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TECHNICAL ADVANCE

Inverted repeat of a heterologous 3′-untranslated region for high-efficiency, high-throughput gene silencing

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Summary

This report describes a method for the easy generation of inverted repeat constructs for the silencing of genes of unknown sequence which is applicable to high-throughput studies. This improved procedure for high-efficiency gene silencing is specific for a target gene, but does not require inverted repeat DNA of the target gene in the construct. The method employs an inverted repeat of the 3′-untranslated region (3′-UTR) of a heterologous gene, and has been demonstrated using the 3′-UTR region of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens*, which is often used as the 3′-UTR for transgene constructs. In a population of independent tomato primary transformants harboring a stably integrated polygalacturonase (*PG*) transgene driven by a constitutive promoter and linked to an inverted repeat of the *nos* 3′-UTR, 51 of 56 primary transformants (91% of the population) showed highly effective post-transcriptional silencing of the *PG* gene, with *PG* mRNA abundance in ripe fruit reduced by 98% or more. The method was also effective in *Arabidopsis*, where two different, relatively uncharacterized plant transcription factors were also targeted effectively. This method has the advantage of ease and rapidity in preparation of the constructs, since a gene of interest can be inserted into a binary vector already containing the promoter and the inverted *nos* domain in a single-cloning step, and does not require any knowledge of the DNA sequence. The approach is suitable for high-throughput gene silencing studies, where it is necessary to investigate the function of hundreds to thousands of uncharacterized genes.

Keywords: double-stranded RNA, gene silencing, inverted repeat, *nos*, RNAi, siRNA.

Introduction

Post-transcriptional gene silencing (PTGS) emerged in the 1980s as an important molecular biology tool for investigating protein function by modulating gene expression, using either antisense (Ecker and Davis, 1986; Rothstein *et al.*, 1987; Sheehy *et al.*, 1988; Smith *et al.*, 1988) or sense constructs (van der Krol *et al.*, 1990; Napoli *et al.*, 1990; Smith *et al.*, 1990). Unfortunately, these approaches to the induction of PTGS generally produce effective gene silencing in no more than 10–20% of a transgenic population (e.g. Hamilton *et al.*, 1998; Smith *et al.*, 2000). In recent years, based on improved understanding of the mechanism of PTGS, efforts have been made to improve the efficiency of gene silencing in order to adapt PTGS for high-throughput applications (e.g. Angell and Baulcombe, 1997).

The discovery that double-stranded RNA (dsRNA) is a more effective inducer of PTGS than either sense or antisense RNA in both plants and animals (Fire *et al.*, 1998; Kennerdell and Carthew, 1998; Sanchez-Alvarado and New-

mark, 1999; Waterhouse *et al.*, 1998) has prompted the development of improved gene-silencing methodology. High-frequency and -level PTGS has been observed by crossing together plants expressing sense and antisense transcripts (Waterhouse *et al.*, 1998), or by expressing sense and antisense transgenes in the same plant under the control of different promoters (Chuang and Meyerowitz, 2000). A great increase in the frequency of PTGS in a population has been obtained by transformation with a construct composed of an inverted repeat of the gene of interest, with or without an intron between the inverted repeat elements (Smith *et al.*, 2000; Waterhouse *et al.*, 1998). However, the technical manipulations necessary to generate constructs to implement these dsRNA strategies limits their routine use as a high-throughput tool for functional genomics.

Recent studies on the mechanism of dsRNA-mediated, sequence-specific RNA degradation (RNA interference, RNAi) suggested an alternative way to implement targeted gene silencing. The key observations include: (i) the function of siRNA molecules (small interfering RNAs of 21–25 nucleotides) as primers for an RNA-dependent RNA polymerase activity (Dalmay *et al.*, 2000; Hamilton and Baulcombe, 1999; Mourrain *et al.*, 2000); and (ii) the degradation of RNA 5′ to a dsRNA region (Han and Grierson, 2002; Sijen *et al.*, 2001). We hypothesized that a molecule consisting of an inverted repeat of a heterologous 3′-untranslated region (3′-UTR) located 3′ of the single-stranded targeting sequence (and thus with a dsRNA region unrelated in sequence to the target RNA) could induce highly efficient degradation of endogenous mRNAs homologous to the transgene. Here, we show that a sequence of interest inserted in a single cloning step into a construct already containing a promoter and an inverted repeat of a nopaline synthase (*nos*) 3′-UTR triggers high-frequency and -efficiency PTGS. This method would therefore seem to be highly suitable for high-throughput gene-silencing studies.

