Plant Biotechnology Journal

Plant Biotechnology Journal (2015), pp. 1–11 doi: 10.1111/pbi.12362

Simple gene silencing using the trans-acting siRNA pathway

Thomas B. Jacobs[1,2,*], Noah J. Lawler[2], Peter R. LaFayette[2,3], Lila O. Vodkin[4] and Wayne A. Parrott[1,2,3]

[1]*Institute for Plant Breeding, Genetics and Genomics, University of Georgia, Athens, GA, USA*
[2]*Center for Applied Genetic Technologies, University of Georgia, Athens, GA, USA*
[3]*Department of Crop and Soil Sciences, University of Georgia, Athens, GA, USA*
[4]*Department of Crop Sciences, University of Illinois, Urbana, IL, USA*

Received 19 November 2014;
revised 14 February 2015;
accepted 19 February 2015.
**Correspondence* (Tel 607-220-9610;
fax 607-254-1242;
email tom.b.jacobs@gmail.com)
Current address: Boyce Thompson, Institute for Plant Research, Ithaca, NY, USA.

Keywords: Plant biotechnology, functional genomics, gene silencing, transgenic crops.

Summary

In plants, particular micro-RNAs (miRNAs) induce the production of a class of small interfering RNAs (siRNA) called trans-acting siRNA (ta-siRNA) that lead to gene silencing. A single miRNA target is sufficient for the production of ta-siRNAs, which target can be incorporated into a vector to induce the production of siRNAs, and ultimately gene silencing. The term miRNA-induced gene silencing (MIGS) has been used to describe such vector systems in *Arabidopsis*. Several ta-siRNA loci have been identified in soybean, but, prior to this work, few of the inducing miRNAs have been experimentally validated, much less used to silence genes. Nine ta-siRNA loci and their respective miRNA targets were identified, and the abundance of the inducing miRNAs varies dramatically in different tissues. The miRNA targets were experimentally verified by silencing a transgenic *GFP* gene and two endogenous genes in hairy roots and transgenic plants. Small RNAs were produced in patterns consistent with the utilization of the ta-siRNA pathway. A side-by-side experiment demonstrated that MIGS is as effective at inducing gene silencing as traditional hairpin vectors in soybean hairy roots. Soybean plants transformed with MIGS vectors produced siRNAs and silencing was observed in the T1 generation. These results complement previous reports in *Arabidopsis* by demonstrating that MIGS is an efficient way to produce siRNAs and induce gene silencing in other species, as shown with soybean. The miRNA targets identified here are simple to incorporate into silencing vectors and offer an effective and efficient alternative to other gene silencing strategies.

Introduction

Induced gene-silencing in plants is an important tool for gene discovery and for the development of novel agronomic or quality traits, such as virus resistance (Tennant *et al.*, 2001; Tricoli *et al.*, 1995), insect resistance (Baum *et al.*, 2007) and altered fatty acid compositions (Flores *et al.*, 2008). The term cosuppression was first used to describe the silencing of a native chalcone synthase gene when a transgenic chalcone synthase was overexpressed in petunia plants (Napoli *et al.*, 1990). It is now known that in plants, gene silencing is initiated by a variety of small RNA (sRNA) molecules that are produced from double-stranded RNA (dsRNA) by the action of DICER-like (DCL) enzymes. Small RNAs are incorporated into Argonaute (AGO) proteins to form RNA-induced silencing complexes (RISCs). The RISCs recognize complementary sequences and induce gene silencing by cleaving mRNA sequences, inhibiting translation or initiating the methylation of DNA. In some gene-silencing pathways, RNA-dependent RNA polymerases (RDR) synthesize a dsRNA molecule from a cleaved transcript. The dsRNA can then be processed into additional sRNA molecules, thus greatly amplifying the original silencing signal (Eamens *et al.*, 2010).

Small RNAs are generally divided into two classes, miRNA and siRNA. The miRNA pathway starts with the transcription of *MIR* genes that produce primary miRNAs which form imperfect, stem-loop structures to create a dsRNA. Primary miRNAs are processed by DCL1, ultimately resulting in a mature miRNA of approximately 21 nt that is incorporated into AGO1 to form a RISC. The RISC directs the cleavage of mRNAs, which are usually degraded by normal cellular processes (Voinnet, 2009). Viruses and inverted repeats can result in long dsRNA molecules that are processed by DCL2 and DCL4 to produce 22-nt and 21-nt siRNAs, respectively. Small interfering RNAs derived from DCL2 and DCL4 typically participate in post-transcriptional gene silencing (PTGS) by directing the cleavage of complementary transcripts (Eamens *et al.*, 2010).

The use of dsRNA has been the cornerstone of current induced gene-silencing strategies reported to date, which rely on the use of hairpin vectors, whereby a portion of the target gene is cloned into a vector in an inverted-repeat orientation and is separated by a loop, which consists of either an intron or a spacer (Miki and Shimamoto, 2004; Wesley *et al.*, 2001). More recently, artificial miRNAs have been used to induce gene silencing (Ossowski *et al.*, 2008; Warthmann *et al.*, 2008). Nevertheless, recent insights into the pathways that plants use

Please cite this article as: Jacobs T.B., Lawler N.J., LaFayette P.R., Vodkin L.O. and Parrott W.A. (2015) Simple gene silencing using the trans-acting siRNA pathway. *Plant Biotechnol. J.*, doi: 10.1111/pbi.12362

© 2015 Society for Experimental Biology, Association of Applied Biologists and John Wiley & Sons Ltd

for gene silencing make it clear that the full potential of these pathways to be used for induced gene silencing has barely been tapped and that it is possible to develop additional gene-silencing systems that are simpler or more efficient than those afforded by current technology.

The trans-acting siRNA pathway is particularly amenable for gene silencing. This pathway is a combination of the miRNA and siRNA pathways and has two different mechanisms, referred to as the one-hit or two-hit models (Fei *et al.*, 2013). In the one-hit model, a single-stranded RNA transcript is first cleaved by AGO1 directed by a miRNA. Unlike the typical miRNA pathway in which the transcript is degraded, RDR6 is recruited to generate a dsRNA molecule from the 3′ cleavage product. DCL4 cleaves the dsRNA every 21 nt from the initial miRNA cleavage point, resulting in a phased production of secondary siRNAs (Figure 1a) (Yoshikawa *et al.*, 2005). The term phasiRNA has been used to refer to secondary siRNAs that are produced in a phased pattern. However, the phasiRNA term is not interchangeable with ta-siRNA. Only when phasiRNAs are shown to target a transcript in *trans*, are they referred to as ta-siRNA (Zhai *et al.*, 2011). PhasiRNAs have been identified in many crops such as tomato (Shivaprasad *et al.*, 2012), tobacco (Li *et al.*, 2012), poplar (Klevebring *et al.*, 2009), legumes (Zhai *et al.*, 2011) and rice (Song *et al.*, 2012). In soybean, several phasiRNA (Hu *et al.*, 2013; Zhai *et al.*, 2011) and five miRNA targets (Hu *et al.*, 2013) were identified, but not experimentally validated.

Only a handful of miRNAs induce the production of ta-siRNAs, and evidence from *Arabidopsis* suggests that 22-nt miRNAs are sufficient to induce the production of ta-siRNAs, while 21-nt versions of the same miRNAs are not (Chen *et al.*, 2010; Cuperus *et al.*, 2010). An additional report has demonstrated that the asymmetry of the initial miRNA/miRNA* duplex, rather than the length of the mature miRNA, is responsible for the recruitment of RDR6 (Manavella *et al.*, 2012).

In the ta-siRNA two-hit model, a transcript with two miR390 target sequences is recognized by AGO7/miR390. The cleaved transcript serves as a template for RDR6 and leads to the production of ta-siRNAs (Axtell *et al.*, 2006). The two-hit model is conserved in most plant species and is likely ancestral to the one-hit model (Xia *et al.*, 2013).

