

TECHNICAL ADVANCE

A 22-nt artificial microRNA mediates widespread RNA silencing in *Arabidopsis*

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SUMMARY

It is known that 22-nucleotide (nt) microRNAs (miRNAs) derived from asymmetric duplexes trigger phased small-interfering RNA (phasiRNA) production from complementary targets. Here we investigate the efficacy of 22-nt artificial miRNA (amiRNA)-mediated RNA silencing relative to conventional hairpin RNA (hpRNA) and 21-nt amiRNA-mediated RNA silencing. *CHALCONE SYNTHASE* (*CHS*) was selected as a target in *Arabidopsis thaliana* due to the obvious and non-lethal loss of anthocyanin accumulation upon widespread RNA silencing. Over-expression of *CHS* in the *pap1-D* background facilitated visual detection of both local and systemic RNA silencing. RNA silencing was initiated in leaf tissues from hpRNA and amiRNA plant expression vectors under the control of an *Arabidopsis RuBisCo small subunit 1A* promoter (SSU). In this system, hpRNA expression triggered *CHS* silencing in most leaf tissues but not in roots or seed coats. Similarly, 21-nt amiRNA expression from symmetric miRNA/miRNA* duplexes triggered *CHS* silencing in all leaf tissues but not in roots or seed coats. However, 22-nt amiRNA expression from an asymmetric duplex triggered *CHS* silencing in all tissues, including roots and seed coats, in the majority of plant lines. This widespread *CHS* silencing required RNA-DEPENDENT RNA POLYMERASE6-mediated accumulation of phasiRNAs from the endogenous *CHS* transcript. These results demonstrate the efficacy of asymmetric 22-nt amiRNA-directed RNA silencing and associated phasiRNA production and activity, in mediating widespread RNA silencing of an endogenous target gene. Asymmetric 22-nt amiRNA-directed RNA silencing requires little modification of existing amiRNA technology and is expected to be effective in suppressing other genes and/or members of gene families.

Keywords: 22 nucleotide, artificial microRNA, phased small-interfering RNA, asymmetric, RNA silencing, *CHALCONE SYNTHASE*, *Arabidopsis*, technical advance.

INTRODUCTION

In plants, diverse populations of small RNAs (sRNAs) are produced by the dicing action of members of the DICER-LIKE (DCL) protein family from structurally distinct double-stranded RNA (dsRNA) precursor transcripts (Xie *et al.*, 2004). These sRNAs act both locally and as mobile signals for the regulation of gene expression, providing sequence specificity to the RNA-induced silencing complex, RISC (Palauqui *et al.*, 1997; Dunoyer *et al.*, 2010; Molnar *et al.*, 2010; Parent *et al.*, 2012).

In *Arabidopsis thaliana* (*Arabidopsis*), small-interfering RNAs (siRNAs) of 21-, 22- and 24-nucleotides (nt) in length

are processed from perfectly self-complementary dsRNA precursors by the hierarchical and redundant activity of DCL4, DCL2 and DCL3 respectively (Deleris *et al.*, 2006; Fusaro *et al.*, 2006). These dsRNAs are produced from either replicating viruses, the activity of plant encoded RNA-DEPENDENT RNA POLYMERASES (RDR) or from inverted repeat encoding genes that are transcribed into hairpin RNA (hpRNA) (Lindbo *et al.*, 1993; Waterhouse *et al.*, 1998). hpRNA transgenes and virus vectors are widely used in the knock-down of homologous transgenes, invading viruses or endogenous transcripts (Watson *et al.*,

2005). However, some instability of hpRNA transgene expression and hpRNA-mediated RNA silencing is observed, particularly in the silencing of endogenous targets, due to self-targeting for transcriptional gene silencing (TGS) and a lack of transitive amplification of the silencing signal from endogenous transcripts (Vaistij *et al.*, 2002; Himer *et al.*, 2003; Dong *et al.*, 2011).

Mature microRNAs (miRNAs) are primarily processed as ~21-nt duplexes from stem-loop miRNA precursor transcripts (pri-miRNA and pre-miRNA) by the action of DCL1 (Park *et al.*, 2002; Reinhart *et al.*, 2002). One strand of the miRNA duplex is preferentially selected by DOUBLE-STRANDED RNA BINDING1 (DRB1, also known as HYPO-NASTIC LEAVES1, HYL1) for loading into ARGONAUTE1 (AGO1), the catalytic core of RISC (Baumberger and Baulcombe, 2005; Eamens *et al.*, 2009). The loaded miRNA guides RISC to identify and subsequently cleave complementary target mRNAs via AGO1 slicer activity (Baumberger and Baulcombe, 2005). Modification of miRNA precursor transcripts to include a desired duplex sequence allows for the efficient and highly specific cleavage of selected targets via artificial miRNA (amiRNA)-directed RNA silencing (Schwab *et al.*, 2006; Eamens *et al.*, 2009, 2011). Aside from a unique class of miRNA that are processed by DCL3 into 24-nt duplexes, miRNAs are not reported to mediate DNA methylation and as such do not self-target for TGS (Wu *et al.*, 2011).

A subset of miRNA/target transcript interactions triggers RDR6-mediated dsRNA synthesis from the cleaved transcript (Peragine, 2004; Vazquez *et al.*, 2004; Allen *et al.*, 2005). The miRNA-directed cleavage site sets the register for phased dsRNA processing by components of the siRNA biogenesis pathway into predominantly 21-nt transacting siRNAs (tasiRNAs) (Allen *et al.*, 2005; Xie *et al.*, 2005; Yoshikawa *et al.*, 2005). The first tasiRNAs to be described were shown to be generated from transcripts with dual target sites for miR390, in a manner dependent on miR390-loaded AGO7 binding the 5' target site without cleavage while the 3' miR390 target site is cleaved (Axtell *et al.*, 2006; Montgomery *et al.*, 2008). Recently, 21- or 22-nt miRNAs derived from precursors that have an asymmetric single-nucleotide bulge in the miRNA/miRNA* duplex have been implicated in tasiRNA biogenesis (Chen *et al.*, 2010; Cuperus *et al.*, 2010; Manavella *et al.*, 2012). These miRNAs derived from asymmetric duplexes trigger phased siRNA (phasiRNA) production from the miRNA cleavage site to the 3' end of a target transcript, despite loading into the same AGO as non-phasiRNA triggering miRNAs, AGO1 (Cuperus *et al.*, 2010). Artificial TAS (aTAS) transcripts that harbour target sites for tasiRNA generating miRNAs mediate effective RNA silencing of complementary targets and have been demonstrated to offer greater sRNA silencing signal mobility than either amiRNAs or hpRNA-derived siRNAs (de la Luz

Gutiérrez-Nava *et al.*, 2008; Tretter *et al.*, 2008; de Felippes *et al.*, 2010, 2012).

