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# Geminivirus sequences as bidirectional transcription termination/polyadenylation signals for economic construction of stably expressed transgenes

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## Abstract

Bidirectional, convergent transcription of transgenes in transgenic plants can occur due to leaky transcription termination of separate convergent genes or from genomic promoters. It might also be engineered with the purpose of generating double-stranded RNA to downregulate genes by RNA interference. We have tested the effects of convergent transcription on expression levels and analysed the potential of geminivirus derived DNA sequences to act as bidirectional transcription termination/polyadenylation signals in transgenes to counteract such negative effects. Convergent, overlapping transcription decreased expression, however, no increased propensity for induction of gene silencing was observed. The geminivirus terminators in both orientations supported efficient expression of single genes and of convergent genes, whereas a unidirectional terminator allowed only reduced expression in this latter situation. Geminivirus terminators could be used as efficient transcription control element for the expression of two genes simultaneously or, with single genes, could afford protection of the gene against unwanted anti-sense transcription from transcription units downstream of the gene, e.g. in the integration locus. Our results also suggest that flanking of a given sequence by two convergent promoters would not be an efficient way to generate double-stranded RNA and induce gene silencing by RNAi.

*Abbreviations:* ACMV – African cassava mosaic virus, MSV – maize streak virus, WDV – wheat dwarf virus

## Introduction

Expression of transgenes is not only controlled by the provided expression signals but also by transgene-external influences. Gene silencing can be responsible for strong reduction of expression and ″position effects″ have been implicated as source for expression variations (Matzke and Matzke 1998). The term ″position effect″ describes the undefined influence which a chromosomal integration locus may have on transgene expression. The molecular basis for the expression variations is largely unknown. The generation of double-stranded RNA has been shown to be one trigger for gene silencing (Angell and Baulcombe 1997; Fire et al. 1998; Waterhouse et al. 1998). This requires that the transgene coding and/or the promoter region is transcribed not only in sense- but also in anti-sense orientation. Such unwanted transcription could generally influence expression by anti-sense RNA interference with RNA stability or usage, or by promoter occlusion, i.e. displacement of transcription factors from promoter regions (e.g. Greger et al. (1998) and references therein). The expression signals for the unintended transcripts have to be derived from the integration locus or have to be accidentally present within the integrated transgene DNA, either

always or only in some integration events after transgene rearrangement (Ingelbrecht et al. 1991). A third possibility would be that the tight configuration of several genes in conventionally used transgene cassettes leads to unwanted transcription of one transgene from promoters of another one. Tandemly organized transgenes are usually separated by plant signals specifying transcription termination and polyadenylation, termed terminators in this manuscript. For plants, little is known about the interplay between transcription termination and polyadenylation (Rothnie 1996). In other eukaryotes RNA polymerase complexes have been detected far downstream of the actual polyadenylation site (e.g. Hansen et al. (1998) and references therein), which in these cases may be chosen independently from transcription termination. The level of this transcriptional readthrough depends on the ability of signal sequences in the terminator to pause the transcription complex (Aranda and Proudfoot 1999). The most frequently used terminator in plant genetic engineering is derived from cauliflower mosaic virus (CaMV). It was shown to be ″leaky″ at least when it was positioned close to the promoter in transient expression experiments (Sanfaçon and Hohn 1990). For the terminator of the CaMV-related rice tungro bacilliform virus the efficiency of RNA 3′ end formation varied with promoter sequences (Klöti et al. 1999) and the plant system used (Rothnie et al. 2001). In other eukaryotes the involvement of the promoter-specified RNA polymerase complex in determination of co- and post-transcriptional RNA processing events has been well documented (reviewed by Hirose and Manley (2000)). This suggests that the proper combination of promoter sequences and processing signals may be important for correct RNA maturation and some combinations may lead to the production of aberrant RNAs.

Naturally, genes on chromosomes are well separated from each other. In tightly arranged transgenes, transcriptional readthrough could cause promoter occlusion or generate antisense RNA from convergently transcribed genes. Interestingly, silencing was much more pronounced with convergently repeated transgenes compared to divergently repeated transgenes in petunias (Stam et al. (1998); reviewed in Muskens et al. (2000)), and a particularly efficient inducer of post-transcriptional silencing, which works reproducibly even as a single copy gene, contains a convergent transcription unit for the selectable marker gene downstream of the silencing GUS gene (Elmayan and Vaucheret 1996).