The one-hit ta-siRNA pathway has been shown to induce the silencing of targeted genes in *Arabidopsis* using transgenic MIGS vectors (de Felippes *et al.*, 2011, 2012). When a vector containing the miRNA target fused to a gene of interest is transformed into a plant cell, siRNAs are produced downstream of the miRNA target and gene silencing of the gene of interest is induced (Figure 1b). In this study, six putative miRNA targets were identified using a sRNA sequencing data set derived from soybean hairy roots. The putative miRNA targets were confirmed by the induction of gene silencing of the *GFP* transgene and two endogenous soybean genes. MIGS is a simple design given that a single 22-nt miRNA target is all that is required to induce the production of siRNAs from target sequences. The miRNA target sequences identified

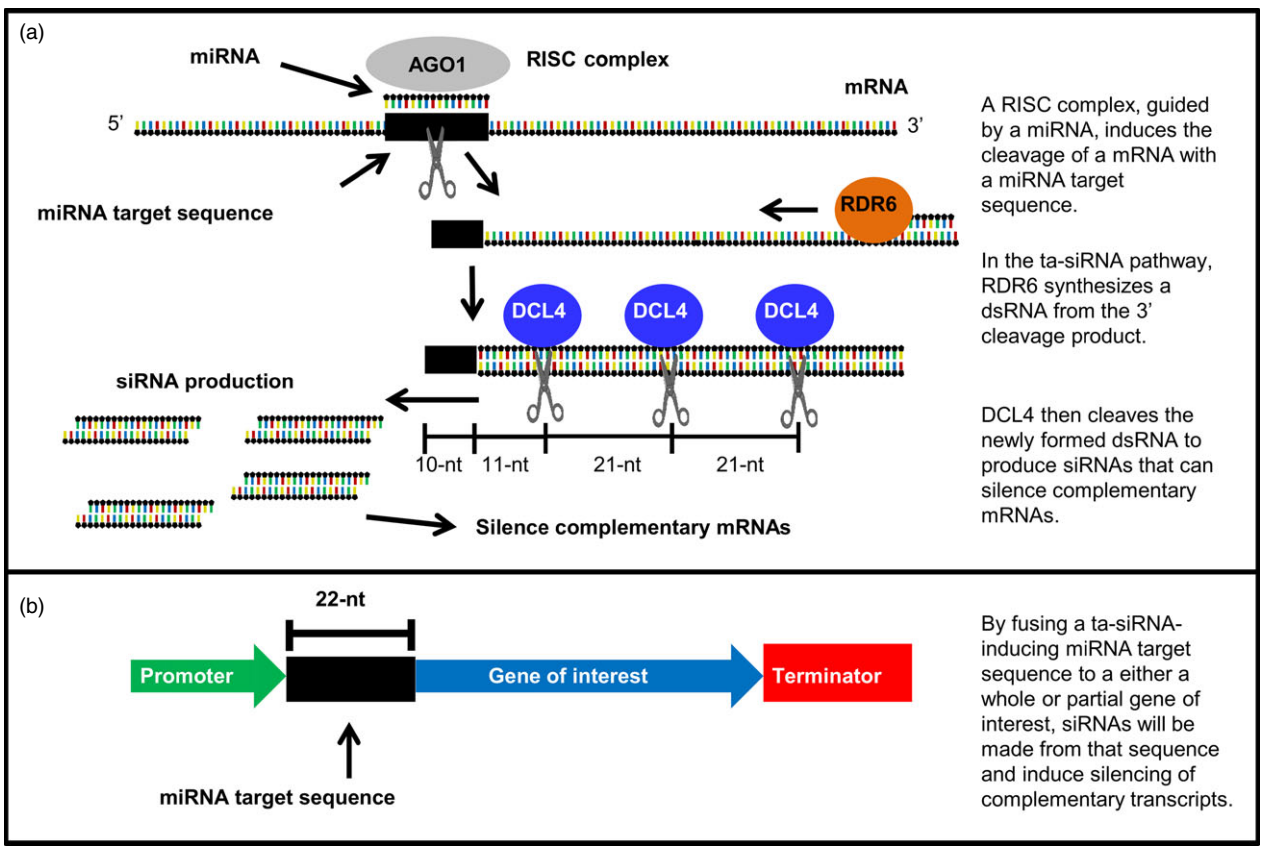


Figure 1 Outline of the ta-siRNA silencing pathway and how to adapt it to a MIGS vector system. (a) The general mechanism of the ta-siRNA pathway that leads to the production of siRNAs. (b) The ta-siRNA mechanism can be co-opted for the production of siRNAs and ultimately gene silencing by the incorporation of a miRNA target sequence.

here adapt MIGS for soybean and potentially other legumes. These results further demonstrate the effectiveness of MIGS for inducing gene silencing in plants.

Results and discussion

Identification of miRNA targets

The goal of this work was to identify putative miRNA targets in soybean and determine whether the sequences can be used to silence genes of interest. As described in the Experimental Procedures section, nine putative *phasiRNA* (*PHAS*) loci corresponding to six unique miRNA targets were identified (Table 1). Of the nine *PHAS* loci, six were previously identified (Hu *et al.*, 2013; Zhai *et al.*, 2011), which suggests the sRNA analysis is correct. All of the putative miRNAs identified here, and corresponding targets, are 22-nt in length, which is consistent with studies in *Arabidopsis* (Chen *et al.*, 2010; Cuperus *et al.*, 2010). One putative miRNA, which is most similar to the peanut (*Arachis hypogaea* L.) miRNA, ahy-miR3514, had not been identified in soybean and potentially targets three *PHAS* loci in soybean hairy roots. The two miRNAs miR1509a and miR1509b.2 are predicted to target the same miRNA target sequence (Table 1). Hu *et al.* also identified the phasiRNA-inducing soybean miRNAs, miR1510 and miR1514, although they report miR1510 as 21 nt and 22 nt and miR1514 as 21 nt (Hu *et al.*, 2013).

Differential accumulation of triggering miRNAs across tissue types

Micro-RNAs have complex expression profiles depending on developmental stage and environmental stress (Carthew and Sontheimer, 2009). To properly use miRNAs for gene silencing, it is important to understand their expression profile. To this end, the abundance of the putative miRNAs was evaluated in immature seed tissues (cotyledon, seed coat and 12–14 day old seed), vegetative tissues (cotyledon from germinating seed, root, stem, shoot tip and leaf) from previously published data sets (Zabala *et al.*, 2012) and hairy roots (this study). The abundance of miR1510a.2 and miR1514a.2 is relatively low [0–20 reads per million sequenced (RPMS)], but consistent levels are present in almost all tissues (Figure 2). In contrast, miR1509b.2 is highly abundant (250–400 RPMS) in all tissues except shoot tips, leaves and hairy roots and peaks at 900 RPMS in root tissue. MiR5770.2 is only detected in soybean hairy roots at the relatively low level of 4 RPMS. These data clearly demonstrate the differential accumulation of the putative miRNAs across multiple tissue types.

The phasiRNA-inducing miRNAs are size variants of known miRNAs

Many of the putative miRNAs identified are size variants (isomiRs) of previously characterized soybean miRNAs. In miRBase (release 18), miR1514a, miR1509b, miR1510a and miR5770 are all annotated as 21-nt miRNAs. Four of the putative 22-nt isomiRs identified here could be derived from the primary miRNAs and are denoted with the .2 notation. The miR1514, miR1509b and miR1510a isomiRs have previously been observed in soybean (Kulcheski *et al.*, 2011).

The miR1510 identified here cannot be produced from the known *miR1510a* and *miR1510b* loci, as a 3′ U is not present in the primary miRNAs. The 3′ uridylation of miRNAs is thought to be part of the miRNA degradation pathway in plants (Li *et al.*, 2005), but it has been suggested that such modifications could serve other unknown biological functions (Wyman *et al.*, 2011). It

Table 1 Summary of putative miRNAs and *PHAS* loci. [A] Identified in Zhai *et al.*, 2011;. [B] Identified in Hu *et al.*, 2013

miRNA Name	Related miRNA	Putative miRNA 5′ -> 3′	miRNA target 5′ -> 3′	*PHAS Loci* Glyma model	Characterization	Coordinates
3514	Ahy-miR3514-3p	UCACCAUUAAGACAGAGACCUU	AAGGTCTCTGTCTTAATGGTGA	Intergenic[A]	Uncharacterized, similar to PPR protein	Gm16 : 30 415 404..30 421 646
				Glyma18 g03980	3′ UTR, protein of unknown function	Gm18 : 2 747 299..2 748 817
				Intergenic[A]	*MIR4409* and *MIR5372*	Gm16 : 5 743 468..5 745 496
5770.2	Gma-miR5770b	UAGGACUAUGGUUUGGACAAGU	TCTTGTCCAAACCATAGTCCAA	Glyma17g02260	Copper amino oxidase	Gm17 : 1 453 405..1 456 485
1510a.2	Gma-miR1510a-3p	UUGUUUUACCUAUUCCACCCAU	ATGGGTGGAATAGGGAAAACAA	Glyma04g39740[A,B]	LRR protein	Gm04 : 45 906 669..45 909 231
1509a	Gma-miR1509a	UUAAUCAAGGAAAUCACGGUCG	CAACCTTGATTTCCTTGATTAA	Intergenic	No information	Gm04 : 43 624 071..43 624 750
1509b.2	Gma-miR1509b	UUAAUCAAGGAAAUCACGGUUG				
1514a.2	Gma-miR1514a	UUCAUUUUUAAAAAUAGGCAUUG	CAATGCCTATTTTTAGAAATGAA	Glyma16g01940[A,B]	No Apical Meristem protein, uncharacterized	Gm16 : 1 443 765..1 449 622
				Glyma07g05360[A,B]	No Apical Meristem protein, uncharacterized	Gm07 : 3 999 218..4 001 905
1510	Gma-miR 1510a-3p	UGUUGUUUUACCUAUUCCACCU	AGGTGGAATAGGAAAAACAACT	Glyma12g27800[A,B]	LRR protein	Gm12 : 31 264 569..31 268 587