These recent findings provide another avenue to exploit endogenous RNA silencing mechanisms through the artificially triggered production of phasiRNAs from targeted transcripts. To evaluate the capacity for endogenous, non-TAS transcripts to support phasiRNA production, and to determine the relative strength and spread of the resulting silencing signal, we took advantage of PAP1/MYB75 (AT1G56650) transcription factor-mediated over-expression of the anthocyanin pathway in the *pap1-D* background (Borevitz *et al.*, 2000; Weigel *et al.*, 2000; Shi and Xie, 2010). In *Arabidopsis pap1-D* plants, we assessed the capacity of various RNA silencing plant expression vectors to mediate widespread silencing of the endogenous *CHALCONE SYNTHASE* (*CHS*, AT5G13930) locus. *CHS* is involved in the initial steps of anthocyanin biosynthesis and is required for accumulation of pigments responsible for the brown colour of wild-type *Arabidopsis* seed coats and the purple colour of leaves and roots in *pap1-D* plants (Shirley *et al.*, 1995). Here we demonstrate that an asymmetric duplex derived 22-nt amiRNA, but not a conventional 21-nt amiRNA, can trigger phasiRNA production from an endogenous, non-TAS transcript. Furthermore, we show that the amplification and diversification of the RNA silencing signal enhances both the strength and spread of the 22-nt amiRNA-mediated silencing phenotype.

RESULTS

Widespread silencing with an SSU promoter-driven hpRNA targeting a GFP transgene

The expression domain of the *Arabidopsis RuBisCo small subunit 1A* (AT1G67090) gene promoter (SSU) was assessed by visual screening of fluorescence in lines transformed with an SSU promoter-driven GFP transgene (SSU_GFP). GFP fluorescence was markedly weaker in SSU_GFP transformants than in plant lines that expressed a *Cauliflower mosaic virus* 35S promoter (35S) driven GFP transgene (35S_GFP). GFP fluorescence in SSU_GFP transformants was also restricted to developing aerial tissues, including the hypocotyl, cotyledons, petioles and leaves (Figure 1a). Despite this low level and localised SSU promoter-driven expression, a hpRNA targeting a 278-bp region of GFP (the central 'F' region in SSU_hp'F', T1 plants, *n* = 2) efficiently silenced the 35S_GFP transgene in all plant tissues of two independent transgenic lines when these alleles were combined by genetic crossing (Figure 1a). This widespread silencing of GFP was observed despite the low level of hpRNA-derived siRNAs detected by northern blotting (Figure 1b). Transitive production of siRNAs corresponding to the 'P' region of GFP (downstream of the hpRNA targeted 'F' region) was readily

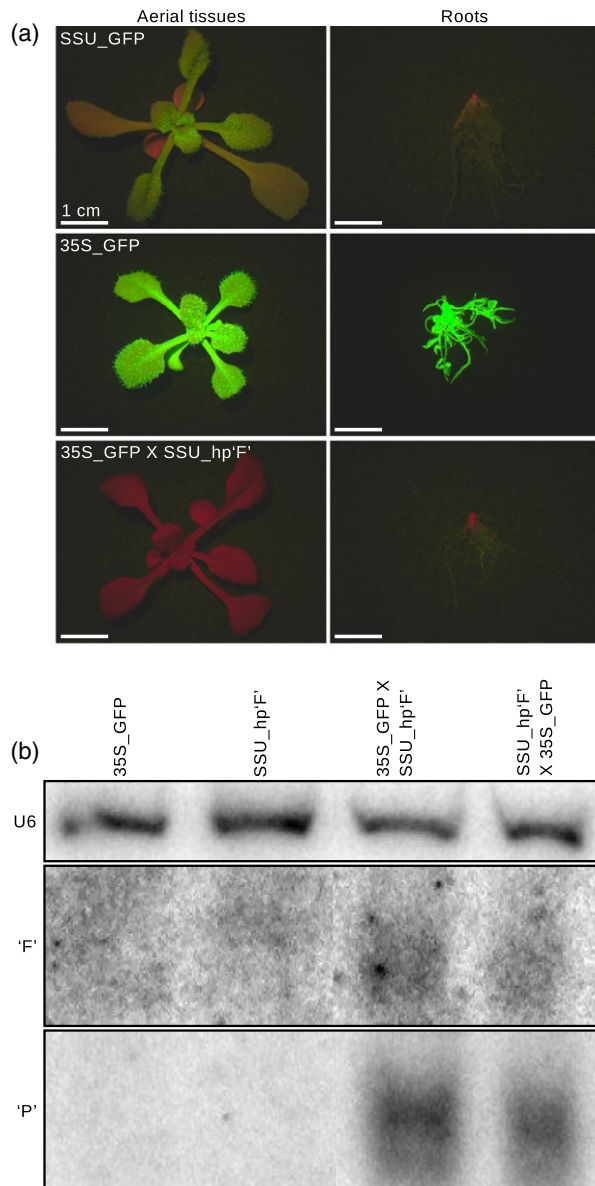


Figure 1. SSU and 35S promoter-driven GFP expression in Arabidopsis and silencing directed by SSU_hp'F'.

(a) GFP fluorescence was restricted to developing aerial tissues including the hypocotyl, cotyledons, petioles and young leaves in SSU_GFP lines, however fluorescence was both ubiquitous and constitutive in 35S_GFP transformants. Genetic crosses were performed between two stably expressing 35S_GFP lines and two SSU_hp'F' T1 plants. F1 progeny that harboured both alleles displayed systemic GFP silencing with only a slightly visible fluorescence in some root tissues of a small number of F1 plants.

