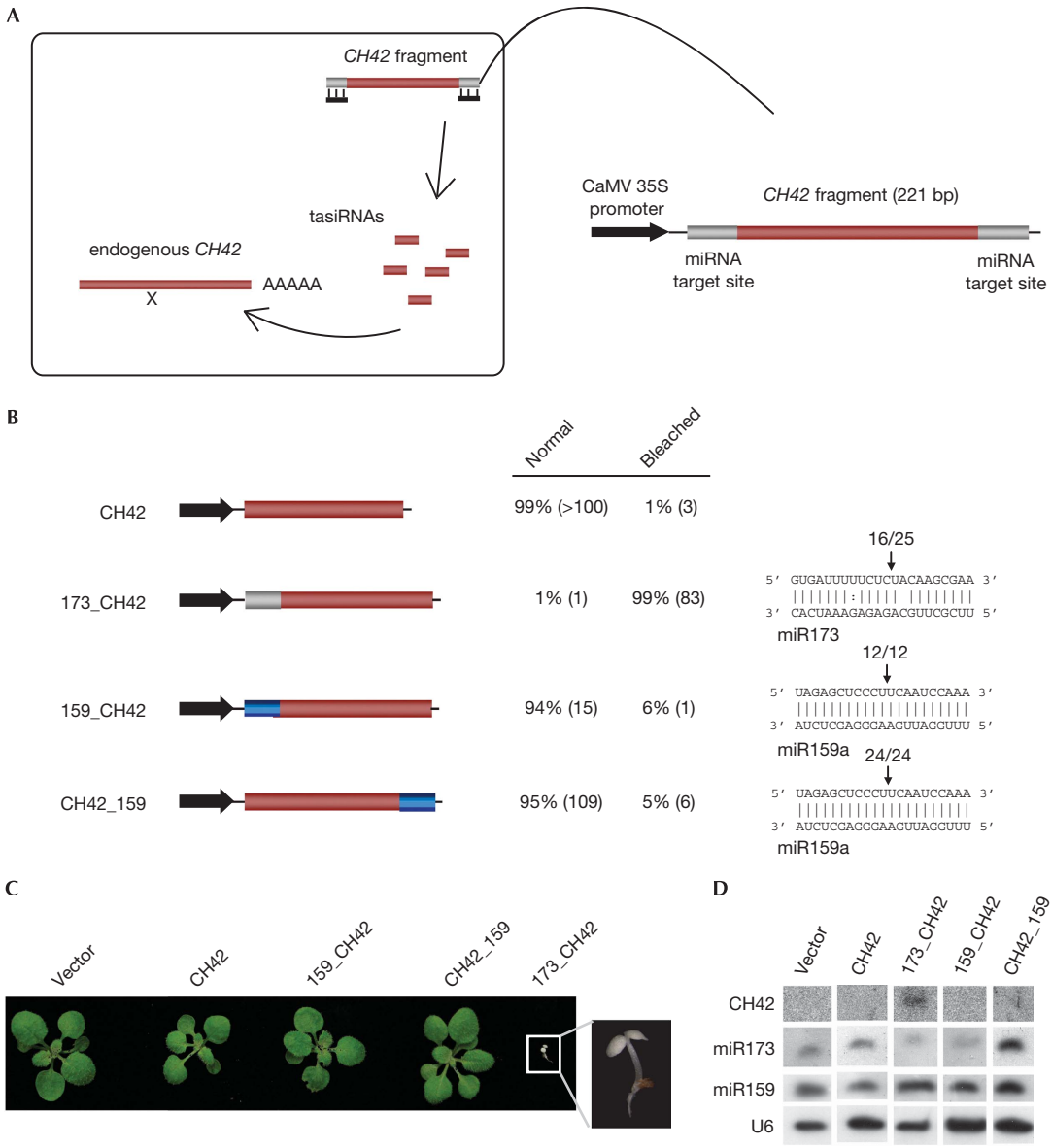

Fig 3 | Sufficiency of the miR173 target site for trans-acting small interfering RNA formation. (**A**) Diagram of *CH42* silencing reporter system. (**B**) Construct diagrams (left), fraction of transgenic plants with bleaching phenotype (middle) and 5′ RACE results (right). *CH42* sequences are indicated in red and miRNA target sites in grey or blue. Numbers in parentheses indicate the number of plants analysed. (**C**) Plants expressing the various *CH42* silencing reporters. (**D**) Small RNA blots; for *CH42*, the fragment present in the silencing reporter was used. CaMV, cauliflower mosaic virus; CH42, CHLORINA 42; miRNA, microRNA; RACE, rapid amplification of cloned ends.

Triggering transitivity in non-*TAS* transcripts with miR173

Although our results so far indicated that *TAS1a* did not contain additional sequences necessary for the production of tasiRNAs, it was still unclear whether miR173 cleavage is the only necessary trigger for tasiRNA biogenesis in *TAS1*. Indeed, it has been suggested that perhaps a second binding element recruits RDR6 in a sRNA-independent manner (Yoshikawa *et al*, 2005). To test the sufficiency of miR173, we developed a *CH42* silencing reporter. In this reporter, a *CH42* fragment is flanked by an miRNA-binding site of choice (Fig 3A). If no secondary sRNAs are formed, expression of such a construct in transgenic plants should be innocuous. The advantage of this reporter is that, apart from ease of scoring the bleaching resulting from the inactivation of *CH42*, perfect phasing is not required for causing a phenotype, resulting in very sensitive detection of secondary sRNA production. In addition, the 221 nucleotide fragment of the *CH42* gene is the same size as the fragment separating the two miR390 target sites in *TAS3*, thus allowing for an appropriate comparison with endogenous *TAS3* (see below).

We introduced an miR173 and an miR159 target site separately upstream from a fragment of *CH42*, and expressed these constructs, 35S:173_CH42 and 35S:159_CH42, in plants. Most of

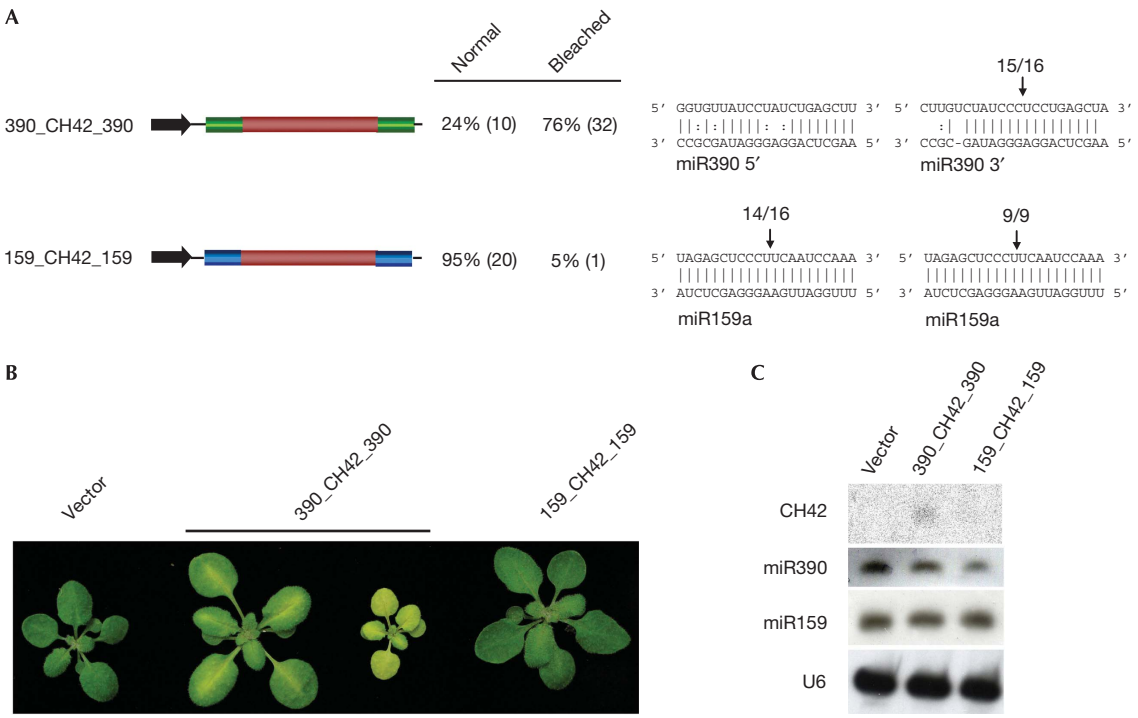

Fig 4 | Dual targeting by miR390, but not miR159, can trigger the formation of small interfering RNA. (**A**) Construct diagrams (left), fraction of transgenic plants with bleaching phenotype (middle) and 5′ RACE results (right). *CH42* sequences are indicated in red and miRNA target sites in green or blue. Cleavage at the downstream miR390 target site could only be mapped using a modified RACE procedure (see Methods). (**B**) Plants expressing the various *CH42* silencing reporters. (**C**) Small RNA analysis of plants expressing *CH42* reporters. CH42, CHLORINA 42; RACE, rapid amplification of cloned ends.

the 35S:173_CH42 plants presented a marked bleaching phenotype, arresting at the seedling stage and finally dying (Fig 3C). By contrast, most of the 35S:159_CH42 plants were normal and did not produce secondary sRNAs (Fig 3B–D). To determine whether miR159 could trigger the production of sRNA upstream from the cleavage site, we also analysed plants expressing a construct in which the miR159 target motif had been placed downstream from the *CH42* fragment (35S:CH42_159). These plants were also phenotypically normal and did not produce detectable secondary sRNAs. We conclude that an miR173 target site is not only necessary but also sufficient for triggering the formation of tasiRNAs.

Triggering transitivity in non-*TAS* transcripts with miR390

Unlike other *TAS* transcripts, *TAS3* transcripts contain two miR390 complementary motifs flanking the tasiRNA-spawning region. Both sites are necessary for the production of tasiRNAs, but the downstream motif can be replaced with other miRNA target sites that result in transcript cleavage (Axtell *et al*, 2006). Nonetheless, it is not clear whether additional sequences in *TAS3* transcripts have a function in triggering the production of tasiRNAs. By using the silencing reporter described above, we generated transgenic plants expressing a *CH42* fragment flanked by the miR390 target motifs found in *TAS3* (35S:390_CH42_390). As a control, the same *CH42* fragment was placed between two genuine miR159 complementary motifs (35S:159_CH42_159). Most of the 35S:159_CH42_159 plants were normal, whereas the 35S:390_CH42_390 plants presented a pale phenotype (Fig 4). Interestingly, bleaching was strongest close to the veins, similar to that already described for atasiRNAs expressed from the *TAS3* backbone and targeting another gene required for the biosynthesis of chlorophyll (Montgomery *et al*, 2008). Our results suggest that the biosynthesis of tasiRNAs from *TAS3* transcripts involves no other specific sequences outside the miR390 target motifs.

DISCUSSION

We have shown that the miR173 target site is sufficient for the production of tasiRNAs at the *TAS1a* locus. miR173 cannot apparently be replaced by an arbitrarily chosen miRNA, and the miR173 effector complex perhaps has unique properties for triggering the formation of tasiRNAs. Our results also indicate that other sequences in the *TAS1* backbone have only a minor function in the biogenesis of tasiRNAs, and that they do not contain any essential feature necessary for the production of tasiRNAs. Similarly, the *TAS3* backbone apparently has only a minor role in triggering the formation of tasiRNAs, based on the fact that miR390 dual targeting is sufficient to initiate the production of secondary sRNAs, a process also known as transitivity. Finally, our data support the idea that transcripts can be routed to tasiRNA production, once certain *TAS* criteria are satisfied.

The two-hit trigger model postulates that a given transcript, once targeted twice by sRNAs, is predisposed for the production of

secondary sRNAs (Montgomery *et al*, 2008). A two-hit trigger has also been invoked to explain transitivity at an overexpressed alien transcript containing a single target motifs that is perfectly complementary to an endogenous miRNA (Axtell *et al*, 2006). However, from genome-wide analyses, it is clear that the presence of two sRNA complementary motifs is not always associated with the production of tasiRNAs (Parizotto *et al*, 2004; Axtell *et al*, 2006). A trivial explanation for this observation could be that the potential triggers are not co-expressed, and that therefore the two-hit trigger situation does not apply. To test explicitly whether two triggers are sufficient for the formation of tasiRNAs, we tested a construct in which two miR159 target sites flanked a fragment of *CH42*. This chimaeric transgene, 35S:159_CH42_159, was much less effective in causing bleaching than the 35S:390_CH42_390 transgene. This is unlikely to be due to insufficient activity of miR159 *per se*, as substituting the downstream miR390 complementary motif in *TAS3* with an miR159 target site does not affect *TAS3* function (Howell *et al*, 2007).

Our results, together with those of Montgomery *et al* (2008), support two conclusions: (1) dual targeting is not sufficient for the formation of tasiRNAs, and (2) only some sRNAs, such as miR173 and miR390, are efficient triggers of transitivity. This raises the question of what makes these miRNAs unique. In the case of *TAS3*, the exclusive interaction of miR390 with AGO7 is the crucial factor that allows the generation of tasiRNAs (Montgomery *et al*, 2008). *A. thaliana* has 10 different AGOs, but their preference for different sRNAs is only known for a subset (Baumberger & Baulcombe, 2005; Qi *et al*, 2005, 2006; Montgomery *et al*, 2008; Takeda *et al*, 2008). What is known is that miR173 does not associate with AGO7, but with AGO1, probably the *Arabidopsis* AGO with the broadest role in sRNA-guided slicing, and to a lesser extent with AGO5, an AGO of unknown function (Mi *et al*, 2008; Montgomery *et al*, 2008). One possibility is that interaction of miR173 with one of the AGOs not yet studied results in an miR390/AGO7-like interaction. However, as *AGO1* is required for the formation of tasiRNAs from the *TAS1* locus, it seems more likely that an miR173/AGO1 complex has a special capacity to recruit another factor required for the production of tasiRNAs. Finally, as we found miR173 to be sufficient to trigger transitivity, our conclusions probably also apply to the miR173-targeted *TAS2* (Baumberger & Baulcombe, 2005).

Another important question is the similarity of the mechanisms of tasiRNA production triggered by miR390/AGO7 and miR173/AGO1. The initial steps are clearly different, as reflected in the different requirements for miRNA targeting. However, both pathways, for *TAS1/2/4* and *TAS3*, converge on the recruitment of SGS3 and RDR6, and subsequent processing by DCL4 (Peragine *et al*, 2004; Allen *et al*, 2005; Gasciolli *et al*, 2005; Xie *et al*, 2005; Yoshikawa *et al*, 2005). That only a subset of AGO1/miRNA complexes, such as AGO1/miR173, can trigger the formation of tasiRNAs might be explained by a change of AGO1 conformation induced by miR173, which would then mimic AGO7/miR390. Structural studies should shed light on these questions.

Finally, we found that constitutive expression of a *CH42* gene fragment linked to an miR173 target site caused a severe *CH42* loss-of-function phenotype. This system could thus present yet another effective approach to gene silencing. It could, for example, be used to create dominant knockouts in non-model systems, by transforming plants with a cassette that expresses miR173 and at the same time contains an miR173 target site next to an outward facing promoter.

METHODS

Generation of transgenic lines. Overlapping PCR was used to replace the siR255 sequence in *TAS1a* (At2g27400) with the atasiSUL sequence, and the miR173 target site in atasiSUL constructs with an miR159 target site. Deletion derivatives were also generated by PCR amplification. The *CH42* (At4g18480) silencing reporters were generated by PCR using oligonucleotide primers that introduced an miRNA target site. For expression in plants behind the CaMV 35S promoter, the pGreen binary vector (Vazquez *et al*, 2004) was used. Transgenes were introduced into accession Col-0 by *Agrobacterium*-mediated transformation (Hellens *et al*, 2000).

RNA analysis. Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). miRNA-guided cleavage site was detected by 5′ RACE (rapid amplification of cloned ends) using modifications of a published protocol (Weigel & Glazebrook, 2002). To detect cleavage at the 3′ miR390 target site in the 390_CH42_390 transcript, a modified RNA adaptor-nested primer was used to amplify specifically products resulting from cleavage at the expected miR390 target motif. For small RNA blots, total RNA was resolved on a 17% PAGE gel under denaturing conditions (7 M urea) and hybridized with DNA probes that had been radioactively labelled, either with ^{32}P-dATP and OptiKinase™ (USB, Cleveland, OH, USA, in the case of oligonucleotide probes) or ^{32}P-dCTP and Prime-a-gene® labelling kit (Promega, Madison, WI, USA, in the case of a DNA fragment probe).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Supplementary Figures for

Triggering tasiRNA formation in *Arabidopsis thaliana*: the role of microRNA miR173

Felipe F. Felippes and Detlef Weigel

5′ATAAACCTAAACCCCTAAGCGGCTAAGCCTGACGTCATATACCAAAAAGAGTAAACATGAGCGC

CGTCAAGCTCTGCAAGTACAATCTCATCTTAACTCAAAAGTTGAGATAGGTTCTTAGATCAGGTTC

CGCCTTTAGATCGAGTCATGGTCTTGTCTGATAGAAAGGTACTTTCTTTTACTTCTCTTGATTAGC

GTCTATAGCTAGATTGAGATCGAGTTTGTGAGATGTTAGGTTCGATATCCCTGTCTATTTGTCACC

AGCCATGTAGGAGTTTCGTCCCTTCCCCTCCCGTCGCCCTCTCTGTTTTTGGTATTCATTGGAATA

CGGAGATATATTTTCAAGAGGAGAAATATTGTTTTGTT**GTGATTTTTCTCTACAAGCGAA**TGAGTC

ATTCATCCTAAGTCCAACATAGCGTTCGATAAGATCTTAGAAAATTATTTTAAGTCTAACATAGCG

TTTGATTGGATCTTAGGAAATTATTAAGTGTCACGGAAATCCCTGAGAAATGGAAGATATCGTGAA

TGATATTTGTAGTAATGGC*GAAACTAGAAAAAGCATTGGA*TATATTCTAGGATATGCAAAAGTTAT

CCTTGAATATGTTCACATTAAATGTTATTTTCTACTTAATGAACAGTTGATGATACAATTATTTTC

TTTAAAATTGTTTCCGTGTAACCAAAACATATTTCAGTATATGCAAAATAAAAAATGGATGTTGGT

ATTCTTATTTTGCAAGGCTTGTAATGGGTGTTGTGTAGTCTCTTTTACAAGGTGTTGTGAAGTCTA

CATGAAGCAAGTCAGCTAATTACATGCATCTTTCACATTGTAATTAATTTGATTTCAATTTTGTAA

TTTTATTTGCTTTTGTGTACCAAAGCTGAAATCAAATTGTTTACAATTTCAATATAAATGATATAA

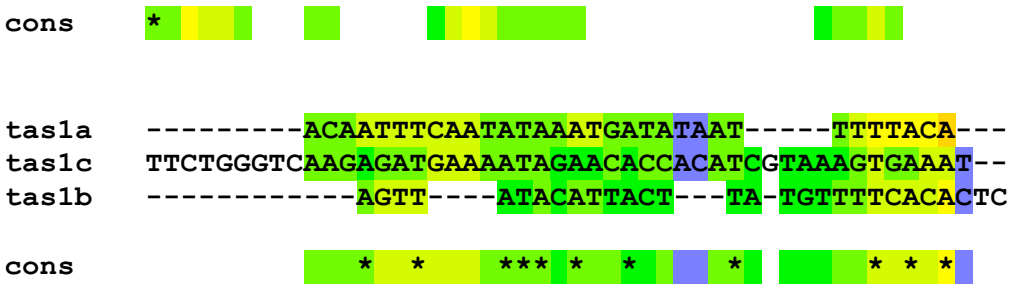
TTTTTACA 3′

Supplementary Figure 1. *TAS1a*-derived artificial tasiSUL (atasiSUL) was engineered by replacing the siR255 for a sequence designed to target specifically *CH42* (green sequence in the box). miR173 target site is represented with a **blue bold** sequence. The sequences underlined refer to the regions deleted for the *TAS1a* studies. The *red and italic* sequence indicates the furthest tasiRNA produce with predicted targets. Arrows refer to the orientation of the tasiRNA.

```
T-COFFEE, Version_5.05(Fri Apr 20 12:53:45 2007)
Cedric Notredame
CPU TIME:17 sec.
SCORE=40
*
 BAD  AVG  GOOD
*
tas1a    :  40
tas1c    :  36
tas1b    :  42

tas1a   ATAAACCTAAACCCCTAAGCGGCTAAGCCTGACGTCATATACCAAAAAGAG-TA
tas1c   --AAACCTAAAC--CTAAACGGCTAAGCCCGACGTCAAATACCAAAAAGAGAAA
tas1b   --AAATCTAAAC--CTAAGCGGCTAAGCCTGACGTCATTTAACAAAAAGAG-TA

cons       *** ******  **** ********** *******  ** *********  *


tas1a   AACATGAGCGCCGTCAAGCTCTGCAAGTACAATCT-------CATCTTAACTCA
tas1c   AACAAGAGCGCCGTCAAGCTCTGCAAATACGATCTGTAAGTCCATCTTAACACA
tas1b   AACATGAGCGCCGTCAAGCTCTGCAACTACGATCTGTAACTCCATCTTAACACA

cons    **** ********************** *** ****     ********* **


tas1a   AAAGTTGAGATAGGTTCTTAGATCAGGTTCCGCCTTTAGATCGAGTCATGGTCT
tas1c   AAAG-TGAGATGGGTTCTTAGATCATGTTCCGCCGTTAGATCGAGTCATGGTCT
tas1b   AAAGTTGAGATAGGTTCTTAGATCAGGTTCCGCTGTTAAATCGAGTCATGGTCT

cons    **** ****** ************* *******  *** ***************


tas1a   TGTCTGATAGAAAGGTACTTTCTTTTACTTCTCTTGATTA------------GC
tas1c   TGTCTCATAGAAAGGTACTTTCGTTTACTTCTTTTGAGTATCGAGTAGAGCGTC
tas1b   TGTCTCATAGAAAGGTACTTTCTTTTACTTCTCTTGAGTA------------GC

cons    ***** ***************** ********* **** **            *


tas1a   GTCTATAGCTAGATTGAGATCGAGTTTGTGAGATGTTAGGTTCGATATCCCTGT
tas1c   GTCTATAGTTAGTTTGAGATTGCGTTTGTCAGAAGTTAGGTTCAATGTCCCGGT
tas1b   TTCTATAGCTAGATTGAGATTGAGGTTTTGAGATATTAGGTTCGATGTCCCGGT

cons     ******* *** ******* * * ** * ***  ******** ** **** **


tas1a   CTATTTGTCACCAGCCATGTAGGAGTTTCGTCCCTTCCCCTCCCGTCGCCCTCT
tas1c   CCAATTTTCACCAGCCATGTGTCAGTTTCGTTCCTTCCCGTCC--------TCT
tas1b   CTATTTGTCACCAGCCATGTGTCAGTTTCGACCAGTCCCGTGC--------TCT
```