Results

To test the effectiveness of an inverted *nos* 3′-UTR in bringing about PTGS, silencing of the *PG* gene of tomato was evaluated. The tomato *PG* gene is expressed at very high levels in ripening fruit, up to 4% of poly(A)$^+$ RNA (DellaPenna *et al.*, 1989), but is not essential for plant development. The *FMV::hsp70::PG::nosIR* construct (Figure 1) consists of (i) a strong constitutive promoter (the *34S* promoter from figwort mosaic virus) joined to a plant leader sequence (from the petunia *hsp70* gene); (ii) a truncated sense *PG* coding sequence; and (iii) an inverted repeat of the 3′-UTR region from the *nos* gene of *Agrobacterium tumefaciens*, with the first repeat element being in the antisense orientation relative to the promoter. Both orientations of the *nos* terminator will be incorporated into the resulting transcript, since the inverted *nos* element will not bring about transcript truncation and polyadenylation of the transcript. The two *nos* repeats are separated by a stuffer region to provide *in vivo* stability, here consisting of part of the 3′ region of the pepper endo-1,4-β-glucanase gene *CaCel1* (Harpster *et al.*, 2002), although any region of neutral DNA should suffice. *CaCel1* is homologous to a tomato gene, *LeCel1* (Lashbrook *et al.*, 1994), with which it shows substantial sequence identity except at the end of the coding region and in the 3′-flanking region. Thus, the region of *CaCel1* used here as a buffer has low sequence identity with the corresponding tomato gene.

The abundance of *PG* mRNA in fruit of a population of 56 independent primary transformant plants stably transformed with the *FMV::hsp70::PG::nosIR* construct was examined by RNA gel blot analysis. Figure 2 shows two representative RNA gel blots of total RNA from light red fruit of these lines hybridized with a labeled *PG* cDNA probe. The first lane of each blot contained RNA from wild-type (untransformed) fruit. The other lanes show RNA from fruit of 33 primary transformants, and RNA from leaves of three of these lines. Of these 33 primary transformants, 30 were strongly suppressed and two were moderately suppressed in *PG* mRNA accumulation. *PG* mRNA was below the level of detection in approximately half of the suppressed individuals, whereas in the other half *PG* mRNA abundance was very low and lanes showed multiple bands typical of the aberrant RNA indicative of mRNA degradation (Han and Grierson, 2002). One plant, number 105, was not silenced and exhibited *PG* mRNA abundance higher than in wild type. *PG* mRNA abundance continues to increase progressively during ripening (DellaPenna *et al.*, 1986), and since fruit were not precisely staged some variability in *PG* mRNA abundance was expected.

Phosphorimager analysis was used to quantify the relative amounts of *PG* mRNA in all the transgenic lines (Figure 3). Silencing of the *PG* gene was observed at a high frequency, and out of a total of 56 primary transformants, 51 showed

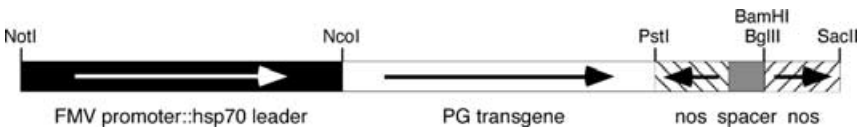


Figure 1. Schematic representation of the *FMV::hsp70::PG::nosIR* construct containing an inverted repeat of the 3′-untranslated region from the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens*. Arrows indicate the orientation of the DNA fragments used to assemble the construct. Restriction sites used in cloning are indicated (note that *Bgl*II and *Bam*HI have cohesive ends, but that ligation together destroys both sites).