Terminators, which prevent readthrough or unintended antisense transcription, should be of value to reduce the expression variation of transgenes (Ingelbrecht et al. 1991). Natural terminators that might have evolved such properties can be found in the genomes of geminiviruses, which harbor on their circular DNA genomes two convergent transcription units (Hanley-Bowdoin et al. 1999). Each of these covers about one half of the genome, one in clockwise orientation, generating an RNA with the same polarity as the single-stranded DNA packaged in virus particles and therefore also termed virion-sense RNA (vRNA) and one in counterclockwise orientation, also termed complementary-sense RNA (cRNA) (Figure 1a). Transcription of both begins in the large intergenic region (LIR) and mature RNAs end in the small intergenic region (SIR). V- and cRNA synthesis is to some degree alternatively regulated during viral infection (Hanley-Bowdoin et al. 1999) but synthesis is not mutually exclusive, therefore, inefficient termination of either transcription process could lead to simultaneous sense- and anti-sense transcription of viral sequences. The SIR contains the elements for RNA polyadenylation and probably also for transcription termination in both directions and therefore should be a bidirectional terminator. We have tested geminivirus terminator sequences from three different viruses for their potential to allow efficient gene expression in transfected rice protoplasts. One of them was also tested in transgenic cell suspension cultures and shown to protect, due to its bidirectional activity, transgenes against antisense transcription.

## Materials and methods

### *Plasmid constructs*

The ubiquitin-promoter-GUS cassette was produced by combining a fragment of the maize ubiquitin 1 gene covering the region from −730 to +1090 [derived from plasmid pUbiCAT (Christensen et al. 1992) after subcloning; numbering with respect to the transcription start] and flanked by engineered *Spe*I (at −730) and *Nco*I plus *Xba*I sites (at +1090) with an *Nco*I-*Xba*I fragment covering the GUS ORF (derived from pRintG, Klöti et al. (1999)). The actin-promoter-LUC cassette was produced by combining a fragment of the rice actin 1 gene (derived from plasmid pAct1F after subcloning; McElroy et al. (1990)) covering the region from −830 to +529 flanked by the same sites