(b) Transitive siRNAs were produced from the GFP CDS 'P' region (downstream of the SSU_hp'F' targeted region) in F1 plants from reciprocal crosses that expressed both the 35S_GFP and the SSU_hp'F'.

detected, a finding that suggested that the observed silencing phenotype was mostly directed by this siRNA class (Figure 1b). The strong systemic silencing response associated with RDR6-dependent transitive siRNA production is

well documented in hpRNA-directed silencing of transgenes (Vaistij *et al.*, 2002).

Silencing is restricted in SSU_hpCHS plants

In contrast with the strong systemic silencing response observed in hpRNA-directed silencing of GFP, a similar SSU promoter-driven hpRNA transgene targeting *CHS* (SSU_hpCHS) triggered only limited silencing in rosette leaves of *pap1-D* transformants (T1 plants, $n = 21$). Anthocyanin accumulation persisted in the hypocotyl, young leaves, petioles, roots and seed coat of *pap1-D*/SSU_hpCHS plants (Figure 2) and appeared to have no effect on anthocyanin accumulation in wild-type *Arabidopsis* (Col-0) transformants (SSU_hpCHS, T1 plants, $n = 19$).

The accumulation of hpRNA-derived *CHS* 'H' region-specific siRNAs in tissues sampled from SSU_hpCHS transformed plants was only detectable in *pap1-D* transformants and is thought to remain below detection sensitivities in Col-0 transformants (Figure 3). Surprisingly, a low level of transitively produced 'S' region-specific siRNAs were also detected in *pap1-D* transformants (Figure 3). These results suggest limited transitive amplification of the silencing signal from the over-expressed endogenous *CHS* transcript in the *pap1-D* background may account for the detectable quantity of both 'H' and 'S' region-specific siRNAs in these plant lines.

Enhanced local silencing in SSU_amiR21-CHS lines

An SSU promoter-driven amiRNA vector was designed to produce symmetric 21-nt duplexes that targeted *CHS* (SSU_amiR21-CHS) in the *Arabidopsis MIR159B* backbone (Eamens *et al.*, 2009, 2011). SSU_amiR21-CHS transformed plants from both Col-0 (T1 plants, $n = 3$) and *pap1-D* backgrounds (T1 plants, $n = 21$) displayed silencing of *CHS* in all aerial tissues except the seed coat (Figure 4). Anthocyanin accumulation persisted in the roots of *pap1-D* transformants, but not to the extent observed in non-transformed controls (Figure 4).

CHS silencing was evident in young leaves and petioles, tissues in which the SSU_hpCHS transgene had caused no observable effect. This finding may suggest that the processing or effector components of the siRNA pathway are limited in these tissues. However, the observation that higher levels of hpRNA expression are able to overcome this limitation, as reported for 35S_hpCHS plants (Figure 2), does not support this explanation. An alternative possibility is that hpRNA-derived siRNAs take longer to accumulate to an effective level than amiRNAs in these tissues.

Widespread silencing in SSU_amiR22-CHS lines

A vector similar to SSU_amiR21-CHS was designed to yield asymmetric 22-nt miRNA/21-nt miRNA* duplexes. Twenty-two nucleotide amiRNA vectors were generated by deleting

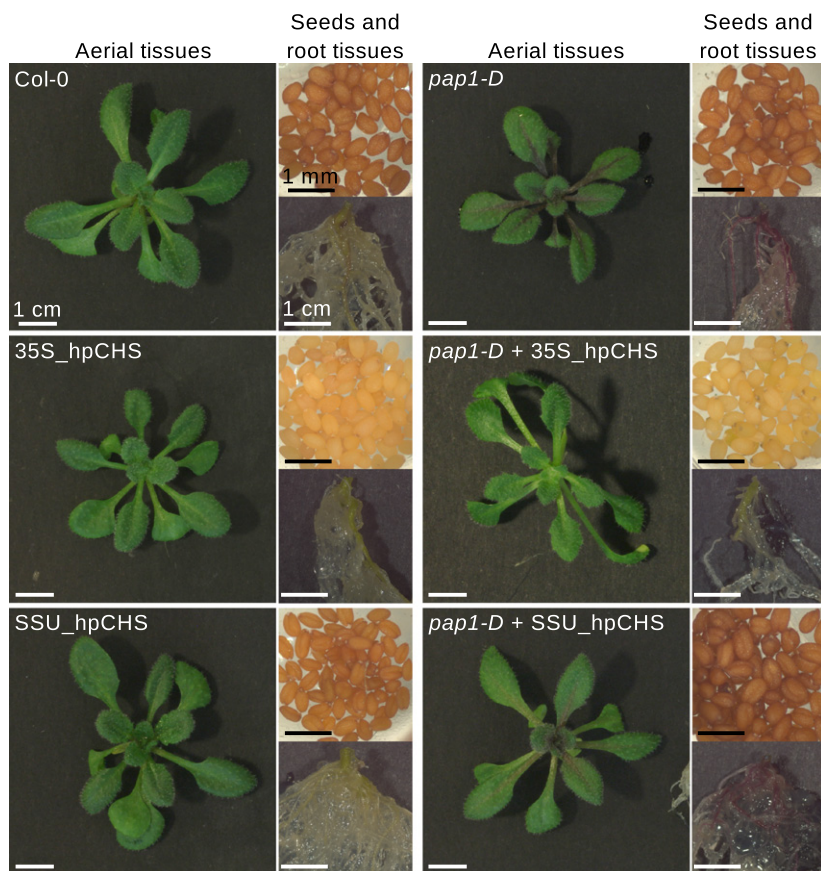


Figure 2. 35S and SSU promoter-driven hpRNA-mediated silencing of *CHS* in Arabidopsis Col-0 and *pap1-D* backgrounds. 35S_hpCHS reduced anthocyanin accumulation in all tissues of Col-0 and *pap1-D* plants. Tissue restricted silencing of CHS was evident in mature leaves of *pap1-D*/SSU_hpCHS plants.