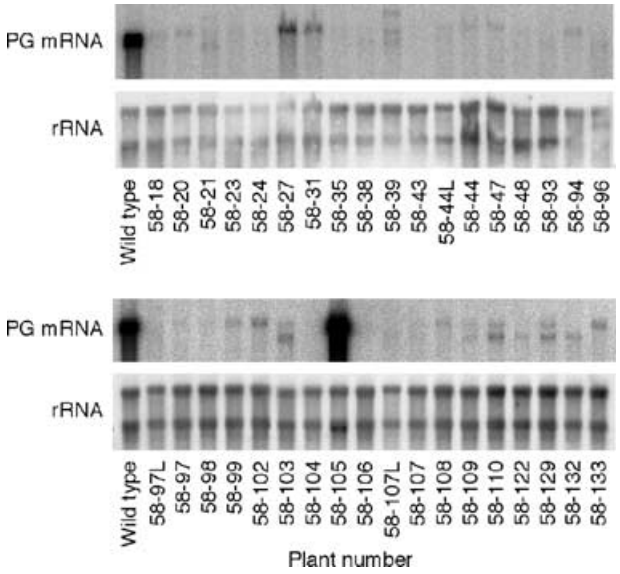


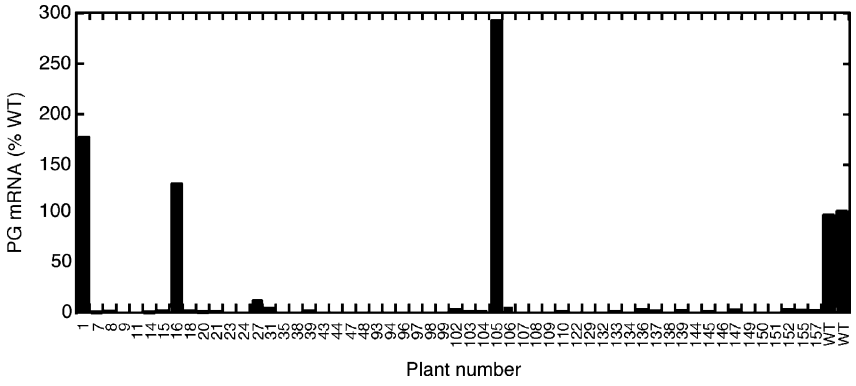
Figure 2. *PG* mRNA abundance in light red fruit of plants transformed with a *FMV::hsp70::PG::nosIR* construct. Total RNA (5 μg per lane) was fractionated by electrophoresis and gel blots were hybridized with a fruit *PG* cDNA probe labeled by random priming. Two representative RNA gel blots are shown, depicting 33 of the 56 independent primary transformants. The three samples labeled L are leaf samples from the corresponding plants. Blots were stripped and re-hybridized with a labeled ribosomal RNA probe.

strong suppression of *PG* mRNA accumulation. Of these 51 strongly silenced lines, 25 plants possessed *PG* mRNA abundance less than 1% of wild type, and 26 lines possessed *PG* mRNA abundance of 1–2% of wild type. The highest level of suppression observed was a 99.8% reduction in PG mRNA accumulation relative to wild type. Further, two lines (numbers 27 and 31) showed moderate suppression, accumulating 13 and 5% of wild-type *PG* mRNA, respectively. Only 3 of the 56 primary transformant lines showed no suppression of *PG* mRNA accumulation.