© 2015 Society for Experimental Biology, Association of Applied Biologists and John Wiley & Sons Ltd,

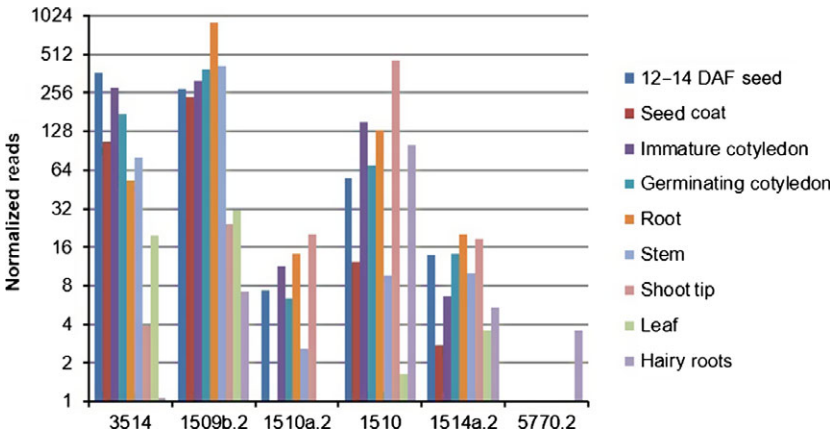


Figure 2 Relative abundance of the 22-nt miRNAs in different soybean tissues. Small RNA reads were obtained from previously published data sets (Zabala *et al.*, 2012) and Gene Expression Omnibus. The number of miRNA reads are normalized to the millions of sequenced reads sequenced (RPMS) and plotted with a log scale. MiR5770.2 was only detected in the hairy root library with four normalized reads. DAF, days after flowering.

is striking that the same isomiR was observed in such a diverse set of tissues, suggesting that 3′ uridylation of miR1510 could be a common post-transcriptional modification.

Consistent and complete silencing of GFP with five miRNA targets

After identifying putative miRNA targets, their ability to induce silencing was tested by targeting a constitutively expressed *GFP* gene (Hernandez-Garcia *et al.*, 2009). All hairy root events from the 1509:GFP, 1510:GFP, 1510a.2:GFP, 1514a.2:GFP and 3514:GFP vectors were strongly silenced, as indicated by a lack of fluorescence, while events from the untargeted GFP controls fluoresced (Figure 3). The abundance of GFP mRNA was reduced, on average, 92%–96% as compared to the levels from the empty-vector control events (Figure 4). The fluorescence of GFP-silenced events was comparable to the background autofluorescence in wild-type controls. While the 5770.2:GFP vector did not appear to induce silencing based on fluorescent imaging, expression was actually reduced 50%, on average, according to qRT-PCR and the fluorescent protein assay (Figure 4). The partial level of silencing observed for this vector may be explained by the observation that miR5770.2 was the least abundant miRNA detected in the tissues sequenced (Figure 2), and only four RPMS could be detected in hairy root tissues. Strong silencing, presumably caused by cosuppression, was observed in one 5770.2:GFP event and in two GFP target-only events, but was absent in empty-vector controls.

Small RNA sequencing and mapping of reads to the GFP transgene produced coverage graphs that show the same pattern of sRNAs for each of the silencing vectors (Figure 5a). Reads from both positive and negative strands indicates that dsRNA was produced and subsequently cleaved by a DCL. In each of the events, tens to thousands of RPMS were produced from both the positive and negative strands (Figure 5b). There is an even distribution of reads between the positive and negative strands, with a slight bias towards the positive strand. The 5770.2:GFP event only had 31 RPMS of GFP sRNAs, which is comparable to the background level of 25 RPMS in the empty-vector control. However, the 5770.2:GFP sRNA reads were distributed across the GFP target, similarly to the other silenced events.

The involvement of the ta-siRNA pathway is evident when looking at the reads that map around the miRNA target sequence. When DCL4 cleaves the dsRNA, the first sRNA molecule contains 10 nt from the miRNA target and 11 nt from the 3′ sequence (Figure 1a). Therefore, the GFP-targeting vectors should produce chimeric reads with 10 nt of the miRNA target and 11-nt GFP. Chimeric reads were observed in all GFP-silenced events, but not in the controls or the cosuppressed 5770.2 events (Figure 5c). These data provide strong evidence that these vectors are using the ta-siRNA pathway and that while the 5770.2 target is not inducing a strong silencing response, it too is likely participating in the ta-siRNA pathway.

The GFP-silencing experiment confirmed that gene silencing and siRNA production could be induced with the identified miRNA targets. The fact that the siRNA patterns were the same for each of the vectors provides strong evidence that the same pathway is being used. The accumulation of siRNAs in a consistent pattern suggests that the siRNAs produced from the transgenic vector can be predicted. In turn, the vector could be designed to avoid or encourage the silencing of closely related genes, depending on the goal. The level of silencing is also uniform indicating that MIGS is a consistent and effective method to induce gene silencing in soybean.

Silencing of two soybean genes with MIGS

MIGS in soybean was further tested by attempting to silence two endogenous genes with the miR1514a.2 target, which was the first miRNA target identified in the analysis. *Glyma02 g43860* encodes a nodulation factor receptor kinase 1α (NFR) that has been shown to control nodule number in soybean, and null mutants fail to produce nodules (Indrasumunar *et al.*, 2011). *Glyma07 g14460* is a putative cytochrome P450 CYP51G1 and is highly expressed in all tissues based on RNA-seq data (Libault *et al.*, 2010). In *Arabidopsis*, null mutants of CYP51G1 are embryo lethal (O'Brien *et al.*, 2005). It is likely that silencing of this gene will be tolerated in hairy roots as no embryos are produced.

All hairy root events transformed with the 1514a.2:P450 vector had an average reduction of 88% for the P450 transcript, as compared to the empty-vector control (Supplemental Figure 1). In contrast, 1514a.2:NFR events had a wide range of expression, from 9 to 108% of empty vector. While the 1514a.2:NFR events had, on average, a 60% reduction in expression compared to the expression of the empty-vector events, they were not significantly different than the NFR target-only events (Figure S1). The production of sRNAs was observed in one of the three NFR target-only events (Table S1) and may explain the reduced transcript levels in these events. The *NFR* gene is expressed at low levels in hairy root tissues, such that it may be difficult to observe a strong silencing effect for this target gene. *NFR* is most highly

© 2015 Society for Experimental Biology, Association of Applied Biologists and John Wiley & Sons Ltd,