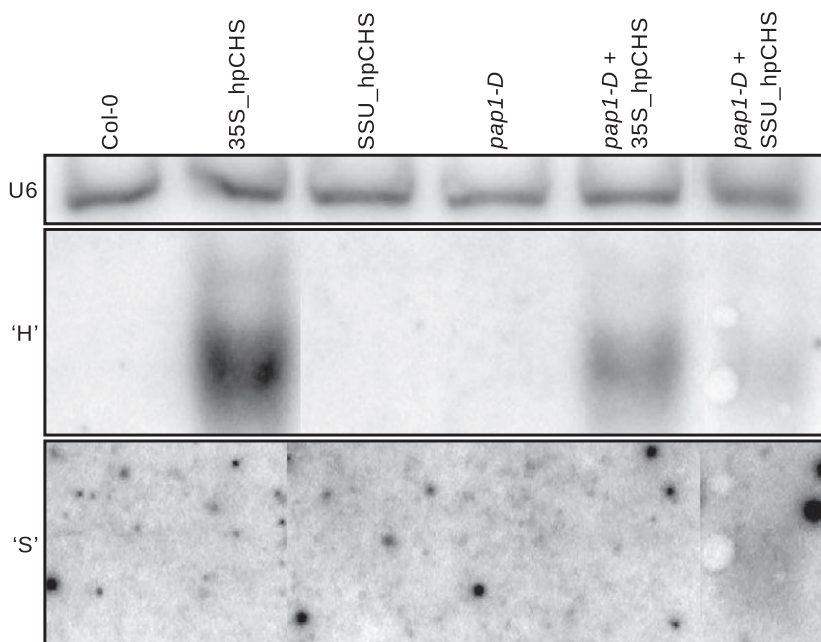


Figure 3. Northern blot analysis of sRNAs in Arabidopsis plants that harboured the 35S_hpCHS and SSU_hpCHS transgenes. 'H' region-specific siRNAs were readily detected in 35S_hpCHS and *pap1-D*/35S_hpCHS plants and to lower levels in the *pap1-D*/SSU_hpCHS line. Transitive, 'S' region-specific siRNAs only accumulated to detectable levels in *pap1-D*/SSU_hpCHS plants.

a single nucleotide from the miRNA* strand sequence, a modification that results in the production of a 22-nt guide strand (Figure S1). RNA silencing was readily obser-

vable in Col-0 (T1 plants, $n = 42$) and *pap1-D* (T1 plants, $n = 27$) backgrounds transformed with the 22-nt, CHS-targeting amiRNA vector (SSU_amiR22-CHS). As with the

SSU_amiR21-CHS vector, *CHS* silencing was observed in all aerial tissues, however *CHS* silencing was also observed in both seed coats and roots of some SSU_amiR22-CHS lines (Figure 4). In the Col-0 background (Col0/SSU_amiR22-CHS plants), 34/42 T1 plants produced at least some yellow seed, including 19 plants that produced yellow seed only. In the *pap1-D* background (*pap1-D*/SSU_amiR22-CHS plants), 24/27 T1 plants produced at least some yellow seed and 12 of these produced yellow seed only. The widespread silencing of *CHS* expression may be mediated by mobile phasiRNAs that are generated in high concentrations in neighbouring tissues. An alternative explanation may be that low levels of SSU promoter expression in the seed coat is only detected due

to the strong silencing response mediated by phasiRNA production in these lines.

Northern blotting confirmed the size of the sRNA silencing signal produced from the two amiRNA vectors (21-nt and 22-nt respectively), and confirmed the transitive production of phasiRNAs from the region downstream of the amiR22-CHS cleavage site (Figure 5). Accumulation of *CHS*-specific phasiRNA species was lower in Col-0 plants, a finding that suggested that phasiRNA production scales with target transcript abundance (Figure 5). The lack of silencing in the seed coats and roots of some SSU_amiR22-CHS lines was associated with lower levels of both the triggering 22-nt amiRNA and the resulting phasiRNAs in both Col-0 and *pap1-D* transformants. These results

Figure 4. Artificial miRNA-directed *CHS* silencing in Col-0 and *pap1-D*.

The 21-nt amiRNA (SSU_amiR21-CHS)-mediated an efficient local silencing response in all aerial tissues. The 22-nt amiRNA (SSU_amiR22-CHS)-mediated widespread silencing, including the seed coats of most plants (34/42 in Col-0/SSU_amiR22-CHS and 24/27 in *pap1-D*/SSU_amiR22-CHS). The SSU_amiR22-CHS transformed plants that did not display systemic silencing expressed a phenotype comparable with that displayed by SSU_amiR21-CHS transformed plants.