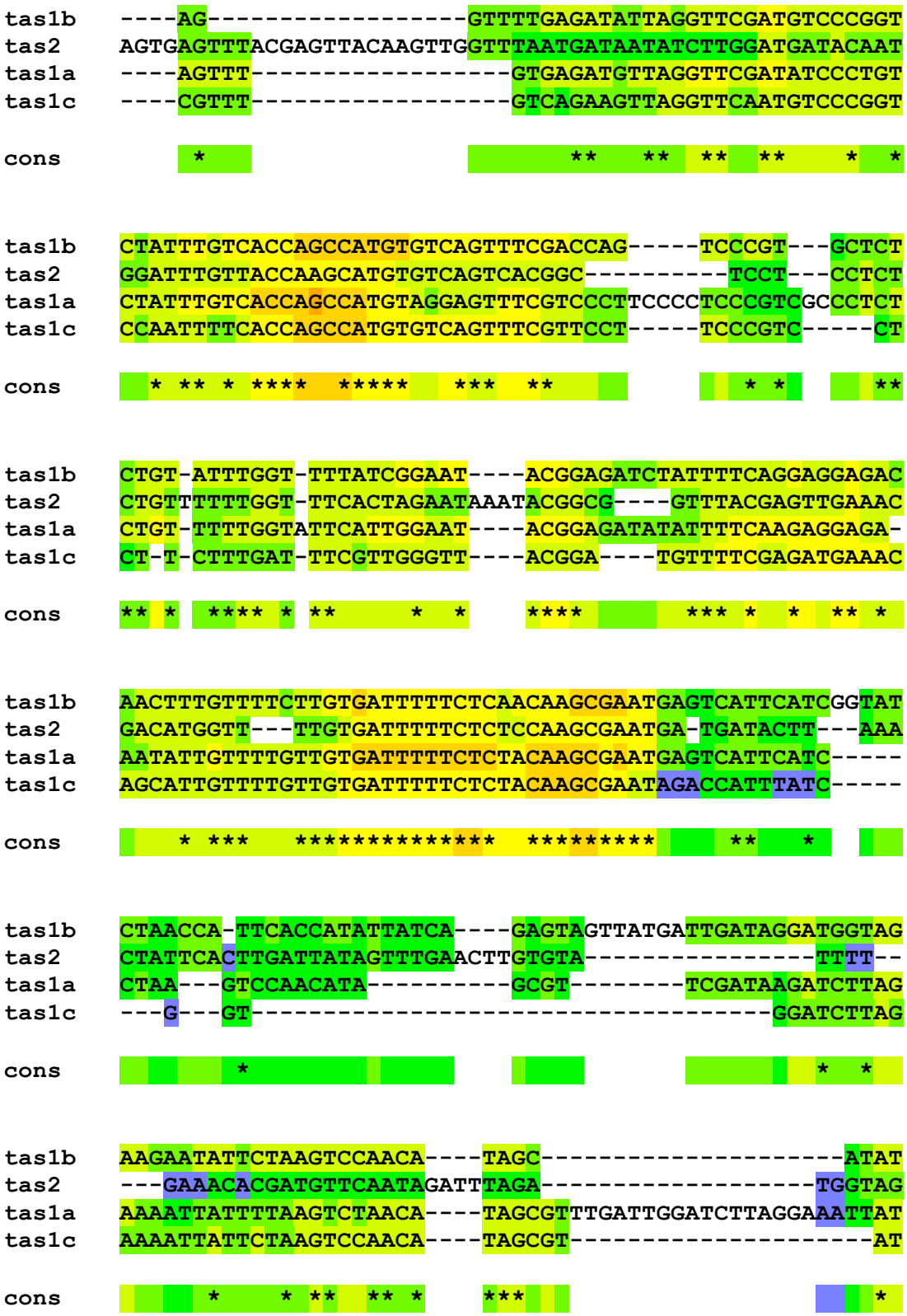
```
cons     *  * **  ************    *******   *   **** * *         ***

tas1a    CTGTTTTTGGTATTCATTGGAATACGGAGATATATTTTCAAGAGGAGA-AATAT
tas1c    ---TCTTTGAT-TTCGTTGGGTTACGGA----TGTTTTCGAGATGAAACAGCAT
tas1b    CTGTATTTGGT-TTTATCGGAATACGGAGATCTATTTTCAGGAGGAGACAACTT

cons     *  * **** *  **  * **  ******  * *****  ** ** * *   *

tas1a    TGTTTTGTTGTGATTTTTCTCTACAAGCGAATGAGTCATTCATCCTAA------
tas1c    TGTTTTGTTGTGATTTTTCTCTACAAGCGAATAGACCATTTATC---G------
tas1b    TGTTTTCTTGTGATTTTTCTCAACAAGCGAATGAGTCATTCATCGGTATCTAAC

cons     ****** ************ **********   **** ***

tas1a    -GTCCAACATA------GCGT--------TCGATAAGATCTTAGAAAATTATTT
tas1c    -GT--------------------------------GGATCTTAGAAAATTATTC
tas1b    CATTCACCATATTATCAGAGTAGTTATGATTGATAGGATGGTAGAAGAATATTC

cons      *          *         *** *****  * ****

tas1a    TAAGTCTAACATAGCGTTTGATTGGATCTTAGGAAATTATTCTAAGTCCAACAT
tas1c    TAAGTCCAACATAGCG--------------------TATTCTAAGTTCAACAT
tas1b    TAAGTCCAACATAGCA--------------------TATTCTAAGTCCAACAT

cons     ****** ********                    ********** ******

tas1a    AGCGTAGAGAAATGGAAGATATCGTGAATGATATTTGTAGTAATGGCGAAACTA
tas1c    ATCGA-------------------CGAA----------------------CTA
tas1b    AGCGTAAAAAATTGGGAGATATCCGGAATGATATT-----------------

cons     * **                   ***                        ***

tas1a    GAAAAAGCATTGGATATATTCTAGGATATGCAAAAGTTATCCTTGAATAT----
tas1c    GAAAAGACATTGGACATATTCCAGGATATGCAAAAGAAAACAATGAATATTGTT
tas1b    ------------------------ATACGTAAAAAAAAA--------------

cons     *** * ****   *

tas1a    --------GTTCACATTAAATGTTATTTTCTACT----TAATGAACAGTTGATG
tas1c    TTGAATGTGTTCA-AGTAAATGAGATTTTCAAGTCGTCTAAAGAACAGTTGCTA
tas1b    TGGGAGATGTCC----GGAATGATATTTG----------------------TA
```

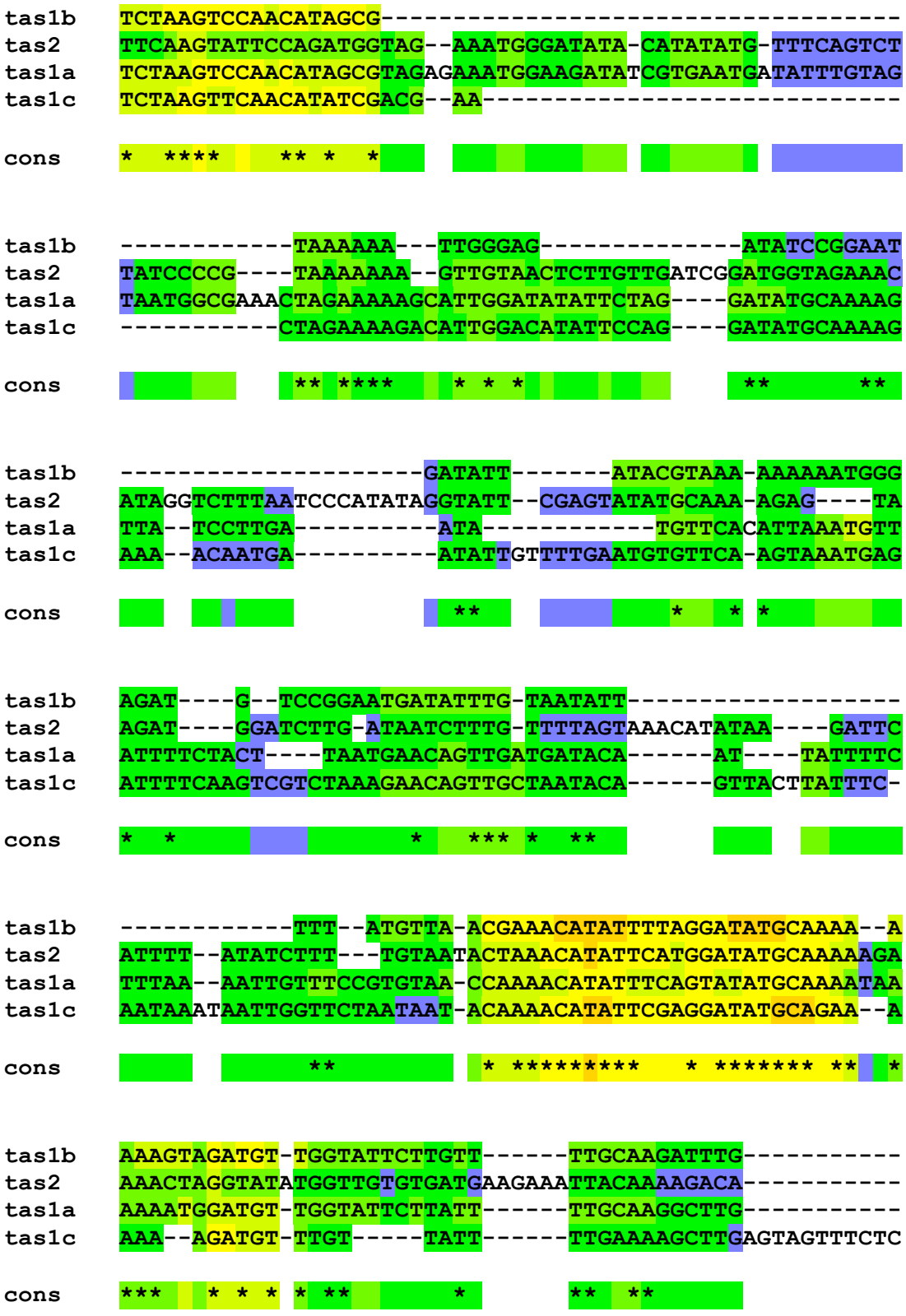
```
cons           ** *      ****  ****                        *

tas1a     ATACAAT----TATTTTCTTTAA--AATTGTTTCCGTGTAACCAAAACATATTT
tas1c     ATACAGTTACTTATTTC-AATAAATAATTGGTTCTAATAATACAAAACATATTC
tas1b     ATATT-------------TTTA--------------TGTTAACGAAACATATTT

cons      ***                 **                    * *********


tas1a     CAGTATATGCAAAATAAAAAATGGATGTTGGTATTCTTATTTTGCAAGGCTTG-
tas1c     GAGGATATGCAGAA--AAAA--AGATGTT-----TGTTATTTTGAAAAGCTTGA
tas1b     TAGGATATGCAAAA--AAAAGTAGATGTTGGTATTCTTGTTTTGCAAGATTTG-

cons       ** ******* **  ****   ******     * ** ***** **   ***


tas1a     ----------TAATGGGTGTTGTGTAG--------TCTCTTTTACAAGGTG---
tas1c     GTAGTTTCTCTCCGAGGTGTAGCGAAGAAGCATCATCTACTTTGTAATGTAATT
tas1b     ----------TAATGGGAGTTGTGTAG--------TCT-TTTTATGATGTG---

cons                *    ** ** * * **        ***  ***   * **


tas1a     TTGTGAA-GTCTACATG----AA--GCAAGTCAGCTAAT-TA-CAT-----GCA
tas1c     TTCTTTATGTTTTCACTTTGTAATTTTATTTGTGTTAATGTACCATGGCCGATA
tas1b     --------------TCATG--AAGTCTA---CCGCCAAT-TA-CATA-----CA

cons                           **    *     *  *** ** ***       *


tas1a     TCTTTCACATTGTAATTAATTT---GA--TTTCAATTT------TGTAAT--TT
tas1c     TCGGTTTATTGAAAGAAAATTTATGTTACTTCTGTTTTGGCTTTGCAATCAGT
tas1b     TCATTCACTTTGTAATTAAATT---GT--CTTCAAGTT------TGTAAT--TT

cons      **  *    *** **  ** **   *    ***   **      ** ***   *


tas1a     TATTTG--CTTTTGTGTACC-------AAAGC--TGAA---ATC--AAATTG-T
tas1c     TATGCTAGTTTTCTTATACCCTTTCGTAAGCTTCCTAAGGAATCGTTCATTGAT
tas1b     TATTTTGT-TTTATGTACC-------AAAAT--C---TAAATTCA--GTTGTT

cons      ***       **  * ****       **            **      *** *


tas1a     TT----------------------------------------------------
tas1c     TTCCACTGCTTCATTGTATATTAAAACTTTACAACTGTATCGACCATCATATAA
tas1b     TACAAC---TT-----GATAACAAA-------------AAAAA-----------
```

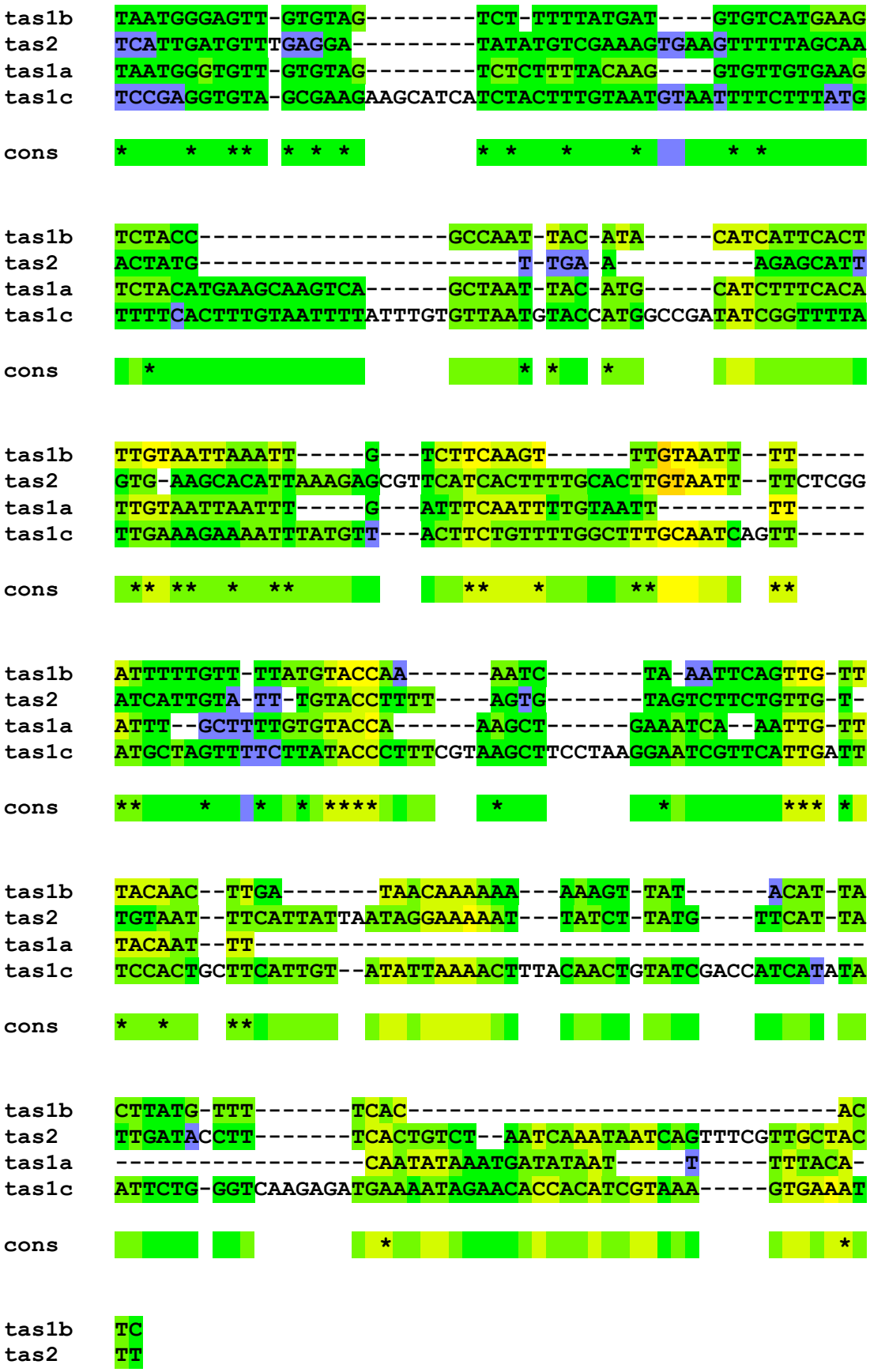


Supplementary Figure 2. Alignment of *TAS1* sequences from *A. thaliana* using T-Coffee. Colors are based on the alignment score generated by the CORE function of T-Coffee (Notredame *et al,* 2000, *J Mol Biol*, **302**, 205-217).

```
T-COFFEE, Version_5.05(Fri Apr 20 12:53:45 2007)
Cedric Notredame
CPU TIME:34 sec.
SCORE=31
*
 BAD  AVG  GOOD
*
tas1b    :  35
tas2     :  24
tas1a    :  33
tas1c    :  30

tas1b    -----AAATCTAAAC--CTAAGCG--GCTAAGC-CTGACGTCATTTAACAAAAA
tas2     AAGAGAAAAATAAGT--ATAAGCGCCGCCAAGCTCTG-----------CAAAGA
tas1a    ---ATAAACCTAAACCCCTAAGCG--GCTAAGC-CTGACGTCATATACCAAAAA
tas1c    -----AAACCTAAAC--CTAAACG--GCTAAGC-CCGACGTCAAATACCAAAAA

cons          *** *** *   *** **  ** **** * *          **** *


tas1b    GAG-TAAACAT--GAGCGCC----GTCAAGCTCTGCAACTACGATCTGTA----
tas2     GA--TCGAGAAAAGAGCCACTTTGGTGAAACACTATAGTTGTGTTGGATTCAGA
tas1a    GAG-TAAACAT--GAGCGCC----GTCAAGCTCTGCAAGTACAATCT-------
tas1c    GAGAAAAACAA--GAGCGCC----GTCAAGCTCTGCAAATACGATCTGTA----

cons     **   * *   ****  *    ** ** * **  *  *    *


tas1b    --ACTCCATCTTAACACAAAAGTTGAGATAGGTT-CTTAGATCAGGTTCCGCTG
tas2     GGACAGAATCTCCTGTCACAC----TGATGGGTTTCGAAGATCAGATTCAGCTG
tas1a    ------CATCTTAACTCAAAAGTTGAGATAGGTT-CTTAGATCAGGTTCCGCCT
tas1c    --AGTCCATCTTAACACAAAAG-TGAGATGGGTT-CTTAGATCATGTTCCGCCG

cons        ****    ** *  *** **** *  ******  *** **


tas1b    TTAAATCGAGTCATGGTCTTGTC--TCATAGAAAGGTACTTT-----CTTTTAC
tas2     TTAGATTGATTCTCCATCTTGTATCCCACTGAAAGGTACTTTTATAGCTAGTCC
tas1a    TTAGATCGAGTCATGGTCTTGTC--TGATAGAAAGGTACTTT-----CTTTTAC
tas1c    TTAGATCGAGTCATGGTCTTGTC--TCATAGAAAGGTACTTT-----CGTTTAC

cons     *** ** ** **    ******   *  ***********     *   * *


tas1b    TTCTCT-TGAGTAG----------CTTC---TATAGC--TAGATTGAGATTG--
tas2     TT-TCTATGAGTAGCCTATCATAGCATCTTCTATAGCTTTAGGTTGGGTTTGGG
tas1a    TTCTCT-TGATTA------------GCGTCTATAGC--TAGATTGAGATCG--
tas1c    TTCTTT-TGAGTA-TCGAGTAGAGCGTCGTCTATAGT--TAGTTTGAGATTG--

cons     ** * * *** **  *   *    *****   *** *** * * *
```


tas1b ----AG------------------GTTTTGAGATATTAGGTTCGATGTCCCGGT
tas2 AGTGAGTTTACGAGTTACAAGTTGGTTTAATGATAATATCTTGGATGATACAAT
tas1a ----AGTTT------------------GTGAGATGTTAGGTTCGATATCCCTGT
tas1c ----CGTTT------------------GTCAGAAGTTAGGTTCAATGTCCCGGT
cons * ** ** ** ** * *
tas1b CTATTTGTCACCAGCCATGTGTCAGTTTCGACCAG-----TCCCGT---GCTCT
tas2 GGATTTGTTACCAAGCATGTGTCAGTCACGGC----------TCCT---CCTCT
tas1a CTATTTGTCACCAGCCATGTAGGAGTTTCGTCCCTTCCCCTCCCGTCGCCCTCT
tas1c CCAATTTTCACCAGCCATGTGTCAGTTTCGTTCCT-----TCCCGTC-----CT
cons * ** * **** ***** *** ** * * ** **
tas1b CTGT-ATTTGGT-TTTATCGGAAT----ACGGAGATCTATTTTCAGGAGGAGAC
tas2 CTGTTTTTTGGT-TTCACTAGAATAAATACGGCG----GTTTACGAGTTGAAAC
tas1a CTGT-TTTTGGTATTCATTGGAAT----ACGGAGATATATTTTCAAGAGGAGA-
tas1c CT-T-CTTTGAT-TTCGTTGGGTT----ACGGA----TGTTTTCGAGATGAAAC
cons ** * **** * ** * * **** *** * * ** *
tas1b AACTTTGTTTTCTTGTGATTTTTCTCAACAAGCGAATGAGTCATTCATCGGTAT
tas2 GACATGGTT---TTGTGATTTTTCTCTCCAAGCGAATGA-TGATACTT---AAA
tas1a AATATTGTTTTGTTGTGATTTTTCTCTACAAGCGAATGAGTCATTCATC-----
tas1c AGCATTGTTTTGTTGTGATTTTTCTCTACAAGCGAATAGACCATTTATC-----
cons * *** ********** ********* ** *
tas1b CTAACCA-TTCACCATATTATCA----GAGTAGTTATGATTGATAGGATGGTAG
tas2 CTATTCACTTGATTATAGTTTGAACTTGTGTA----------TTTT--
tas1a CTAA---GTCCAACATA----------GCGT--------TCGATAAGATCTTAG
tas1c ---G---GT----------GGATCTTAG
cons * * *
tas1b AAGAATATTCTAAGTCCAACA----TAGC----------ATAT
tas2 ---GAAACACGATGTTCAATAGATTTAGA----------TGGTAG
tas1a AAAATTATTTTAAGTCTAACA----TAGCGTTTGATTGGATCTTAGGAAATTAT
tas1c AAAATTATTCTAAGTCCAACA----TAGCGT----------AT
cons * * ** ** * *** *