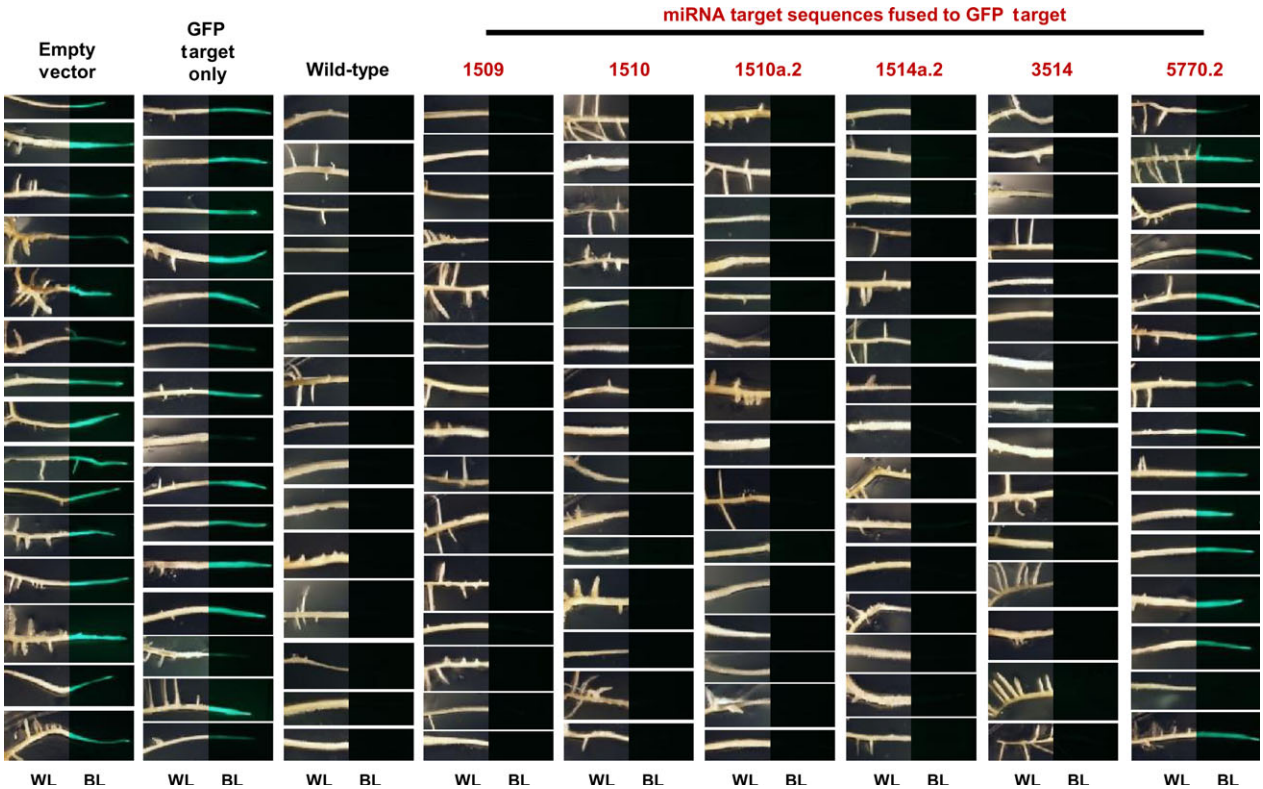


Figure 3 Gene silencing of GFP in hairy roots. Each root is an individual transgenic event, and blue light (BL) images are overlain white light (WL) images. Silencing is evident by roots that do not fluoresce under BL. Some silencing is observed in the control GFP target-only treatment, but all events are silenced for five of the six miRNA recognition sequences used.

expressed in lateral roots and is up-regulated by *Bradyrhizobium japonicum* inoculation (Indrasumunar *et al.*, 2011); therefore, it may be necessary to inoculate to detect silencing. By comparison, the *P450* gene is highly and constitutively expressed; therefore, it may be a better reporter for detecting gene silencing.

Small RNA sequencing was performed on three events from each of the 1514a.2: target and control vectors (Table S1). Small RNA reads were detected in all of the 1514a.2: target events. Less than ten RPMS were observed in the target-only control events, and these are likely background levels. One NFR target-only event produced a large number of sRNAs (4606 RPMS), which is likely due to cosuppression. Furthermore, two-thirds of the sRNAs derived from the 1514a.2:NFR vector map to the positive strand. As only reads from the negative strand are expected to induce silencing, this strand bias may also explain why the 1514a.2:NFR vector is less effective at reducing transcript levels compared to the P450- and GFP-silencing vectors.

The coverage graphs show that sRNA production mainly occurs 3′ from the miR1514a.2 target and ends in the terminator (Figure S2). Independent events from the same silencing vectors have similar patterns of sRNAs. These results demonstrate that MIGS can also be used to silence endogenous genes in soybean in an efficient and predictable manner.

MIGS vectors are as effective as hairpin vectors at inducing gene silencing

A side-by-side experiment was conducted to compare silencing efficiency with hairpin and MIGS vectors in soybean hairy roots. PCR for the vector components (MIGS target or hairpin arms) revealed that many of the hairpin events were missing one hairpin arm or the other resulting in incomplete hairpins (Table S2). Incomplete hairpins should not produce dsRNA, and therefore, not be able to induce gene silencing. While the percentage of incomplete hairpins was not consistent between experimental replicates, on average, 18%–43% of hairpin events were incomplete, whereas, on average, only 4%–8% of the MIGS events were missing the silencing vector (Table S2). Events with incomplete hairpins are routinely observed, and the frequency can vary considerably for different hairpin vectors and the transformation method used (data not shown). Yet, there is only one report in the literature describing the loss of hairpin-vector components (Sunitha *et al.*, 2012).

Expression analysis from events containing complete vectors demonstrated that the MIGS and hairpin vectors reduced expression levels by approximately 90% for both the GFP and the NFR targets (Figure S3). While the 1514a.2:P450 vector reduced the expression by 95%, the hairpin-P450 vector reduced the expression by 70% and was not significantly different than the empty-vector control. These results demonstrate that the MIGS vectors are at least equivalent to hairpin vectors at inducing gene silencing in soybean hairy roots. Given the multiple cloning steps required to produce hairpin vectors (see Methods) and the reduced number of events that contain complete hairpins, MIGS vectors offer a simple and effective alternative.

MIGS in transgenic soybean plants

Hairy roots are a quick model system to study RNAi phenomena in soybean, but they cannot be used to generate whole plants. To

© 2015 Society for Experimental Biology, Association of Applied Biologists and John Wiley & Sons Ltd,

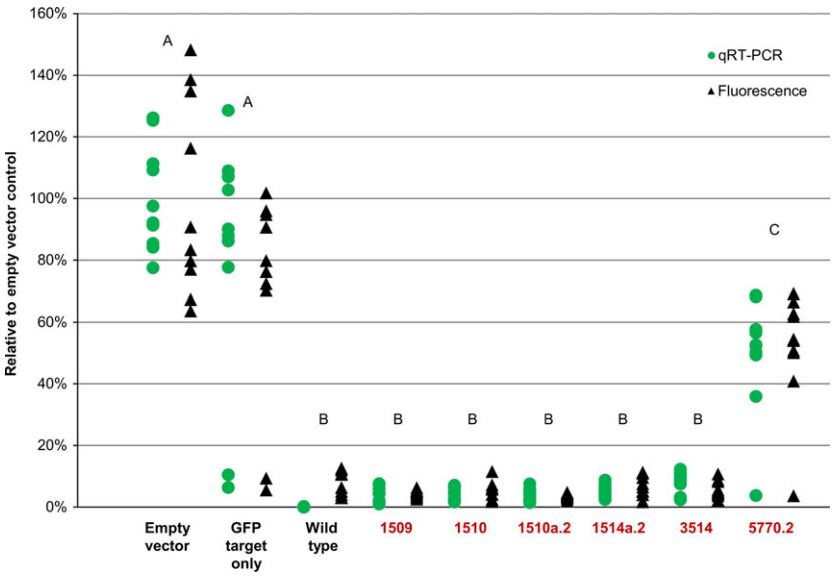
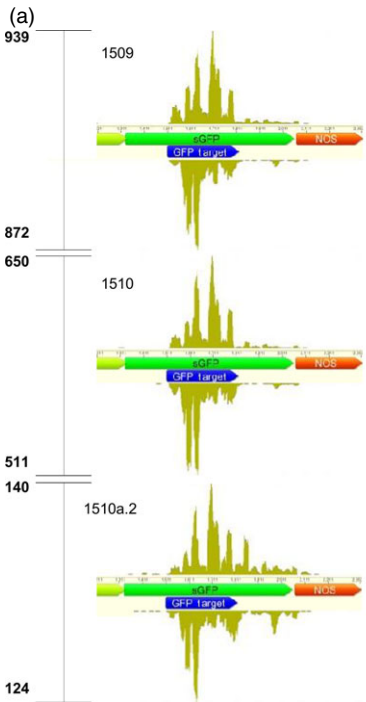


Figure 4 QRT-PCR and fluorescent protein quantification of hairy roots. Each point represents a single transgenic event. All values were normalized to the empty-vector control. GFP mRNA and protein levels are approximately 5%–15% of the empty-vector control, consistent with images in Figure 3. These results show that the miR5770.2:GFP vector reduced GFP expression by approximately 50%. Means were separated using Student's *t*-test $\alpha = 0.05$, $n = 10$. Groups with the same letter are not significantly different. Results are the same for qRT-PCR and fluorescent protein quantification.