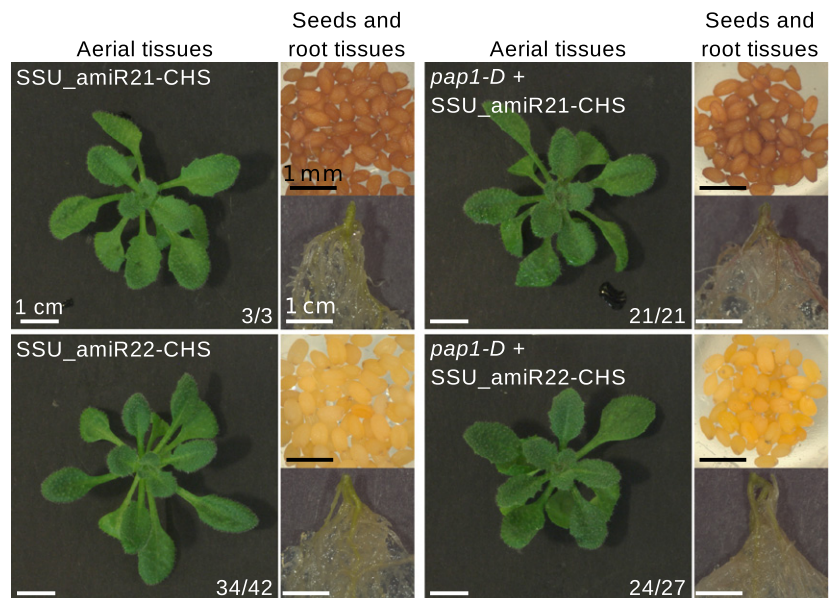
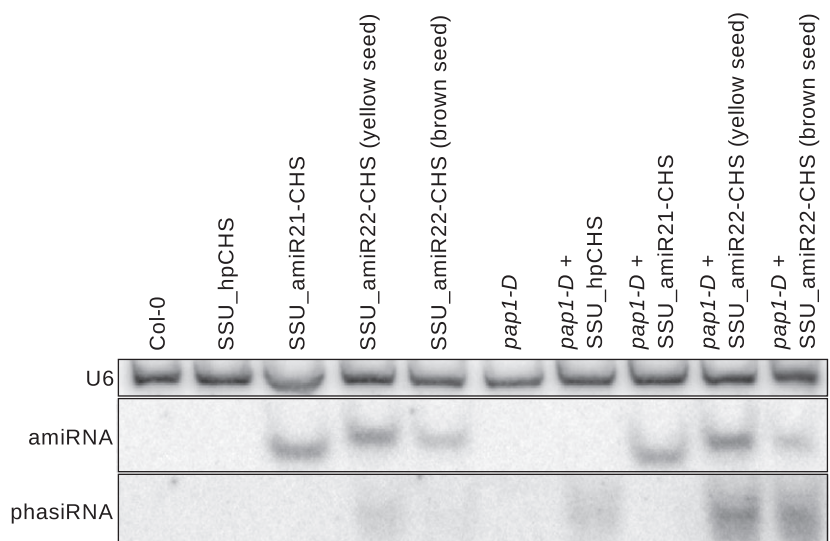


Figure 5. Northern blot analysis of sRNA accumulation in SSU_amiR21-CHS and SSU_amiR22-CHS plants.

Among the amiRNA expressing lines, the accumulation of sRNAs specific to the sequence downstream of the amiRNA cleavage site (phasiRNA accumulation) was only detected SSU_amiR22-CHS transformed lines. SSU_amiR22-CHS transformed lines that displayed widespread *CHS* silencing (yellow seed) accumulated both the 22-nt amiRNA and phasiRNAs to higher levels than lines that displayed only local silencing (brown seed). The SSU_hpCHS transgene contains sequences that overlap with the phasiRNA probe, however these sequences were only detected in the *pap1-D* background.



mirror the observed efficacy of amiRNAs in local silencing and suggest a requirement for a strong local silencing signal to mediate a widespread silencing response (Figure 5).

Characterisation of phasiRNA production by sRNA sequencing

Sequencing of sRNA-only fractions confirmed the accumulation of hpRNA-derived 'H' region siRNAs, and transitively produced 'S' region siRNAs in the *pap1-D* background expressing the SSU_hpCHS vector (Figure 6 and Table S2). No abundant 'C' region (5' of 'H')-specific siRNAs were detected, and indicated a predominantly 5' to 3' transitive spread (Figure 6 and Table S2). In *pap1-D*/SSU_amiR21-CHS and *pap1-D*/SSU_amiR22-CHS lines, the most abundant sRNA reads corresponded to the predicted amiRNA guide strands (Figure 6 and Table S3). In contrast with SSU_amiR21-CHS, the SSU_amiR22-CHS vector triggered production of abundant phasiRNAs 3' from the miRNA target site (Figure 6 and Table S4). These phasiRNAs were demonstrated to be in phase with the predicted amiRNA-directed cleavage site, and further validated the 22-nt amiRNA as the trigger for their production (Table S5).

22-nt amiRNA-mediated widespread CHS silencing requires RDR6 activity

To confirm that phasiRNA production was responsible for the widespread silencing phenotype observed in SSU_amiR22-CHS lines, plants that expressed strong amiRNA-directed silencing phenotypes were genetically crossed

with the RDR6 defective plant line, *rdr6* (*sgs2-1*) (Elmayan *et al.*, 1998; Mourrain *et al.*, 2000). F2 progeny, confirmed to be homozygous for *rdr6* and to also harbour the SSU_amiR22-CHS transgene, displayed local silencing as observed in SSU_amiR21-CHS lines (Figure 7a). However, anthocyanin accumulation persisted in roots and seed coats in the *rdr6* background despite localised miRNA-directed RNA silencing (Figure 7a). The phasiRNA dependent nature of the widespread silencing phenotype was further validated by the lack of detectable phasiRNA production in all plants that harboured the RDR6 mutation (Figure 7 b).

DISCUSSION

hpRNA-mediated silencing

The expression domains observed in SSU_GFP expressing plant lines overlapped closely with those previously reported for a similar promoter sequence, a 1971-bp region upstream of the *Arabidopsis RuBisCo small subunit 1A* (*AT1G67090*) locus (Sawchuk *et al.*, 2008). This promoter fragment was reported to direct expression in cotyledons, leaves, flowers, siliques and in the root tips of developing embryos and young seedlings. Expression was also reported to be conspicuously absent from the vasculature, including progenitor preprocambial cells (Sawchuk *et al.*, 2008). Despite the restricted expression of the SSU promoter, complete systemic silencing of a constitutively expressed 35S promoter-driven GFP transgene was

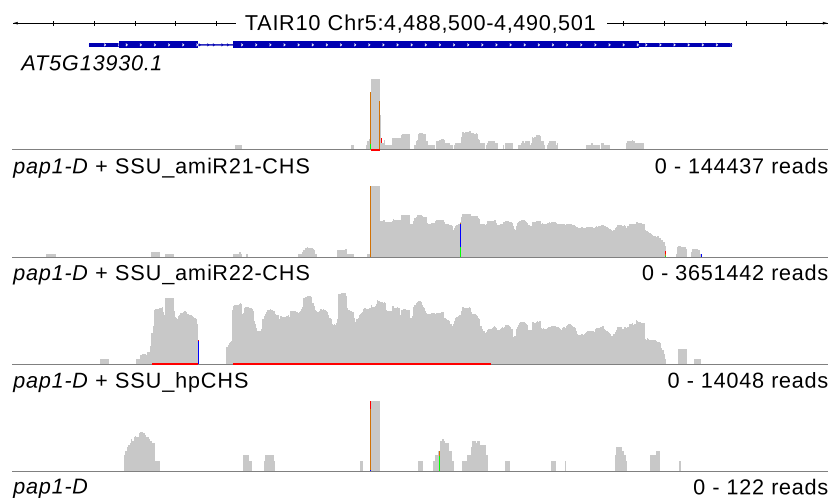


Figure 6. Mapping of CHS-specific sRNAs in *pap1-D*/SSU_amiR21-CHS, *pap1-D*/SSU_amiR22-CHS and *pap1-D*/SSU_hpCHS plants.

