New Construct Approaches for Efficient Gene Silencing in Plants

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An important component of conventional sense, antisense, and double-strand RNA-based gene silencing constructs is the transcriptional terminator. Here, we show that this regulatory element becomes obsolete when gene fragments are positioned between two oppositely oriented and functionally active promoters. The resulting convergent transcription triggers gene silencing that is at least as effective as unidirectional promoter-to-terminator transcription. In addition to short, variably sized, and nonpolyadenylated RNAs, terminator-free cassette produced rare, longer transcripts that reach into the flanking promoter. These read-through products did not influence the efficacy and expression levels of the neighboring hygromycin phosphotransferase gene. Replacement of gene fragments by promoter-derived sequences further increased the extent of gene silencing. This finding indicates that genomic DNA may be a more efficient target for gene silencing than gene transcripts.

The unidirectional and unperturbed transcription of either genes or gene fragments from promoter to terminator can trigger posttranscriptional silencing of target genes. Initial expression cassettes for posttranscriptional gene silencing in plants comprised a single gene fragment positioned in either the antisense (Shewmaker et al., 1992; McCormick et al., 2003) or sense (van der Krol et al., 1990) orientation between regulatory sequences for transcript initiation and termination. In Arabidopsis (Arabidopsis thaliana), recognition of the resulting transcripts by RNA-dependent RNA polymerase leads to the production of double-stranded (ds) RNA (Dalmay et al., 2000). Cleavage of this dsRNA by Dicer-like (Dcl) proteins such as Dcl4 yields 21-nucleotide small interfering RNAs (siRNAs; Hamilton and Baulcombe, 1999; Dunoyer et al., 2004). These siRNAs complex with proteins including members of the Argonaute (Ago) family to produce RNA-induced silencing complexes (Morel et al., 2002; Liu et al., 2004). The RNA-induced silencing complexes then target homologous RNAs for endonucleolytic cleavage.

More effective silencing constructs contain both a sense and antisense component, producing RNA molecules that fold back into hairpin structures (Waterhouse et al., 1998; Smith et al., 2000). The high dsRNA levels produced by expression of inverted repeat transgenes were hypothesized to promote the activity of multiple Dcls. Analyses of combinatorial Dcl knockouts in Arabidopsis supported this idea and also identified Dcl4 as one of the proteins involved in RNA cleavage (Gasciolli et al., 2005; Xie et al., 2005).

Although constructs that lack a promoter element failed to induce gene silencing (Waterhouse et al., 1998), the requirement of termination sequences has not been studied extensively. Here, we demonstrate that terminators are only important for the efficacy of conventional gene silencing constructs. A new type of expression cassette that contains either gene or promoter fragments between oppositely oriented promoters was found to trigger silencing at least as effectively as conventional constructs. Construct efficacy was correlated with convergent collisional transcription that resulted in the production of pools of variably sized RNAs.

RESULTS

The Role of Terminators in Conventional Expression Cassettes for Gene Silencing

The requirement of terminator elements in gene silencing was studied by transforming a transgenic tobacco (Nicotiana tabacum) plant that constitutively expressed the β-glucuronidase (gus) gene (see “Materials and Methods”) with unidirectional silencing constructs (Fig. 1). Two control constructs contained a single 305-bp gus gene fragment inserted in the antisense (pSIM755) or sense (pSIM718) orientation between the 35S promoter of Cauliflower mosaic virus (P35S) and the terminator of the nopaline synthase (nos) gene. Introduction of these constructs into gus tobacco triggered low frequencies of gene silencing (3%–6%) that are typical for antisense and sense approaches (see, for example, Smith et al., 2000; Singh et al., 2000; Table I). Employment of a construct containing both a sense and antisense copy of the gene fragment between promoter and terminator (pSIM374) confirmed the much greater frequency of gene silencing that is obtained...
with hairpin RNAs (Waterhouse et al., 1998; Table I). In contrast, the use of terminator-free constructs representing antisense (pSIM758) and sense (pSIM140) approaches did not yield any silenced plants, and the silencing frequency of a terminator-free construct designed to produce hairpin RNA (pSIM777) was only one-sixth that of pSIM374 (Fig. 1; Table I).