tas1b TCTAAGTCCAACATAGCG----------
tas2 TTCAAGTATTCCAGATGGTAG--AAATGGGATATA-CATATATG-TTTCAGTCT
tas1a TCTAAGTCCAACATAGCGTAGAGAAATGGAAGATATCGTGAATGATATTTGTAG
tas1c TCTAAGTTCAACATATCGACG--AA----------
cons * **** ** * *
tas1b ----------TAAAAAA---TTGGGAG----------ATATCCGGAAT
tas2 TATCCCCG----TAAAAAAA--GTTGTAACTCTTGTTGATCGGATGGTAGAAAC
tas1a TAATGGCGAAACTAGAAAAAGCATTGGATATATTCTAG----GATATGCAAAAG
tas1c ----------CTAGAAAAGACATTGGACATATTCCAG----GATATGCAAAAG
cons ** **** * * * ** **
tas1b ----------GATATT-------ATACGTAAA-AAAAAATGGG
tas2 ATAGGTCTTTAATCCCATATAGGTATT--CGAGTATATGCAAA-AGAG----TA
tas1a TTA--TCCTTGA----------ATA----------TGTTCACATTAAATGTT
tas1c AAA--ACAATGA----------ATATTGTTTTGAATGTGTTCA-AGTAAATGAG
cons ** * * *
tas1b AGAT----G--TCCGGAATGATATTTG-TAATATT----------
tas2 AGAT----GGATCTTG-ATAATCTTTG-TTTTAGTAAACATATAA----GATTC
tas1a ATTTTCTACT----TAATGAACAGTTGATGATACA------AT----TATTTTC
tas1c ATTTTCAAGTCGTCTAAAGAACAGTTGCTAATACA------GTTACTTATTTC-
cons * * * *** * **
tas1b ----------TTT--ATGTTA-ACGAAACATATTTTAGGATATGCAAAA--A
tas2 ATTTT--ATATCTTT---TGTAATACTAAACATATTCATGGATATGCAAAAAGA
tas1a TTTAA--AATTGTTTCCGTGTAA-CCAAAACATATTTCAGTATATGCAAAATAA
tas1c AATAAATAATTGGTTCTAATAAT-ACAAAACATATTCGAGGATATGCAGAA--A
cons ** * ********* * ******* ** *
tas1b AAAGTAGATGT-TGGTATTCTTGTT------TTGCAAGATTTG----------
tas2 AAACTAGGTATATGGTTGTGTGATGAAGAAATTACAAAAGACA----------
tas1a AAAATGGATGT-TGGTATTCTTATT------TTGCAAGGCTTG----------
tas1c AAA--AGATGT-TTGT-----TATT------TTGAAAAGCTTGAGTAGTTTCTC
cons *** * * * * ** * ** **


tas1b TAATGGGAGTT-GTGTAG--------TCT-TTTTATGAT----GTGTCATGAAG
tas2 TCATTGATGTTTGAGGA---------TATATGTCGAAAGTGAAGTTTTTAGCAA
tas1a TAATGGGTGTT-GTGTAG--------TCTCTTTTACAAG----GTGTTGTGAAG
tas1c TCCGAGGTGTA-GCGAAGAAGCATCATCTACTTTGTAATGTAATTTTCTTTATG
cons
tas1b TCTACC-----------------GCCAAT-TAC-ATA-----CATCATTCACT
tas2 ACTATG----------------------T-TGA-A----------AGAGCATT
tas1a TCTACATGAAGCAAGTCA------GCTAAT-TAC-ATG-----CATCTTTCACA
tas1c TTTTCACTTTGTAATTTTATTTGTGTTAATGTACCATGGCCGATATCGGTTTTA
cons
tas1b TTGTAATTAAAATT-----G---TCTTCAAGT------TTGTAATT--TT-----
tas2 GTG-AAGCACATTAAAGAGCGTTCATCACTTTTGCACTTGTAATT--TTCTCGG
tas1a TTGTAATTAATTT-----G---ATTTCAATTTTGTAATT--------TT-----
tas1c TTGAAAGAAAATTTATGTT---ACTTCTGTTTTGGCTTTGCAATCAGTT-----
cons
tas1b ATTTTTGTT-TTATGTACCAA------AATC-------TA-AATTCAGTTG-TT
tas2 ATCATTGTA-TT-TGTACCTTTT----AGTG-------TAGTCTTCTGTTG-T-
tas1a ATTT--GCTTTTGTGTACCA------AAGCT------GAAATCA--AATTG-TT
tas1c ATGCTAGTTTTCTTATACCCTTTCGTAAGCTTCCTAAGGAATCGTTCATTGATT
cons
tas1b TACAAC--TTGA-------TAACAAAAAA---AAAGT-TAT------ACAT-TA
tas2 TGTAAT--TTCATTATTAATAGGAAAAAT---TATCT-TATG----TTCAT-TA
tas1a TACAAT--TT---
tas1c TCCACTGCTTCATTGT--ATATTAAAACTTTACAACTGTATCGACCATCATATA
cons
tas1b CTTATG-TTT-------TCAC-------------------------------AC
tas2 TTGATACCTT-------TCACTGTCT--AATCAAATAATCAGTTTCGTTGCTAC
tas1a ----------------CAATATAAATGATATAAT-----T-----TTTACA-
tas1c ATTCTG-GGTCAAGAGATGAAAATAGAACACCACATCGTAAA-----GTGAAAT
cons
tas1b TC
tas2 TT

Supplementary Figure 3. Alignment of *TAS1* and *TAS2* sequences from *A. thaliana* using T-Coffee. Colors are based on the alignment score generated by the CORE function of T-Coffee (Notredame *et al,* 2000, *J Mol Biol*, **302**, 205-217).

5.1.3 "Comparative analysis of non-autonomous effects of tasiRNAs and miRNAs in *Arabidopsis thaliana*"

Felipe Fenselau de Felippes, Felix Ott and Detlef Weigel

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doi:10.1093/nar/gkq1240

Comparative analysis of non-autonomous effects of tasiRNAs and miRNAs in *Arabidopsis thaliana*

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ABSTRACT

In plants, small interfering RNAs (siRNAs) can trigger a silencing signal that may spread within a tissue to adjacent cells or even systemically to other organs. Movement of the signal is initially limited to a few cells, but in some cases the signal can be amplified and travel over larger distances. How far silencing initiated by other classes of plant small RNAs (sRNAs) than siRNAs can extend has been less clear. Using a system based on the silencing of the *CH42* gene, we have tracked the mobility of silencing signals initiated in phloem companion cells by artificial microRNAs (miRNA) and *trans*-acting siRNA (tasiRNA) that have the same primary sequence. In this system, both the ta-siRNA and the miRNA act at a distance. Non-autonomous effects of the miRNA can be triggered by several different miRNA precursors deployed as backbones. While the tasiRNA also acts non-autonomously, it has a much greater range than the miRNA or hairpin-derived siRNAs directed against *CH42*, indicating that biogenesis can determine the non-autonomous effects of sRNAs. In agreement with this hypothesis, the silencing signals initiated by different sRNAs differ in their genetic requirements.

INTRODUCTION

Plants produce a variety of small RNAs (sRNAs), including microRNAs (miRNAs), small interfering RNAs (siRNAs) and *trans*-acting siRNAs (tasiRNAs), to regulate many different processes, such as development, stress and nutritional responses, chromatin structure and pathogen defense (1–5). A common theme in sRNA biogenesis is the processing of a double stranded RNA (dsRNA) by DICER-LIKE (DCL) enzymes into 21–24 nt long molecules. The sRNAs are then loaded onto one of several ARGONAUTE (AGO) proteins that drive transcriptional or post-transcriptional gene silencing (3,6–9).

SiRNAs are produced from perfectly-paired dsRNAs with endogenous (transposons, repetitive sequences) or exogenous (virus, transgenes) origins (3,7,8), while miRNAs originate from endogenous transcripts that include an imperfect foldback. Different from the other classes of sRNAs, a miRNA precursor often spawns just one functional sRNA. MiRNAs can trigger cleavage of target transcripts, or interfere with their translation (9). In the case of *TAS* targets, miRNA-initiated cleavage primes the synthesis of dsRNA by RNA DEPENDENT RNA POLYMERASE 6 (RDR6) and SUPPRESSOR OF GENE SILENCING 3 (SGS3), followed by DCL4-dependent processing of the dsRNA into 21 nt long tasiRNAs (10–15).

An important property of plant siRNAs is their non-cell autonomous activity. Even before the association of gene silencing with sRNAs was recognized, it became clear that co-suppression and post-transcriptional gene silencing (PGTS) could spread from one part of the plant to the other (16–18). Systemic silencing is transmitted via the phloem and it is dependent on RDR6 for amplification and reception of the silencing signal in other tissues (19–23). Silencing triggered by siRNAs likely moves from one cell to the other via plasmodesmata, channels that connect the cytoplasm of adjacent cells (16,20,23,24). In a first step, duplexes of 21 nt long siRNAs produced by DCL4 move 10 to 15 cells from their production site (24–26). In some cases, the primary silencing signal can spread further, relying on an RDR6- and SILENCING DEFECTIVE 3 (SDE3)-dependent amplification mechanism that supports the production of secondary siRNAs (24). Although amplification of the silencing signals is preferentially triggered by foreign RNAs, such as those derived from transgenes or from viruses (22,24), there are endogenous hairpin loci that behave very similarly (27). Furthermore, additional factors required for cell-to-cell movement of siRNA-triggered silencing include RDR2, the NRPD1a subunit of RNA polymerase IVa and CLASSY1, a SNF2 domain-containing protein (25,28,29). Grafting and deep sequencing of small RNA pools have revealed that endogenous 24 nt siRNAs can travel long distances in the plant (27,30).

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While the mobility of siRNAs and its consequences are well documented, less is known about the mechanisms underlying non-autonomous effects of other classes of sRNAs, such as miRNAs and tasiRNAs. Several experiments with miRNA sensors and tissue-specific expression of natural or artificial miRNAs have indicated that the non-autonomous effects of most miRNAs do not extend very far (31–36). There are, however, several notable exceptions. MiR399 acts as a long distance signal in phosphate homeostasis (37), while miR390 accumulates in different tissues than its precursor (38). In addition, miRNAs have been detected in the phloem sap of several species (39,40). Since the phloem cells are enucleate and cannot produce RNAs, such miRNAs would need to be delivered from other cells such as phloem companion cells. Similarly, several strong lines of evidence indicate that miR165 and miR166 can move radially within the root, and thereby contribute to the patterning of root tissues (36). Finally, the precursor of tasiRNAs that regulate *AUXIN RESPONSE FACTOR3* (*ARF3*) is transcribed in a narrow domain at the adaxial side of the leaf, but the mature tasiRNAs accumulate in a gradient that extends through much of the leaf (41,42).

While mobility of a variety of small RNAs is now accepted, their non-autonomous effects appear to differ. For example, movement of miRNAs appears to be much more limited than that of siRNAs (31–36). Because the investigated sRNAs differed in sequence in previous work, it has been difficult to disentangle the effects of sRNA sequence from the consequences of different sRNA histories due to divergent biogenesis mechanisms. We have compared sRNAs of identical sequence, but generated by either the miRNA or tasiRNA pathway. We show that similar to siRNAs, the silencing effects of miRNA can spread 10 to 15 cells from phloem companion cells to mesophyll cells, while a tasiRNA of the same sequence has much more far-reaching non-autonomous effects. Importantly, the genetic requirements for the mobile silencing signals triggered by miRNAs, tasiRNAs and siRNAs differ.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana Columbia (Col-0) is referred to as wild type. The *dcl2 dcl3 dcl4* ('*dcl234*'), *dcl1-100*, *rdr6*-15, *rdr2*-1 and *nrpd1a*-3 mutants and the *SUC2:3xYFP* transgenic lines have been previously described (43–47). Mutants expressing atasiR-SUL, amiR-SUL and siR-SUL were selected from F_2 plants by PCR-based genotyping for the transgene and the mutations. F_1 hybrids containing both *SUC2:amiR-SUL* and *SUC2:3xYFP* were isolated by double antibiotic selection.

Transgenic lines

The sRNA targeting the *SUL* homolog *CH42* (At4g18480), UUAAGUGUCACGGAAAUCCCU, was designed with the WMD tool (33,48). Overlapping PCR was used to replace the mature miRNA and miRNA* in the MIR319a (AT4G23713), MIR156c (AT4G31877), MIR164b (AT2G47585) and MIR167a (AT3G22886) backbones. The same approach was used to generate the atasiR-SUL constructs, by replacing siR255 in the three members of the *TAS1* family, *TAS1a* (AT2G27400), *TAS1b* (AT1G50055) and *TAS1c* (AT2G39675), respectively (48). For the siR-SUL construct, we cloned the same *CH42* fragment (TAIR9 coordinates chromosome 4, 10, 202, 162-10, 202, 350) in both sense and antisense orientation into the pHANNIBAL vector (49). All constructs were shuttled into a modified version of the pGreen vector (50) containing the *CaMV35S* and *SUC2* promoters (51–53). Binary constructs (Supplementary Table S3) were introduced into *Agrobacterium tumefaciens* strain ASE (54), which was used for floral dip transformation (55) (see Supplementary Data for additional details).

RNA analysis

Total RNA was isolated from two-week old plants using TRIZOL (Invitrogen, Carlsbad, CA, USA). sRNA blots were prepared by resolving 10–20 µg of total RNA on a 17% PAGE gel under denaturing conditions (7 M urea) and subsequent transfer to a positively charged nylon membrane. Membranes were hybridized with DNA oligonucleotide probes that had been radioactively labeled with γ-^{32}P-ATP and OptiKinase^{TM} (USB, Cleveland, OH, USA). For detection of sRNAs derived from the siR-SUL construct, we employed a DNA probe consisting of the *CH42* fragment in the RNAi triggering vector, which was labeled with α-^{32}P-dCTP using the Prime-a-genes kit (Promega, Madison, WI, USA). cDNA for RT–PCR was synthesized with the RevertAid^{TM} First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada). See Supplementary Table S4 for probes.

Small RNA sequencing

Small RNA libraries were constructed following a protocol described elsewhere (56) with modifications (Supplementary Data) and sequenced on the Illumina GAII platform (San Diego, CA, USA). Two independent libraries (biological replicates) were analyzed for the amiR-SUL and atasiR-SUL lines. The sRNA sequence tags were filtered and mapped back to the *A. thaliana* reference genome using SHORE (57), yielding 5.7–6.5 million aligned sRNA tags. We then calculated coverage graphs allowing or disallowing up to two mismatches to the *CH42* locus. The effect of excluding repetitive matches was investigated, but found to be negligible (data not shown). We tested the significance of the secondary sRNA population observed in the *SUC2:atasi-SUL* line as follows. First, we defined a 500-bp region for the *CH42* locus where secondary sRNAs were highly increased (Chr4:10201701.10202200, excluding the amiR/atasi-SUL region). We then determined the total number of reads for this region in both samples, which were 99 in *SUC2:atasiR-SUL* and 22 in *SUC2:amiR-SUL*. Next, starting from this region, we divided the genome in both directions, in 500-bp bins, counted the total sRNA reads in the two lines and calculated the fold change for each bin with more than 60 reads across both lines (50% of that in

the *CH42* bin). To avoid division by zero, we added a pseudo count of one to each bin.

Microscopy

YFP expression and natural florescence of chlorophyll were analyzed with a Leica MZ FLIII microscope (Leica Microsystems, Wetzlar, Germany) fitted with wide- and band-pass YFP filters and an AxioCam HRc (Zeiss, Jena, Germany) digital camera with Zeiss AxioVision software version 3.1.

RESULTS

Non-autonomous effects of miRNAs

To investigate movement of a silencing signal, we employed sRNAs targeting *CHLORINA42* (*CH42*), the *A. thaliana* homolog of tobacco *SULPHUR* (*SUL*). Inactivation of *CH42* causes bleaching of green plant tissues (58), resulting in an easily-scorable phenotype. We targeted *CH42* with an artificial miRNA, amiR-SUL (33). We compared the effects of amiR-SUL with those of siRNAs spawned from a transcribed inverted repeat of *CH42* sequences (siR-SUL) (49). Both constructs were introduced into *A. thaliana* plants under the control of the *SUCROSE-PROTON SYMPORTER 2* (*SUC2*) promoter, which confers strong expression in phloem companion cells (51,52).

Himber and colleagues (24) have shown that the effects of siRNAs can extend 10 to 15 cells from their production site. Consistent with this report, there was prominent bleaching of green mesophyll cells along the leaf veins in *SUC2:siR-SUL* plants (Figure 1A). A very similar phenotype was seen in *SUC2:amiR-SUL* plants, suggesting that silencing initiated by miRNAs spreads over a range comparable of that of siRNAs. Closer analysis of chlorophyll autofluorescence in *SUC2:amiR-SUL* plants confirmed that the bleached regions extended beyond the veins (Figure 1B). To determine directly how far the silencing had spread beyond the cells in which the *SUC2* promoter is active, we crossed *SUC2:amiR-SUL* to a plant expressing three tandem copies of yellow florescent protein in the *SUC2* domain (*SUC2:3xYFP*) (43). The large size of 3xYFP traps it inside cells, allowing precise localization of *SUC2* promoter activity. The bleached area around the veins in *SUC2:amiR-SUL* was indeed much larger than the *SUC2* expression domain (Figure 1C). Most plants carrying the *SUC2:amiR-SUL* construct presented different degrees of bleaching around the vascular tissue (Figure 1D, Supplementary Figure S1). The levels of amiR-SUL were positively correlated with the severity of bleaching (Figure 1E), but the extent of the bleached area around the vasculature was similar in all lines.

While miRNAs are produced mainly through the action of DCL1, several factors, such as secondary structure of the pre-miRNA and the tissue where the miRNA is expressed, can lead to miRNA precursors being processed by different DCLs, resulting in the production of siRNAs instead (59–61). Therefore, the non-autonomous effects in *SUC2:amiR-SUL* plants might be not caused by true miRNA-mediated silencing, but through

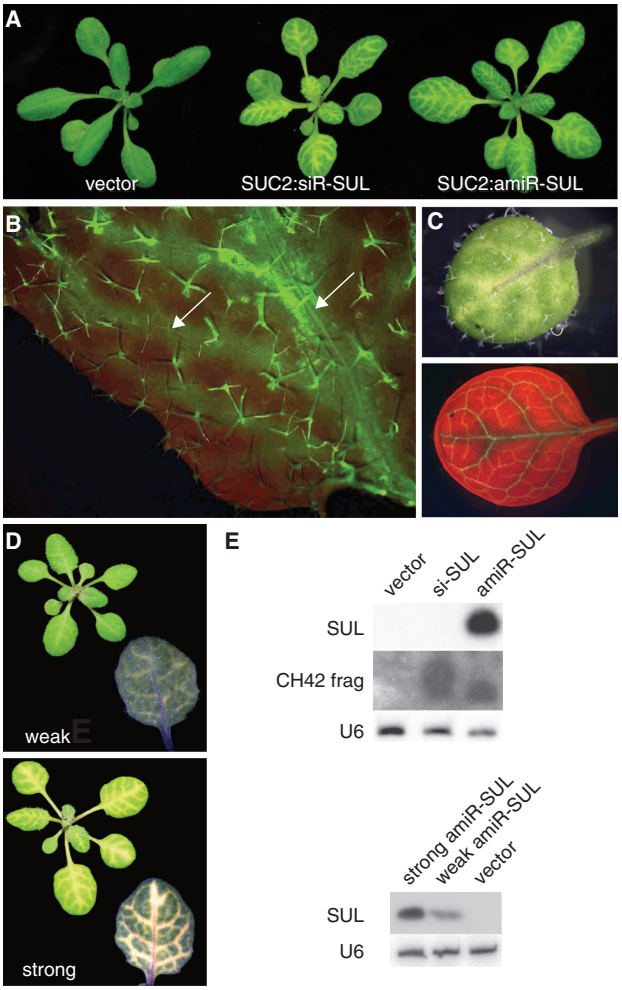

Figure 1. Spreading of miRNA-triggered silencing from phloem companion cells. (**A**) *SUC2:amiR-SUL* and *SUC2:siR-SUL* plants present similar bleaching patterns. (**B**) UV-induced red chlorophyll autofluorescence is suppressed in bleached areas, which appear light green in a *SUC2:amiR-SUL* leaf. Arrows point to leaf veins. (**C**) *SUC2:amiR-SUL SUC2:3xYFP* leaf. Top, visible light; bottom, UV fluorescence. Bright green YFP signal is more restricted than the bleached areas that are dark. (**D**) Comparison of mild and severely bleached plants. A single leaf is shown in detail. (**E**) sRNA blots probed with an oligonucleotide specific for amiR-SUL (SUL) or a *CH42* fragment (CH42 frag). U6 was used as loading control.

siRNAs. To examine this possibility, we transformed *dcl234* triple mutants with the *SUC2:amiR-SUL* construct. Inactivation of *DCL2*, *DCL3* and *DCL4* did not affect the bleaching phenotype (Figure 2A). As a control, we introduced the *SUC2:amiR-SUL* construct into *dcl1* plants by crossing. In these plants, no bleaching occurred (Supplementary Figure S2).