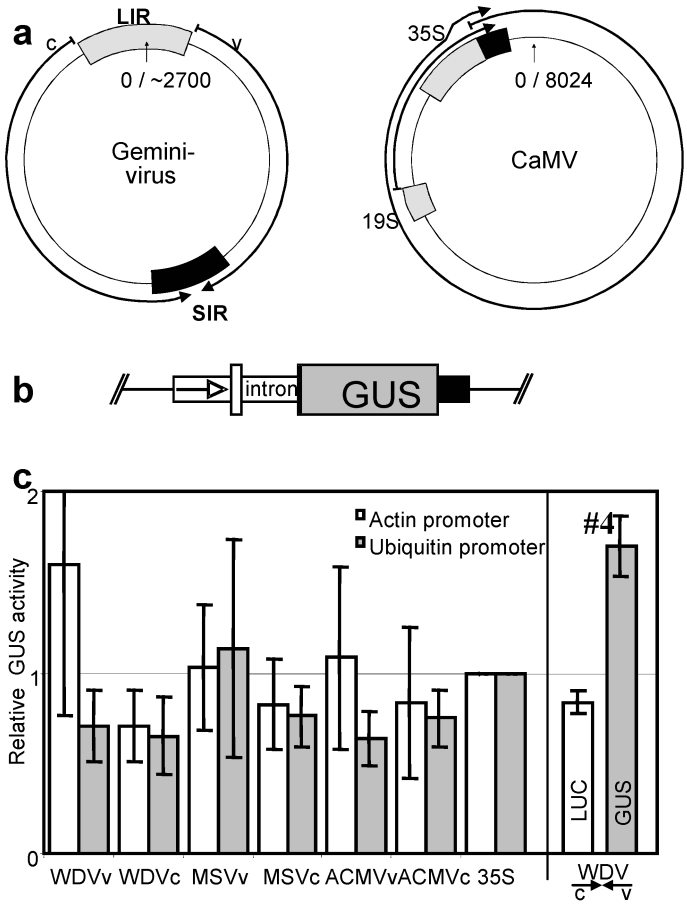


*Figure 1.* Comparison of transcription termination/polyadenylation sequences from geminiviruses and CaMV in transient gene expression. **a**: Simplified draft of Geminivirus and CaMV transcription unit organization. Circular DNA genomes are presented as thin circles, transcripts as thick arrows, promoter sequences by gray boxes, transcription termination sequences by black boxes. Geminiviruses produce convergent virion sense (v-) and complementary sense (c-) transcripts. LIR: large intergenic region; SIR: small intergenic region; 19S/35S: major transcripts of CaMV with the approximate location of their corresponding promoter sequences. **b**: Relevant part of plasmids used for transformation. The GUS reporter gene was driven by the Actin or Ubiquitin promoter (arrow) including their native first exon and first intron and terminated by one of the geminivirus SIRs in either direction or the CaMV 35S terminator (black box). **c**: Results of transient protoplast transfection assays. Respective GUS activities are shown relative to the corresponding 35S terminator construct. Geminivirus SIRs were tested in both orientations [e.g. WDV (v) and WDV (c)] and in combination with the Actin promoter (white columns) or the Ubiquitin promoter (gray columns). In the rightmost panel, construct #4 with two convergent genes (Figure 2) was tested relative to constructs #5 and 6 (Figure 2). The results represent the mean of 5 independent transformation experiments, the standard deviations are indicated.

as above with an *Nco*I-*Xba*I fragment covering the LUC ORF (derived from plasmid pGL3, Promega).

Geminivirus transcription terminator fragments were generated by PCR and covered positions 1178–1375 for ACMV DNA A (Gene bank accession no. X17095), 1015–1212 for MSV (X01633) or 1092–1359 for WDV (X82104). The CaMV terminator was derived from plasmid pDH51 (Pietrzak et al. 1986). All terminator fragments were flanked by *Nhe*I and *Xba*I sites by primer design or subcloning. Due to the complementarity of *Spe*I-, *Xba*I- and *Nhe*I-sites, the different reporter and terminator cassettes could be combined in simple cloning steps to yield the plasmids shown in Figures 1 and 2. The plasmid backbone includes matrix associated regions (MAR) and sites for cassette excision by *I-Sce*I and has been described in Bliffeld et al. (1999). More details are available upon request.

The plasmid encoding the aph4 gene for selection of transgenic cell lines contained the same CaMV35S promoter-aph4ORF cassette as pHCintG (Klöti et al. 1999), but subcloned in pBluescript (Stratagene).