An important signal for RNA polyadenylation is the near upstream element (NUE) with consensus sequence 5’-AAUAAA, located between 13 and 30 nucleotides upstream of the cleavage site (Loke et al., 2005). Elimination of the corresponding DNA sequence in terminator elements was predicted to negatively affect mRNA 3’-end processing and, consequently, reduce the efficacy of silencing. This concept was tested by substituting the nos terminator of pSIM374 with a derivative element lacking the NUE element (mT). Use of the resulting vector pSIM376 was, indeed, shown to decrease the frequency of gus gene silencing if compared to the original construct ($P = 0.05$; Table I). Collectively, our data demonstrate an important link between conventional gene silencing and mRNA 3’-end processing. Effective silencing appears to be associated with the ability of the construct to process its transcripts.

**Terminator-Free Silencing Constructs Containing Inverted Repeats**

To study whether gene silencing could be established by new types of expression cassettes, we generated terminator-free constructs that contained two oppositely oriented promoters (Fig. 1). The first convergent transcription vector pSIM717 contained two copies of the gus gene fragment positioned as inverted repeat between $P_{35S}$ and the figwort mosaic virus (FMV) promoter ($P_{FMV}$). Surprisingly, retransformation of gus-expressing tobacco plants with this construct resulted in a high frequency of gene silencing. This frequency was at least as high as that of the corresponding conventional silencing construct pSIM374 ($P = 0.02$; Table I; data not shown). Transformation with the second vector pSIM756, which is identical to pSIM717 except that the gus gene fragments are oriented as divergent repeat, also yielded similar gene silencing frequencies. This finding indicates that the orientation of the inverted repeat does not play an important role in establishing gene silencing.

Because $P_{FMV}$ of pSIM717 and pSIM756 was identical to the promoter driving the original gus gene expression cassette, gene silencing could have been induced by promoter targeting. This possibility was excluded by evaluating vector pSIM754, which contains $P_{FMV}$ operably linked to $P_{35S}$. None of the tested

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**Figure 1.** Silencing constructs. A blue arrow indicates the position and orientation of the gus gene fragment used as trigger for gene silencing; a green box between arrows indicates a spacer. 35S, CMV 35S promoter; FMV, FMV35S promoter; FMV', FMV35S promoter lacking TATA box; Ubi, promoter of the potato ubiquitin-7 gene; T, nos gene terminator; mT, modified nos terminator lacking a NUE element; pA, synthetic poly(A) tail. A pink arrow depicts a fragment of the tobacco Ppo gene; an orange box indicates the intron of the gus gene. The expression cassette for the hpt selectable marker gene was oriented in such a way that the terminator of this cassette would not function in 3’-end processing of transcripts produced by the silencing constructs.
double-transformed tobacco plants displayed a reduced level of gus expression (Table I).

The stability of pSIM717-mediated gene silencing was assessed by assay groups of approximately 25 T1 and T2 plants derived from three randomly chosen transformants. Progeny plants that were PCR positive for both the gus gene and the silencing construct displayed a similar level of gus silencing as determined for T0 plants, indicating that terminator-free gene silencing is, at least in the T1 and T2 generation, not gradually diminished (data not shown).

The molecular basis of pSIM717-mediated gene silencing was studied by isolating RNA from double transformants. This RNA was used as template for reverse transcription (RT) PCRs with the primer combinations pr1 to pr3 and pr1 to pr4 (Fig. 2A). These experiments demonstrated that effective silencing of the gus gene in plants such as 717-36 was correlated with successful amplification of both cDNAs, indicative for the production of dsRNA (Fig. 2, A and B; data not shown). In contrast, amplification of only one of the cDNAs in plants 717-13 (with pr1–pr3) and 717-5 (with pr1–pr4) was associated with a lack of detectable gene silencing (Fig. 2B). Similar results were obtained for silenced plants transformed with the pSIM717-derivative vector pSIM715, which contained a larger intron separating the two gus gene fragments (Fig. 2B; data not shown).