In some cases, miRNA-triggered cleavage of targets can initiate transitive action of the miRNA, in which the cleaved target transcript is converted to dsRNA by RDR6 and subsequently processed into secondary siRNAs by DCLs (31,62). To test whether the cell-autonomous effect of amiR-SUL was due to transitivity, we crossed *SUC2:amiR-SUL* to *rdr6*-15 mutants, which do not generate secondary siRNAs. The *SUC2:amiR-*

SUL-induced bleaching phenotype was unaffected by the *rdr6* mutation (Figure 2B). The presence of the *rdr6* mutation was confirmed both by the gross phenotype, and by the absence of tasiR255 production (Figure 2B and C). Taken together, these results suggest that the mobile silencing triggered by the *SUC2:amiR-SUL* is due to bona fide miRNA action.

A potential concern when using transgenes to characterize an endogenous mechanism is that expression levels much higher than those seen for endogenous miRNAs contribute to the observed effects. While commonly employed for assays of non-autonomous action of proteins or sRNAs (25,27,28,35,43,63), the *SUC2* promoter is known to be strong (52,64,65). Abnormally high expression of a miRNA under control of the *SUC2* promoter might saturate the processing machinery. This could in turn result in miRNA processing through pathways that are not DCL1 dependent. We therefore compared the expression levels of amiR-SUL to endogenous miRNAs by deep sequencing of the sRNA population. As reported in Supplementary Table S1, many miRNAs were expressed more strongly than amiR-SUL, with steady-state levels of some being more than two orders of magnitude higher. We conclude that our system reflects the natural action of the sRNA machinery.

Non-autonomous miRNA effects are not precursor specific

In phloem sap of the *A. thaliana* relative *Brassica napus*, a distinct subset of plant miRNAs has been identified (39,40), raising the possibility that only certain miRNA precursors can produce miRNAs that leave the cell of origin. To test the effects of the precursor, if any, on non-autonomous effects of the mature miRNA, we produced amiR-SUL from different miRNA precursors. We engineered the MIR156c, MIR164b and MIR167a precursors to produce the same mature miRNA sequence as our original amiR-SUL construct, which was in the MIR319a backbone; the corresponding constructs were named amiR-SUL_156, amiR-SUL_164 and amiR-SUL_167 (Supplementary Figure S3). We chose miR156, because it represents one of the families found in phloem sap (39,40). We chose MIR164 and MIR167, because it has been suggested that amiRNAs produced from these backbones in phloem companion cells do not have non-autonomous effects (35).

To determine the efficiency of amiRNA production from the different precursors, we first expressed these from the CaMV 35S promoter (53). Like plants that expressed amiR-SUL ubiquitously from the MIR319a precursor, *35S:amiR-SUL_156* and *35S:amiR-SUL_167* plants were very small and strongly bleached, like the original *35S:SUC2:amiR-SUL* lines (Figure 3A). *35S:amiR-SUL_164* plants were larger, with variable bleaching, flowered normally and were fertile (Figure 3A). RNA blots indicated that amiR-SUL was only very inefficiently processed from the MIR164 precursor, even though it was expressed at a similar level as the other precursors (Figure 3B and C).

Similarly, *SUC2:amiR-SUL_156* and *SUC2:amiR-SUL_167* plants were strongly bleached, like the original *SUC2:amiR-SUL* lines (Figure 3A), but *SUC2:amiR-SUL_164* plants were largely normal. While our results suggest that there are no specific miRNA precursor requirements for non-autonomous miRNA effects, the absence of extensive bleaching in *SUC2:amiR-SUL_164* plants, apparently due to inefficient miRNA processing,

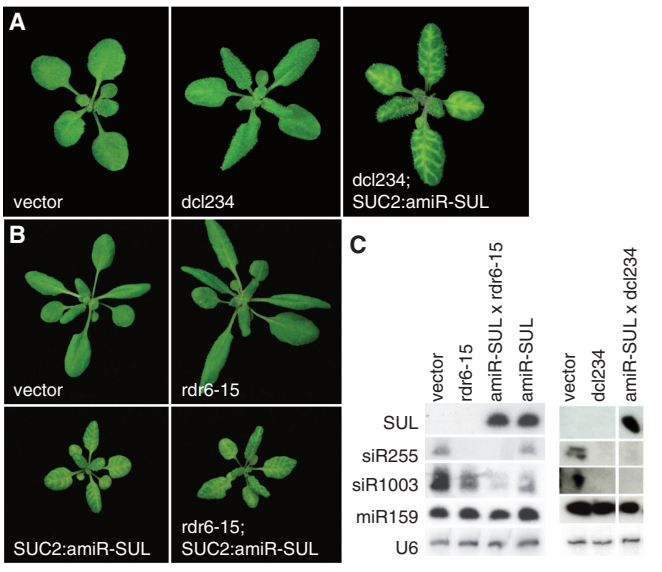

Figure 2. Confirmation of amiR-SUL-triggered silencing. (**A**) amiR-SUL production in *dcl234* triple mutant background. (**B**) RDR6-independent spreading of amiR-SUL-triggered silencing. (**C**) sRNA blots. Probes are indicated on the right. siR255 production is RDR6 and DCL4-dependent, siR1003 is DCL3-dependent but RDR6-independent. MiR159 was used as an additional control. Note characteristic leaf shape of *rdr6* and *dcl234* mutants in (A) and (B).

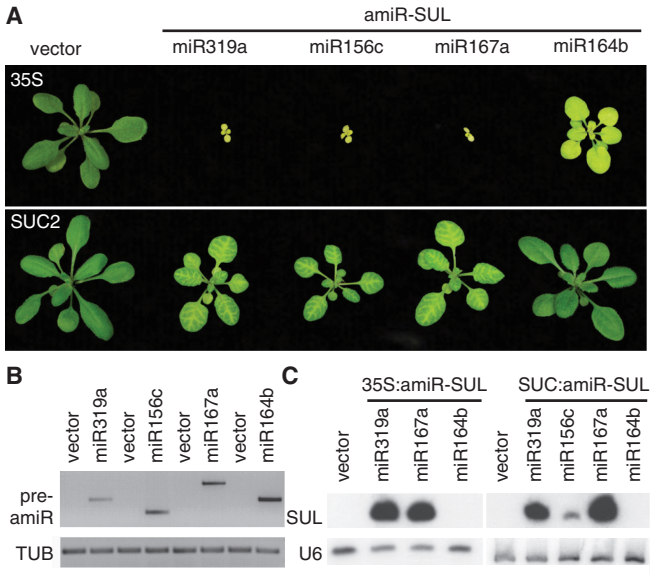

Figure 3. Effect of *MIRNA* backbone on spreading of the silencing signal. (**A**) Whole-rosette phenotypes of plants expressing amiR-SUL from different precursors, with promoters indicated on the left. (**B**) Precursor expression monitored by RT–PCR with *β-TUBULIN-2* (*TUB*) as control. (**C**) sRNA blots.

indicates that expression levels are important in determining non-autonomous effects, consistent with non-selective movement of the silencing signal, similar to what appears to be the default for many proteins (66,67).

Non-autonomous effects of tasiRNAs

TasiRNAs, which like miRNAs are normally 21 nt long, are generated from *TAS* precursor transcripts. The phase of tasiRNA formation is determined by the miRNA cleavage event that triggers tasiRNA formation (12). This feature allows the design of artificial tasiRNAs (atasiRNAs) with specific sequences (68–70). We have previously developed a *TAS1a* derivative that produces an atasiRNA, atasiR-SUL_1a, with the same sequence as our amiR-SUL (48), therefore allowing a direct comparison of both sRNAs. The *TAS1b* and *TAS1c* derivatives atasiR-SUL_1b and atasiR-SUL_1c also produce the same sRNA.

SUC2:atasiR-SUL transgenic plants were much more severely affected than *SUC2:amiR-SUL* plants. In the most extreme cases, the phenotype of *SUC2:atasiR-SUL* plants began to approach that of *35S:atasiR-SUL* plants, with pervasive bleaching throughout the entire leaf and much reduced stature (Figure 4A and Supplementary Figure S4). In weaker lines, which were more intensely bleached around the veins than in the remainder of the leaf, the boundary between affected and unaffected tissue was nevertheless much more diffuse than in *SUC2:amiR-SUL* plants (Figure 4A). The phenotypic differences between *SUC2:atasiR-SUL* and *SUC2:amiR-SUL* plants suggest that the biogenesis pathway of an sRNA, rather than its expression levels, has a major effect on its non-autonomous activity. This hypothesis was corroborated by a direct comparison of mature sRNA accumulation in the *SUC2:atasiR-SUL* and *SUC2:amiR-SUL* lines, with the first having much lower levels (Figure 6B).

SiRNA-triggered silencing can spread across long distances, by means of an RDR6-dependent amplification mechanism termed transitivity (24). Unfortunately, one cannot test directly RDR6-dependence of non-cell autonomous tasiRNA effects, because tasiRNA generation itself requires RDR6 (10,11). We therefore designed an atasiR-SUL in which the two or three last nucleotides, respectively, do not pair with the target transcript (atasiR-SUL_2mm and atasiR-SUL_3mm) (Supplementary Data), based on a proposal (62) that this reduces 3′ 5′ transitivity, which depends on priming by the sRNA. Both *SUC2:atasiR-SUL_2mm* and *SUC2:atasiR-SUL_3mm* plants suffered from the same widespread bleaching as the original *SUC2:atasiR-SUL* plants (Supplementary Figure S5). This experiment, however, does not address the possibility of 5′ 3′ or priming-independent transitivity. We therefore sequenced the 19 to 25 nt sRNA population around the *CH42* locus in these transgenic lines (Figure 5A). We found very few novel sRNAs matching the *CH42* locus in *SUC2:siR-SUL* and *SUC2:amiR-SUL* plants that did not correspond to the sRNAs generated from the triggering transgene (Figure 5B and Supplementary Figure S6). Although the most abundant species was still the original atasiR-SUL trigger, the level of novel sRNAs was more than four times higher in *SUC2:atasiR-SUL* compared to *SUC2:amiR-SUL* plants (Supplementary Table S2). To confirm that this variation was not fortuitous, we compared the ratios of sRNAs reads between these two lines for different regions of the genome. We identified 1971 bins of length 500 bp in which the sum of the read counts for both samples was at least 50% of the read count for the *CH42* bin. Only 60 bins (3%) had a read ratio between the two lines equal or higher than half of the ratio at the *CH42* locus, and only 14 (0.7%) had a similar or higher ratio (Supplementary Figure S7). This comparison suggested that the increase in secondary sRNAs in the *SUC2:atasi-SUL* line is indeed significant and that transitivity has potentially a role in tasiRNA spreading.

Genetic requirements for non-autonomous effects of different sRNAs

Mutations in several genes, including *RDR2*, *NRPD1a* and *CLASSY* compromise the non-autonomous effects of sRNAs (28,29). To determine whether the different classes of sRNAs rely on the same genetic system for spreading of the silencing signal, we crossed siR-SUL, amiR-SUL and atasiR-SUL producing lines to *rdr2*-1 and *nrpd1a*-3 mutants. As expected from previous work (28,29), the bleaching in *SUC2:siR-SUL* plants was completely suppressed in both mutant backgrounds. In contrast, bleaching triggered by *SUC2:amiR-SUL* and *SUC2:atasiR-SUL* was not affected in these mutants (Figure 6A), indicating that the non-autonomous effects of the different sRNAs have differential genetic requirements.

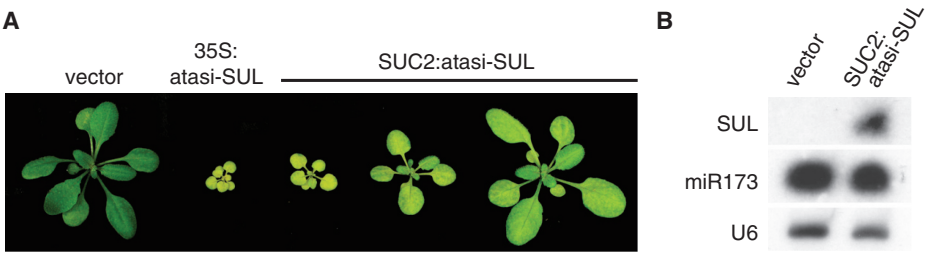

Figure 4. Non-autonomous effects of of atasiR-SUL. (**A**) Whole-rosette phenotype of *SUC2:atasiR-SUL_1c*. (**B**) sRNA blots. MiR173, which triggers *TAS1* processing, was used as an additional control.

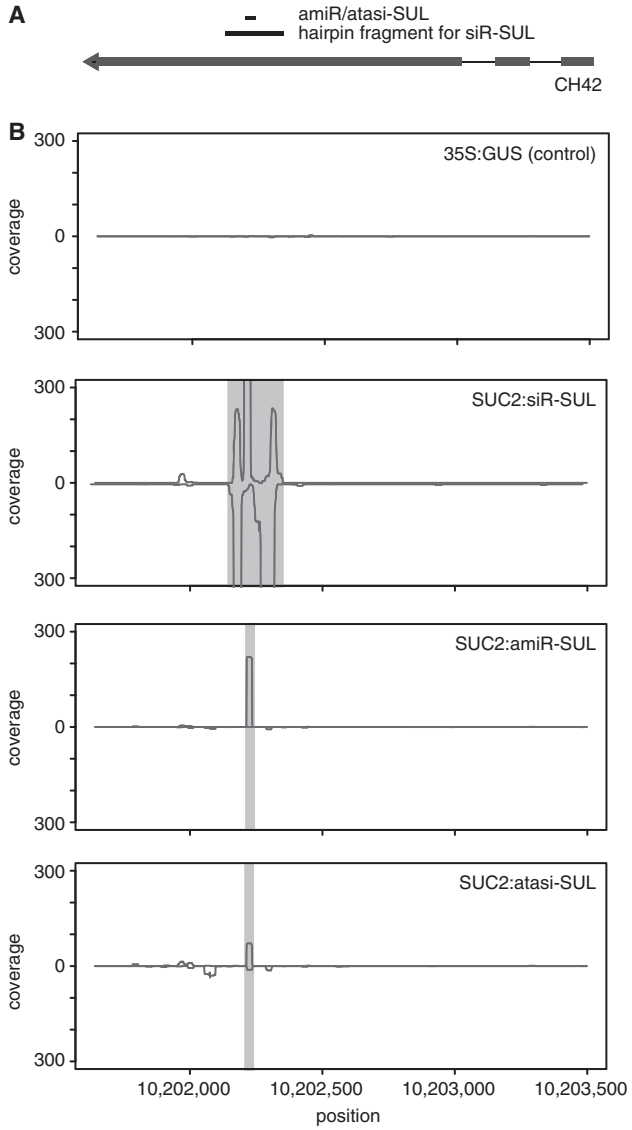

Figure 5. Secondary sRNAs at the *CH42* locus. (**A**) Diagram of *CH42* locus. Exons are indicated as thick lines. Regions targeted by primary sRNAs from siR-SUL and amiR/atasiR-SUL transgenes are shown. (**B**) Small RNA populations at the *CH42* locus. About 19–26 nt sRNAs, with a maximum of two mismatches (as in amiR/atasi-SUL), are shown. See Supplementary Figure S6 for perfect-match sRNAs only. Grey regions indicate origin of primary siR-SUL and amiR/atasiR-SUL, respectively.

DISCUSSION

Here, we have documented that the silencing effects of an amiRNA, amiR-SUL, can extend 10 to 15 cells from the site of its production in phloem companion cells, which is in the same range as observed for transgene-derived siRNAs. In contrast to siRNAs (25,28,29), the non-autonomous effects of amiR-SUL do not depend on RDR2 and NRPD1a. While the specific precursor from which the amiRNA is processed does not seem to be essential for non-autonomous silencing, the biogenesis pathway through which a 21 nt sRNA is generated plays a crucial role, since the silencing effects of an atasiR-SUL of identical sequence as amiR-SUL extend much further.

Recently, Tretter *et al.* (35) examined non-autonomous effects of sRNAs, using sRNAs targeting *PHYTOENE DESATURASE* (*PDS*), downregulation of which produces a similar phenotype as *CH42*/*SUL* knockdown. They reported that expression of siRNAs, but not amiRNAs, under indirect control of the *SUC2* promoter via the LhG4 transactivator (71), resulted in bleaching beyond the veins. One reason for apparent failure to detect amiRNA non-autonomy could be relatively low sRNA expression levels due to the LhG4 system, which is known to suffer from variable efficacy (71,72). Such a scenario is in line with our finding that efficiency of amiRNA processing affects the detection of non-autonomous effects, similar to what has been reported before for siRNAs (25,29). In support of this explanation, Tretter *et al.* (35) did observe non-autonomous effects after simultaneous expression of two amiRNAs under direct control of the *SUC2* promoter, which caused a very similar phenotype as seen in the majority of our *SUC2:amiR-SUL* lines.

Perhaps our most intriguing finding is that amiR-SUL and atasiR-SUL, despite having identical sequences, caused distinct silencing phenotypes in our system. Which factors could be responsible for defining how far sRNA-triggered silencing spreads? The most obvious difference is the pathway that generates the sRNA. Mallory *et al.* (73) have reported a case in which siRNAs produced from inverted repeats could spread systemically, while siRNAs for the same target, but derived from viral amplicons, were not able to move. It is likely that different DCLs and co-factors, which load AGO-containing RNA induced silencing complexes (RISCs), or AGOs and their co-factors, play a major role in defining the range of the silencing signal (9). This is at least indirectly supported by the different genetic requirements for miRNA- and tasiRNA-triggered silencing signals in our system.

Given several recent reports in which production and effect of sRNAs were directly examined (27,30), it seems likely that the mobile signal is the triggering sRNA itself. Differences in biogenesis could impact the production of secondary sRNAs, which in turn can mediate mobility of the silencing signal (24). We have detected more secondary sRNAs in tasiR-SUL than in amiR-SUL or siR-SUL expressing plants, showing that limited transitivity might contribute to the spreading of tasiRNA silencing.

In contrast to siR-SUL, transmission of the silencing signal triggered by an amiRNA or an atasiRNA does not rely on RDR2 and NRPD1a. Genetic screens using two different trigger loci have previously identified these two factors as being required for movement of siRNA-silencing signals (25,28,29). Some observations (28) indicate that RDR2 and NRPD1a act downstream of siRNA production, either by supporting the translocation of the silencing signal or its reception in other cells. Smith and colleagues (29), on the other hand, suggested that both proteins are involved in the amplification and/or generation of the signal. In any case, that the non-autonomous effects of amiR-SUL and atasiR-SUL are insensitive to loss of RDR2 or NRPD1a shows that not

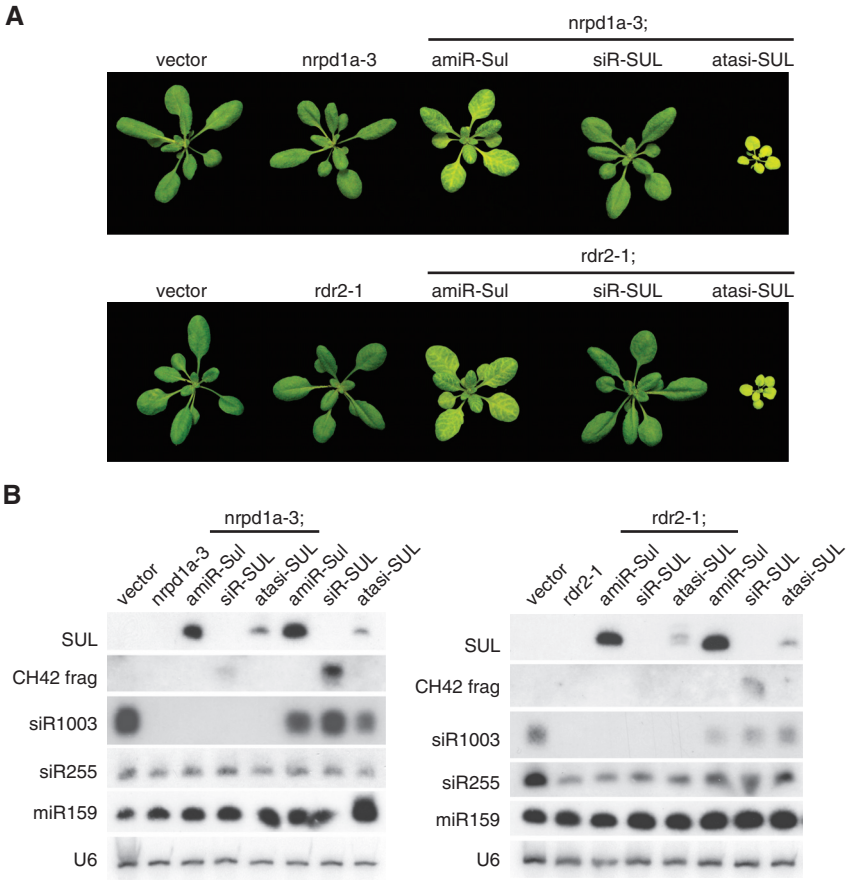

Figure 6. Differential genetic requirements for non-autonomous effects of amiR-SUL and atasiR-SUL initiated silencing. (**A**) Whole-rosette phenotypes. (**B**) sRNA blots.

all sRNAs require these two factors for transmission of their effects to neighboring cells.

Together with previous studies (31–38), our work highlights that non-autonomous action of miRNAs is likely to be context-dependent. One of the responsible factors might be the expression level of an miRNA. Among mutants that alter cell-to-cell spreading of siRNA-mediated gene silencing, those with more extensive movement of the silencing signal also have higher siRNA levels, while one of the classes lacking non-autonomous siRNA effects no longer accumulates 21 nt siRNAs (25). The same correlation has been observed for systemic movement of siRNAs, where higher copy number of the triggering transgene may lead to more efficient systemic acquired silencing (20). As discussed above, this could be one of the reasons why different levels of non-autonomy have been detected for the same amiRNAs (35).

Plants expressing higher levels of amiR-SUL present stronger bleaching, but silencing does not appear to spread further than in more weakly bleached lines. Nonetheless, expression levels still seem to be an important feature, since it affects the extent to which the neighboring cells are affected. Corroborating this idea, there are various types of published evidence for non-cell autonomy of 13 of the 19 miRNA families that are expressed more highly than amiR-SUL in *SUC2:amiR-SUL* plants (Supplementary Table S1) (36–40).