In order to test the broader applicability of using an inverted *nos* terminator for high-frequency, -efficiency PTGS, gene suppression was examined using different transgenes, under the control of a different promoter, and in a different species. The genes used were: (i) the *Arabidopsis* transcription factor designated *T10P11.13* (GenBank accession numbers AC002330 for nucleotides, AAC78259 for the encoded protein), which is of the helix–loop–helix type; and (ii) the *Arabidopsis* transcription factor *Athb-1*, which is of the homeodomain leucine zipper type (Aoyama *et al.*, 1995; Ruberti *et al.*, 1991). These were assembled into constructs from which the transcription factor coding sequences were transcribed from the cauliflower mosaic virus *35S* promoter with the petunia *Cab22L* leader, and linked to the *nos* inverted repeat terminator. These constructs and empty binary vector were used to transform *Arabidopsis thaliana* plants. Plants transformed with the *35S::Cab22L::Athb-1::nosIR* construct showed smaller leaves with serrated edges, and a rosette of reduced diameter compared to controls (Figure 4). Plants transformed with the *35S::Cab22L::T10P11.13::nosIR* construct showed a stronger phenotype, with very small leaves and a much smaller rosette diameter than controls (Figure 4). The number of T-DNA insertions in these lines was not determined, but in other studies the number of T-DNA loci did not correlate with the extent of PTGS (data not shown). The phenotype of smaller rosette diameter was seen at a relatively high frequency of transformed plants, five out of eight lines transformed with the *35S::Cab22L::T10P11.13::nosIR* construct and two out of four lines transformed with the *35S::Cab22L::Athb-1::nosIR* construct (Figure 4), although this was a lower frequency than gene silencing induced by the *FMV::hsp70::PG::nosIR* construct (Figure 3). Since suppression of these transcription factors resulted in a severe growth phenotype, it is possible that complete silencing of these genes is lethal. If high-level suppressants were not found due to lethality and only plants showing partial suppression of the transcription factors were viable, the actual frequency of PTGS may have been much higher than detected.

To confirm that transformation with the *35S::Cab22L::T10P11.13::nosIR* and *35S::Cab22L::Athb-1::nosIR* const-

Figure 3. Relative *PG* mRNA abundance in a population of 56 independent primary transformant tomato plants containing a stably integrated *FMV::hsp70::PG::nosIR* transgene. Fruit *PG* mRNA abundance was quantified from RNA gel blots in arbitrary units by phosphorimager analysis, and expressed relative to wild-type (untransformed) fruit.



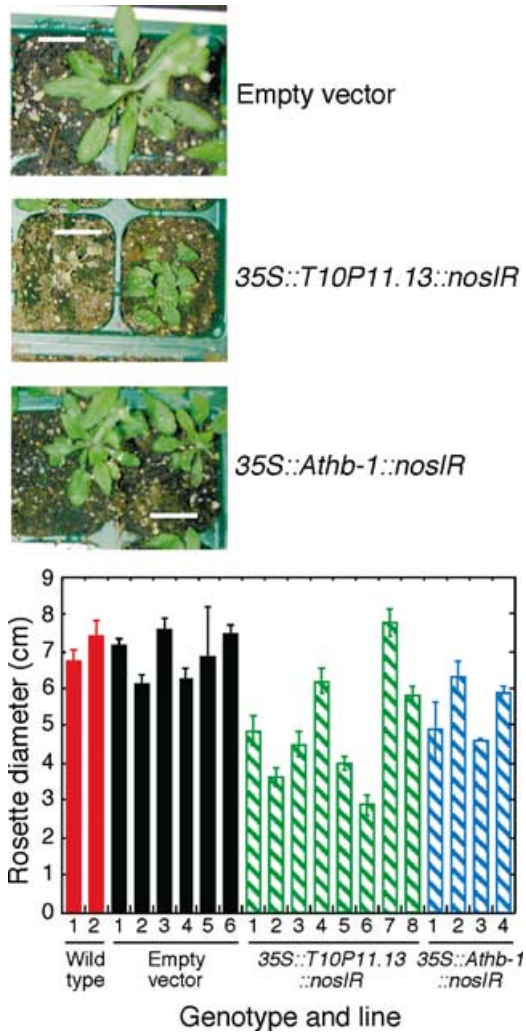


Figure 4. Phenotype of 25-day-old *Arabidopsis* plants transformed with the *35S::Cab22L::T10P11.13::nosIR* and *35S::Cab22L::Athb-1::nosIR* constructs, relative to control plants transformed with empty binary vector. The top panels show gross plant morphology (empty vector transgenic line 5, *35S::Cab22L::T10P11.13::nosIR* transgenic line 2, *35S::Cab22L::Athb-11::nosIR* transgenic line 3). The white scale bar in the photographs represents 3 cm. The histogram shows rosette diameter for these transgenic lines and for wild-type untransformed controls measured 25 days after planting. Measurements are mean ± SE from a minimum of six homozygous or hemizygous sibling plants per line.

