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Technical Note

A versatile method to prevent transcriptional gene silencing in *Arabidopsis thaliana*

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Abstract Transcriptional gene silencing (TGS) is a phenomenon by which transgenes that share homology in their promoter regions are inactivated. TGS is known to be reduced by using different promoters to drive the expression of each transgene. However, in order to perform a large-scale genetic screen in which overexpression lines are mutagenized by T-DNA tagging and therefore harbor two types of transgene, it is critical to develop a technique that consistently blocks TGS. Here, I report a versatile method that completely prevents TGS in transgenic Arabidopsis. A seedling morphology-based assay demonstrated that TGS could be significantly diminished by using different terminator sequences for each transgene. Furthermore, it was suggested that TGS might be reduced if the orientations of the two T-DNA sequences were reversed relative to each other. By combining these strategies, I showed that TGS was completely blocked in over 50,000 T1 plants. These findings present a thorough and versatile method to prevent TGS that is potentially applicable to various plant species and is expected to be used in diverse situations, from basic to applied research fields.

Key words: *Arabidopsis thaliana*, gene silencing, phytochrome, T-DNA tagging, TGS.

The introduction of additional genes into overexpression lines induces the inactivation of the first transgene. This phenomenon presents a serious problem for genetic screens in which overexpression lines are mutagenized by T-DNA tagging. In such cases, although the possibility cannot be excluded that post-transcriptional gene silencing (PTGS) that is based on homology in mRNA sequences may also be involved, the inactivation of the transgene is most likely due to transcriptional gene silencing (TGS), which occurs in the presence of sequence homology in the promoter regions that are used to overexpress a gene of interest in the first T-DNA and to express a selectable marker gene in the second T-DNA. Although TGS can be substantially reduced by using different promoters to drive the expression of each transgene (Neuhuber et al. 1994; Thierry and Vaucheret 1996), a technique that completely prevents TGS is needed in order to facilitate large-scale genetic screens. In this study, I have developed a procedure to block TGS, while designing a large-scale genetic screen to identify downstream components of phytochrome B (phyB) signal transduction.

PhyB is the major photoreceptor of light-grown plants and the phyB polypeptide consists of two domains, the photosensory N-terminal domain and the C-terminal domain, which has nuclear localization and dimerization activities. Because the C-terminal domain contains a kinase motif, phyB is believed to transduce the signal via the kinase activity inherent in the C-terminal domain. However, a green fluorescent protein (GFP)-fusion of the N-terminal domain of phyB (NG), that has been dimerized and localized to the nucleus by the activities of β-glucuronidase (GUS) and nuclear localization signal (NLS), respectively (NG-GUS-NLS), has been shown to be even more active than full-length phyB (Matsushita et al. 2003). This finding demonstrates that phyB transduces the signal from its N-terminal domain and prompted me to conduct a forward genetic screen to identify novel components of the signaling pathway that function downstream of the phyB N-terminal domain. The biggest obstacles to forward genetic screens are the redundancy of gene functions, and the subtle nature of the phenotypes exhibited by many recessive mutants. In this study, I sought to overcome these issues by

Abbreviations: BAR, glufosinate resistance gene; *CaMV*, *Cauliflower mosaic virus*; cry1, cryptochrome 1; cW, continuous white light; GFP, green fluorescent protein; GUS, β-glucuronidase; LB, T-DNA left border; masP, *Agrobacterium tumefaciens* mannopine synthase promoter; NG, N-terminal domain of phyB fused to GFP; NLS, nuclear localization signal; nosT, *Agrobacterium tumefaciens* nopaline synthase terminator; NPTII, neomycin phosphotransferase II; ocsT, *Agrobacterium tumefaciens* octopine synthase terminator; p35S, *CaMV* 35S promoter; phyB, phytochrome B; RB, T-DNA right border; t35S, *CaMV* 35S terminator; TGS, transcriptional gene silencing.

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exaggerating the mutant phenotype in order to facilitate its detection.