Alignments were performed allowing for 1 mismatch (indicated by vertical coloured lines) due to the single-nucleotide mismatch in the SSU_amiR22-CHS duplex structure. Absolute read values are presented with a Log10 scaled y-axis with a sample specific range defined on the right below each plot. Horizontal red bars beneath each sample plot represent the region of homology to the predicted transgene derived sRNAs (amiR21-CHS, amiR22-CHS or hpCHS). The SSU_amiR21-CHS-expressing line accumulated few siRNAs from the region downstream of the amiRNA-directed cleavage site compared with the SSU_amiR22-CHS-expressing line. The SSU_hpCHS-expressing line accumulated mostly hpRNA-derived siRNAs, although some siRNAs accumulated from the downstream region. The *pap1-D* background reported some reads including 122 counts corresponding to the SSU_amiR22-CHS derived miRNA. This situation is thought to be due to the abundance of this read in the SSU_amiR22-CHS-expressing line and some error in de-multiplexing of Illumina barcodes.

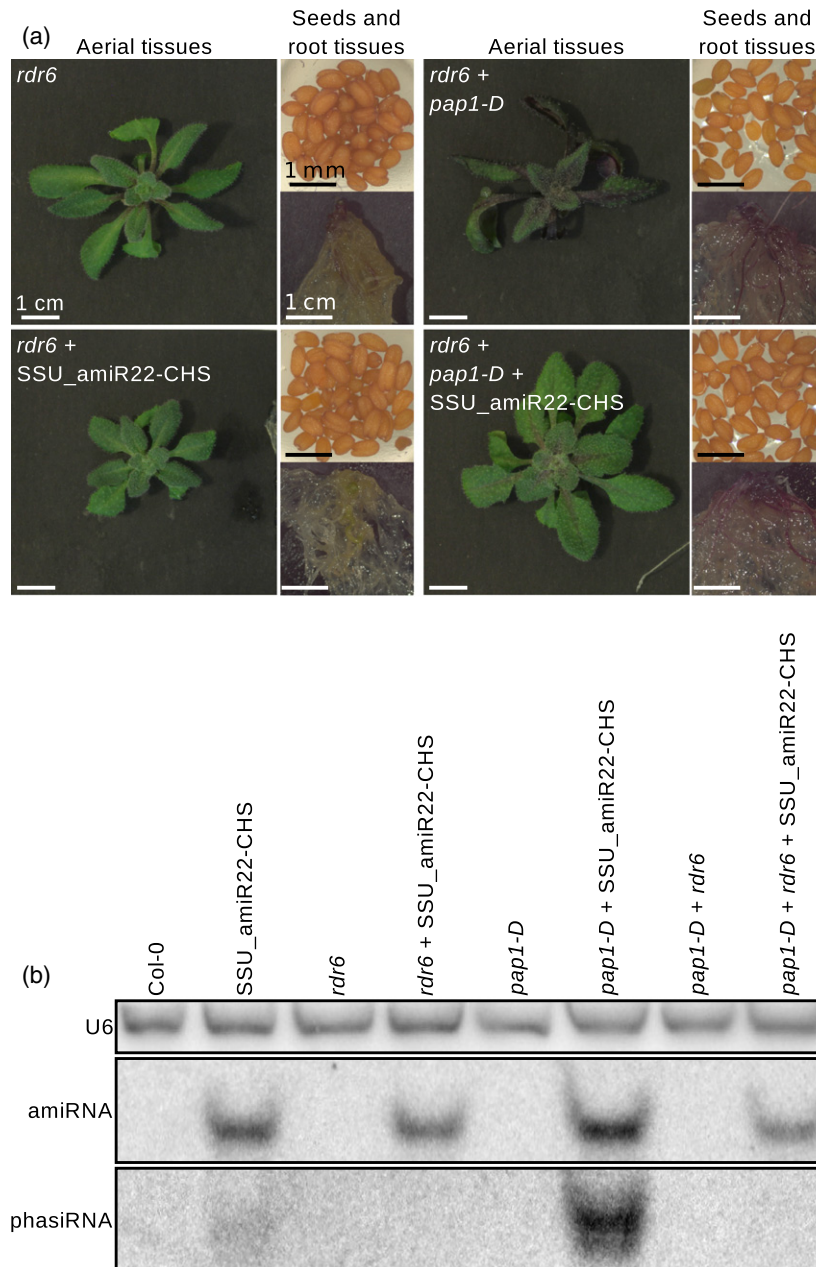


Figure 7. Widespread *CHS* silencing is dependent on RDR6 catalysed dsRNA production and associated phasiRNA accumulation.

(a) SSU_amiR22-CHS expression mediated a local, but not a systemic, silencing response in the *rdr6* mutant background. A SSU_amiR22-CHS line in the *pap1-D* background that displayed the systemic silencing phenotype was crossed with an *rdr6* mutant (*sgs2-1*) line. The systemic silencing phenotype was lost in F2 plants that were homozygous for the *rdr6* mutant allele, despite harbouring the SSU_amiR22-CHS transgene and displaying a strong local silencing phenotype. (b) Northern blot assessment of sRNA accumulation, amiRNA and phasiRNAs, in the presence or absence of RDR6 activity.

triggered by the SSU_hp'F' construct. This systemic silencing was associated with the transitive production of siRNAs from the region 3' of the hpRNA targeted fragment, as reported previously (Braunstein *et al.*, 2002; Vaistij *et al.*, 2002).