A second factor affecting miRNA non-autonomy could relate to time and place of expression. Both selective and non-selective intercellular mobility of molecules are affected by the tissue and developmental stage of the plant (66,74–76). In addition, trafficking of the silencing signal may depend on the cell type. RNAi initiated in epidermal cells has been shown to spread only locally, while expression of the same RNAi trigger in an entire leaf engenders systemic silencing (77). It is possible that, compared to other cell types, miRNAs expressed in phloem companion cells, as in this study, can more easily initiate non-autonomous silencing, or move themselves to adjacent cells, e.g. because phloem companion cells contain factors that promote non-autonomous behavior.

A third, less often considered possibility could be tissue- or cell type-specific processing of the precursor. Some miRNA precursors that are mainly processed by DCL1 in leaves can be processed by DCL3 in inflorescences, where they spawn a distinct class of miRNAs that are 23 to 25 nt long (61). Spreading of silencing triggered by amiR-SUL and atasiR-SUL is not due to the sRNA sequence, but more likely caused by biogenesis factors or effectors engaged in the miRNA and tasiRNA pathways. In analogy, the production of miRNAs through tissue-specific pathways could result in differential non-autonomous effects.

In summary, we propose that the question of cell-autonomy versus non-autonomy of sRNAs does not have a simple answer, but rather that it is contingent on several circumstances that include time, place and level of expression, which may interact with biogenesis and translocation pathways in a complex manner. Depending on the setting, miRNA behavior might therefore range from strictly cell-autonomous action, to local spreading that generates morphogenetic gradients, and even long-distance systemic silencing (36–38,42). The apparent behavior of tasiRNAs might be even more complex, as the non-autonomous effect of tasiRNA might depend both on tasiRNA-specific factors and on the action of the upstream triggering miRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Supporting Information for

Comparative analysis of non-autonomous effects of tasiRNAs and miRNAs in *Arabidopsis thaliana*

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SUPPLEMENTARY METHODS

Transgenes

The amiR-SUL_156, amiR-SUL_164 and amiR-SUL_167 constructs were generated by replacing the respective mature miRNA sequence with the same siRNA sequence targeting *CH42* as used in the original amiR-SUL transgene using the *MIR319a* precursor. The complementary strand was replaced by a sequence that preserves the original binding structure and the final folding of the precursor (Figure S2). RNA structure was predicted with the RNAfold web tool using standard settings (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The final foldback sequences are presented below, with the amiRNA in **underlined bold letters**, and the amiRNA* in *underlined italics*. Lower case indicates mismatched positions.

amiR-SUL_156

5′-GACAAATTTTAAGAGAAACGCATAGAAAC**TTAAGTGTCACGGAAATCCC**ACAAAGG
CACTTTGCATGTTCGATGCATTTGCTTCTCTTGC*GGGATTTCCaGTGAtCACTTAA*GAT
TCCGGCTCCGATTCGGTCCC-3′

amiR-SUL_164

5′-GGTGTGTGATGAGCAAGA**TTAAGTGTCACGGAAATCCCT**TTACTAGCTCATATATA
CACTCTCACCACAAATGCGTGTATATATGCGGAATTTTGTGATATAGATGTGTGTGTGT
GTTGAGTGTGATGATATGGATGAGTTAGTTC*ATGGGTTTCCGATACACATAA*TCATGAC
CACTCCACCTTGGTGA-3′

amiR-SUL_167

5′-TGAACAGAAAAATCTCTCTTTCTCTTTCTTGATCTGCTACGGTGAAGTCTATGGTG
CACCGGCATCTGA**TTAAGTGTCACGGAAATCCCT**ATTAGCTTTCTTTATCCTTTGTTGT
GTTTCATGACGATGGTTAAGAGATCAGTCTCGAT*AGGGATTTCTGAGACATTTAA*CCCG
TTGACTGTCGCACCCTTCTATAAACCCTAAATTTTCTCTCTATCTTTTTTAGTTTGATT
TTCAAGACACTTTGTTTCTCAATCTTCAGTCTGATTTTGTGAGCTTACTTCTCTTTCTG
AGGCTATATATATTTCTTTTTCATAAGAATTTTCACTATATTGTTGTGTGTATGTGGAT
CTGAAGAATTTATGTTTTGTTAGGGTTATAGAGTTGCATGACTTTAGGAAACCCTAATT
TGGTCTTTGACAAATTGAGTATATTTTTTTGGGTTATAAATATGAAAATCTTCTTATGA
TGAAGCTAGGAAAGAGGAGCTTTGTTTTTTTTACAAAGATTATGGAATTTAGCAAATTC
AGTTAATTATCTATAATTACGTTTTTGGGGATCTCAAGATAGACTTCCTAGTTCTTCTT
TTTACTCATATCTGTtttaagaaaagactttcatgtacatttttttatttttttggct
tatgttagtttagggtttctgggaagaagttgaaactgaagttgtacactattgataca
tatgtagtttatataagttcgactaaagattttggagtttatatatagcagttgcccg
gagctcaaactctacttcctgatagattaaccacagatatgctg-3′

Atasi-SUL based on *TAS1b* and *TAS1c* (atasi-SUL_1b and atasi-SUL_1c, respectively) were generated as described for atasiSUL-1a (1). The final sequences are shown below. The sRNA targeting *CH42* is shown in **underlined bold letters**, and the miR173 target site in *underlined italics*.

Atasi-SUL_1b

5′-AAATCTAAACCTAAGCGGCTAAGCCTGACGTCATTTAACAAAAAGAGTAAACATGA
GCGCCGTCAAGCTCTGCAACTACGATCTGTAACTCCATCTTAACACAAAAGTTGAGATA
GGTTCTTAGATCAGGTTCCGCTGTTAAATCGAGTCATGGTCTTGTCTCATAGAAAGGTA
CTTTCTTTTACTTCTCTTGAGTAGCTTCTATAGCTAGATTGAGATTGAGGTTTTGAGAT
ATTAGGTTCGATGTCCCGGTCTATTTGTCACCAGCCATGTGTCAGTTTCGACCAGTCCC
GTGCTCTCTGTATTTGGTTTTATCGGAATACGGAGATCTATTTTCAGGAGGAGACAACT
TTGTTTTCTT*GTGATTTTTCTCAACAAGCGAA*TGAGTCATTCATCGGTATCTAACCATT
CACCATATTATCAGAGTAGTTATGATTGATAGGATGGTAGAAGAATATTCTAAGTCCAA
CATAGCATA**TTAAGTGTCACGGAAATCCCT**AAAAATTGGGAGATATCCGGAATGATATT
ATACGTAAAAAAAAATGGGAGATGTCCGGAATGATATTTGTAATATTTTTATGTTAACG
AAACATATTTTAGGATATGCAAAAAAAAGTAGATGTTGGTATTCTTGTTTTGCAAGATT
TGTAATGGGAGTTGTGTAGTCTTTTTATGATGTGTCATGAAGTCTACCGCCAATTACAT
ACATCATTCACTTTGTAATTAAATTGTCTTCAAGTTTGTAATTTTATTTTTGTTTTATG
TACCAAAATCTAAATTCAGTTGTTTACAACTTGATAACAAAAAAAAAGTTATACATTAC
TTATGTTTTCACACTC-3′

Atasi-SUL_1c

5′-AAACCTAAACCTAAACGGCTAAGCCCGACGTCAAATACCAAAAAGAGAAAAACAAG
AGCGCCGTCAAGCTCTGCAAATACGATCTGTAAGTCCATCTTAACACAAAAGTGAGATG
GGTTCTTAGATCATGTTCCGCCGTTAGATCGAGTCATGGTCTTGTCTCATAGAAAGGTA
CTTTCGTTTACTTCTTTTGAGTATCGAGTAGAGCGTCGTCTATAGTTAGTTTGAGATTG
CGTTTGTCAGAAGTTAGGTTCAATGTCCCGGTCCAATTTTCACCAGCCATGTGTCAGTT
TCGTTCCTTCCCGTCCTCTTCTTTGATTTCGTTGGGTTACGGATGTTTTCGAGATGAAA
CAGCATTGTTTTGTT*GTGATTTTTCTCTACAAGCGAA*TAGACCATTTATCGGTGGATCT
TAGAAAATTA**TTAAGTGTCACGGAAATCCCT**TTCTAAGTTCAACATATCGACGAACTAG
AAAAGACATTGGACATATTCCAGGATATGCAAAAGAAAACAATGAATATTGTTTTGAAT
GTGTTCAAGTAAATGAGATTTTCAAGTCGTCTAAAGAACAGTTGCTAATACAGTTACTT
ATTTCAATAAATAATTGGTTCTAATAATACAAAACATATTCGAGGATATGCAGAAAAAA
AGATGTTTGTTATTTTGAAAAGCTTGAGTAGTTTCTCTCCGAGGTGTAGCGAAGAAGCA
TCATCTACTTTGTAATGTAATTTTCTTTATGTTTTCACTTTGTAATTTTATTTGTGTTA
ATGTACCATGGCCGATATCGGTTTTATTGAAAGAAAATTTATGTTACTTCTGTTTTGGC
TTTGCAATCAGTTATGCTAGTTTTCTTATACCCTTTCGTAAGCTTCCTAAGGAATCGTT
CATTGATTTCCACTGCTTCATTGTATATTAAAACTTTACAACTGTATCGACCATCATAT
AATTCTGGGTCAAGAGATGAAAATAGAACACCACATCGTAAAGTGAAAT-3′

For the generation of the siR-SUL construct, a 189 bp fragment of the *CH42* cDNA was used (positions 822 to 1010 of the coding region), with the following sequence:

5′-GCTTAGGCCACAGCTTCTTGATCGGTTTGGTATGCATGCACAAGTAGGGACGGTTA
GAGATGCTGATTTACGGGTCAAGATTGTTGAAGAGAGAGCTCGTTTCGATAGTAACCCA
AAGGATTTCCGTGACACTTACAAAACCGAGCAGGACAAGCTTCAAGACCAGATTTCAAC
TGCTAGGGCAAACCT-3′

sRNA libraries

50 µg of total RNA from 2-3 week old plants was extracted. sRNAs were enriched using the mirVana miRNA isolation kit (Applied Biosystems/Ambion, Austin, TX, USA) according to manufacturer's instructions. Enriched sRNAs were resolved on a 15% 19:1 acrylamide:bisacrylamide gel with 8 M urea and 0.5 x TBE, and a region corresponding to 19 to 25 nucleotides was extracted. The sample was transferred to chromatography paper using a semi-dry blot transfer (Bio-Rad, Hercules, CA, USA). The paper was placed in a spin-X column (Corning/Costar, Lowell, MA, USA), washed three times with low salt buffer (10 mM Tris, pH 7.6; 1 mM EDTA, pH 8.0; 100 mM NaCl) and eluted by incubating the paper for 15 min with 200 µL of High Salt Buffer (10 mM Tris, pH 7.6; 1 mM EDTA, pH 8.0; 1 M NaCl; 50 mM L-arginine). Eluates were precipitated with 20 µg glycogen and 2.5 volumes 100% ethanol at -20°C overnight. A second precipitation round was carried out by adding 0.6 volumes of 5 M NH_4OAc, 2.5 volumes of 100% ethanol and incubation at -80°C for two hours. Ligation of 5' and 3' adaptors was performed as described (2), but with gel purification and sample elution steps as described above in between each ligation step. The RNA/adaptor sample was used as template for cDNA synthesis using the Fermentas kit (Fermentas, Burlington, Canada) according the manufacturer's instructions. PCR amplification was performed as described (2).

Sequencing and data analysis

We sequenced two sRNA libraries (biological replicates, only one replicate shown) for each of the SUC2:amiR-SUL and SUC2:atasi-SUL lines, and one library for each of the SUC2:siR-SUL, SUC2:amiR-SUL; rd6-15 lines and the 35S:GUS line that was used as a control. The raw sequence tags were input to SHORE (3) for quality filtering and sequencing adapter removal. We then utilized GenomeMapper (4) for matching the resulting sRNA tags to the *A. thaliana* reference sequence. The best matching loci allowing for up to two sequence mismatches were reported for each 21-30mer tag, and allowing for up to one mismatch for 15-20mer tags.

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SUPPLEMENTARY TABLES

Table S1. miRNAs with expression levels higher than amiR-SUL.

Locus identifier	Gene	Fold amiR-SUL read count	Evidence for non-cell autonomy
AT5G10945	miR156d	153.9	(5,6)
AT1G66783	miR157a	5.1	(6)
AT1G66795	miR157b	5.1	
AT3G18217	miR157c	5.1	
AT1G73687	miR159a	8.3	(5,6)
AT1G18075	miR159b	1.6	
AT1G48267	miR161	9.3	
AT2G47585	miR164a	46.3	(6)
AT5G01747	miR164b	46.4	
AT5G27807	miR164c	3.1	
AT1G01183	miR165a	33.5	(7)
AT4G00885	miR165b	32.5	
AT2G46685	miR166a	32.2	(6,7)
AT3G61897	miR166b	24.4	
AT5G08712	miR166c	24.4	
AT5G08717	miR166d	24.4	
AT5G41905	miR166e	24.4	
AT5G43603	miR166f	24.4	
AT5G63715	miR166g	24.4	
AT3G22886	miR167a	19.8	(5); (6)
AT3G63375	miR167b	19.8	
AT4G19395	miR168a	42.5	(6)
AT5G45307	miR168b	42.4	
AT1G53687	miR169e	1.2	(6)
AT3G14385	miR169f	1.7	
AT1G11735	miR171b	2.7	(5);
AT1G62035	miR171c	1.4	
AT2G28056	miR172a	22.0	(6)
AT5G04275	miR172b	22.0	
AT2G38325	miR390a	6.0	(6,8)
AT5G58465	miR390b	6.0	
AT5G60408	miR391	5.0	None
AT2G10606	miR396a	4.1	None
AT2G03445	miR398a	2.8	None
AT2G47015	miR408	2.9	(6)
AT4G24415	miR824a	2.9	None
AT2G26211	miR825a	2.0	None

Table S2. Read counts of secondary sRNAs in the region of the *CH42* locus located distally to the amiR/atasi-SUL.

Line	Strand	Raw reads	Reads per million
SUC2:amiR-SUL	plus	10	1.53656
	minus	13.342857142857	2.0502
	total	23.342857142857	3.58676
SUC2:atasi-SUL	plus	34	5.98235
	minus	69.4	12.211
	total	103.4	18.1934

Table S3. Key to construct names.

Construct	Name	Primary vector
35S:*amiR-SUL*	pFF169	pGreen
35S:*amiR-SUL_156*	pFF259	pHB
35S:*amiR-SUL_164*	pFF473	pGreen
35S:*amiR-SUL_167*	pFF474	pGreen
35S:*atasi-SUL_1c*	pFF273	pHB
35S:*GUS*	pFF087	pGreen
SUC2:*amiR-SUL*	pFF168	pGreen
SUC2:*amiR-SUL_156*	pFF287	pGreen
SUC2:*amiR-SUL_164*	pFF469	pGreen
SUC2:*amiR-SUL_167*	pFF470	pGreen
SUC2:*siR-SUL*	pFF189	pGreen
SUC2:*atasi-SUL_1a*	pFF329	pGreen
SUC2:*atasi-SUL_1b*	pFF294	pGreen
SUC2:*atasi-SUL_1c*	pFF301	pGreen
SUC2:*atasi-SUL_2mm*	pFF471	pGreen
SUC2:*atasi-SUL_3mm*	pFF472	pGreen

Table S4. Oligonucleotide sequences.

Purpose	Sequence
Small RNAs	
amiR-SUL/atasi-SUL	TTA AGT GTC ACG GAA ATC CCT
atasi-SUL 2mm	TTA AGT GTC ACG GAA ATC CCA
atasi-SUL 3mm	TTA AGT GTC ACG GAA ATC GCA
Probes for sRNA blots	
amiR-/atasi-SUL	AGG GAT TTC CGT GAC ACT TAA
U6 (loading control)	GCT AAT CTT CTC TGT ATC GTT CC
siR255	TAC GCT ATG TTG GAC TTA GAA
siR1003	ATG CCA AGT TTG GCC TCA CGG TCT
miR159a	TAG AGC TCC CTT CAA TCC AAA
miR173	GTG ATT TCT CTC TGC AAG CGA A
RT-PCR	
miR156c precursor, forward	GAC AAA TTT TAA GAG AAA CGC ATA G
miR156c precursor, reverse (terminator based)	CGC ATA TCT CAT TAA AGC AGG
miR164b precursor, forward	GAA GGT GTG TGA TGA GCA AG
miR164b precursor, reverse (terminator based)	CGC ATA TCT CAT TAA AGC AGG
miR167a precursor, forward	AGG GAT TTC TGA GAC ATT TAA CCC GTT GAC TGT CGC ACC CTT
miR167a precursor, reverse (terminator based)	CGC ATA TCT CAT TAA AGC AGG
miR319a precursor, forward	GAA GAG ATT TCC GTG TCA CTT ATT CAC AGG TCG TGA TAT G
miR319a precursor, reverse (terminator based)	CGC ATA TCT CAT TAA AGC AGG
β-TUBULIN-2, forward	GAG CCT TAC AAC GCT ACT CTG TCT GTC
β-TUBULIN-2, reverse	ACA CCA GAC ATA GTA GCA GAA ATC AAG
Genotyping	
DCL2 wild-type allele, forward	GGC TGA GAT ACC TCA AGG TGG TTT

DCL2 wild-type allele, reverse	CCT CTC CGG AAG TCT TCC ACA ATT
dcl2-1 mutant allele, forward	GGC TGA GAT ACC TCA AGG TGG TTT
dcl2-1 mutant allele, reverse	TGG TTC ACG TAG TGG GCC ATC G
DCL3 wild-type allele, forward	CCT GAA GAG CGT GAA GGA G
DCL3 wild-type allele, reverse	AGC TTT GGA GAT ACA TGC CCA G
dcl3-1 mutant allele, forward	CCT GAA GAG CGT GAA GGA G
dcl3-1 mutant allele, reverse	TGG TTC ACG TAG TGG GCC ATC G
DCL4 wild-type allele, forward	TCT TGT TGG GCT GGA CGT TG
DCL4 wild-type allele, reverse	TAG CGC GCT CAA GTT CAG AG
dcl4-2 mutant allele, forward	TCT TGT TGG GCT GGA CGT TG
dcl4-2 mutant allele, reverse	GAC CAT CAT ACT CAT TGC TGA TCC ATG
RDR6 wild-type allele, forward	TGA ATC CAT TCC TGA ACA AGC
RDR6 wild-type allele, reverse	CAA TGC AAC CTC ATC TTG GAT G
rdr6-15 mutant allele, forward	TGA ATC CAT TCC TGA ACA AGC
rdr6-15 mutant allele, reverse	TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C
RDR2 wild-type allele, forward	ACA CAT TAG GAC TAA CAA ATT TAC C
RDR2 wild-type allele, reverse	ATG GTG TCA GAG ACG ACG ACG AAC CGA TCA AC
r*dr2*-1, forward	ACA CAT TAG GAC TAA CAA ATT TAC C
r*dr2*-1, reverse	TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA C
NRPD1a wild-type allele, forward	TTA ATG TTC TTC ATG CGG GAC
NRPD1a wild-type allele, reverse	AAA AGG GAT CAA AAC GAG ACG
nrpd1a-3 mutant allele, forward	TTA ATG TTC TTC ATG CGG GAC
nrpd1a-3 mutant allele, reverse	ATT TTG CCG ATT TCG GAA C
siR-SUL, forward	AAA GAA TTC GCT TAG GCC ACA GCT TCT TG
siR-SUL, reverse	CTT CGT CTT ACA CAT CAC TTG TCA
amiR-SUL, forward (*SUC2* promoter)	CCA CTC TTC CTC TTC CTC CAC C
amiR-SUL, reverse	GAA GGG ATT TCC GTG ACA CTT AAT CAA AGA GAA TCA ATG A
atasi-SUL_1c, forward (*SUC2* promoter)	CCA CTC TTC CTC TTC CTC CAC C

atasi-SUL_1c, reverse	AGG GAT TTC CGT GAC ACT TAA TAA TTT TCT AAG ATC CAC

SUPPLEMENTARY FIGURES

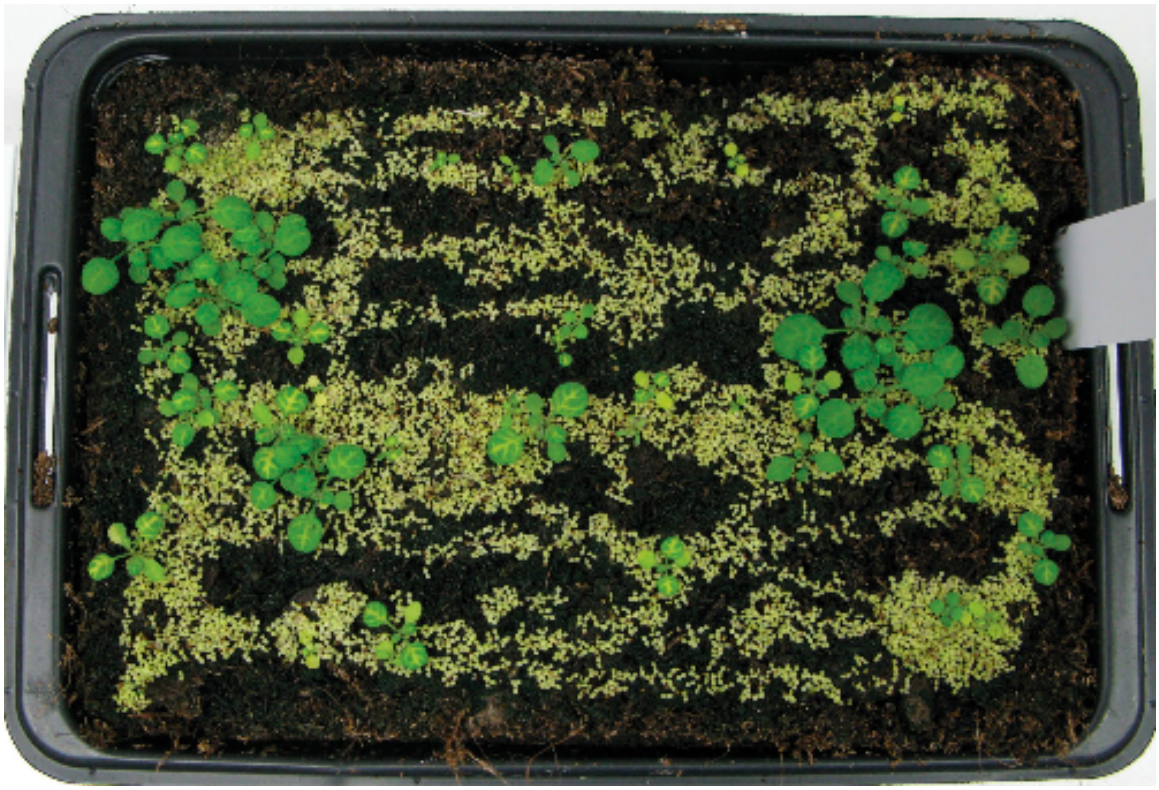

Figure S1. Phonotypic variation among primary transformants of *SUC2:amiR-SUL* line.