(b)

Vector	Positive strand	Negative strand	Total
1509	3898	3154	7052
1510	2492	1998	4490
1510a.2	692	516	1208
1514a.2	3272	2336	5608
5770	23	8	31
3514	481	332	813
GFP	4	0	4
Empty vector	22	2	24

(c)

```
                    10-nt
                    ▼------>
                    NNNNNNNNNNNNNCTCGTGACCACCTTCAC
1509 (152)          CCTTGATTAACTCGTGACCACC
1509 (20)           CCTTGATTAACTCGTGACCAC
1510 (25)           AAAAACAACTCTCGTGACCACC
1510a.2 (20)          GAAAACAACTCGTGACCACCT
1514a.2 (112)       TAGAAATGAACTCGTGACCACC
1514a.2 (52)        TAGAAATGAACTCGTGACCAC
3514 (3)            TTAATGGTGACTCGTGACCACC
5770.2 (2)          CATAGTCCAACTCGTGACCACC
```

Figure 5 Mapping of sRNAs to the targeted GFP gene. (a) Small RNA reads map to the GFP target sequence (blue arrow) and the downstream GFP and NOS sequences. Small RNAs accumulate in similar patterns for each of the targeting vectors. Scale bars are normalized to the millions of reads sequenced per library (reads per million). (b) The normalized number of small RNA reads that mapped to events transformed with each MIGS vector or control. (c) The chimeric MIGS vector sequence with the miRNA target as N's and the GFP sequence in green. The ta-siRNA inducing miRNA should initiate a cleavage event 10-nt from the 3′ end of the miRNA target site ultimately producing chimeric small RNAs. Chimeric reads from each sequenced ta-siRNA-silenced event are aligned below, and the total number of reads observed for each small RNA species is in parentheses. Chimeric reads were not observed in the GFP or empty-vector control reads.

determine whether MIGS is effective in whole plants, the vectors 1514a.2:NFR and 1514a.2:P450 were transformed into soybean. Small RNA sequencing of T0 leaf tissue from three 1514a.2:P450 events confirmed the production of siRNAs (Table S3). Small RNA reads that mapped to the P450 mRNA show the same pattern of siRNAs that was observed in hairy roots (Figure S4).

In the T1 generation, a range of gene silencing was observed in lines containing the 1514a.2:P450 vector. Five lines have a consistent, although nonsignificant, reduction in P450 expression of 30%–50% (Figure 6, lines 24, 11, 4, 47 and 18). Small RNA sequencing of the 1514a.2:P450 lines shows that siRNAs were produced in all of the sequenced events, and the abundances ranged from 56 RPMS to 5866 RPMS (Table 2). No correlation between siRNA abundance and level of silencing was observed. Line 11 had the second lowest P450 expression, while lines 7 and 33 had approximately 500–1800 more RPMS than line 11. A stronger level of silencing was observed in the soybean hairy roots than in the leaves of T1 plants. As P450 null mutants are embryo lethal, the expectation is therefore that any plant recovered from somatic embryos or seed will need to have attenuated expression, as opposed to complete elimination of expression. Alternatively, the different levels of silencing could be due to differences in tissue type (leaf vs. hairy root).

Expression analysis and nodule counts were performed on three 1514a.2:NFR lines at the T1 generation. The qRT-PCR analysis from root tissues showed that line E9 consistently expressed the silencing construct, line E2 did not express at all, and only one plant from line E10 expressed the silencing

© 2015 Society for Experimental Biology, Association of Applied Biologists and John Wiley & Sons Ltd,

Table 2 Small RNA reads from 1514a.2:P450 T1 events mapped to the *P450* target gene. Reads are normalized to the millions of reads sequenced per library (reads per million)

Line_T1 plant	Millions of reads	Positive strand	Negative strand	Total
7_1	1.226	116	1119	1235
7_2	1.368	173	1519	1692
11_1	1.179	171	842	1013
11_2	0.828	132	627	759
12_1	0.825	5	90	95
12_2	1.269	6	50	56
24_1	1.183	934	4932	5866
24_2	0.978	689	3151	3840
33_1	1.089	522	2515	3037
33_2	0.997	366	2312	2678
Jack	0.837	0	0	0

construct (data not shown). As with the hairy root data, reduction in NFR expression was inconsistent between experiments. There was a significant 48% reduction in NFR expression in line E9 for rep 1, but there was no significant difference in expression for rep 2 (Figure S5). No reduction in nodule numbers was observed (data not shown). The two NFR mutants, *rj1* and *nod49*, are recessive, loss-of-function mutants (Indrasumunar *et al.*, 2011), so while line E9 had reduced levels of NFR, it may not be low enough to recapitulate the phenotype. Small RNA sequencing was performed on the 1514a.2:NFR lines and, as expected, sRNAs targeting the NFR gene were detected in line E9 and the expressing line E10 plant, in patterns similar to those observed in hairy roots (Table S4).

In hindsight, the selection of *P450* and *NFR* as targets was not the best choice for a proof-of-concept experiment in transgenic plants. While siRNAs were produced from each of the MIGS vectors, and silencing was observed in some T1 lines, there were no observable phenotypes. A potential complication could be tissue-specific accumulation of miR1514a.2 in specific tissues or cell types which could induce silencing in a particular subset of cells, while other cells would be expressing at wild-type levels. Another consideration could be the low levels of expression of miR1514a.2 in leaf tissues (Figure 2). The use of other, highly abundant miRNA targets (i.e. 1509b.2) may lead to greater levels of silencing. Nonetheless, these data do support the potential of using MIGS vectors to generate specific siRNAs for gene silencing.

Practical considerations

MIGS vectors are dependent on the ta-siRNA-inducing miRNA. To use this system in a different plant species, the miRNAs and corresponding targets must be identified, or a miRNA that induces the production of secondary siRNAs would need to be co-expressed (de Felippes *et al.*, 2012). The use of endogenous miRNAs is preferred as it simplifies vector construction and avoids potential off-target effects caused by the ectopic expression of an exogenous miRNA. A number of *PHAS* loci have been described in other plant species, so it should be simple to identify the inducing miRNAs and produce silencing vectors in a similar manner. Furthermore, the sRNA deep-sequencing results suggest that the putative soybean miRNAs have some level of tissue specificity. The tissue-specific profile for each of the different miRNAs will need to be well characterized to ensure silencing in the tissue type of interest.

The lack of inverted repeats in MIGS vectors makes them very easy to construct and amenable to high-throughput cloning. To facilitate vector construction, binary silencing vectors have been made that contain a multiple cloning site next to each of the six reported miRNA targets. This vector series, called pGmute, is available from Addgene (plasmids 47 025, 55 768–55 772).

MIGS is an effective and efficient gene-silencing tool for plant research. Here, six soybean miRNA targets were identified that can be used to induce ta-siRNA for a specific target in a transgenic system. Incorporating the miRNA targets into transgenic vectors led to consistent gene silencing in hairy roots and transgenic soybean plants. The simple vector design makes cloning and transformation straightforward. The predictable patterns of sRNAs that are produced and the specific expression profiles of the miRNA triggers may add a level of control not as easy to achieve with other gene-silencing systems.

Experimental procedures

Identification of putative ta-siRNA loci

Putative *PHAS* loci were identified by aligning small RNA sequences from a soybean cultivar 'Jack' hairy root library to the soybean genome (Glyma v1.0) using Geneious version 5.4

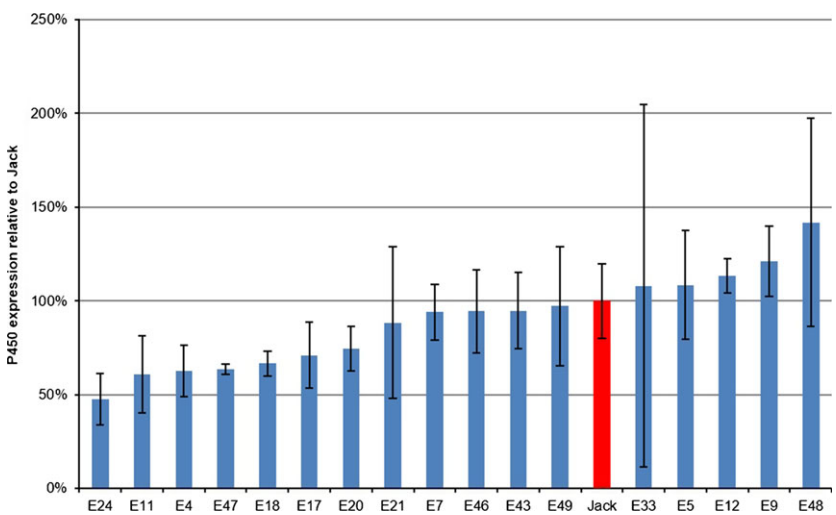


Figure 6 QRT-PCR of *P450* in T1 plants. Results are an average of two T1 plants (blue bars) and are normalized to the expression of two Jack plants (red bar). Error bars are standard deviation. P450 transcript levels are consistently reduced by 30%–50% in individual plants from events 24, 11, 4 and 47.