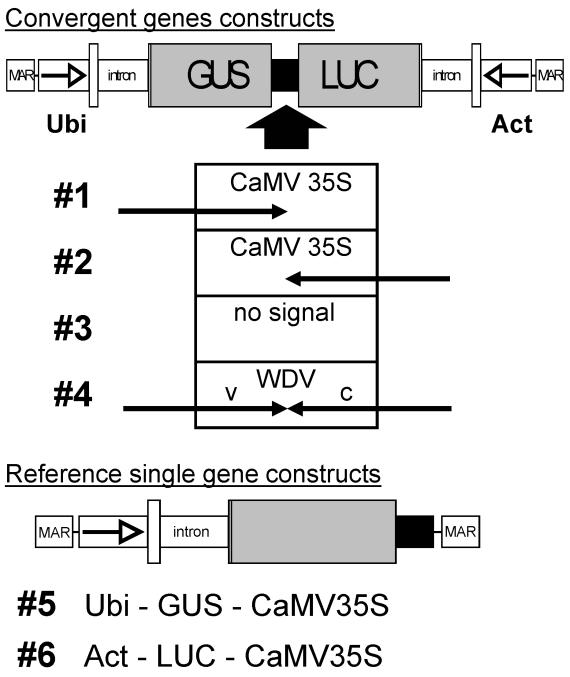


*Figure 2.* Constructs used to assess the effect of antisense transcription on reporter gene expression. Two different classes of linearized constructs were used: Constructs with convergent genes include the transcription units of GUS and LUC driven by the Ubiquitin (Ubi) or the Actin (Act) promoter (arrow) with their native first exon and intron. Constructs #1 to #4 differ only by the nature of the terminator (black box) used: #1 with a CaMV 35S signal terminating the GUS transcription unit, #2 with the same signal in the opposite direction terminating the LUC gene, #3 with no terminator, and #4 with the bidirectional SIR from WDV. Single gene reference constructs included the GUS coding sequence with the Ubiquitin promoter (#5) or the LUC coding sequence with the Actin promoter (#6), both with the CaMV 35S terminator (black box). All sequences are flanked by matrix attachment regions (MAR).

*Protoplast preparation and transfection*

Protoplasts from *Oryza sativa* line Oc were isolated from a cell suspension culture and transfected as described previously (Chen et al. 1994) with modifications: Suspension cells were cultured in MS medium (Murashige and Skoog 1962) plus 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 6% sucrose and 2.5 mM 2-morpholinoethanesulfonic acid (MES). Digestion was performed in 3% cellulase Onozuka R-10, 1% macerozyme R-10 (both Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan), 8% mannitol and 1 mM $CaCl_2$ for 16 h with slight shaking at 30 °C. The protoplasts were transformed by the PEG method with 10 $\mu$g plasmid DNA and harvested 16 h later. The protoplasts were analysed for activity of $\beta$-glucuronidase with 4-methylumbelliferyl-$\beta$-D-glucuronide (MUG) (Jefferson et al. 1987) and luciferase with a Luciferase Assay System following the provider's instructions (Promega Corporation, Madison, USA).

*Rice transformation by particle bombardment*

*Oryza sativa* suspension cells were transformed by particle bombardment essentially as described (Burkhardt et al. 1997). Suspension cells were plated on solid MS medium supplemented with 2 mg/l 2,4-D and 12% sucrose 1 h prior to bombardment. Per shot 0.5 mg gold particles were coated with 100 ng (reporter genes) and 50 ng (selection) linearised DNA. Line Oc cells were harvested 16 h post bombardment either for analysis of transient reporter gene expression with the histological GUS assay using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc, Biosynth, Switzerland) (Jefferson et al. 1987) or with a quantitative luciferase and $\beta$-glucuronidase activity assay, or streaked on solid MS medium supplemented with 2 mg/l 2,4-D, 6% sucrose, 2.5 mM MES and 50 mg/l hygromycin B. After 3 weeks of selection, culture plates were stained for GUS and expressing colonies scored to evaluate the transformation efficiency or sampled (up to 30 per construct) and further cultured in liquid medium containing 50 mg/l hygromycin B for 4 weeks. The activity of $\beta$-glucuronidase and luciferase was scored twice for each suspension culture.

Transformed *Oryza sativa* line TP309 suspension cells were directly transferred to MS1 (Burkhardt et al. 1997) containing 30 mg/l hygromycin B 24 h post-bombardment. Resistant calli were maintained on solid selective MS1 medium for up to 6 month. Glucuronidase and luciferase of the individual lines was measured six and seven weeks post-bombardment and again six months post-bombardment.

*Southern blot analysis*

DNA was extracted with Nucleon Phytopure Plant DNA extraction kit (Amersham Life Science, England). Southern blot analysis of transgenic TP309 lines was done according to ″The DIG System User's Guide for Filter Hybridization″ (Roche). DNA was cut with *Hind*III (single cut 5′ of GUS start codon) and subsequently hybridized with probes for the GUS, the LUC and the Aph4 coding regions.