RNA gel-blot analyses using a gus gene fragment amplified with primers pr1 and pr2 showed that convergent transcription in fully silenced plants such as 717-12 and 717-36 (Fig. 2, C and D), and 715-19, 715-38, and 715-55 (Fig. 2D) resulted in both 3′-to-approximately 10-fold reduced levels of gus gene transcripts and the production of new RNAs that were produced by the silencing construct and shorter than the distance between the two driver promoters (Fig. 2, C and D). In contrast, a lack of detectable gene silencing in, for instance, plants 717-7, 717-8, 717-9, 717-10, and 717-13 was linked to less than 2-fold reduced gus gene transcript levels (Fig. 2, C and D). These plants produced only limited amounts of the new small RNAs (Fig. 2, C and D). The production of relatively large amounts of variably sized (approximately 0.2–1.0 kb) transcripts in silenced plants was confirmed with a probe specific for the silencing construct (Fig. 2E). Silenced plants such as 715-19 and 715-38 also produced rare, approximately 1-kb transcripts that extended into P35S and could be visualized after extended exposure (Fig. 2F). The absence of similar transcripts extending into P35S (Fig. 2G) suggests that this promoter is stronger and dominant over P35S.

The above-described studies had shown that terminator-free sense (pSIM140) and antisense (pSIM758) constructs did not trigger gene silencing effectively. We therefore assumed that the rare read-through transcripts of pSIM715 would not interfere with the expression of neighboring genes. To test this hypothesis, we compared the efficacy of the hygromycin phosphotransferase (hptII) selectable marker gene of pSIM717 with that of pSIM374. Two weeks after infection with Agrobacterium strains carrying the binary vectors, tobacco explants had developed comparable numbers of hygromycin-resistant calli (Fig. 3A). The apparent lack of an inadvertent regulatory effect of the terminator-free expression cassette on the neighboring hptII gene was confirmed by carrying out real-time PCR. Figure 3B shows similar levels of hptII transcript for 6-week-old pSIM374 and 717 plants that displayed gus gene silencing. Each group of plants contained one individual with a lower expression level. This variation within the groups is probably due to position-integration effects.

We studied whether the products of collisional transcription were polyadenylated by performing RT-PCRs on the RNA of silenced plants. Control amplifications were carried out with an oligo(dT) reverse primer together with pr1. As expected, this reaction yielded a strong band that was confirmed by sequencing to represent the polyadenylated 3′ region of the gus transcript (Fig. 4). However, amplification with the oligo(dT) and pSIM717-specific pr4 primer combination yielded only small quantities of cDNA that visualized on agarose gels as a faint smear (Fig. 4). Sequence analysis confirmed that none of 20 cDNAs isolated from this smear corresponded to transcripts produced by the silencing construct (data not shown). Similar results were obtained by sequencing weak bands amplified with oligo-T and pr3 (Fig. 4; data not shown). Our results indicate that collisional transcription of inverted repeats triggers effective gene silencing through the production of variably sized and generally nonpolyadenylated RNAs.

### Table I. Constructs for transcript-targeted gene silencing

<table>
<thead>
<tr>
<th>Construct</th>
<th>TER Approach</th>
<th>Target</th>
<th>Plants Assayed</th>
<th>Silencing Frequency</th>
</tr>
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</table>
| pSIM755     | Y UD antisense gus RNA | 36 | 3%
| pSIM718     | Y UD sense gus RNA | 34 | 6%
| pSIM374     | Y UD hairpin gus RNA | 107 | 48%
| pSIM758     | N UD antisense gus RNA | 29 | 0%
| pSIM140     | N UD sense gus RNA | 36 | 0%
| pSIM777     | N UD hairpin gus RNA | 36 | 8%
| pSIM376_y  | N UD sense gus RNA | 35 | 29%
| pSIM717     | N CV hairpin gus RNA | 147 | 62%
| pSIM756     | N CV hairpin gus RNA | 37 | 51%
| pSIM754     | N CV promoter gus promoter | 38 | 0%
effective (8%) than the single gene fragment of vector pSIM772 (3%; Table II). An additional 4- to 5-fold increase was obtained by employing expression cassettes containing four direct repeats (pSIM787 and pSIM1111; Table II). These results demonstrate that convergent transcription of four direct repeats can be as effective as that of an inverted repeat and suggest that the complementary RNAs produced by convergent transcription hybridize efficiently to produce dsRNA.