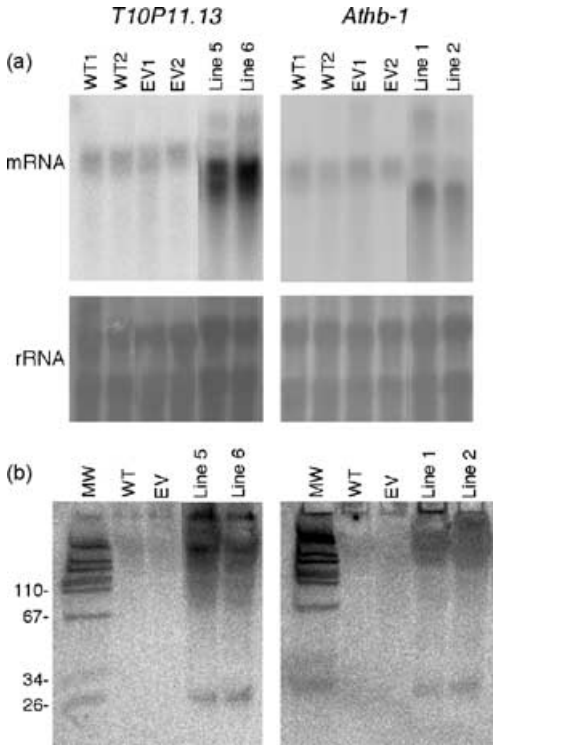


Figure 5. Analysis of *Arabidopsis* transformants for abundance of *T10P11.13* and *Athb-1* mRNA and oligoribonucleotides. In both (a) and (b), plants were either wild-type ecotype Columbia (WT), transformed with empty binary vector (EV), or transformed with the *35S::Cab22L::T10P11.13::nosIR* construct (left hand panels) or the *35S::Cab22L::Athb-1::nosIR* construct (right hand panels).
(a) mRNA abundance. RNA gel blot of 10 μg total RNA per lane hybridized with a random-primed labeled cDNA prepared from the open reading frame of *T10P11.13* or *Athb-1* as indicated. Lower panels show staining of the gel blot with methylene blue to assess equal loading of lanes.
(b) Detection of 21–25-mer RNA fragments. RNA (50 μg total RNA) was separated and hybridized with labeled probes as described (Hamilton and Baulcombe, 1999). Molecular weight (MW) markers were derived from pGEM-7ZF^{+} after digestion with *Hpa*II and labeling of the fragments with α-[^{32}P]-dCTP and the Klenow fragment of DNA polymerase I.

ructs was bringing about PTGS of the corresponding endogenous genes, mRNA accumulation of these genes was examined (Figure 5). In transgenic lines of both constructs the abundance of the corresponding mRNA was substantially reduced, although not absent, with a strong cross-hybridizing band of lower molecular weight derived from the transgene (Figure 5a). Examination of the mRNA from these transgenic lines revealed the presence of small 21–25 nucleotide siRNA fragments (Figure 5b), which is indicative of PTGS (Hamilton and Baulcombe, 1999). Separate experiments showed that overexpression of the *T10P11.13* or *Athb-1* genes did not result in a morphological phenotype (data not shown).

Discussion

Since it is an objective of the plant research community to determine the functions of all *Arabidopsis* genes by the end of this decade, high-throughput methods for gene silencing are desirable. Such a method is presented in this report, in which gene sequences are incorporated into a vector containing an inverted repeat of the 3′-UTR of the nopaline synthase gene from *A. tumefaciens*, in order to provide a dsRNA region at the 3′-end of the transcript. This method, which we term *s*ilencing by *h*eterologous 3′-*UTR*s (SHUTR), has been shown to operate effectively in *Arabidopsis*

thaliana and *Lycopersicon esculentum*, using different promoters, suggesting the generality of this approach. We anticipate that this method will work equally well with any heterologous 3′-UTR sequence providing a polyadenylation element.