## Results

### *Efficiency of geminivirus terminators for gene expression in transfected protoplasts*

Terminators can influence expression levels probably by contributing sequences to the 3′ untranslated regions (3′UTR) of the mRNA and thus by affecting RNA stability or translation. It has been shown that different terminators can cause up to 100 fold variations in the efficiency of transgene expression (An et al. 1989; Ingelbrecht et al. 1989; Ali and Taylor 2001). It was therefore of interest whether geminivirus terminators support efficient gene expression. For the analysis, we constructed 14 different expression cassettes containing a β-glucuronidase (GUS; Jefferson et al. (1987)) open reading frame (ORF) under the transcriptional control of either the maize ubiquitin 1 promoter (including the native first exon and intron; Christensen et al. (1992)) or the rice actin1 promoter (also with its first exon and and intron; McElroy et al. (1990)), and containing different terminators (Figure 1b). The terminators were derived from the CaMV 35/19S transcription unit or comprised the SIRs of the A component of African cassava mosaic virus (ACMV), of wheat dwarf virus (WDV), or of maize streak virus (MSV) (Figure 1a). The geminivirus terminators were inserted in both orientations. Expression efficiency was assessed in transfected rice protoplasts (Figure 1c). All expression cassettes with geminivirus or CaMV terminators were found to produce similar levels of GUS activities. Compared to the CaMV terminator, at most two-fold higher or lower activities could be obtained with the geminivirus terminators. For the ubiquitin promoter, the orientation of the terminator was unimportant, for the actin promoter, slightly higher expression was observed for constructs with the WDV or ACMV terminator in virion-sense (v) orientation compared to the complementary-sense (c) orientation (Figure 1c). The results show that the RNA 3′ ends generated at the geminivirus terminators function as efficiently in gene expression as those generated by the CaMV 35S terminator, i.e. they do not contain destabilizing sequences or interfere otherwise with transient gene expression.

### *The WDV SIR as bidirectional terminator in convergent transcription units*

One potential usefulness of the geminivirus terminators for genetic engineering would be the property to terminate two convergent genes with a single sequence element, allowing for a more effective transgene design. Various gene expression constructs with such transcription units were prepared. Convergent genes for GUS, driven by the maize ubiquitin promoter (UbiGUS), and firefly luciferase (LUC), driven by the rice actin 1 promoter (ActLUC), were separated either by the unidirectional CaMV 35S terminator in both possible orientations (Figure 2, constructs #1 and #2), no terminator (#3), or the WDV terminator (#4); for comparison, the same genes terminated by the CaMV signal were used as ″single gene″ constructs (#5, #6). To assess the expression of the genes in the different contexts, a number of transient expression experiments in rice Oc protoplasts or in bombarded rice Oc suspension cells were performed. In both transient expression systems, efficient expression of both genes on construct #4 was observed (Figure 1c). Both convergent genes were expressed similarly to single genes with either the CaMV terminator or with the WDV terminator.

Ingelbrecht et al. (1989) observed differences in the effects of terminator sequences between transiently and stable expressed transgenes. We therefore compared expression from constructs #4 to 6 also in transgenic rice callus lines from bombarded rice Oc suspension cells or immature embryos of rice TP309 either in batches of transformed calli (results not shown) or in a large number of individual calli (Figure 3). In total, very similar average expression levels as observed in transient expression were also found in transgenic rice cell lines and the distribution of expression levels of individual callus lines was almost identical for constructs #4, #5 and #6 (Figure 3).

These results show that the WDV terminator allows efficient expression also in the context of convergent genes, i.e. if any effect of terminator leakyness in construct #4 existed, it must be small. The terminator is useful for construction of such expression units.

### *Convergent, overlapping transcription units in transient expression experiments*

We have compared expression from convergent genes separated by the bidirectional WDV terminator, by only one unidirectional CaMV terminator or by no terminator at all (Figure 2). With all these constructs, besides #4 with the WDV terminator, the two transcription units overlap to some extent, mimicking the situation of transgene transcription from a promoter