Terminator-free silencing constructs can also be designed to express the intron of a target gene. The silencing construct of vector pSIM782 contains an inverted repeat consisting of two copies of the intron of the gus gene inserted between P35S and PFMV. Though only 3% of plants transformed with this construct were partially silenced, it is interesting that targeting of transient and nuclear pre-mRNAs can, in fact, result in silencing of the corresponding gene (Table II).
Multigene Silencing Constructs

Conventional gene silencing constructs have in some cases been used to simultaneously down-regulate the expression of multiple genes (Halpin et al., 2001). The inverted repeats of such expression cassettes consisted of sense and antisense copies of two or more different gene fragments. Because transcripts produced by constructs such as pSIM717 were often shorter than the distance between the two driving promoters, we reasoned that distal gene fragments would be less effective than centrally located fragments in triggering gene silencing. This hypothesis was confirmed by generating two binary vectors with sense and antisense gene fragments from both the gus and tobacco polyphenol oxidase (Ppo) genes inserted between two promoters (Table II). Vector pSIM774 contained the gus gene fragments in distal positions with the fragments from the Ppo gene positioned near the center of the expression cassette. In contrast, vector pSIM775 contained the central spacer flanked by gus gene fragments. Analyses of double-transformed tobacco plants demonstrated that the frequency of gus silencing was lower for pSIM774 than for pSIM775 ($P = 0.007$; Table II). These results demonstrate that the efficacy of gene silencing depends, indeed, on the position of the trigger gene fragments within the terminator-free collisional transcription (TFCT) construct. Most likely, the outer sequences are less frequently transcribed into RNAs that fold back into hairpins.

Promoter Substitutions Influence Silencing Efficacy

To test the promoter specificity of TFCT silencing constructs, new vectors were constructed that contained a sense and antisense fragment of the gus gene inserted between various convergent promoter combinations. Histochemical analyses of retransformed gus plants demonstrated that the expression cassette of pSIM771, which combines the P$_{35S}$ with the promoter of the potato (Solanum tuberosum) ubiquitin-7 (P$_{Ub7}$) gene (Garbarino et al. 1995), triggered a high overall frequency of gus silencing (43%) approaching that previously determined for pSIM717 (Tables I and II). In contrast, the use of two P$_{35S}$ elements in pSIM770 dramatically lowered the silencing efficacy to 8% only (Table II). The poor efficacy of this expression cassette may be due to methylation of P$_{35S}$. Although not studied as part of the work described here, the accessibility of P$_{35S}$ to methylation was frequently observed by others (Meyer et al., 1992; Dieguez et al., 1998). Another combination of identical promoters for TFCT constructs was tested as well. Vector pSIM789 contains two copies of P$_{FMV}$ and triggered gene silencing about as frequently (46%) as pSIM717 (Table II; Fig. 5). Collectively, our data demonstrate that the efficacy of TFCT-based gene silencing is to a large degree dependent on the choice of promoter combination.

TFCT-Mediated Gene Silencing of Endogenous Genes Expressed in Potato Tubers

We also studied whether TFCT expression cassettes could be exploited to down-regulate the expression of endogenous genes. For this purpose, three new vectors targeting the potato tuber-expressed Ppo gene were constructed (Fig. 6A). The control vector pSIM217 represented a conventional silencing approach and...
comprised both a sense and antisense 154-bp fragment of the untranslated trailer of the potato tuber-expressed Ppo gene (Rommens et al., 2004) inserted between the promoter of the granule-bound starch synthase (Gbss) gene and the nos gene terminator. Vector pSIM764 was identical to pSIM217 except that the terminator was replaced by a second copy of the Gbss promoter. The third vector, pSIM765, contained the two Ppo gene fragments inserted in the opposite orientation between two convergent Gbss promoters. Agrobacterium strains carrying the various binary vectors were used to transform the potato variety Ranger Russet, and three copies of 21 PCR positive lines for each construct were allowed to set tubers in the greenhouse. Subsequent biochemical assays of three tubers per line demonstrated that all lines displayed reduced Ppo activity (Fig. 6, B and C). On average, the activity was lower in pSIM764 plants (36.8% ± 2.5% of wild-type levels) than in pSIM217 controls (49.5% ± 3.9%; $P = 0.007$). The opposite orientation of Ppo gene fragments in pSIM765 resulted in an average activity of 41.2% ± 3.9%.