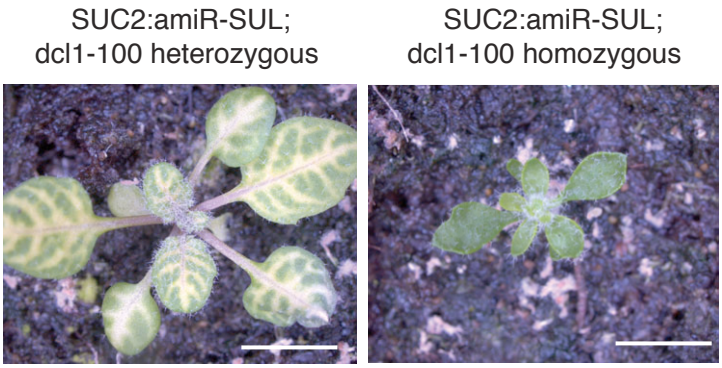

Figure S2. *SUC2:amiR-SUL* in *dcl1-100* background. On the left, a heterozygous plant showing the characteristic bleaching. In the right panel, a homozygous plant lacking the bleaching.

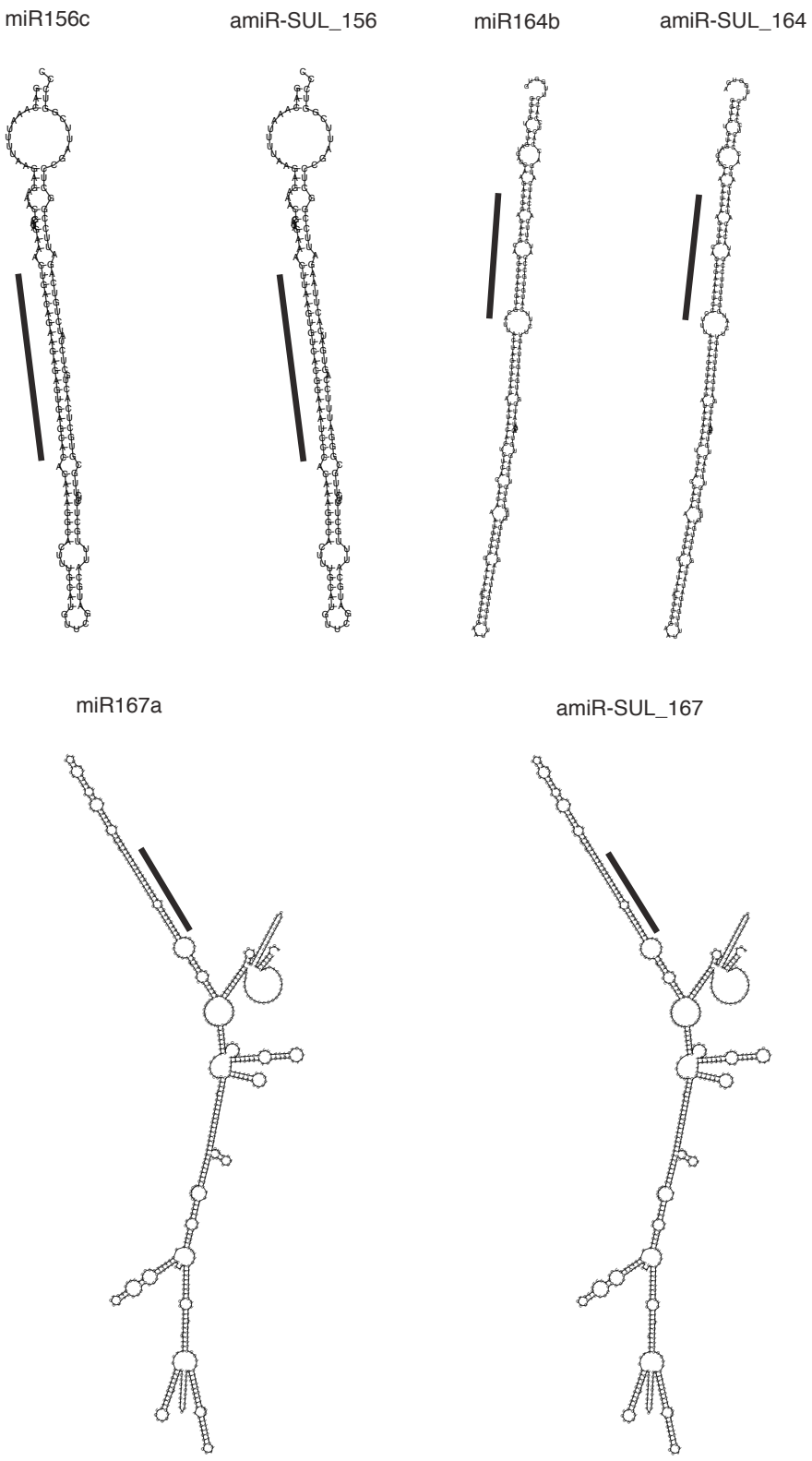

Figure S3. *In silico* RNA folding of amiR-SUL foldbacks and their progenitors. Black lines indicate the mature miRNAs.

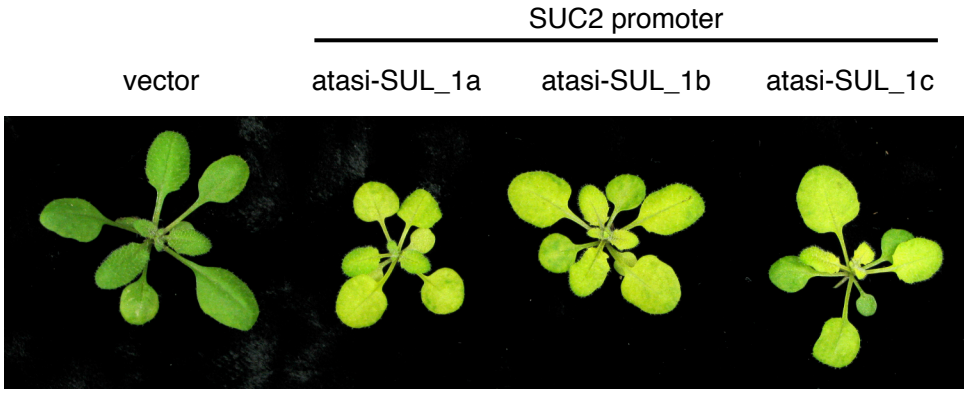

Figure S4. Non-autonomous effects of atasiR-SUL produced from different *TAS1* family backbones.

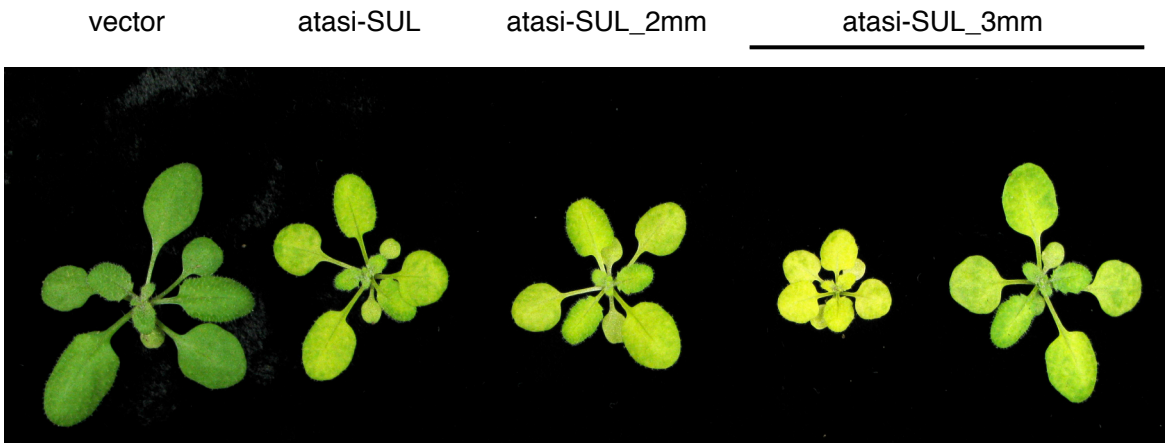

Figure S5. Primed transitivity does not play a major role in tasiRNA movement. *SUC2:atasi-SUL_2mm* and *SUC2:atasi-SUL_3mm* plants express atasiRNAs with the two or three last nucleotides, respectively, unpaired to the target. Both have the same phenotype as the original *SUC2:atasi-SUL* lines. *35S:GUS* plants were used as a vector control.

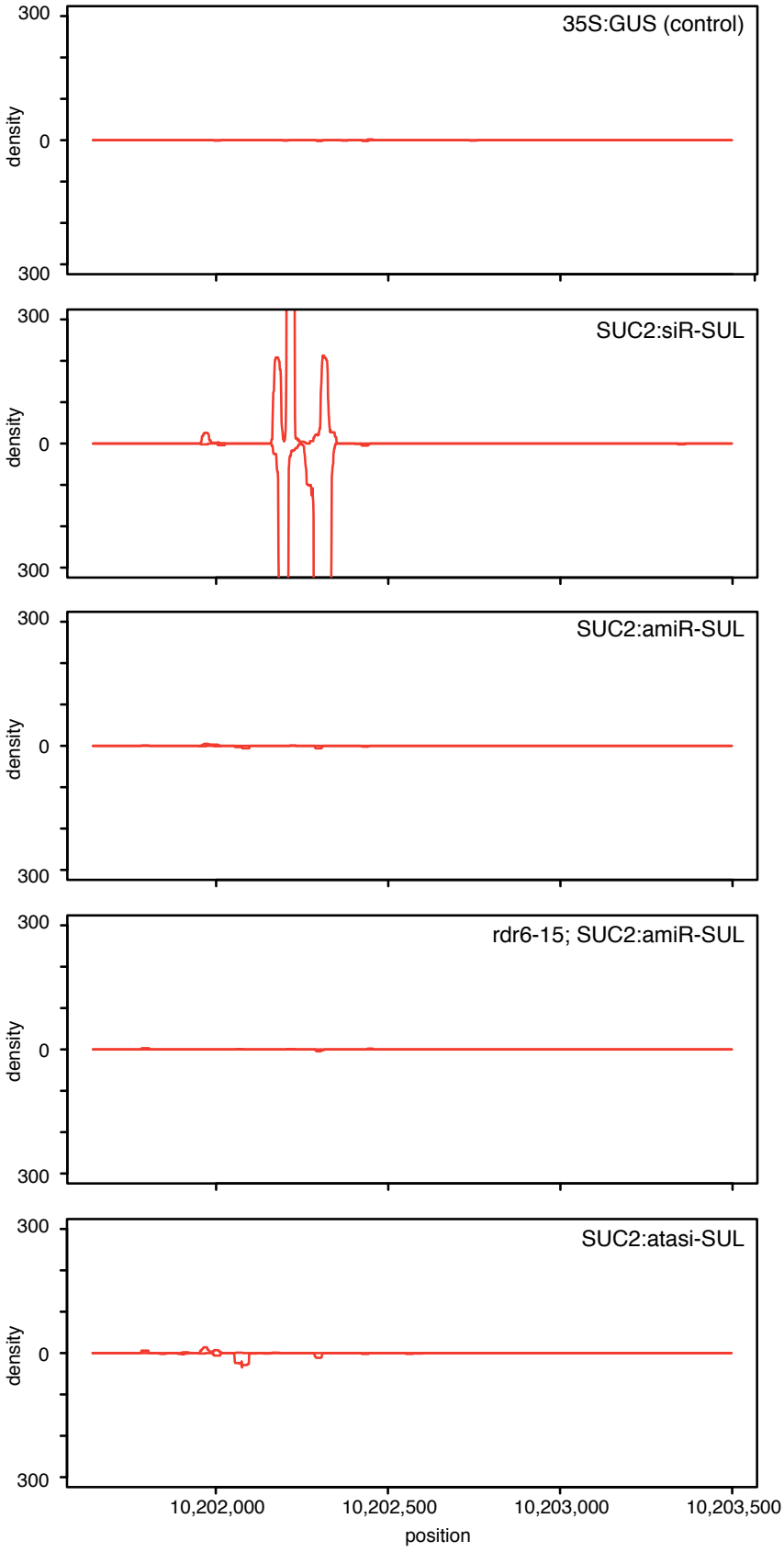

Figure S6. Perfect-match sRNA populations at the *CH42* locus.
Note the absence of the amiR-SUL and atasi-SUL, which contain mismatches to *CH42*; compare to Fig. 5*B* in main text. Top and bottom indicate reads from either strand of the DNA.

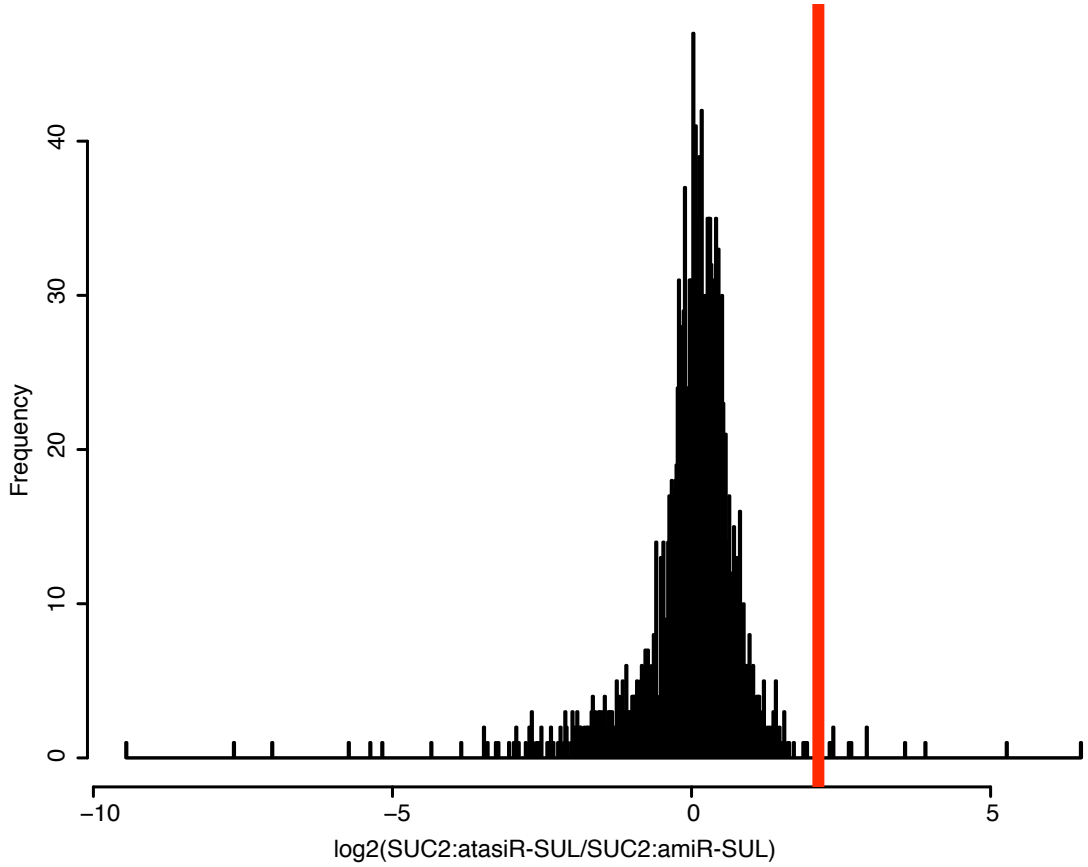

Figure S7. Distribution of ratios of reads in *SUC2:atasi-SUL* over *SUC2:amiR-SUL* between 500 bp regions of the genome that had at least 60 reads in both lines combined. The red bar indicates the ratio at the *CH42* locus.

5.1.4 "MIGS: an efficient gene silencing approach for plant functional genomics"

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MIGS: an efficient gene silencing approach for plant functional genomics.

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Abstract

Gene silencing is a powerful tool for functional genomics in both animal and plants. Virus-induced gene silencing (VIGS), hairpin RNA interference (hpRNAi) and artificial microRNA (amiRNA) are the most popular gene silencing tools in plants. However, all of them have their own limitations. Here, we introduce a novel method, named MiRNA Induced Gene Silencing (MIGS). MIGS is based on another class of small RNAs, called transacting small interfering RNA (tasiRNAs). We show that introduction of miR173 binding site in front of a protein coding gene is sufficient to trigger secondary tasiRNAs production and subsequently induce gene silencing in *Arabidopsis thaliana*. MIGS can be reliably used for silencing a single gene, as well as for multiple genes of different identities. In addition, we show that MIGS can be widely applied to other plant species by co-expression of miR173.

Introduction

The ability to downregulate the expression of a gene is an important part of modern biology, either for biotechnological reasons or as a tool to study different aspects in basic research. The discovery of small RNAs (sRNAs) and their role in gene silencing (also referred as RNA interference, RNAi) has revolutionized our capacity to manipulate gene expression. sRNAs are 19-24 nt long molecules resulted of the enzymatic processing of a double-stranded RNA (dsRNA) by specific RNAses that in plants are called DICER-LIKE (DCL). Together with proteins, these sRNAs form the RISC complex, which in turn will cause silencing of the target gene by either affecting transcription (transcriptional gene silencing, TGS) or mRNA stability/translation (post-transcriptional gene silencing, PGTS)[1,2]. Depending on the nature of the precursor, plants sRNAs can be divided in two main classes, the small interfering RNAs (siRNAs) or micro RNAs (miRNAs). siRNAs originate from perfect complementary dsRNA and correspond to different regions of the precursor. miRNAs, on the other hand, are product of endogenous transcripts that contain self-complementary regions that can fold, forming imperfect dsRNA regions that are usually processed to one main mature miRNA[3-5].

The first methods to silence genes through sRNAs were based on the production of siRNAs from dsRNA with perfect complementarity. Among them, Virus Induced Gene Silencing (VIGS) and hairpin RNAi (hpRNAi) are the most successful[6,7]. The principle how VIGS works is based on the replication mechanism of RNA virus, which during this process can be found as dsRNA, and therefore is easily processed by DCLs resulting in the production of siRNAs. Usually, a sequence of the target gene is add to the virus genome or part of it, and them transferred to plants either using the own virus or

Agrobacterium as vector[8-10]. hpRNAi is a more direct method that requires the cloning of a sequence in sense and antisense direction, connected by a linker. Once expressed, this transcript can easily assume a dsRNA configuration, and consequently, be targeted by DCLs[7,11,12]. More recently, a new method based on miRNA has been developed, where an sRNA, designed to target a gene of interest, replaces the original miRNA in the precursor transcript, resulting in an artificial miRNA (amiRNA)[6,13]. All these techniques present advantages and disadvantages, with its use being usually a question of preference.

Plants have a unique sub-class of siRNA called trans-acting siRNA (tasiRNA). tasiRNA generation starts when a TAS transcript is targeted by a miRNA. The cleavage driven by the miRNA triggers a process where the TAS transcript will serve as template for the synthesis of a dsRNA by an RNA-dependent polymerase (RDR), which is readily processed by DCLs[2,4,5]. The reason why TAS transcripts are driven to tasiRNA production instead of degradation, as the majority of miRNAs targets, has been one of the central questions in sRNA biology. At first, it has been shown that miR173, which targets *TAS1* and *TAS2*, is specifically necessary to trigger tasiRNA production in the genes of these families. In addition, it has been shown that miR173 cleavage is sufficient to initiate transitivity, a process resulting in the production of secondary sRNAs[14,15]. Recently, it was reported that the ability to trigger secondary sRNA production of some miRNAs, such as miR173, is related to its size of 22 nt, instead of the regular 21 nt found for most of the miRNAs in plants [16,17].

We have used miR173 ability in starting secondary sRNA production to develop a new tool for efficient silencing of plants genes, which we named MiRNA-Induced Gene Silencing (MIGS). We present here prove of concept of this technique, comparing its

efficiency to loss of function mutants. In addition, we show that MIGS can be used successfully to silence more then one gene, without necessity of any degree of relationship among them. Finally, we have developed a set of plasmid for easy and rapid use of MIGS.

Results

MIGS as an Easy and Efficient Method for Gene Silencing

In a previous study, we found that miR173-directed cleavage of a chimeric fragment of *CHLORATA42* (*CH42*) with miR173 binding site was sufficient to induce silencing of the endogenous copy of *CH42*[15]. This result suggests that miR173 binding site can be used as an universal trigger to produce miRNA cleavage-dependent secondary tasiRNAs. To test this hypothesis, we overexpressed the cDNA fragments of *AGAMOUS* (*AG*), *EARLY FLOWERING 3* (*ELF3*), *FT* and *LEAFY* (*LFY*) with miR173 binding site in front, in *Arabidopsis thaliana* (Figure 1A). *AG* is a homeotic gene involved in the proper flower development. Knockout of *AG* causes flower defect, with petals and sepals being generated instead of the reproductive organs and flower buds producing multiple flowers [18,19] (Bowman et al., 1989). *ELF3* encodes a protein involved in the circadian clock and one of the phenotypes associated to its lost of function is the increase in the hypocotyl elongation during short days conditions[20]. *FT* is involved with the control of flowering time and act as a florigen triggering the change from vegetative to reproductive phase[21-23]. Lost of *FT* leads to late flowering plants. *LFY* is a transcription factor expressed in the inflorescence primordia and is important for a proper flowering transition[24-26].

Downregulation of *LFY* causes developmental abnormalities such as increase of the number of ramifications in the main shoot and unfertile plants[24].