In our assays, the SSU promoter-driven expression of hpCHS did not display systemic spread of endogenous *CHS* silencing in either the wild-type or the upregulated

pap1-D background. This finding is in contrast with the systemic RNA silencing observed when the endogenous *NITRATE REDUCTASE* (*NIA*) was upregulated in *Nicotiana tabacum* (Palauqui and Vaucheret, 1998). Importantly, however, transactively produced siRNAs were detected in the *pap1-D* background and suggested that high levels of expression are likely to promote a systemic silencing response.

Transitively produced siRNAs were only detected in lines that expressed the SSU_hpCHS, and not in lines that expressed a similar hpRNA fragment under the control of the 35S promoter. It is possible that the strong 35S promoter-driven silencing signal prevents the accumulation of transitively produced siRNAs, probably due to a higher rate of primary siRNA-directed degradation of targeted transcripts. These targeted transcripts may otherwise serve as a template for RDR6 catalysed dsRNA production. In these experiments, the detection of transitive siRNAs in the presence of the relatively weak SSU promoter-driven hpCHS (compared with the constitutive and ubiquitous 35S_hpCHS) was further facilitated by the high level of *CHS* expression in the *pap1-D* background.

SSU_hpCHS-mediated silencing was observed in all tissues of mature leaves, including the vasculature, despite this region being reported to lack SSU promoter-driven expression (Sawchuk *et al.*, 2008). This result is probably due to the 10–15 cell spread of the transitivity independent local silencing signal (Himber *et al.*, 2003).

21-nt amiRNA-mediated RNA silencing

Intriguingly, silencing of *CHS* was not observed in petioles and young leaves of lines that expressed the SSU_hpCHS vector, a result that may be attributed to restricted SSU promoter expression (Sawchuk *et al.*, 2008). However, SSU promoter-driven amiRNA (SSU_amiR21-CHS)-mediated *CHS* silencing was efficient in clearing anthocyanin from these tissues in transformed plants. This result suggests an inefficiency of hpRNA-derived siRNA production and/or reduced siRNA-directed RNA silencing efficiency as compared with amiRNA-directed silencing, at least in these tissues. The efficient silencing in SSU_amiR21-CHS lines was still limited in its distribution, with persistent anthocyanin accumulation in roots and seed coats, reflecting limited or absent SSU promoter expression in these tissues.

22-nt amiRNA-mediated silencing

The limited distribution of SSU promoter-driven amiRNA and hpRNA-mediated *CHS* silencing was overcome by the use of a 22-nt amiRNA that triggers phasiRNA production. The 22-nt amiRNA itself functions effectively in local silencing, as evidenced in *rdr6* mutants that displayed similar silencing distribution to 21-nt amiRNA lines.

The production of phasiRNAs in SSU_amiR22-CHS lines was shown to mediate widespread silencing of *CHS*, indicated by clearing of anthocyanin from roots and seed coats in many of these lines. This enhanced silencing may be attributed to the greater mobility of phasiRNAs (Tretter *et al.*, 2008; de Felippes *et al.*, 2010) and/or the additive effect of both amiRNA- and phasiRNA-directed target mRNA cleavage.

It was noted that accumulation of phasiRNAs was greater in the *pap1-D* background, suggesting the production of

this silencing signal is in proportion to target transcript abundance. This scaling of phasiRNA production would be expected to reduce the likelihood of interference with developmentally important regulation that might occur with saturation of components of the silencing machinery, such as AGO1 (Martínez de Alba *et al.*, 2011). As such, the relative efficiency and dynamic response of 22-nt amiRNA-directed silencing may be advantageous over hpRNA- and conventional amiRNA-directed silencing strategies that rely on the production of high levels of primary sRNAs.

CONCLUSIONS

The limited distribution of SSU promoter-driven 21-nt amiRNA- and hpRNA-mediated RNA silencing of an endogenous target gene, *CHS*, was overcome by the use of a 22-nt amiRNA vector. In addition to highly efficient miRNA-directed RNA silencing, 22-nt amiRNAs trigger the production of transitive and reportedly highly mobile phasiRNAs. 22-nt amiRNA-directed phasiRNA production also offers the advantages of a heterogeneous sRNA population allowing multiple target complementarity not possible with conventional amiRNA vectors. This simple modification to existing amiRNA-directed silencing technologies should facilitate the triggering of widespread and efficient RNA silencing of target genes and/or gene families in diverse species.

EXPERIMENTAL PROCEDURES

Vectors

The constructs used in this paper are outlined in the supplementary materials (Figure S2). Amplicons were generated with the Expand Long Template PCR system (Roche Diagnostics, Sydney, Australia) and region-specific primers (Table S1), and cloned into the pGEM-T Easy cloning vector (Promega, Sydney, Australia) for restriction enzyme digestion-based cloning.

The 35S_GFP transgene was constructed in the pORE04 backbone and carries the 35S promoter and GFP CDS between *NotI* and *KpnI* sites and the HSP terminator between *MluI* and *NcoI* sites (Coutu *et al.*, 2007; Nagaya *et al.*, 2009). The SSU_GFP vector consists of the expression cassette from pBSF12 inserted into pORE04 (*NheI/EcoRI* digest) with the *sfa8* fragment including KDEL (*BamHI/XhoI* digest) replaced with the GFP CDS (Tabe *et al.*, 1995; Christiansen *et al.*, 2000; Coutu *et al.*, 2007). The SSU_GFP expression cassette includes the SSU promoter (1720-nt of upstream sequence from *Arabidopsis RuBisCo small subunit 1A* (AT1G67090) finishing 5-nt upstream of the start codon) and a *Nicotiana tabacum* RuBisCo small subunit terminator sequence. Vector p3-SSU carries the SSU promoter released from SSU_GFP (*BamHI/SalI* digest) and directionally cloned into pORE03 (Coutu *et al.*, 2007).