A second gene that was targeted for silencing was the potato tuber-expressed phosphorylase-L (PhL) gene. Conventional silencing approaches have shown that lowered PhL gene expression triggers reduced Glc accumulation in cold-stored tubers (Kawchuk et al., 1999). These results were confirmed with vector pSIM216, which contains an inverted repeat consisting of two untranslated leader sequences inserted between the same regulatory elements as used for pSIM216 (Fig. 6D). The TFTC vector pSIM847 differs from pSIM216 in that the inverted repeat is positioned between the Gbss promoter and the promoter of the potato ADP-Glc pyrophosphorylase gene (du Jardin and Berhin, 1991). Transgenic tubers containing this construct displayed a level of reduced Glc accumulation that was, on average, at least similar to that for pSIM216 (Fig. 6E). Because Glc levels provide only an indirect indication of the efficacy of gene silencing, we also studied transcript levels in randomly chosen pSIM216 and pSIM847 tubers. This analysis confirmed that the two different constructs triggered similar reductions in PhL gene expression (Fig. 6F). In both cases, PhL expression levels were about 20-fold lower than those of control plants containing only the selectable marker gene. Thus, endogenous genes can be silenced effectively through convergent transcription.

**Table II. Alternative constructs for transcript-targeted gene silencing**

<table>
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<tr>
<th>Construct</th>
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<th>Approach</th>
<th>Target</th>
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**Promoter-Targeted Gene Silencing**

To compare the frequencies of gene-based silencing constructs with constructs targeting promoter sequences, we produced two vectors containing one (pSIM1112) and two (pSIM773) copies of PFMV which also drives the gus target gene, between the convergent driver $P_{35S}$ and $P_{Ubi7}$ elements (Fig. 1). Compared to the above-described vector pSIM772, which contains a single copy of the gus gene fragment, exploitation of pSIM1112 and pSIM773 yielded higher frequencies of gene silencing (Tables II and III). These results indicate that promoter sequences can be more effective than gene-derived sequences in triggering gene silencing. Furthermore, the much stronger activity of pSIM773 compared to pSIM1112 demonstrates the importance of dsRNA generation for promoter-targeted gene silencing approaches.

Interestingly, all silenced pSIM773 plants lacked any gus expression (Fig. 7A). This phenotype is different from the generally partial silencing that is triggered by gene-based silencing constructs (Fig. 7; data not shown) and indicates that gene silencing may be accomplished more effectively by targeting promoters than through

![Figure 5. pSIM789 plants. Histochemically stained leaf punches of plants containing both the gus gene and the silencing construct of pSIM789. Boxed leaf samples depict stained gus positive (top) and wild-type (bottom) plants.](image-url)
RNA degradation. Progeny analyses showed that the level of silencing triggered by pSIM773 is stable in at least the T1 and T2 generations (Fig. 7A; data not shown). The efficacy of promoter-targeted silencing is not limited to constructs that contain the target promoters in a convergent orientation. Similar frequencies (77%) were obtained when these promoters were placed in the opposite (divergent) direction in pSIM1120 (Table III; Fig. 7B).

The pSIM773-derived vector pSIM788 only contained the target promoter sequences upstream from the TATA box. Although this promoter fragment was shown to be nonfunctional (data not shown), pSIM788 also triggered effective gus gene silencing (Table III; Fig. 7C). This finding demonstrates, for the first time, that nonfunctional target promoter fragments can activate transgene silencing if expressed to produce dsRNA. We also found that replacement of one of the two driver promoters of pSIM773 by a terminator element (pSIM1101) still provided effective silencing. In this case, some of the retransformed plants displayed only a partial gene silencing phenotype (Table III; Fig. 7D). This result indicates that the presence of the terminator element may have influenced the plant’s silencing response.

DISCUSSION

Terminator-Free Gene-Based Silencing Constructs

One aspect of the work presented here relates to the significance of a terminator element in silencing constructs. Although little is understood about the processes and sequences that govern transcriptional termination, the nos terminator that was used in our studies is known to contain mRNA 3’-end processing signals required for cleavage and polyadenylation (Depicker et al., 1982). Removal of this element greatly reduced the efficacy of constructs that contained a promoter element driving the expression of gene-derived...
sense, antisense, or inverted repeat structures. This result implies that 3’-end processing has a positive effect on the ability of transcripts to function as substrates for Dcl proteins. In fruit flies (Drosophila melanogaster), the Ago protein associated with siRNA-directed silencing (Ago2) was shown to play a role in poly(A) length maintenance (Siomi et al., 2005). Depletion of Ago2 resulted in mRNA stabilization and shortened poly(A) tails. A similar link between mRNA 3’-end processing and silencing would explain the importance of the terminator element in conventional constructs for gene silencing in plants.