A feature of the inverted *nos* construct is that the inverted repeat portion needs to be made only once, and then any known or unknown cDNA can be inserted into the vector between the promoter and inverted *nos* terminator to give an inverted repeat construct with a simple one-step cloning. This method could be used with a collection of previously cloned genes, or with cDNA libraries. A number of cloning strategies or recombinational methods could be used to create a set of silencing constructs derived from a collection of full-length coding sequence clones, including amplifying by PCR to attach cloning sites, or by attaching linkers to cDNA fragments. The preparation of a set of silencing constructs from a cDNA library is more complex, since silencing requires the presence of the heterologous *nos* inverted repeat UTR region at the 3′ terminus of the mRNA, and it is likely that cDNA library clones possessing a natural plant polyadenylation sequence and a poly(A) tract would not be suitable. However, cDNA libraries prepared using random hexamers rather than the standard oligo d(T) to prime first strand synthesis would consist of cDNAs generally lacking poly(A) tracts, which would be ideal for this system since full length clones are not required and the orientation of the cloned fragment is not important. The presence of duplicate restriction sites in the cDNA of interest additional to the ones used for cloning is not a problem, provided that a sufficiently long piece of the target gene cDNA is cloned into the vector. It is thus not necessary to determine the full sequence of the cDNA of interest in order to make an inverted repeat construct for gene silencing, or even to obtain any sequence information at all.

Previous studies of transgene-induced PTGS in plants have suggested that gene silencing is initiated in the 3′ region of the target gene (English *et al.*, 1996; Han and Grierson, 2002; Sijen *et al.*, 1996). Thus, our presumption is that the dsRNA resulting from the inverted *nos* polyadenylation signal in the 3′ region of the transcript triggers RNA degradation that includes not only the upstream transgene but is also sufficient to induce sequence-specific degradation of endogenous mRNAs homologous to the transgene targeting sequence. Sijen *et al.* (2001) have shown that the initiation of RNAi in the dsRNA triggering sequence of a transcript can result in the silencing of a second transcript that has no homology to the initial dsRNA trigger but shares upstream sequence in common with the first transcript. This phenomenon, demonstrated in *Caenorhabditis elegans*, has been dubbed 'transitive RNAi' (Sijen *et al.*, 2001). Our results indicate that 3′ → 5′ directed transitive RNAi is also operative in plants. Transitive RNAi appears to occur when siRNA molecules created against the primary triggering sequence prime synthesis of new dsRNA, mediated by an RNA-dependent RNA polymerase, in the 5′ → 3′ direction on the antisense strand, that thus extends in the 5′ direction of the transcript. The newly synthesized dsRNA leads to the production of more siRNA, which amplifies and triggers further gene silencing (Han and Grierson, 2002; Sijen *et al.*, 2001).

Other studies have also described the silencing of sequences heterologous to the primary, PTGS-triggering dsRNA region. Heterologous silencing was first reported by Hamilton *et al.* (1998), who used an inverted repeat of a 79-bp fragment of the 5′-UTR of the tomato *ACO1* gene to target degradation of the endogenous *ACO1* transcript. They found that the *ACO2* transcript, which has limited sequence identity to *ACO1* in the 5′-UTR but extensive sequence identity in the coding sequence, was also targeted for degradation. This is consistent with evidence that transitive RNAi operates in both the 5′ → 3′ and 3′ → 5′ directions in plants (Vaistij *et al.*, 2002). However, Wesley *et al.* (2001) found no improvement in sense silencing of a GUS gene using a construct in which an inverted repeat sequence is 5′ to a GUS sense sequence. These conflicting observations call for further analysis of 5′ → 3′ transitive RNAi in plants.

Angell and Baulcombe (1997) have described an alternative approach to high-throughput gene silencing, in which a viral system is adapted to produce transgenic plants that can express high levels of double-stranded viral RNA (Amplicon). However, this method may need to be adapted to work effectively in different host plant species. In contrast, the SHUTR method, in principle, can be used for functional genomics approaches with any plant species as long as a transformation method of reasonably high efficiency exists.