© 2015 Society for Experimental Biology, Association of Applied Biologists and John Wiley & Sons Ltd,

(Biomatters Ltd., Auckland, New Zealand). Regions with a high coverage of reads were evaluated by a number of criteria. First, the sRNA reads had to be primarily 21 nt in length, consistent with DCL4 activity. Second, the reads had to be from both positive and negative strands, which indicates the presence of dsRNA. Finally, a single species of sRNA (presumably a miRNA) had to align 5′ to the majority of the aligned sequences, as such alignment can indicate the possibility of a miRNA inducing the production of the siRNAs. To get putative miRNAs to align to the reference sequence, the miRNA alignments allowed for mismatches. *PHAS* loci were also identified by aligning all miRNA from the Fabaceae deposited in miRBase release 18.0. Regions with sRNAs adjacent to possible miRNA cleavage sites were evaluated following the criteria outlined above. Once putative miRNA targets were identified, they were compared to the soybean genome via BLAST to identify other potential *PHAS* loci. Finally, the counts for small RNAs with perfect match to the 22-nt putative miRNAs were determined from previously published data sets (Zabala *et al.*, 2012) and Gene Expression Omnibus sample accession GSM1010368 to find their abundances in different tissues.

Vector design

The binary vector p201N uses an *npt*II selectable cassette under the control of the *Solanum tuberosum* Ubi3 promoter and terminator (Joshi *et al.*, 2005) and was used for all hairy root experiments. The GmUbi promoter (Hernandez-Garcia *et al.*, 2009) drives the expression of the silencing and control cassettes and is terminated by the *Pisum sativum* rbcS terminator (An *et al.*, 1985). Gene-silencing targets were created by amplifying the target regions from cDNA or DNA of the gene to be silenced with the miR1514a.2 target fused to the forward primer (Table S5). Control vectors were created with the same primers, except with the miRNA target sequence omitted. The amplicons were inserted into the p201N vector via its *Asc*I and *Avr*II restriction sites. Additional miRNA targets were fused to the GFP target using the same cloning scheme.

For hairpin vectors, a soybean *FAD3* intron (Siminszky *et al.*, 2005) was inserted between the GmUbi and rbcs terminator. The *FAD3* intron is flanked by *Asc*I and *Swa*I restriction sites on the 5′ end and *Bam*HI and *Avr*II on the 3′ end. Gene targets were amplified with forward primers with an *Asc*I and *Avr*II 5′ tail and reverse primers with a *Swa*I and *Bam*HI 5′ tail. The hairpin arms were added in two successive cloning steps. First, the 3′ arm of the hairpin was inserted between the rbcS terminator and FAD3 intron with the *Avr*II and *Bam*HI restriction sites. The 5′ arm was then added with the *Asc*I and *Swa*I restriction sites. Due to an internal *Bam*HI site within the NFR target, the *Bgl*II restriction site was used in place of the *Bam*HI site in the NFR reverse primer as *Bgl*II and *Bam*HI produce identical DNA overhangs.

For biolistic transformation of soybean, a pSMART HC Kan (Lucigen Corporation, Middleton WI, accession number AF532107) cloning vector was modified to contain a *hygromycin phosphotransferase* gene under the control of the Ubi3 promoter and terminator and the meganuclease I-*Ppo*I site and is referred to as pSPH2. The gene-silencing cassettes 1514a.2:P450 and 1514a.2:NFR were moved into pSPH2 as an I-*Ppo*I fragment. Prior to bombardment, the vectors were digested with *Pac*I to release the hygromycin resistance and gene-silencing cassettes from the vector backbone.

The pGmute series of vectors were created by inserting annealed oligos into the p201N vector using *Asc*I and *Bam*HI restriction sites. For each miRNA target, oligo pairs (see Table S5) were designed such that after denaturing and re-annealing, inserts contain the miRNA target with the *Asc*I, *Bam*HI and *Avr*II multiple cloning site on the 3′ end. The vectors are available from Addgene (plasmids 47025, 55768–55772).

Hairy root transformation of soybean

Soybean 'Jack' seeds were transformed with *Agrobacterium rhizogenes* strain K599 (Cho *et al.*, 2000) with slight modifications from the protocol as previously described (Cho *et al.*, 2000). Soybean seeds were surface sterilized and germinated for approximately 1 week on a filter paper wetted with ½ MSO liquid medium (½ MS salts, B5 vitamins, 30 g/L sucrose) (Murashige and Skoog, 1962). *Agrobacterium* from glycerol stocks was streaked out on YM medium (Lin, 1994) supplemented with 50 mg/L kanamycin and grown for 2 days at 28 °C. The *Agrobacterium* was resuspended in 600 μL of phosphate buffer (PB, 0.01 M Na_2HPO_4, 0.15M NaCl, pH 7.5) + 100 μM acetosyringone to an $O.D._{600}$ of 0.5–0.8. Soybean cotyledons were prepared similarly as for cot-node transformation (Olhoft *et al.*, 2003); the root and lower hypocotyl were removed from the cotyledons, leaving approximately 5 mm of hypocotyl. The apical shoot and hypocotyl were cut longitudinally to produce two cotyledons with a short hypocotyl piece. After the meristem was removed, 1-mm deep cuts were made on the adaxial surface of the cotyledons using a scalpel dipped in the solution of *Agrobacterium*. Cotyledons were cocultivated with the *Agrobacterium* for 3 days on filter paper wetted with 2 mL of ½ MSO + 100 μM acetosyringone. Cotyledons were then transferred onto medium consisting of: ½ MS salts, 2 g/L Phytagel (Sigma-Aldrich Co. St. Louis, MO) and 500 mg/L timentin. Each root was considered an individual event and selected on ½ MSO solid medium with 10 mg/L of geneticin (G418). No-vector control roots were grown on ½ MSO medium without G418. Those roots that grew on ½ MSO + G418 medium were considered transformation events, and a 2-cm portion of a root tip was collected for CTAB DNA extraction (Murray and Thompson, 1980). PCR was performed to confirm the insertion of the gene of interest using primers in the promoter and terminator (Table S5). After selection and PCR verification, roots were grown on individual plates for 2 weeks. Root tissue was then harvested, frozen in liquid nitrogen and stored at −80 °C.

RNA isolation

Tissues were harvested from culture plates or plants in the greenhouse and immediately frozen in liquid nitrogen. Samples were ground with a mortar and pestle in liquid nitrogen or with a stainless steel BB and extraction buffer, in a 2010 Geno/Grinder® (Spex Sample Prep, Metuchen, NJ); 100 mg of tissue was used for each extraction. Total RNA was extracted with Tri-Reagent® (Life Technologies™, Grand Island, NY) according to the manufacturer's instructions.

qRT-PCR

Total RNA was treated with Turbo DNase™ (Life Technologies™, Grand Island, NY) to remove all contaminating DNA. Then, 100 ng of treated total RNA was used as the template in the Go Taq® 1-Step RT-qPCR system (Promega, Madison, WI). The qRT-PCRs were performed in triplicate in a Light Cycler 480II (Roche Diagnostics, Indianapolis, IN) using: 37 °C for 15 min; 95 °C for 10 min; 40 cycles (95 °C for 10 s; 60 °C for 30 s; 72 °C for 30 s); and a melt-curve analysis from 60 °C to 95 ° at a ramp rate of

0.11 °C/s. The melt-curve analysis was used to confirm the specificity of the qRT-PCR. Next, qRT-PCR amplicons were sequenced to confirm that it was the target gene that was amplified. The metalloprotease amplicon (Libault *et al.* 2008) was used to normalize expression of each of the target genes. A list of primers used and amplicon efficiencies can be found in Table S5. Finally, ΔCt values for each event were calculated by the LightCycler® 480 SW 1.5.1 program using the advanced relative quantification analysis.

GFP protein quantification

At the time of harvest, 100 mg of root tissues was ground in a 2-mL microcentrifuge tube in PB. Samples were stored at −80 °C. GFP was quantified using a Synergy 2 plate reader (BioTek Instruments, Inc., Winooski, VT) according to (Remans *et al.*, 1999). Protein concentrations were measured by a Bradford assay (Bradford, 1976). Raw relative fluorescent units (RFU) were normalized by protein concentration.

GFP imaging

After selection on ½ MSO + G418, root tips were imaged with an Olympus MVX10 microscope with a GFP filter cube and DP controller version 2.2.1.227 (Olympus America Inc., Center Valley, PA) imaging software. Blue light images were taken with a 5-ms exposure.