Interestingly, the need for a terminator element is circumvented by placing the gene-derived trigger sequences between two convergent promoters. Our results indicate that the resulting transcripts are not polyadenylated. In addition to RT-PCR experiments, further studies on such RNAs are currently ongoing. The activation of gene silencing by transcripts that did not undergo full 3’-end processing is not without precedent. In petunia, cosuppression of the chalcone synthase gene was correlated with an accumulation of nonpolyadenylated chalcone synthase RNA (Metzlaff et al., 1997). Similarly, the initiation of silencing of the MuDR/Mu transposition system in maize (Zea mays) coincided with nonpolyadenylated RNA production (Rudenko et al., 2003). Thus, aberrant transcripts produced by convergent transcription, cosuppression, and transposable elements may represent highly accessible substrates for Dcl activity, possibly after processing by factors involved in the recognition of such RNAs. Convergent transcription constructs display about the same efficacy as conventional promoter-to-terminator constructs in suppressing target genes. We also found that the two different construct approaches were most effective if used to produce dsRNA. These resemblances imply an involvement of similar Dcl proteins in transcript cleavage.

To some extent, the organization of convergent transcription constructs resembled that of rare transformation events where two T-DNAs that contain a conventional silencing construct integrate at the same locus in a divergent orientation. Read-through transcription might in some cases produce hairpin RNAs that trigger gene silencing. For instance, a transgenic tomato (Lycopersicon esculentum) line silenced for the polygalacturonase gene contains two oppositely oriented T-DNA inserts at the same locus. RNA gel-blot analyses of this line might suggest the presence of mRNAs that extend beyond the terminators (Sanders and Hiatt, 2005). However, there may be alternative explanations for this phenomenon. By studying transgenic plants containing multiple copies of a target gene, gene silencing appeared to be linked to copy number rather than inverted repeats (Lechtenberg et al., 2003). Convergent transcription constructs have been used previously for Dicer-dependent gene silencing in mammalian cells. In contrast to our approach, these constructs still contained the gene-derived sequences linked to five consecutive thymine residues that function in transcript termination (Tran et al., 2003).

The new construct approaches described here may facilitate efforts to fine-tune the regulation of gene silencing. For instance, it may be possible to specifically silence certain genes in cold-stored potato tubers by using a silencing construct that contains both a cold-inducible and tuber-specific promoter. Most RNAs that were produced through convergent transcription had sizes that are shorter than the distance between the two convergent promoters. However, we did detect transcripts that extended into one of the driver promoters. This finding was unexpected because hairpin transcripts are susceptible to Dicer, and transcripts lacking a poly(A) tail would be predicted to be unstable. Given the poor efficacy of terminator-free antisense constructs such as pSIM758, the production of rare long transcripts is unlikely to inadvertently affect the expression of neighboring genes. Indeed, we did not find convergent transcription to lower hptIII gene expression levels in pSIM717 plants.

In addition to the use of exon-derived sequences as trigger for gene silencing, we also demonstrated the efficacy of constructs containing intron DNA. This finding indicates that nuclear pre-mRNAs can function as target for degradation. A similar retention of RNAs was associated with MuDR/Mu silencing.

Figure 7. Promoter-targeted gene silencing. Representative histochemically stained leaf punches are shown for pSIM773 (A), pSIM1120 (B), pSIM788 (C), and pSIM1101 (D). Partially silenced leaf punches are shown in a green box.
by the promoter-based silencing constructs may have processing of at least some of the transcripts produced terminator of an adjacent expression cassette. The earlier expressed cassettes either contained a terminator or were functionally associated with the terminator. The earlier expressed cassettes either contained untranslated leader sequences. Another unique aspect influenced, at least in part, by their ability to express efficacy of these earlier constructs could have been stream from their transcription start (Mette et al., 1999, 2000; Kanno et al., 2004, 2005). Thus, the silencing constructs lack any sequences that represent functional or nonfunctional promoters and can be oriented as convergent or divergent repeat.

The convergent transcription of inverted repeats containing target promoter sequences triggers a high frequency of complete gene silencing. The efficacy of this approach suggests that ds promoter RNAs activate a pathway different from that triggered by gene-based methods. Based on silencing pathways that operate at the level of the nuclear genome, promoter-derived dsRNAs are likely to trigger RNA-mediated DNA methylation (Wassenegger et al., 1994; Chan et al., 2005). The Dcl protein involved in this process may be Dcl3, which produces siRNA of the 24-nt size class that target cytosine methylation (Xie et al., 2004). Proteins involved in this de novo methylation include cytosine methyltransferases such as Drm1, Drm2, Met-1, and Cmt3. The latter two enzymes are involved in the maintenance of CG, CNG, and CNN methylation, respectively (Jones et al., 2001; Lindroth et al., 2001; Chan et al., 2005), and may explain the complete knockout phenotypes in, for instance, pSIM773 plants. Replacement of one of the driver promoters by a terminator negatively affects the efficacy of gene silencing with some plants still displaying the full gene silencing typical for the corresponding terminator-free constructs, but others are only partially silenced.