In summary, our results indicate that efficient, high-throughput gene silencing can be achieved by attaching an inverted repeat of any 3′-UTR functional in a host cell (as long as the 3′-UTR is unrelated to any host sequence) to the 3′-end of a target sequence and controlling expression of this transcript with a strong constitutive promoter. It is relatively simple to design vectors incorporating a strong constitutive promoter and a 3′-UTR inverted repeat into which suitable cDNA libraries can readily be cloned. The SHUTR method using such vectors should greatly facilitate the rapid determination of gene function for predicted genes in *Arabidopsis*, rice and other plants.

Experimental procedures

Plasmid constructs

A portion of plasmid pPG1.9 (kind gift of Robert Fischer, University of California Berkeley), containing a cDNA clone of the entire open

reading frame of tomato fruit *PG*, was amplified using PCR with sense primer 5′-CTGTTCAATCCATGGTTCC-3′ and antisense primer 5′-GAAGATCTATACTGCAGATTAATAATTATAC-3′. The resulting 1070 bp PCR product contained a truncated derivative of the *PG* gene in which the DNA coding for 111 amino acids had been deleted from the 5′-region, and into which were introduced an *Nco*I site at the 5′-end and a *Pst*I site immediately downstream of the translation stop codon, and retaining an existing *Bgl*II site distal to the engineered *Pst*I site. The PCR fragment was blunted by treatment with T4 DNA polymerase then cloned into a plasmid vector containing the *nos* 3′-UTR, and a clone with the *nos* 3′-UTR fused downstream of the *PG* cDNA was selected. The resulting intermediate plasmid was digested with *Pst*I and *Bgl*II, and a 375-bp *Bam*HI–*Pst*I fragment derived from a *CaCel1::nos* construct (Harpster *et al.*, 2002) was ligated into the plasmid in the antisense orientation, between the *PG* gene and the sense *nos* 3′-UTR. The *CaCel1::nos* fragment was composed of 115 bp of the end of the coding region of the pepper *CaCel1* gene and a 260-bp *nos* 3′-UTR. This resulted in the *PG* open-reading frame being followed downstream by a *nos* 3′-UTR in the antisense orientation, a 115-bp stuffer region of pepper *CaCel1* cDNA in the antisense orientation, and a second *nos* 3′-UTR in the sense orientation (Figure 1). The *PG::nosIR* construct was released from the vector by digestion with *Nco*I and *Sac*II, and ligated into another vector containing the FMV *34S* promoter with *hsp70* leader. The FMV *34S* promoter is a constitutive promoter of similar strength to the cauliflower mosaic virus *35S* promoter (Sanger *et al.*, 1990). The completed *FMV::hsp70::PG::nosIR* construct was released by digestion with *Not*I and *Sac*II, blunted by treatment with T4 DNA polymerase and ligated into the *Sma*I site of binary vector SVS297, which contains a *nos::nptII* gene conferring resistance to kanamycin. This plasmid was introduced into *A. tumefaciens* strain ABI for transformation into tomato plants.

The coding sequences of the *Arabidopsis* transcription factors *Athb-1* and *T10P11.13* were amplified by PCR from plasmid templates, at the same time adding an *Nco*I site at the translation start and an *Xba*I site after the translation stop. The PCR products were digested with *Nco*I and *Xba*I then were ligated into an intermediate vector between the CaMV *35S* promoter with *Cab22L* leader and a version of the *nos* inverted repeat 3′-UTR with additional restriction sites. The *35S::Cab22L::Athb-1::nosIR* and *35S::Cab22L::T10P11.13::nosIR* constructs were ligated into binary vector AR4741, which carries a *Group2:surB* selectable marker gene conferring resistance to chlorsulfuron, and introduced into *A. tumefaciens* strain ABI.

Plant transformation

Cotyledons of tomato (*L. esculentum* Mill. cv. T53, a proprietary inbred of Seminis Vegetable Seeds) were transformed using *A. tumefaciens* strain ABI harboring the *FMV::hsp70::PG::nosIR* construct, as described (Yoder *et al.*, 1988). Regenerated transformed seedlings were selected by growth on media containing kanamycin, rooted and 56 independent transformants were grown to maturity in a greenhouse.