The 'F' sequence (oligos GFP'F'-F and GFP'F'-R_CACC) of the GFP CDS was cloned into pENTR-D via the use of TOPO Cloning technology (Life Technologies, Melbourne, Australia). Subsequently, the 'F' fragment was recombined into pHELLSGATE12 (Helliwell and Waterhouse, 2003) using LR Clonase II enzyme

mix (Life Technologies) to create the inverted repeat vector, pHG12-hpF'. The 'F' inverted repeat was released as a *NotI* fragment and inserted into a similarly digested p3-SSU to create vector SSU_hpF'.

A central region of the *CHS* CDS, termed the 'H' region, was amplified from a clone of the *CHS* CDS with two sets of primers that contained different restriction endonuclease overhangs at their 5' termini (CHS'H'-F_*KpnI*/CHS'H'-R_*XhoI* and CHS'H'-F_*Clal*/CHS'H'-R_*BamHI*, Table S1). These amplicons were cloned into pOP, a modified pORE03 vector that harboured the PDK intron from pHANNIBAL (Wesley *et al.*, 2001) between *XhoI* and *SpeI* sites, using the terminal restriction sites to produce pOP_hpCHS. The inverted repeat was transferred from pOP_hpCHS to p3-SSU between *MluI* and *XhoI* sites to create vector SSU_hpCHS.

The Arabidopsis *MIR159B* backbone of pBlueGreen (Eamens *et al.*, 2009; Eamens and Waterhouse, 2011), including the *LacZ* gene for blue/white selection was released by *XhoI* and *Clal* digestion and directionally cloned into a *Sall* and *Clal* digested p3-SSU vector to create vector SSU_amiR. amiR21-CHS and amiR22-CHS amplicons (CHSmiR-R with CHSmiR-F or CHSmiR22-F respectively) were digested with *Lgl* and cloned into the similarly digested SSU_amiR vector to produce vectors SSU_amiR21-CHS and SSU_amiR22-CHS respectively (Eamens and Waterhouse, 2011). Artificial miRNA primer design for entry into pBlueGreen vectors is automated at <http://www.p-bluegreen.com>.

Plant lines

The *pap1-D* line (Borevitz *et al.*, 2000; Weigel *et al.*, 2000; Shi and Xie, 2010) was obtained from Mandy Walker (CSIRO Plant Industry, Adelaide). Arabidopsis transformation was performed by *Agrobacterium tumefaciens* (*Agrobacterium*)-mediated transformation (Clough and Bent, 1998; Martinez-Trujillo *et al.*, 2004). The 35S_hpCHS line is transformed with pHANNIBAL-hpCHS as described previously (Wesley *et al.*, 2001). Selection for pORE04 derived vectors was performed on MS media that contained 20 mg L⁻¹ kanamycin sulfate and for pORE03 derived vectors on soil by spraying with 0.2 g L⁻¹ Basta™. In the *pap1-D* background, selection of plant lines that harboured the SSU_hpCHS, SSU_amiR21-CHS and SSU_amiR22-CHS was conducted by visual screening for silencing phenotypes. The *sgs2-1* (*rd6*) mutant was obtained from Hervé Vaucheret, (INRA, Versailles, France).

Total RNA extraction and quantification

Total RNA was extracted from aerial tissues of 3-week-old plants grown on MS media using TRIzol™ Reagent (Life Technologies, USA) as per manufacturer's instructions with modifications as described by Smith and Eamens (2012).

Northern blot

Twenty micrograms (20 µg) of total RNA was separated on 15% polyacrylamide gels by electrophoresis as described by Smith and Eamens (2012). For ribonucleotide probes, the corresponding sequence cloned into pGEM-T Easy cloning vector (Promega) was transcribed with either T7 or SP6 polymerase (Promega) in the presence of α-³²P UTP. The resulting transcripts were hydrolyzed with 200 mM carbonate buffer to yield fragments of ~50-nt lengths. U6 and amiRNA oligoprobes were prepared from oligos U6 and amiRNA-CHS by 3' end-labelling with α-³²P CTP using terminal deoxynucleotide transferase (Fermentas). Images of exposed northern blot filters were cropped and realigned for figure presentation.

sRNA sequencing and data analysis

Small RNA sequencing was performed by the Australian Genome Research Facility (AGRF, Melbourne, Australia) in an Illumina Hi-Seq instrument using the Illumina CASAVA pipeline version 1.8.2. Short RNAs were sequenced as 100-nt single end reads from *pap1-D*/SSU_hpCHS (~11 million (M) reads) *pap1-D*/SSU_amiR21-CHS (~18 M reads) and *pap1-D*/SSU_amiR22-CHS (~17 M reads). A *pap1-D* plant was included in these analyses as a control (15 M reads). Data quality was assessed using fastqc (Babraham Bioinformatics, <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) prior to further analysis. Adaptor sequences and reads with lengths shorter than 18-nt were trimmed using fastx_clipper from the FASTX-Toolkit (Hannon Lab, http://hannonlab.cshl.edu/fastx_toolkit). Alignments were performed using bowtie (Langmead *et al.*, 2009) against the prebuilt *Arabidopsis thaliana* TAIR10 index. Alignments were sorted and indexed using SAMtools (Li *et al.*, 2009). UEA sRNA toolkit (Stocks *et al.*, 2012) was run locally against the TAIR10 cDNA representative gene model to assess phasing from the predicted 22-nt amiRNA cleavage site (Chen *et al.*, 2007). The sequence data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE49792 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49792>).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Oligonucleotide primer and probe sequences used in this study.

Table S2. sRNA accumulation in the *pap1-D* background expressing the SSU_hpCHS.

Table S3. amiRNA accumulation in the *pap1-D* background expressing SSU_amiR21-CHS and SSU_amiR22-CHS.

Table S4. Distribution of sRNA accumulation in the *pap1-D* background expressing SSU_amiR21-CHS and SSU_amiR22-CHS.

Table S5. Phasing analysis of SSU_amiR22-CHS directed phasiRNA production.

Figure S1. A single nucleotide (U) was deleted (–) from the oligonucleotide corresponding to the amiRNA* strand (blue) resulting in a predicted asymmetric 'bubble' in the duplex structure.

Figure S2. Diagram of the constructs used in these experiments.

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