Small RNA sequencing and assembly

Small RNA libraries were prepared from 1 μg of total RNA using Illumina's TruSeq™ small RNA library kit. The MiSeq was used for small RNA sequencing according to the manufacturer's instructions. Raw reads were separated by barcodes using the MiSeq Reporter software. Fastq files were imported into Geneious, which was used trim the adapters and select reads 18–25 nt in length for assembly. The reads were assembled to the respective silencing or control cassettes and to the target mRNA using the following parameters: Gaps not allowed, word length 18, index word length 13, ignore words repeated more than five times, maximum mismatches per read 0% and maximum ambiguity 4.

Statistical analysis

The ΔCt values were normalized to the mean ΔCt values of the empty-vector control events (ΔΔCt method). Protein quantification data were processed likewise. The normalized qRT-PCR and protein quantification values were analysed with JMP® Pro 9.0.2 (SAS Institute Inc., Cary, NC), using the Fit Y by X function, with the vector as the X variable and the normalized values as the Y response variable(s). A one-way ANOVA was performed to ensure a significant difference between the results from the different vectors.

Biolistic transformation of soybean

Biolistic transformation of soybean was performed as previously described (Hancock *et al.*, 2011). PCR was used to confirm the presence of transgenes in T0 plants. Small RNA analysis was performed on three independent 1514a.2:P450 events. RNA was extracted from immature leaves of T0 plants growing in the greenhouse.

Nodulation assay

T1 seeds were germinated in 32-cell pack trays containing a mix of Fafard 3B (Sun Gro Horticulture Canada Ltd., Agawam, MA), field soil and sand. After 2–3 weeks, seedlings were removed from the trays, and the roots were washed and nodules counted. Tissue from lateral roots was collected for RNA extraction, and leaf tips were taken for DNA extraction. Null segregants for the transgene were removed from the analysis. Two replications were performed approximately 1 month apart, and each replicate was processed on a single day.

1514:P450 transgenic plants

Young, unexpanded leaves were taken from T0 1514:P450 plants growing in the greenhouse and used for RNA extraction. T1 seeds from transgenic lines and 'Jack' controls were germinated in the laboratory under sterile conditions and transplanted to the greenhouse. After 3 weeks of growth, young, unexpanded leaves were taken for DNA and RNA extractions. RNA was extracted from two T1 plants per line that tested positive for the transgene.

Acknowledgements

This work was supported by the United Soybean Board and State and Federal monies allocated to the Georgia Agricultural Experiment Stations. We would like to thank John Finer from The Ohio State University for the GFP soybean line.

Conflict of interest

The authors T.B.J., L.O.V and W.A.P. are currently pursuing patent PCT/US2013/044267 on the described miRNA target sequences.

References

An, G., Watson, B.D., Stachel, S., Gordon, M.P. and Nester, E.W. (1985) New cloning vehicles for transformation of higher-plants. *EMBO J.* **4**, 277–284.

Axtell, M.J., Jan, C., Rajagopalan, R. and Bartel, D.P. (2006) A two-hit trigger for siRNA biogenesis in plants. *Cell*, **127**, 565–577.

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., Vaughn, T. and Roberts, J. (2007) Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* **25**, 1322–1326.

Bradford, M.M. (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Carthew, R.W. and Sontheimer, E.J. (2009) Origins and mechanisms of miRNAs and siRNAs. *Cell*, **136**, 642–655.

Chen, H.M., Chen, L.T., Patel, K., Li, Y.H., Baulcombe, D.C. and Wu, S.H. (2010) 22-nucleotide RNAs trigger secondary siRNA biogenesis in plants. *Proc. Natl Acad. Sci. USA*, **107**, 15269–15274.

Cho, H.J., Farrand, S.K., Noel, G.R. and Widholm, J.M. (2000) High-efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. *Planta*, **210**, 195–204.

Cuperus, J.T., Carbonell, A., Fahlgren, N., Garcia-Ruiz, H., Burke, R.T., Takeda, A., Sullivan, C.M., Gilbert, S.D., Montgomery, T.A. and Carrington, J.C. (2010) Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in *Arabidopsis*. *Nat. Struct. Mol. Biol.* **17**, 997–1003.

Eamens, A., Curtin, S.J. and Waterhouse, P.M.. (2010) *RNA Silencing in Plants*. Springer, 233 Spring Stree, New York, NY 10013, United States.

Fei, Q.L., Xia, R. and Meyers, B.C. (2013) Phased, secondary, small interfering RNAs in posttranscriptional regulatory networks. *Plant Cell*, **25**, 2400–2415.

de Felippes, F.F., Ott, F. and Weigel, D. (2011) Comparative analysis of non-autonomous effects of tasiRNAs and miRNAs in *Arabidopsis thaliana*. *Nucleic Acids Res.* **39**, 2880–2889.

© 2015 Society for Experimental Biology, Association of Applied Biologists and John Wiley & Sons Ltd,

de Felippes, F.F., Wang, J.W. and Weigel, D. (2012) MIGS: miRNA-induced gene silencing. *Plant J.* **70**, 541–547.

Flores, T., Karpova, O., Su, X.J., Zeng, P.Y., Bilyeu, K., Sleper, D.A., Nguyen, H.T. and Zhang, Z.J. (2008) Silencing of Gm*FAD3* gene by siRNA leads to low alpha-linolenic acids (18: 3) of fad3-mutant phenotype in soybean *Glycine max* (Merr.). *Transgenic Res.* **17**, 839–850.

Hancock, C.N., Zhang, F., Floyd, K., Richardson, A.O., LaFayette, P., Tucker, D., Wessler, S.R. and Parrott, W.A. (2011) The rice miniature inverted repeat transposable element *mPing* is an effective insertional mutagen in soybean. *Plant Physiol.* **157**, 552–562.

Hernandez-Garcia, C.M., Martinelli, A.P., Bouchard, R.A. and Finer, J.J. (2009) A soybean (*Glycine max*) polyubiquitin promoter gives strong constitutive expression in transgenic soybean. *Plant Cell Rep.* **28**, 837–849.

Hu, Z., Jiang, Q.Y., Ni, Z.Y., Chen, R., Xu, S. and Zhang, H. (2013) Analyses of a *Glycine max* degradome library identify microRNA targets and microRNAs that trigger secondary siRNA Biogenesis. *J. Integr. Plant Biol.* **55**, 160–176.

Indrasumunar, A., Searle, I., Lin, M.H., Kereszt, A., Men, A., Carroll, B.J. and Gresshoff, P.M. (2011) *Nodulation factor receptor kinase 1 alpha* controls nodule organ number in soybean (*Glycine max* L. Merr). *Plant J.* **65**, 39–50.

Joshi, M., Niu, C., Fleming, G., Hazra, S., Chu, Y., Nairn, C.J., Yang, H.Y. and Ozias-Akins, P. (2005) Use of green fluorescent protein as a non-destructive marker for peanut genetic transformation. *In Vitro Cell Dev. Biol. Plant.* **41**, 437–445.

Klevebring, D., Street, N.R., Fahlgren, N., Kasschau, K.D., Carrington, J.C., Lundeberg, J. and Jansson, S. (2009) Genome-wide profiling of populus small RNAs. *BMC Genom.* **10**, 620.

Kulcheski, F.R., de Oliveira, L.F.V., Molina, L.G., Almerao, M.P., Rodrigues, F.A., Marcolino, J., Barbosa, J.F., Stolf-Moreira, R., Nepomuceno, A.L., Marcelino-Guimaraes, F.C., Abdelnoor, R.V., Nascimento, L.C., Carazzolle, M.F., Pereira, G.A.G. and Margis, R. (2011) Identification of novel soybean microRNAs involved in abiotic and biotic stresses. *BMC Genom.* **12**, 17.

Li, J.J., Yang, Z.Y., Yu, B., Liu, J. and Chen, X.M. (2005) Methylation protects miRNAs and siRNAs from a 3 '-end uridylation activity in *Arabildopsis*. *Curr. Biol.* **15**, 1501–1507.

Li, F., Pignatta, D., Bendix, C., Brunkard, J.O., Cohn, M.M., Tung, J., Sun, H.Y., Kumar, P. and Baker, B. (2012) MicroRNA regulation of plant innate immune receptors. *Proc. Natl Acad. Sci. USA* **109**, 1790–1795.

Libault, M., Thibivilliers, S., Bilgin, D.D., Radwan, O., Benitez, M., Clough, S.J. and Stacey, G. (2008) Identification of four soybean reference genes for gene expression normalization. *Plant Genome*, **1**, 44–54.

Libault, M., Farmer, A., Joshi, T., Takahashi, K., Langley, R.J., Franklin, L.D., He, J., Xu, D., May, G. and Stacey, G. (2010) An integrated transcriptome atlas of the crop model *Glycine max*, and its use in comparative analyses in plants. *Plant J.* **63**, 86–99.