Arabidopsis thaliana ecotype Columbia were transformed by *A. tumefaciens* strain ABI harboring the *35S::Cab22L::T10P11.13::nosIR* or *35S::Cab22L::Athb-1::nosIR* constructs, or harboring empty binary vector as a transformation control, using the floral dip method (Clough and Bent, 1998). Seeds were grown in soil under a 24-h day regime at 25°C. At approximately 14 days from planting they were sprayed thrice at intervals of 48 h with Telar herbicide (13.4 mg l^{-1}), at which time wild-type seedlings planted as controls and untransformed seedlings died.

RNA analysis

Tomato fruit were harvested at the light red ripening stage (4–7 days after the breaker stage, which is the beginning of ripening), locules and seeds were discarded and pericarp tissue was frozen in liquid nitrogen and stored at −80°C. RNA was prepared by powdering pericarp tissue in liquid nitrogen, extracting with phenol/chloroform and precipitating with lithium acetate as described (Dunsmuir *et al.*, 1987). Aliquots of 5 μg total RNA were separated by electrophoresis in 1.2% agarose and 10% formaldehyde denaturing gels, then blotted to nylon membranes (Duralon-UV, Stratagene, La Jolla, CA) following the manufacturer's instructions and cross-linked by irradiation with ultra-violet light. RNA gel blots were hybridized with a radioactively labeled probe prepared from the cDNA of the tomato *PG* gene using random nucleotide hexamers, α-[^{32}P]-dCTP and the Klenow fragment of DNA polymerase I. Hybridization was in Robbins hybridization buffer (7% SDS, 0.25 M Na_2HPO_4, 1% BSA, 1 mM EDTA, pH 7.4) at 65°C overnight, and the blot was subsequently washed in 0.1× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate) and 0.1% SDS at 65°C three times, then exposed to X-ray film. Blots were additionally exposed to phosphorimager plates so that relative *PG* mRNA abundance could be quantified. To determine that lanes were equally loaded, gel blots were stripped by washing twice with boiling 0.1% SDS, 0.1× SSC, then re-hybridized with a probe prepared as above from pTA-71, a plasmid containing a cDNA of wheat ribosomal RNA (Gerlach and Bedbrook, 1979).

RNA was prepared from leaves of *Arabidopsis* plants using the Trizol reagent (Life Technologies, Gaithersburg, MD) and following the manufacturer's instructions. RNA was examined on RNA gel blots essentially as above, using labeled cDNA probes synthesized from the *T10P11.13* and *Athb-l* coding sequences using random nucleotide hexamers, α-[^{32}P]-dCTP and the Klenow fragment of DNA polymerase I. RNA gel blots were stained with methylene blue to ensure that lanes had been equally loaded. The presence of 21–25-mer oligoribonucleotides in RNA preparations from lines exhibiting PTGS was detected after separation in 15% polyacrylamide gels and blotting to nylon membranes essentially as described (Hamilton and Baulcombe, 1999), using a hybridization temperature of 40°C and washes in 2× SSC, 0.1% SDS at a temperature of 50°C, except that the probe was a labeled, denatured cDNA rather than a riboprobe.

Phenotype analysis of Arabidopsis *transformants*

Silencing of the *Arabidopsis* transcription factors *T10P11.13* and *Athb-1* resulted in reduced leaf expansion and a rosette of smaller diameter. Independent transformants resistant to chlorsulfuron, containing either the *35S::T10P11.13::nosIR* or *35S::Athb-1::nosIR* inverted repeat constructs or empty binary vector, were allowed to self pollinate and seeds collected. Approximately 12 seeds per line of the following generation were grown in soil and selected by spraying with chlorsulfuron herbicide as above. The surviving homo- and hemizygotes were grown further, then measurements of rosette diameter were made 25 days after planting, taking the mean of four measurements per plant at 45° angle to each other across the diameter of the rosette to allow for uneven leaf development. Measurements were made on all surviving plants (a minimum of six) per line.

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