Transgenic plants carrying the *AG* fragment behind the miR173 target site (*35S:173ts_AG*) showed a similar phenotype as *ag-2*, a loss-of-function allele of *AG* mutant (Figure 1B). Compared to wild type, *35S:173ts_ELF3* seedlings had an indistinguishable phenotype as *elf3-9* mutant with elongated hypocotyls (Figure 1C). The same is true for LFY and FT, as *35S:173ts_LFY* and *35S:173ts_FT* displayed nearly the same phenotypes as *LFY* and *FT* mutants (Figure 1*D* and *E*).

The fact that miR173-direct cleavage was able to trigger secondary sRNA production (Fig 2A) and caused effective gene silencing in different tissues, such as seedlings (*ELF3*), primordia (*LFY*), flowers (*AG*) and leaves (*FT*) (Figure 2*B*), corroborates our hypothesis that MIGS can be use as an efficient approach for gene silencing in *A. thaliana*.

Multiple Gene Silencing by MIGS

We then tested whether MIGS could also be used to silencing two independent genes at the same time. To this end, we generated two transgenic plants that overexpressed the chimeric *AG-FT* fragments behind miR173 binding site in different order (Figure 3A). In addition, we chose *AG* and *ELF3* as another combination.

Both *35S:173ts_AG_FT* and *35S:173ts_FT_AG* flowered late in long day as *ft-10* mutant and had a similar floral patterning defects as *ag-2* plants (Figure 3B). However, we only observed a strong gene silencing effect in the situation which the respective fragment is located in the proximal position, suggesting an inefficient production of

sRNAs from gene fragments placed distally. Indeed, the amount of sRNAs derived from the distal fragment was decreased (Figure S1). *35S:173ts_AG_ELF3* and *35S:173ts_ELF3_AG* plants showed the same phenotype as *ag* and *elf3* mutants, although, at least for *AG*, the same positional effect was observed (Figure 3C).

Since the positional effect is likely due to the distance between the fragment and miR173 binding site, we tested whether the addition of another miR173 target site in front of the second fragment could recover the silencing efficiency. Plants carrying the construct *35S:173ts_AG_173ts_FT*, which has miR173 binding sites in front of both fragments, were generated (Figure 3D). As expected, introduction of a second miR173 binding site was sufficient to cause strong silencing of *AG* and *FT* when the respective fragments were located in the distal position (Figure 3D). Taken together, we conclude that MIGS can be successfully used for down-regulation of multiple genes at the same time.

Species-wide Usage of MIGS

MIGS requires miR173 as a trigger. Unfortunately, miR173 is a species-specific miRNA, which is only present in *A. thaliana* and a few closed-related species and some citrus [27-30]. However, as miRNA processing and tasiRNA generating machinery is well conserved among plants[5], it is possible to apply MIGS to other plant species by co-expression of miR173. To this end, we infiltrated *Nicotiana benthamiana* leaves with the Agrobacteria harboring *35S:3x_YFP* and *35S:173ts_YFP,* in the presence or absence of the miR173 (*35S:miR173*). As seen in Figure 4, co-expression of *35S:173ts_YFP* and *35S:miR173* caused a strong decrease of YFP florescence. This result suggests that MIGS may be applied to any plant species by co-expression of miR173.

A collection of plasmids for convenient use of MIGS

Compared to other gene silencing tools, generation of MIGS constructs is relatively easy. To facilitate the use of MIGS, we made a convenient and high throughput collection of MIGS plasmids. All these plasmids are based on the pGreen binary vector[31] and Gateway compatible, named as MIGS1, 2, 3, 4 and 5, followed by .1 or .2 according to their plant resistance marker, BASTA or kanamycin respectively (Figure 5).

MIGS1 series is characterized by the presence of miR173 binding site between *CaMV 35S* promoter and attR recombination site. This series allows the direct cloning of a fragment by recombination. Although our results together with sRNA sequencing data (ASRP, http://asrp.cgrb.oregonstate.edu/db/) suggest that miR173 is highly and constitutively expressed, we can't rule out the possibility that miR173 is under-expressed in the specific tissue or organs. To avoid this potential inconvenience, we developed another three series of plasmids, MIGS2-4. All the plasmids in these series consist of an expression cassette with miR173 coding sequence under a constitutive promoter, *UBQ11*. Unlike MIGS1 and MIGS2 series, MIGS3 series does not contain miR173 binding site behind *35S* promoter. Another option is available in MIGS4 series, which possesses a multiple cloning site for cloning the desirable tissue-specific promoter.

Finally, we generated a fully customized vector, MIGS5. This plasmid has a promoterless miR173 expression cassette near to one of the recombination sites and the Rubisco (RBSC) terminator in the other extremity. In this way, it is possible to use Multisite Gateway to, in on reaction, add a promoter of choice for expression of both, the miR173 and the MIGS, as well as the MIGS construct itself (Figure 5B).

Discussion

We have developed a novel method for gene silence, which we called MIGS. This method is based in the unique feature of miR173 in triggering secondary sRNA production after cleavage of its target. MIGS can be use successfully to silence one or more gene, regardless of their identities. In addition we have developed a set of vectors for convenient and inter-specie use.

Among the methods available for gene silencing in plants, VIGS, hpRNAi and amiRNAs are the most commonly used. MIGS has the advantage over the three methods regarding the practicality for its designed. With a single PCR step, miR173 target site can be fused to the sequence of interest, which can be cloned to any binary vector of choice. Alternatively, a fragment or the whole target gene can be recombine into one of our gateway compatible MIGS vectors. VIGS on the other hand, requires the use of specific viral vectors, many of them being restricted to one or few species[9,32]. In the case of hpRNAi, a fragment of the gene of interest need to be placed as an inverted repeated[12], which without the help of expensive cloning technologies, can be quite a long and tedious process. Likewise, the designed of amiRNAs requires multiple steps PCR for replacement of the mature miRNA in the precursor backbone[13].

Another great feature of MIGS is its ability to silence multiple genes. We have shown that fragment of different genes flanked by the miR173 target site can be put together in the same construct and drive silencing of more than one gene, which are not necessarily related. The same idea can also be used for VIGS, however this has the disadvantage of producing some of the symptoms associated to the virus infection[7]. For

multiple genes silencing using hpRNAi or amiRNA, co-transformation of multiple constructs, one for each gene to be silenced, would need to be performed. The designed of hpRNAi with fused fragments (one for each target gene) is theoretical possible, however, the size of such inverted repeat could be a limitation. amiRNAs can be designed to target more than one gene, however, the chance to get high efficient amiRNAs is greatly reduced by the number of target genes involved[6,13]. In addition, the characteristics of miRNA itself are a limiting factor, since most of the miRNAs spawn mainly one mature sRNA[3].

There are some issues that need to be considered when using MIGS, most of them common to VIGS and hpRNAi. The three techniques are based on the use of a gene fragment to trigger silencing. This sequence is the template for the dsRNA that will be processed by DCL; given origin to a diverse mixture of sRNAs. The first aspect to be taken in consideration is regarding the length of the gene fragment. We have tested two different size ranges, fragments with approximately 200 nt and 500 nt. Both sizes were suitable for trigger gene silencing; however phenotype of T1 population was more consistent when the bigger fragment was used (data not shown). Usually, VIGS and hpRNAi systems also make use of similar size range, with most fragments ranging from 300 to 800 nt long[9]. Nonetheless, fragments as small as 23 and 98 nt (for VIGS and hpRNAi, respectively) have been reported to trigger silencing[12,33]. It is likely that the same apply to MIGS, however experimental support is still needed.

A second consideration is the sequence of the fragment to be used. Similar to VIGS and hpRNAi, a variable set of sRNAs is produced from the template dsRNA. If the sequence chose is part of a conserved gene region, it is quite likely that the sRNAs

generated will cause silencing of not only the intended target, but also any other sequence that share this conserved region in its sequence. While this can be desirable in some situations, such as the silencing of gene families or genes with similar function, this off targeting is, in its majority, unwanted. In order to guarantee gene specificity to MIGS, it is necessary to select regions with poor conservancy. 5' and 3' untranslated regions (UTRs) are usually less conserved and therefore might be a good fragment choice. Accordingly, Wesley and colleagues[12] have shown that 5' and 3' UTRs are both good target options for hpRNAi.

In this aspect, amiRNAs have a clear advantage over the other methods, since a highly specific sRNA can be designed[13]. However, in order to do so it is necessary the prior knowledge of the plant genome in which one wants to work with. This is not a limitation for MIGS, since all the information needed for the construct designed is a fragment of the gene to be silenced. Likewise, VIGS utilization can be limited in some species due to the incompatibility of the viral vector and the plant species[7-9]. We have shown that MIGS is effective in *A. thaliana*, but also its production can be triggered in other species by co-expression of the miR173.

We also have developed a set of plasmids for convenient use of MIGS, not only in *A. thaliana*, but in other species as well. By positioning the miR173 target site just after the 35S promoter and before the recombination site we allow the fast cloning of sequences already found in gateway system. This plasmid could also be used in large scale silencing screenings of genes found in gateway compatible libraries. We have also developed a vector containing the miR173 cassete for convenient co-transformation of the miRNA and the MIGS in the specie of interest.

We have previously showed that flaking a fragment of the *CH42* gene with two miR390 target site could also drive silencing of the endogenous gene copy[15]. Therefore it is highly probable that other miRNAs involved in tasiRNA production could be used for trigger MIGS. However, in this specific case, MIGS based on the miR390 will likely not be an interesting system. miR390 is dependent on ARGONAUTE 7 (AGO7), one of the components of RISC. AGO7 expression, and most likely miR390 activity, is limited to the vascular system[34]. Therefore, constitutive expression of AGO7 would be required. This overexpression of AGO7 could cause disturbance in the normal plant physiology, since miR390/TAS3 system is involved in the juvenile to adult phase transition[35-38]. On the other hand, miR173 appears as a suitable trigger for MIGS. miR173 is a non-conserved miRNA, which usually is considered to have low or no role in the plant physiology[28,30]. Indeed, target search for tasiRNAs produced from *TAS1* and *TAS2* results in genes which seems to play a minor role to the plant[39-44]. In accordance, we could not detect any collateral phenotype associated to the overexpression of miR173 in *A. thaliana* (data not shown). However we cannot discard that, in *A. thaliana*, overexpression of miR173 in some situations could result in unwanted outcomes that could mislead the interpretation of MIGS silencing. With the discovery that miRNAs, which are 22 nt in length could start transitivity[16,17], one could envision a artificial MIGS system relying on a 22 nt amiRNA. This amiRNA would have to be designed to be neutral to the genotype where it would be applied, having as unique target, the sequence placed in front of the fragment referent to the gene to be silenced.

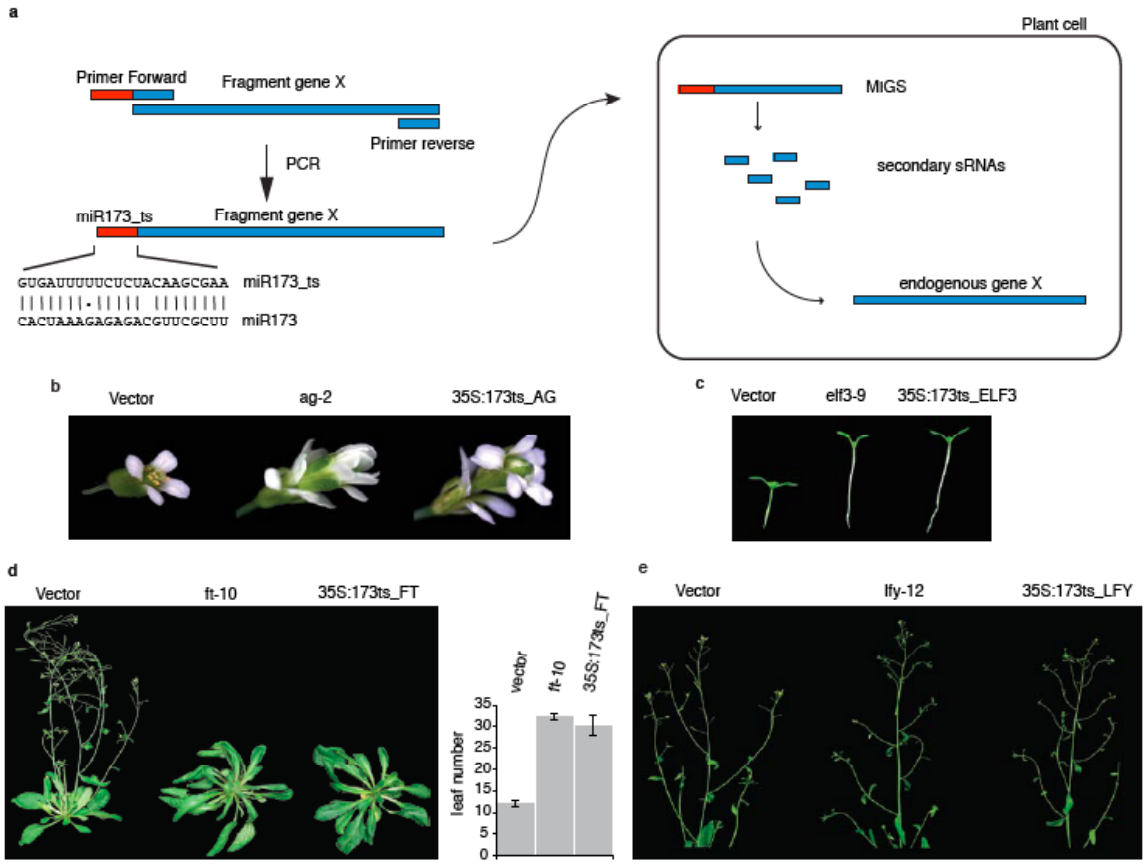

Figure 1. Silencing of single genes using MIGS. A) Scheme exemplifying the concept behind MIGS. Both, miR173 and miR173 target site (miR173_ts) are given in detail; b) Detail of the flower from a plant expressing MIGS targeting *AG* (35S:173ts_AG) compared to *AG* loss-of-function mutant; c) MIGS designed to target *ELF3* (35S:173ts_ELF3) compared to the respective mutant; d) MIGS targeting *FT* (35S:173ts_FT) results in late flowering plants similar to *ft-10*. Number of leaves before flowering is given for each line; e) Silencing of *LFY* caused by loss-of-function mutation and MIGS targeting (35S:173ts_LFY) is shown. In all cases, the vector line corresponds to a 35S:GUS transgenic plant, which was used as negative control.

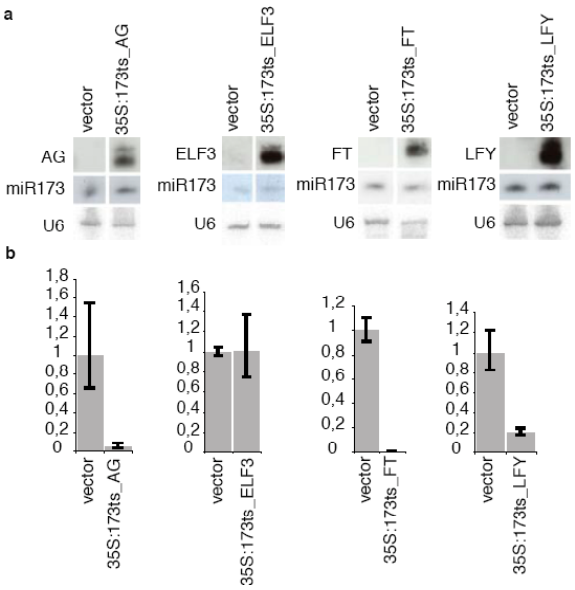

Figure 2. Molecular characterization of the single gene MIGS lines. A) sRNA blots from the different lines used. U6 was used as loading control; b) Expression of the respective target genes was measured by qRT-PCR. Average deviation is given.

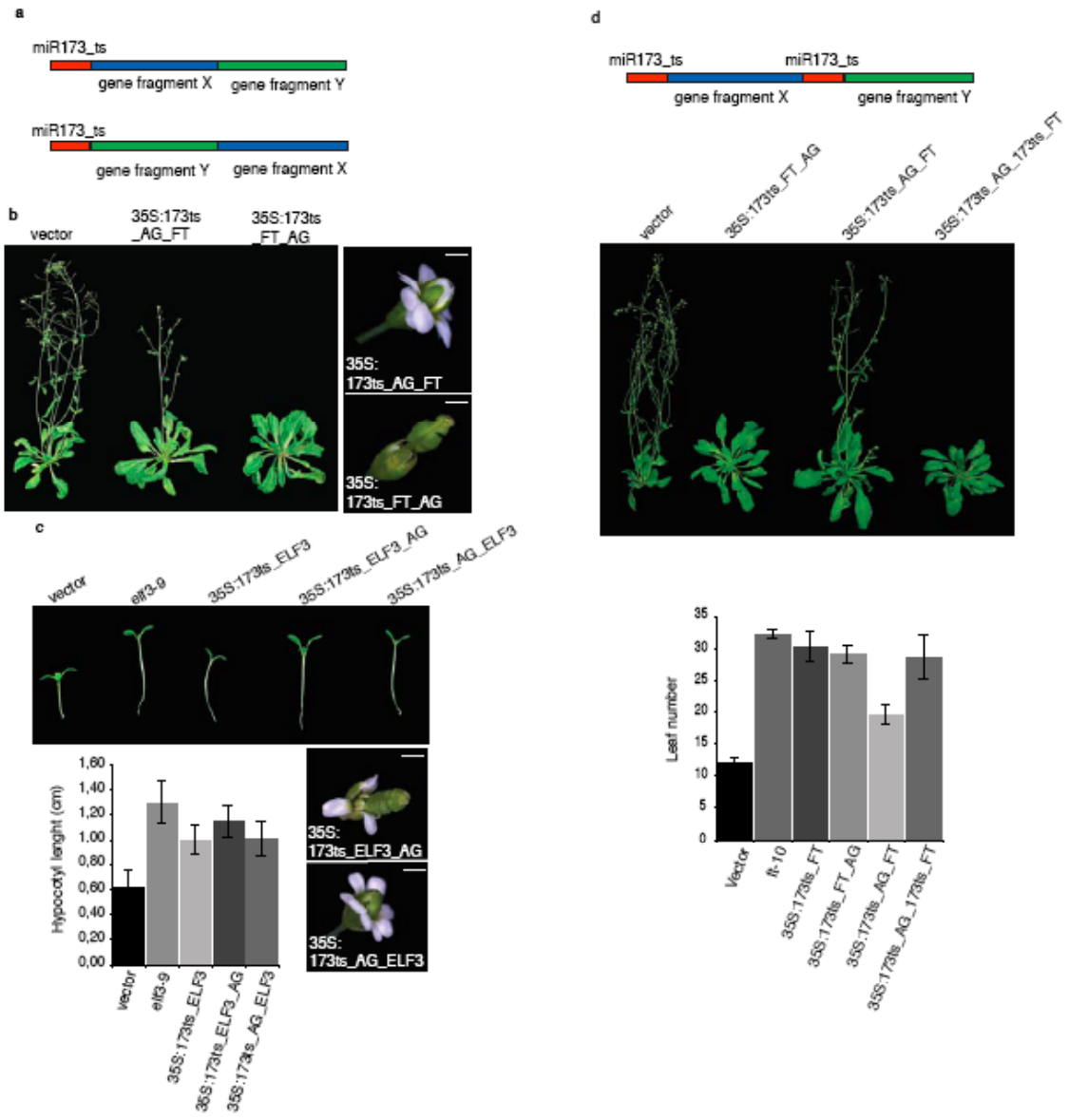

Figure 3. MIGS targeting multiple genes. A) Scheme showing the first strategy used for multiple gene silencing; b) Plants expressing MIGS for simultaneous silencing of *AG* and *FT*. Note the intermediate phenotype resulted when the fragment of the target gene is located in the distal position in the MIGS construct, c) *AG* and *ELF3* silencing triggered by the same MIGS construct. *ELF3* downregulation does not seem to be affected by positional effect, while *AG* silencing is more efficient when the gene fragment is located next to the miR173 target site. Hypocotyl length is given; d) Efficient

multiple gene silencing is achieved when miR173 target site is placed in front of each gene fragment in the MIGS construct. Flowering time is given by the leave number before flowering. Note that data relative to vector, *ft-10* and 173ts_FT is the same shown in figure 1.

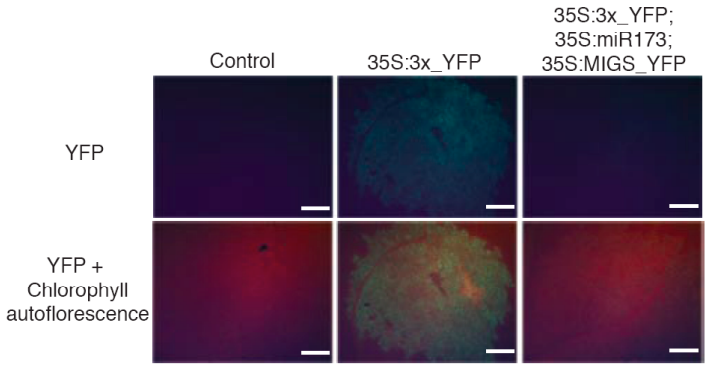

Figure 4. Agroinfiltration of MIGS in *N. benthamiana* leaves. Silencing of YFP can be seen after co-infiltration of a MIGS construct targeting *YFP* and the miR173. Control corresponds to leaves infiltrated only with infiltration buffer.