A transgenic Arabidopsis line expressing highly functional NG-GUS-NLS in the *cry1phyB* double mutant background was used as the parental line for the genetic screen. The *cry1phyB* double mutant is devoid of both phyB and cryptochrome 1 (cry1), a blue light photoreceptor that has overlapping functions with phyB. Seedling de-etiolation, which involves the inhibition of hypocotyl elongation and cotyledon expansion, is the most striking example of a light response that is governed by phyB and cry1. Thus, the *cry1phyB* double mutant has an extremely long hypocotyl under continuous white light (cW) (Figure 1). On the other hand, the transgenic line overexpressing NG-GUS-NLS in this double mutant background exhibited an even shorter hypocotyl than wild-type plants under cW (Figure 1). Hence, if this transgenic line is used as the parental line, the difference in hypocotyl length between this line and the *cry1phyB* double mutant (9.9 mm, Figure 1) would be greater than that between the wild type and the *phyB* mutant (2.2 mm, Figure 1), and the mutant phenotype involving phyB signaling would therefore be exaggerated. It is also important to note that NG-GUS-NLS lacks the C-terminal domain, which allows for the isolation of mutants specifically involved in the signaling of the phyB N-terminal domain.

In this study, mutations were introduced by T-DNA tagging. This mutagenesis method facilitates the cloning of genes responsible for mutants with only subtle phenotypes, because the cloning technique relies on the known sequences in the T-DNA region, and requires much less time and effort than map-based cloning. However, TGS is predicted to occur, since the same *Cauliflower mosaic virus* (*CaMV*) 35S promoters are used for overexpressing *NG-GUS-NLS* in the first T-DNA construct and the glufosinate resistance gene (*BAR*) in the second T-DNA, which is used for tagging mutagenesis. The genetic screen would be hampered by this TGS, because inactivation of *NG-GUS-NLS* results in a long-hypocotyl phenotype that is indistinguishable from that of the intended mutants. Therefore, to avoid TGS completely, I examined the effect of using various combinations of terminator sequences and altering the direction of the sequence within the T-DNA, as well as that of the promoter sequences, in the first and second T-DNAs.

First, to minimize the number of introduced promoters and terminators in a line, the *cry1phyB* double mutant was transformed, by means of *Agrobacterium*-mediated floral dip (Clough and Bent 1998), with the binary vector pPZP200/RB-p35S-*NG-GUS-NLS*-nosT-LB, which contains the T-DNA right border (RB), *CaMV* 35S promoter (p35S), *NG-GUS-NLS*, *Agrobacterium tumefaciens* nopaline synthase terminator (nosT), and T-DNA left border (LB) in a pPZP200 backbone (Hajdukiewicz et al. 1994). The resultant transgenic line was used as a parental line for the genetic screen. In a representative homozygous line, I used Southern blot analysis to confirm that a single copy T-DNA was inserted in a single locus (data not shown). In this line, *NG-GUS-NLS* was overexpressed under the control of the 35S promoter without using any selectable marker genes; transformants were selected and a homozygous T3 line was established based solely on the presence of the de-etiolation phenotype (i.e., short hypocotyls and large cotyledons).

This parental line was then transformed with the pPZP200/RB-p35S-*BAR*-nosT-LB binary vector, and the resultant T1 seeds were sown on soil containing 30 μg ml^{-1} glufosinate. Of 312 T1 plants that showed resistance to glufosinate, 170 exhibited an obvious reduction in de-etiolation (i.e., longer hypocotyls; silencing rate = 54.4%) (Figure 2). GUS staining was either abolished or markedly reduced in all of the long-hypocotyl T1 plants examined (Figure 3), indicating a decrease in the amount of NG-GUS-NLS protein in these lines. Consistently, I also observed a marked reduction in GFP fluorescence in these lines (data not shown). These results suggest that the long-hypocotyl phenotype of the T1 plants is a marker of reduced *NG-GUS-NLS* expression due to TGS. Furthermore, because the level of seedling de-etiolation is known to be sensitive to the amount of phyB protein present (Wester et al. 1994), the morphology-based assay used in this study is considered to be sensitive enough to assess the severity of TGS.

I examined whether TGS could be avoided or minimized by replacing the promoter or terminator sequences in the T-DNA tagging vector with other sequences. I transformed the parental line with pPZP200/RB-masP-*BAR*-nosT-LB, in which the promoter is replaced with the *Agrobacterium tumefaciens* mannopine synthase promoter (masP), and found that all of the 295 T1 plants that showed resistance to glufosinate exhibited a level of de-etiolation that was typical of the parental plants (silencing rate = 0%) (Figure 2). This finding is consistent with the previous observation that TGS can be reduced by using different promoters to drive the expression of each transgene (Neuhuber et al. 1994; Thierry and Vaucheret 1996). Then, I replaced the nosT terminator with the *Agrobacterium tumefaciens* octopine synthase terminator (ocsT) to yield pPZP200/RB-p35S-*BAR*-ocsT-LB, and performed a similar analysis. In this case, 10 out of 172 T1 plants exhibited the long-hypocotyl phenotype (silencing rate = 5.8%) (Figure 2). These results indicate that replacing either the promoter or the terminator sequence alone could dramatically reduce TGS.