In summary, we have shown that expression cassettes lacking a terminator but containing two convergent promoters can be used to induce effective gene silencing. The efficacy of these constructs implies that silencing can be activated by a pool of nonprocessed transcripts of variable size. The use of two different promoters makes it possible to fine tune the regulation of gene silencing.

**MATERIALS AND METHODS**

**Development and Use of a gus-Expressing Tobacco Line**

A transfer DNA only containing an expression cassette for the gus gene flanked by T-DNA borders was introduced into tobacco (*Nicotiana tabacum*) by employing a novel marker-free transformation method (Weeks and Rommens, 2003). Explant material for retransformation was obtained from propagated 6-week-old tissue culture material derived from a single homozygous T1 plant. Silencing constructs were introduced into the T1 plant as described previously (Rommens et al., 2004). Gus expression levels were determined by performing at least three independent assays per plant. Histochemical and fluorometric assays were performed as described previously (Jefferson et al., 1987; Lin et al., 1994).

**Construction of Expression Cassettes**

The gus gene fragment used as trigger for gene silencing represents the sequences at position 1292-1596 of GenBank accession number S69414. Silencing constructs were inserted into the T-DNA of a binary vector next to an expression cassette for the hptII selectable marker gene in such a way that the
associated terminator of the expression cassette was not operably linked to the silencing constructs. The P35S element was produced synthetically and corresponds to nucleotides 6,368 to 6,949 of accession X06166. A truncated promoter derivative lacking TATA box and downstream sequences comprises the sequence from 6,368 to 6,887.

RNA Analysis

TRIZol (Invitrogen) was used to isolate RNA according to the manufacturer’s recommendations. The following primers were used for RT-PCRs: 5′-CCA CGC GTA AAC TCG ACC CCG TGC TGC (pr1), 5′-ATG CAC ACT GAT CAT CCT CAC TCC AC (pr2), 5′-TGG TGT TTT TGG TAT TGC ATG TTC TGC (pr3), and 5′-TGG AGG AAG TGA TGA AAA GAT ACC AGG (pr4). Gel-blot analyses were carried out using Hybon-N™ membranes (Amersham Biosciences). A 294-bp gus gene fragment amplified with primers pr1 and pr2 was used as probe to visualize the transcripts produced by expression of the gus gene and silencing constructs. Additional probes targeted an intron derived from the potato (Solanum tuberosum) Gbss gene that separates the two gus gene fragments of vectors such as pSIM717, amplified with 5′-CCT GCA GAA GCT ACA GAT GAA C and 5′-GAC GCT ACA GGT GAT GAT CCT TC (256 bp); P35S, amplified with 5′-GGT TGA GTA TCT GAT GAT CCT TC (582 bp). Of the solid fraction, the change of OD410 was determined over time.

Ppo Assays

The levels of Ppo enzyme activity were compared with wild-type levels by mixing the pulverized tubers (1 g) of plants that had been grown for 6 weeks in the greenhouse for 1 h in 50 mL MOPS buffer at pH 6.5 (5 mL). After precipitation of the solid fraction, the change of OD410 was determined over time.

Glucose Determination in Cold-Stored Potato Tubers

Tubers of plants that had been grown for 6 weeks in the greenhouse were harvested and incubated for 1 month at 4°C. After this cold storage, Glc levels were determined by using a Glc oxidase/peroxidase reagent (Megazyme).

Acknowledgments

We are grateful to Dr. Kathy Swords for fruitful discussion and a critical review of the manuscript, and Scott Simplot and Bill Whitacre for continued support. We also thank Dr. William Belknap for providing the potato ubiquitin-7 promoter. Kristine Barney, Joanna Owen, and Lynda Zhang are acknowledged for excellent technical assistance.

Received April 17, 2006; revised May 26, 2006; accepted June 4, 2006; published June 9, 2006.

LITERATURE CITED


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