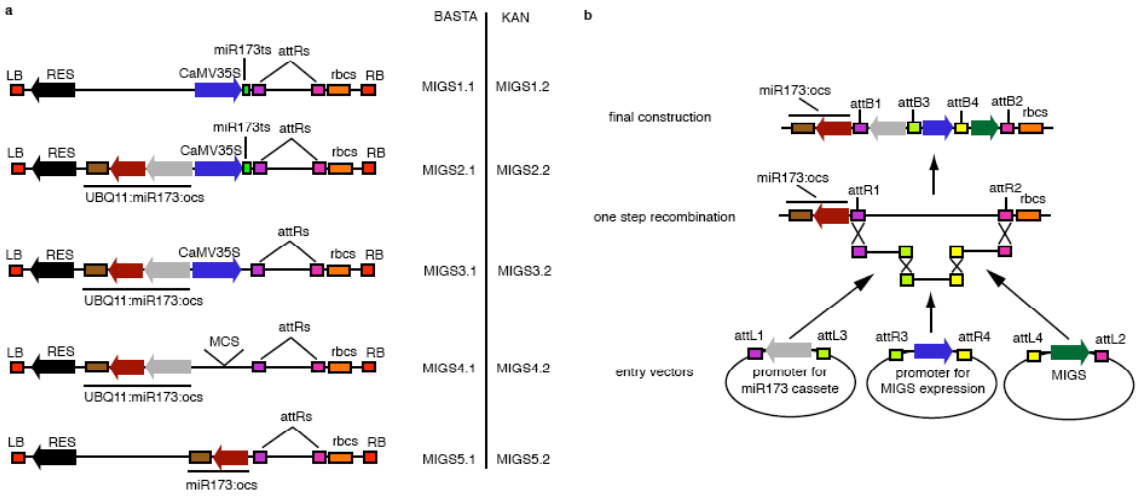

Figure 5. Vector collection for MIGS usage. a) Detail of the T-DNA region of each of the MIGS plasmids. T-DNA borders are indicated by LB (left border) and RB (right border). RES refers to the resistance marker. Vectors names are given for both, plasmids with a BASTA or kanamycin (KAN) resistance marker; b) Detail on the strategy for cloning in MIGS5 plasmids.

Material and Methods

Plant material

The plants of *A. thaliana*, accession Columbia (Col-0), and *Nicotiana benthamiana* were grown under long day (16h light:8 h dark) or short day (8 h light:16 h light) conditions. a*g-2*, *elf3-9*, *ft-10* and *lfy-12* mutants were described elsewhere[45-48].

Transgenic plants

For MIGS designed to silence one gene, miR173 target site was added in front of a 200-500 nt fragment of the target gene by PCR using cDNA as template. Specific primers were used, with forward primer having the sequence corresponding to the miR173 target site. Overlapping PCR was used to fuse the different gene fragments found in MIGS constructs targeting multiple genes. Details on the constructs as well the primers used can be found in Supplementary materials. MIGS constructs were clone into a modified version of pGreen vector[31] under the expression of the CaMV 35S promoter and used to transform *A. thalinana* plants[49] or in agroinfiltration of *N. benthamiana* leaves[50]. For the generation of the MIGS plasmid collection, *UBQ11* promoter, miR173 and OCS terminator were amplified by PCR, and fused by overlapping PCR. The resultant cassette was cloned in the modified version of pGreen vector. miR173 target site was added behind the CaMV 35S promoter using PCR. Detailed information about the plasmids can be obtained upon request.

Plant molecular analysis

Total RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA). For sRNA blots, 2,5 to 10 μg of RNA was resolved in a 17% acryl amide gel with UREA (7M). Samples were transferred to a positively charged nylon membrane and used in the hybridization. Probes for detection of sRNAs derived from the MIGS constructs were labeled with α ^{32}P-dCTP using the Prime-a-genes kit (Promega, Madison, WI, USA). Specific probes for U6 and miR173 detection were labeled with γ ^{32}P-ATP and OptiKinase™ (USB, Cleveland, OH, USA). For qRT-PCRs, 1μg of RNA was used for cDNA synthesis using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada). Probes and primers are given in Supplementary Information.

Microscopy

YFP and natural florescence of chlorophyll was visualized using a Leica MZ FLIII microscope (Leica Microsystems, Wetzlar, Germany) fitted with wide- and band-pass YFP filters and equipped with an AxioCam HRc (Zeiss, Jena, Germany) digital camera.

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Support Information for

MIGS: an efficient gene silencing approach for plant functional genomics.

Felipe Fenselau de Felippes, Jia-Wei Wang & Detlef Weigel

1) Details on the MIGS construct used in this work. miR173 target site is given underlined. In the dual gene targeting MIGS constructs, the second gene fragment is shown in ***italic and bold***. 35S:MIGS_YFP was generated using MIGS1.1 plasmid. Sequence belonging to the vector is indicated by small caps.

a) 35S:173ts_AG

GTGATTTTTCTCTACAAGCGAATCTTCTCTAGCCGTGGTCGTCTCTATGAGTA
CTCTAACAACAGTGTAAAAGGGACTATTGAGAGGTACAAGAAGGCAATATCG
GACAATTCTAACACCGGATCGGTGGCAGAAATTAATGCACAGTATTATCAAC
AAGAATCAGCCAAATTGCGTCAACAAATAATCAGCATACAAAACTCCAACAG
GCAATTGATGGGTGAGACGATAGGGTCAATGTCTCCCAAAGAGCTCAGGAAC
TTGGAAGGCAGATTAGAGAGAAGTATTACCCGAATCCGATCCAAGAAGAATG
AGCTCTTATTTTCTGAAATCGACTACATGCAGAAAAGAGAAGTTGATTTGCAT
AACGATAACCAGATTCTTCGTGCAAAGATAGCTGAAAATGAGAGGAACAATC
CGAGTATAAGTCTAATGCCAGGAGGATCTAACTACGAGCAGCTTATGCCACC
ACCTCAAACGCAATCTCAACCGTTTGATTCACGGAATTATTTCCAAGTCGCGG
CATTGCAACCTAACAATCACCATTACTCATCCGCGGGTCGCCAAGACCAAAC
CGCTCTCCAGTTAGTGTAATATAGGCTGAAGGAAATGGCC

b) 35S:173ts_ELF3

GTGATTTTTCTCTACAAGCGAATCTGATGATTCGATGGTGGATTCTATATCCA
GCATAGATGTCTCTCCCGATGATGTTGTGGGTATATTAGGTCAAAAACGTTTC
TGGAGAGCAAGGAAAGCCATTGCCAATCAACAAAGAGTATTTGCTGTTCAAC
TATTTGAGTTGCACAGACTGATTAAGGTTCAAAAACTTATTGCTGCATCACCG
GATCTCTTGCTCGATGAGATCAGTTTTCTTGGAAAAGTTTCTGCTAAAAGCTA
TCCAGTGAAGAAGCTCCTTCCATCAGAATTTCTGGTAAAGCCTCCTCTACCAC
ATGTTGTCGTCAAACAAAGGGGTGACT

c) 35S:173ts_FT

GTGATTTTTCTCTACAAGCGAACTGGAACAACCTTTGGCAATGAGATTGTGTG
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TTCGACAGCTTGGCAGGCAAACAGTGTATGCACCAGGGTGGCGCCAGAACTT
CAACACTCGCGAGTTTGCTGAGATCTACAATCTCGGCCTTCCCGTGGCCGCAG
TTTTCTACAATTGTCAGAGGGAGAGTGGCTGCGGAGGAAGAAGACTTTAGAT
GGCTTCTTCCTTTATAACCAATTGATATTGCATACTCTGATGAGATTTATGCAT
CTATAGTATTTTAATTTAATAACCATTTTATGATACGAGTAACGAACGGTGAT
GATGCCTATAGTAGTTCAATATATAAGTGTGTAATAAAAATGAGAGGGGGAG
GAAAATGAG

d) 35S:173ts_LFY

GTGATTTTTCTCTACAAGCGAAACGCCGTCATTTGCTACTCTCCGCCGCTGGT
GATTCCGGTACTCATCACGCTCTTGATGCTCTCTCCCAAGAAGATGATTGGAC
AGGGTTATCTGAGGAACCGGTGCAGCAACAAGACCAGACTGATGCGGCGGG
GAATAACGGCGGAGGAGGAAGTGGTTACTGGGACGCAGGTCAAGGAAAGAT
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CGGCAACGGAGGTAGTGGTTTGGGGACAGAGAGACAGAGGGAGCATCCGTT
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e) 35S:173ts_AG_FT

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TGGCAATGAGATTGTGTGTTACGAAAATCCAAGTCCCACTGCAGGAATTCATCG
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f) 35S:173ts_FT_AG

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GAAAATGAG***TCTTCTCTAGCCGTGGTCGTCTCTATGAGTACTCTAACAACAGT***
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CGGATCGGTGGCAGAAATTAATGCACAGTATTATCAACAAGAATCAGCCAAATT
GCGTCAACAAATAATCAGCATACAAAACTCCAACAGGCAATTGATGGGTGAGA
CGATAGGGTCAATGTCTCCCAAAGAGCTCAGGAACTTGGAAGGCAGATTAGAG
AGAAGTATTACCCGAATCCGATCCAAGAAGAATGAGCTCTTATTTTCTGAAATC
GACTACATGCAGAAAAGAGAAGTTGATTTGCATAACGATAACCAGATTCTTCGT
GCAAAGATAGCTGAAAATGAGAGGAACAATCCGAGTATAAGTCTAATGCCAGG
AGGATCTAACTACGAGCAGCTTATGCCACCACCTCAAACGCAATCTCAACCGTT
TGATTCACGGAATTATTTCCAAGTCGCGGCATTGCAACCTAACAATCACCATTA

CTCATCCGCGGGTCGCCAAGACCAAACCGCTCTCCAGTTAGTGTAATATAGGC TGAAGGAAATGGCC

g) 35S:173ts_AG_173ts_FT

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GACAATTCTAACACCGGATCGGTGGCAGAAATTAATGCACAGTATTATCAAC
AAGAATCAGCCAAATTGCGTCAACAAATAATCAGCATACAAAACTCCAACAG
GCAATTGATGGGTGAGACGATAGGGTCAATGTCTCCCAAAGAGCTCAGGAAC
TTGGAAGGCAGATTAGAGAGAAGTATTACCCGAATCCGATCCAAGAAGAATG
AGCTCTTATTTTCTGAAATCGACTACATGCAGAAAAGAGAAGTTGATTTGCAT
AACGATAACCAGATTCTTCGTGCAAAGATAGCTGAAAATGAGAGGAACAATC
CGAGTATAAGTCTAATGCCAGGAGGATCTAACTACGAGCAGCTTATGCCACC
ACCTCAAACGCAATCTCAACCGTTTGATTCACGGAATTATTTCCAAGTCGCGG
CATTGCAACCTAACAATCACCATTACTCATCCGCGGGTCGCCAAGACCAAAC
CGCTCTCCAGTTAGTGTAATATAGGCTGAAGGAAATGGCCGTGATTTTTCTCT
ACAAGCGAA***CTGGAACAACCTTTGGCAATGAGATTGTGTGTTACGAAAATCCA AGTCCCACTGCAGGAATTCATCGTGTCGTGTTTATATTGTTTCGACAGCTTGGC AGGCAAACAGTGTATGCACCAGGGTGGCGCCAGAACTTCAACACTCGCGAGTT TGCTGAGATCTACAATCTCGGCCTTCCCGTGGCCGCAGTTTTCTACAATTGTCA GAGGGAGAGTGGCTGCGGAGGAAGAAGACTTTAGATGGCTTCTTCCTTTATAA CCAATTGATATTGCATACTCTGATGAGATTTATGCATCTATAGTATTTTAATTTA ATAACCATTTTATGATACGAGTAACGAACGGTGATGATGCCTATAGTAGTTCAA TATATAAGTGTGTAATAAAAATGAGAGGGGGAGGAAAATGAG***

h) 35S:173ts_ELF3_AG

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GATCTCTTGCTC***TCTTCTCTAGCCGTGGTCGTCTCTATGAGTACTCTAACAACA GTGTAAAAGGGACTATTGAGAGGTACAAGAAGGCAATATCGGACAATTCTAAC ACCGGATCGGTGGCAGAAATTAATGCACAGTATTATCAACAAGAATCAGCCAA ATTGCGTCAACAAATAATCAGCATACAAAACTCCAACAGGCAATTGATGGGTGA***

i) 35S:173ts_AG_ELF3

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AAGAATCAGCCAAATTGCGTCAACAAATAATCAGCATACAAAACTCCAACAG
GCAATTGATGGGTGA***TCTGATGATTCGATGGTGGATTCTATATCCAGCATAGA TGTCTCTCCCGATGATGTTGTGGGTATATTAGGTCAAAAACGTTTCTGGAGAGC AAGGAAAGCCATTGCCAATCAACAAAGAGTATTTGCTGTTCAACTATTTGAGTT GCACAGACTGATTAAGGTTCAAAAACTTATTGCTGCATCACCGGATCTCTTGCT C***

j) 35S:MIGS_YFP

GTGATTTTTCTCTACAAGCGAAtctagaggatcacaagtttgtacaaaaaagcaggctttcgaattcca
agcttgcccACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGC
GATGCCACCTACGGCAAGCTGACCCTGAAGCTGATCTGCACCACCGGCAA
GCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGGGCTACGGCCTGC
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GCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCACTACCAGCA
GAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACC
TGAGCTACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCA
CATGGTCCTGCTGGAGTTCGTG

2) Probes for detection of sRNAs originated from MIGS constructs.

a) *AG*

TCTTCTCTAGCCGTGGTCGTCTCTATGAGTACTCTAACAACAGTGTAAAAG
GGACTATTGAGAGGTACAAGAAGGCAATATCGGACAATTCTAACACCGG
ATCGGTGGCAGAAATTAATGCACAGTATTATCAACAAGAATCAGCCAAAT
TGCGTCAACAAATAATCAGCATACAAAACTCCAACAGGCAATTGATGGGT
GAGACGATAGGGTCAATGTCTCCCAAAGAGCTCAGGAACTTGGAAGGCA
GATTAGAGAGAAGTATTACCCGAATCCGATCCAAGAAGAATGAGCTCTTA
TTTTCTGAAATCGACTACATGCAGAAAAGAGAAGTTGATTTGCATAACGA
TAACCAGATTCTTCGTGCAAAGATAGCTGAAAATGAGAGGAACAATCCGA
GTATAAGTCTAATGCCAGGAGGATCTAACTACGAGCAGCTTATGCCACCA
CCTCAAACGCAATCTCAACCGTTTGATTCACGGAATTATTTCCAAGTCGCG
GCATTGCAACCTAACAATCACCATTACTCATCCGCGGGTCGCCAAGACCA
AACCGCTCTCCAGTTAGTGTAATATAGGCTGAAGGAAATGGCC

b) *ELF3*

TCTGATGATTCGATGGTGGATTCTATATCCAGCATAGATGTCTCTCCCGAT
GATGTTGTGGGTATATTAGGTCAAAAACGTTTCTGGAGAGCAAGGAAAGC
CATTGCCAATCAACAAAGAGTATTTGCTGTTCAACTATTTGAGTTGCACAG
ACTGATTAAGGTTCAAAAACTTATTGCTGCATCACCGGATCTCTTGCTCGA
TGAGATCAGTTTTCTTGGAAAAGTTTCTGCTAAAAGCTATCCAGTGAAGA
AGCTCCTTCCATCAGAATTTCTGGTAAAGCCTCCTCTACCACATGTTGTCG
TCAAACAAAGGGGTGACT

c) *FT*

CTGGAACAACCTTTGGCAATGAGATTGTGTGTTACGAAAATCCAAGTCCC
ACTGCAGGAATTCATCGTGTCGTGTTTATATTGTTTCGACAGCTTGGCAGG
CAAACAGTGTATGCACCAGGGTGGCGCCAGAACTTCAACACTCGCGAGTT
TGCTGAGATCTACAATCTCGGCCTTCCCGTGGCCGCAGTTTTCTACAATTG
TCAGAGGGAGAGTGGCTGCGGAGGAAGAAGACTTTAGATGGCTTCTTCCT
TTATAACCAATTGATATTGCATACTCTGATGAGATTTATGCATCTATAGTA
TTTTAATTTAATAACCATTTTATGATACGAGTAACGAACGGTGATGATGCC
TATAGTAGTTCAATATATAAGTGTGTAATAAAAATGAGAGGGGGAGGAA
AATGAG

d) *LFY*

ACGCCGTCATTTGCTACTCTCCGCCGCTGGTGATTCCGGTACTCATCACGC
TCTTGATGCTCTCTCCCAAGAAGATGATTGGACAGGGTTATCTGAGGAAC
CGGTGCAGCAACAAGACCAGACTGATGCGGCGGGGAATAACGGCGGAGG
AGGAAGTGGTTACTGGGACGCAGGTCAAGGAAAGATGAAGAAGCAACAG
CAGCAGAGACGGAGAAAGAAACCAATGCTGACGTCAGTGGAAACCGACG
AAGACGTCAACGAAGGTGAGGATGACGACGGGATGGATAACGGCAACGG
AGGTAGTGGTTTGGGGACAGAGAGACAGAGGGAGCATCCGTTTATCGTA
ACGGAGCCTGGGGAAGTGGCACGTGGCAAAAAGAACGGCTTAGATTATC
TGTTCCACTTGTACGAACAATGCCGTGAGTTCCTTC

Table S1. Key to construct names.

Construct	Name
35S:GUS	FF087
35S:173ts_AG	FF494
35S:173ts_ELF3	FF516
35S:173ts_FT	FF493
35S:173ts_LFY	FF492
35S:173ts_AG_FT	FF523
35S:173ts_FT_AG	FF538
35S:173ts_AG_173ts_FT	FF524
35S:173ts_ELF3_AG	FF520
35S:173ts_AG_ELF3	FF521
35S:MIGS_YFP	FF597
35S:miR173	FF251
35S:3xYFP	SW241
MIGS1.1	JW493
MIGS1.2	JW640
MIGS2.1	FF570
MIGS2.2	FF573
MIGS3.1	FF537
MIGS3.2	FF574
MIGS4.1	FF571
MIGS4.2	FF575
MIGS5.1	FF572
MIGS5.2	FF576

Table S2. Oligonucleotide sequences.

Purpose	Sequence
MIGS generation	
35S:173ts_AG	GTG ATT TTT CTC TAC AAG CGA ATC TTC TCT AGC CGT GGT CGT
	GGC CAT TTC CTT CAG CCT AT
35S:173ts_ELF3	GTG ATT TTT CTC TAC AAG CGA ATC TGA TGA TTC GAT GGT GGA
	AGT CAC CCC TTT GTT TGA CG
35S:173ts_FT	GTG ATT TTT CTC TAC AAG CGA ACT GGA ACA ACC TTT GGC AAT
	CTC ATT TTC CTC CCC CTC TC
35S:173ts_LFY	GTG ATT TTT CTC TAC AAG CGA AAC GCC GTC ATT TGC TAC TCT
	GAA GGA ACT CAC GGC ATT GT
35S:173ts_AG_FT	GTG ATT TTT CTC TAC AAG CGA ATC TTC TCT AGC CGT GGT CGT
	ATT GCC AAA GGT TGT TCC AGG GCC ATT TCC TTC AGC C
	GGC TGA AGG AAA TGG CCC TGG AAC AAC CTT TGG CAA T
	CTC ATT TTC CTC CCC CTC TC
35S:173ts_FT_AG	GTG ATT TTT CTC TAC AAG CGA ACT GGA ACA ACC TTT GGC AAT
	CGA CCA CGG CTA GAG AAG ACT CAT TTT CCT CCC CCT CT
	AGA GGG GGA GGA AAA TGA GTC TTC TCT AGC CGT GGT CG
	GGC CAT TTC CTT CAG CCT AT
35S:173ts_AG_173ts_FT	GTG ATT TTT CTC TAC AAG CGA ATC TTC TCT AGC CGT GGT CGT
	TTC GCT TGT AGA GAA AAA TCA CGG CCA TTT CCT TCA GCC TA
	TAG GCT GAA GGA AAT GGC CGT GAT TTT TCT CTA CAA GCG AAC TGG AAC
	CTC ATT TTC CTC CCC CTC TC
35S:173ts_ELF3_AG	GTG ATT TTT CTC TAC AAG CGA ATC TGA TGA TTC GAT GGT GGA
	CGA CCA CGG CTA GAG AAG AGA GCA AGA GAT CCG GTG AT
	ATC ACC GGA TCT CTT GCT CTC TTC TCT AGC CGT GGT CG
	TCA CCC ATC AAT TGC CTG TTG
35S:173ts_AG_ELF3	GTG ATT TTT CTC TAC AAG CGA ATC TTC TCT AGC CGT GGT CGT
	TCC ACC ATC GAA TCA TCA GAT CAC CCA TCA ATT GCC TGT T
	AAC AGG CAA TTG ATG GGT GAT CTG ATG ATT CGA TGG TGG A
	GAG CAA GAG ATC CGG TGA TGC

35S:MIGS_YFP	ACG TAA ACG GCC ACA AGT TC
	CAC GAA CTC CAG CAG GAC CAT G
sRNA blots	
U6 (loading control)	GCT AAT CTT CTC TGT ATC GTT CC
miR173	GTG ATT TCT CTC TGC AAG CGA A

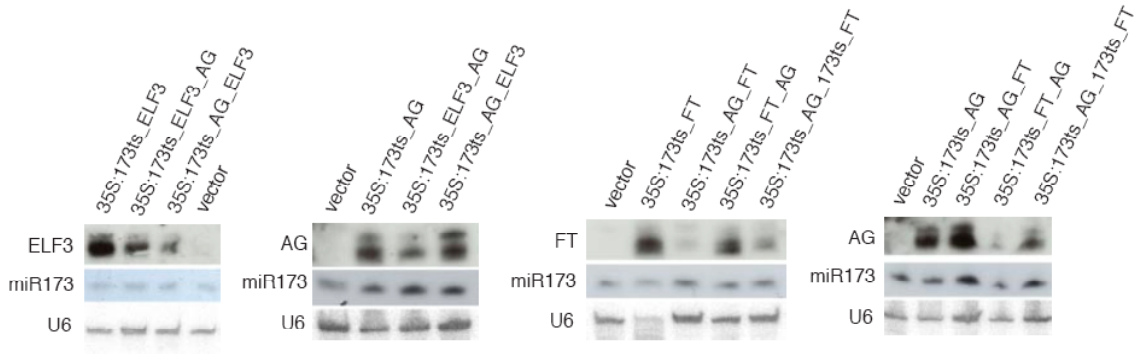

Figure S1. Molecular characterization of lines expressing MIGS designed for dual gene targeting. Note the low levels of sRNA derived from the gene fragment located at the second position and the partial recovering caused by the addition of a second miR173 target site. Vector refers to a 35S:GUS line used as transformation control. U6 was used as loading control.

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5.3 Curriculum vitae

Personal information

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2005-2010 PhD student in Molecular Biology at the Max Planck Institute for Developmental Biology, Tübingen, Germany.

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1999-2003 Bachelor degree in Biology, emphasis on Molecular, Cell and Functional biology at the UFRGS, Porto Alegre, Brazil.
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1996-1998 High school at the Colégio Militar de Brasília, Brasília, Brazil.