Lin, J.J. (1994) optimization of the transformation efficiency of agrobacterium-tumefaciens cells using electroporation. *Plant Sci.* **101**, 11–15.

Manavella, P.A., Koenig, D. and Weigel, D. (2012) Plant secondary siRNA production determined by microRNA-duplex structure. *Proc. Natl Acad. Sci. USA*, **109**, 2461–2466.

Miki, D. and Shimamoto, K. (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant Cell Physiol.* **45**, 490–495.

Murashige, T. and Skoog, F. (1962) A Revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular-weight plant DNA. *Nucleic Acids Res.* **8**, 4321–4325.

Napoli, C., Lemieux, C. and Jorgensen, R. (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in *trans*. *Plant Cell*, **2**, 279–289.

O'Brien, M., Chantha, S.C., Rahier, A. and Matton, D.P. (2005) Lipid signaling in plants. Cloning and expression analysis of the obtusifoliol 14alpha-demethylase from *Solanum chacoense* Bitt., a pollination- and fertilization-induced gene with both obtusifoliol and lanosterol demethylase activity. *Plant Physiol.* **139**, 734–749.

Olhoft, P.M., Flagel, L.E., Donovan, C.M. and Somers, D.A. (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. *Planta*, **216**, 723–735.

Ossowski, S., Schwab, R. and Weigel, D. (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. *Plant J.* **53**, 674–690.

Remans, T., Schenk, P.M., Manners, J.M., Grof, C.P.L. and Elliott, A.R. (1999) A protocol for the fluorometric quantification of mGFP5-ER and sGFP(S65T) in transgenic plants. *Plant Mol. Biol. Rep.* **17**, 385–395.

Shivaprasad, P.V., Chen, H.M., Patel, K., Bond, D.M., Santos, B. and Baulcombe, D.C. (2012) A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. *Plant Cell*, **24**, 859–874.

Siminszky, B., Gavilano, L., Bowen, S.W. and Dewey, R.E. (2005) Conversion of nicotine to nornicotine in *Nicotiana tabacum* is mediated by *CYP82E4*, a cytochrome P450 monooxygenase. *Proc. Natl Acad. Sci. USA*, **102**, 14919–14924.

Song, X.W., Li, P.C., Zhai, J.X., Zhou, M., Ma, L.J., Liu, B., Jeong, D.H., Nakano, M., Cao, S.Y., Liu, C.Y., Chu, C.C., Wang, X.J., Green, P.J., Meyers, B.C. and Cao, X.F. (2012) Roles of DCL4 and DCL3b in rice phased small RNA biogenesis. *Plant J.* **69**, 462–474.

Sunitha, S., Shivaprasad, P.V., Sujata, K. and Veluthambi, K. (2012) High frequency of T-DNA deletions in transgenic plants transformed with intron-containing hairpin RNA genes. *Plant Mol. Biol. Rep.* **30**, 158–167.

Tennant, P., Fermin, G., Fitch, M.M., Manshardt, R.M., Slightom, J.L. and Gonsalves, D. (2001) Papaya ringspot virus resistance of transgenic rainbow and SunUp is affected by gene dosage, plant development, and coat protein homology. *Eur. J. Plant Pathol.* **107**, 645–653.

Tricoli, D.M., Carney, K.J., Russell, P.F., McMaster, J.R., Groff, D.W., Hadden, K.C., Himmel, P.T., Hubbard, J.P., Boeshore, M.L. and Quemada, H.D. (1995) Field-evaluation of transgenic squash containing single or multiple virus coat protein gene constructs for resistance to cucumber mosaic-virus. *Bio-Technol.* **13**, 1458–1465.

Voinnet, O. (2009) Origin, biogenesis, and activity of plant microRNAs. *Cell*, **136**, 669–687.

Warthmann, N., Chen, H., Ossowski, S., Weigel, D. and Herve, P. (2008) Highly specific gene silencing by artificial miRNAs in rice. *PLoS ONE*, **3**, 10.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G. and Waterhouse, P.M. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* **27**, 581–590.

Wyman, S.K., Knouf, E.C., Parkin, R.K., Fritz, B.R., Lin, D.W., Dennis, L.M., Krouse, M.A., Webster, P.J. and Tewari, M. (2011) Post-transcriptional generation of miRNA variants by multiple nucleotidyl transferases contributes to miRNA transcriptome complexity. *Genome Res.* **21**, 1450–1461.

Xia, R., Meyers, B.C., Liu, Z.C., Beers, E.P., Ye, S.Q. and Liu, Z.R. (2013) MicroRNA superfamilies descended from miR390 and their roles in secondary small interfering RNA biogenesis in eudicots. *Plant Cell*, **25**, 1555–1572.

Yoshikawa, M., Peragine, A., Park, M.Y. and Poethig, R.S. (2005) A pathway for the biogenesis of trans-acting siRNAs in *Arabidopsis*. *Genes Dev.* **19**, 2164–2175.

Zabala, G., Campos, E., Varala, K.K., Bloomfield, S., Jones, S.I., Win, H., Tuteja, J.H., Calla, B., Clough, S.J., Hudson, M. and Vodkin, L.O. (2012) Divergent patterns of endogenous small RNA populations from seed and vegetative tissues of *Glycine max*. *BMC Plant Biol.* **12**, 26.

Zhai, J.X., Jeong, D.H., De Paoli, E., Park, S., Rosen, B.D., Li, Y.P., Gonzalez, A.J., Yan, Z., Kitto, S.L., Grusak, M.A., Jackson, S.A., Stacey, G., Cook, D.R., Green, P.J., Sherrier, D.J. and Meyers, B.C. (2011) MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. *Genes Dev.* **25**, 2540–2553.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 QRT-PCR of the *P450* and *NFR* genes in soybean hairy roots. Each point represents a single transgenic event. Expression is relative to the empty-vector control. Groups with the same letter are not significantly different. P450 transcript levels are significantly reduced in the 1514a.2:target events compared to target-only and empty-vector controls. NFR transcript levels are

significantly reduced in the 1514a.2:target and target-only events compared to the empty-vector controls. Means were separated using Student's *T*-test, $\alpha = 0.05$.

Figure S2 Mapping of sRNA reads to MIGS vectors and mRNA targets P450 and NFR. Scale bars are normalized to the millions of reads sequenced per library (RPMS).

Figure S3 QRT-PCR of hairy root events transformed with hairpin and 1514a.2:target vectors. Each point represents a single transgenic event. Grey bars are the average of the expression values. Within each target, vectors labelled with the same letter are not significantly different. Means were separated with Tukey–Kramer HSD ($\alpha = 0.05$). For hairpin GFP $n = 6$, 1514a.2:GFP $n = 9$, Jack GFP $n = 9$; hairpin NFR $n = 6$, 1514a.2:NFR $n = 9$, Jack NFR $n = 9$; hairpin P450 $n = 5$, 1514a.2:P450 $n = 10$, Jack P450 $n = 9$.

Figure S4 Assembly of sRNAs to the transgenic vector and target mRNA from T0 leaf tissue. Scale bar is RPMS.

Figure S5 QRT-PCR of the NFR gene in T1 1514a.2:NFR events. A significant difference was only detected in rep 1 ($P < 0.0073$). Groups labelled with the same letter are not significantly different. Means were separated with Tukey-Kramer HSD ($\alpha = 0.05$). Rep1, E2 $n = 2$, E9 $n = 6$, E10 $n = 12$, Jack $n = 9$; Rep2, E2 $n = 3$, E9 $n = 4$, E10 $n = 11$, Jack $n = 7$. Error bars are standard error.

Table S1 Small RNAs mapping to *P450* and *NFR* target genes. Reads are normalized to the millions of reads sequenced (RPMS) per library. The number of normalized reads mapping to either the positive or negative strand are indicated. Only reads from the negative strand are expected to induce silencing.

Table S2 Presence and absence of vector components from hairy root events transformed with 1514a.2: target and hairpin vectors. Hairpin (hp) and MIGS (1514) vectors targeting GFP, NFR, and P450 were used to produce hairy root events. PCR was used to determine incorporation the respective vector components; either the MIGS target or hairpin arms.

Table S3 Small RNA reads from 1514a.2:P450 T0 events mapped to the P450 target gene. Reads are normalized to the millions of reads sequenced per library (RPMS).

Table S4 Small RNA reads from 1514a.2:NFR T1 events mapped to the NFR target gene. The Line_1 plants are from rep1, and the line_2 plants are from rep2. Reads are normalized to the millions of reads sequenced per library (RPMS).

Table S5 Primers used in this study. Restriction sites in bold, miRNA recognition sequences are underlined.

© 2015 Society for Experimental Biology, Association of Applied Biologists and John Wiley & Sons Ltd,