Next, I investigated whether TGS is influenced by the direction of the sequences within the T-DNA region. The sequences within the T-DNA region of all of the T-DNA

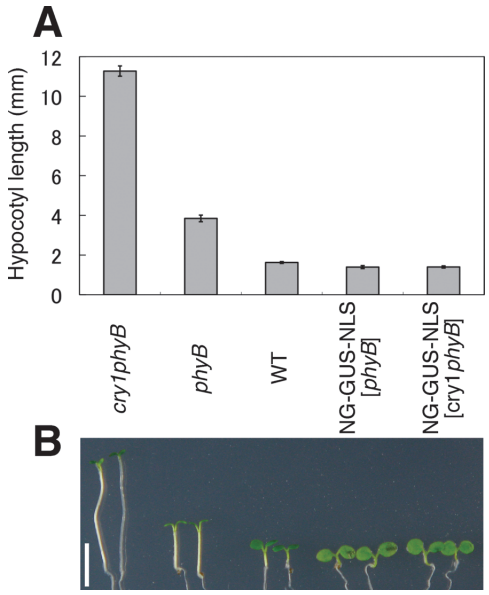

Figure 1. De-etiolation phenotype of the parental line. (A) Hypocotyl lengths of the Arabidopsis *cry1phyB* double mutant, *phyB* mutant, wild-type Col-0 (WT), and transgenic lines overexpressing NG-GUS-NLS in the *phyB* or *cry1phyB* mutant background. The rightmost bar represents the parental line used in this study. Seedlings were grown for 6 d under continuous white light ($32\ \mu mol\ m^{-2}\ s^{-1}$) and hypocotyl lengths were determined. Experimental details are as previously reported (Oka et al. 2004; Oka et al. 2008; Usami et al. 2007). The data are presented as the mean ± SE ($n=25$). (B) Images of the seedlings analyzed in (A). Scale bar = 5 mm.

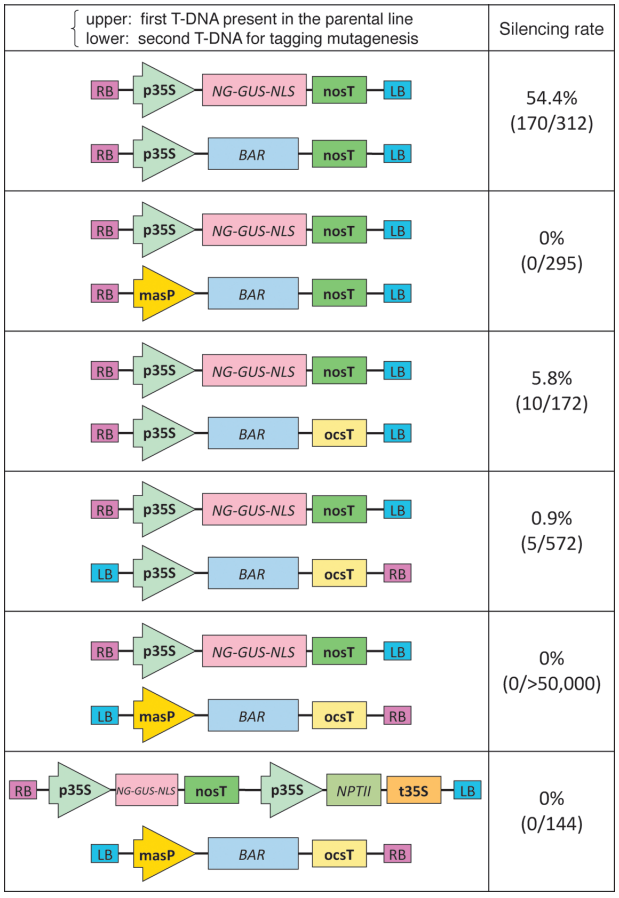

Figure 2. Diagrams of the T-DNA constructs used and the rate of TGS. In each pair, the upper construct depicts the first T-DNA, which was harbored in the parental line, and the lower construct depicts the second T-DNA, which was used for tagging mutagenesis (left column). The parental line was transformed with the second T-DNA, and the percentage of the resultant T1 plants that displayed a long-hypocotyl phenotype is shown as a silencing rate (right column). The number of long-hypocotyl T1 plants and the total number of T1 plants are in parentheses.

tagging vectors analyzed above had the same orientation as that of the parental line. Therefore, I inverted the sequence between the RB and LB in pPZP200/RB-p35S-*BAR*-ocsT-LB to yield pPZP200/LB-p35S-*BAR*-ocsT-RB, and examined the effect of this inversion on TGS. The resultant T1 plants exhibited a silencing rate of only 0.9%, i.e., only five out of 572 T1 plants had the long-hypocotyl phenotype (Figure 2). Thus, reversing the direction of the sequence within the T-DNA region relative to that in the parental line appears to reduce the occurrence of TGS.

Combining all of these strategies, I constructed pPZP200/LB-masP-*BAR*-ocsT-RB, in which both the promoter and terminator sequences were replaced and the orientation of the sequence between the RB and LB was reversed, and conducted a large-scale transformation of the parental line with this tagging vector. Of the more than 50,000 T1 plants that I have observed to date, not a single individual has exhibited the long-hypocotyl phenotype. Therefore, I have demonstrated that this strategy of T-DNA construction prevents TGS, at least at the morphological level. By screening the more than 50,000 lines of the T2 generation and characterizing a large number of recessive mutants that display mildly reduced light responses, I expect to identify novel components of the signaling pathway that function downstream of the phyB N-terminal domain.

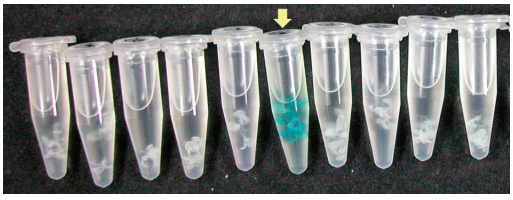

Figure 3. NG-GUS-NLS protein level, as revealed by GUS staining. The parental line harboring RB-p35S-*NG-GUS-NLS*-nosT-LB was transformed with the binary vector pPZP200/RB-p35S-*BAR*-nosT-LB, and the resultant T1 plants that showed a long hypocotyl were examined for GUS activity. Two-week-old light-grown plants were stained overnight with the substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, basically according to Tsugeki et al. (2009). The yellow arrow indicates the parental T0 plant, and all the others are long-hypocotyl T1 plants.

If this strategy is to be widely used, it should also be effective when an overexpression line bearing a drug-resistance marker gene is used as the host line. Therefore, I introduced pPZP200/LB-masP-*BAR*-ocsT-

RB into the transgenic line harboring RB-p35S-*NG-GUS-NLS*-nosT-p35S-*NPTII*-t35S-LB, which overexpresses *NG-GUS-NLS* and neomycin phosphotransferase II (*NPTII*, a gene that confers kanamycin resistance), in the *phyB* mutant background (t35S, *CaMV* 35S terminator). Among the 144 resultant T1 plants, not a single individual displayed a long-hypocotyl phenotype (silencing rate=0%) (Figure 2). This result suggests that the strategy is versatile enough to be applicable to general overexpression lines that harbor selectable marker genes.

The frequency of TGS can also be reduced by introducing mutations such as *ddm1* (Jeddeloh et al. 1998), *mom1* (Amedeo et al. 2000), and *met1* (Morel et al. 2000) into the parental line. However, TGS is not completely blocked in these mutants (Amedeo et al. 2000; Aufsatz et al. 2002). Moreover, the introduction of the mutations requires time-consuming crossing events, and such mutants are only available in limited species. In contrast, the method introduced in this current study is thorough, convenient, and potentially applicable to various plant species.

In cases where the same promoter sequences have to be used to express more than one transgene in a line, e.g., when the 35S promoter is used to overexpress some gene in T-DNA insertional mutants such as SALK (Alonso et al. 2003), FLAG (Samson et al. 2002), and GABI (Rosso et al. 2003) lines, all of which contain the 35S promoter (Daxinger et al. 2008), the strategy presented in this study would be useful because it is able to reduce the occurrence of TGS significantly by using different terminator sequences and/or by reversing the direction of T-DNA sequences.

In summary, in the process of establishing a genetic screen system that could exaggerate a mutant phenotype, I developed a versatile method to completely avoid TGS. In addition to changing the promoter sequence that drives expression of the second transgene, different terminator sequences were used for each transgene and the orientations of the two T-DNA sequences were reversed relative to each other. This strategy of T-DNA construction is also generally applicable when overexpressing multiple genes simultaneously in a stable transgenic line, and is expected to be widely used in diverse situations, from basic to applied research fields.

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