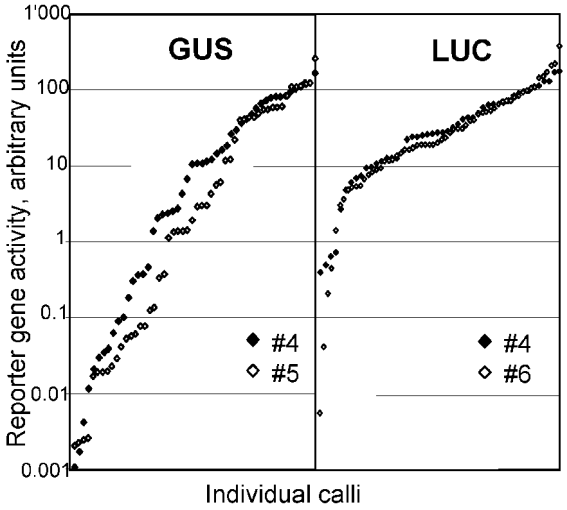


*Figure 3.* Comparison of expression level distribution in stably transformed TP309 callus lines. Construct #4 (Figure 2) with the convergent GUS and LUC genes terminated with the bidirectional WDV SIR (black diamonds) was compared with single gene constructs (open diamonds) with only GUS (#5, left panel), or only LUC (#6, right panel). GUS expression below 0.01 may not be different from background. Dependent lines are included. The expression level is shown in arbitrary units. For each construct the lines were sorted according to their expression level and distributed along the x-axis. (Note that a small horizontal shift of the distribution can be caused by small differences of the numbers of clones with a certain expression level, but does not reflect a fundamental difference).

located downstream and in anti-sense orientation, as it might occur at some integration loci.

We expected strong negative effects of anti-sense transcription since anti-sense RNA generated in *trans* can strongly reduce expression even in transient expression systems (Fütterer et al. 1993). However, in the presence of a second, convergent, non-terminated gene the CaMV 35S terminated UbiGUS or ActLUC genes both were expressed at only a slightly lower level than the WDV terminator version in transient expression (#1 for the terminated GUS gene and #2 for the terminated LUC gene; Table 1), indicating that the potential anti-sense transcription had no drastic effect on a terminated gene. The non-terminated genes of each pair were usually expressed with lower but still considerable efficiency (compare #1 and 3 for GUS expression and #2 and 3 for LUC expression; Table 1). It is likely that in the absence of a proper terminator, other sequences can act as at least partially efficient termination signals. Linearization of the terminatorless construct #3 in the LUC coding region abolished GUS activity in transient expression,

*Table 1.* Average GUS (G) and LUC (L) expression and the GUS/LUC ratio in batches of transiently (T) or stable (S) transformed rice cells. Expression from constructs #1 to 3 is given relative to construct #4 for which GUS and LUC expression were defined as 100% and the G/L ratio accordingly as 1; terminated genes are shown in bold. The number of treatments (each with three repetitions) or individually analysed calli is indicated in square brackets in the first column.

| Constructs: | #1 | | | #2 | | | #3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | **G** | L | G/L | G | **L** | G/L | G | L | G/L |
| T (protoplasts) [6] | 69 | 24 | 2.9 | 39 | 45 | 0.9 | 22 | 8 | 2.8 |
| T (Oc) [8] | 80 | 23 | 3.5 | 18 | 60 | 0.3 | 17 | 6 | 2.8 |
| T (Oc[1]) [4] | 24 | 6 | 4.0 | 9 | 35 | 0.3 | 24 | 4 | 6.0 |
| S (Oc[1]) [4] | 96 | 26 | 3.7 | 34 | 67 | 0.5 | 36 | 7 | 5.1 |
| S (Oc) [20-30] | 31 | 7 | 4.4 | 16 | 57 | 0.3 | 16 | 6 | 2.7 |
| S (TP309)[16-49] | 71 | 13 | 5.5 | 23 | 119 | 0.2 | 53 | 33 | 1.6 |
| Average (transient) | 58 | 18 | 3.2 | 22 | 43 | 0.5 | 21 | 6 | 3.5 |
| Average (stable) | 66 | 15 | 4.4 | 24 | 81 | 0.3 | 35 | 15 | 2.3 |

[1)] transient and stable expression from the same transformation experiment.

while the same linearization of #4 had no negative effect on expression (results not shown), suggesting that sequences in the LUC gene are important for GUS expression in construct #3 but not in #4. Since the nonterminated UbiGUS genes in constructs #2 and #3 are expressed equally well, the degree of overlapping transcription seems to be unimportant in this case. In contrast, a potentially longer overlap inhibits expression of the nonterminated ActLUC genes in #1 and #3 stronger (Table 1).

From these experiments it appears that the absence of a terminator is probably more detrimental for the respective gene expression than potential anti-sense transcription.

### *Observations from stable transformation experiments with convergent gene constructs*

The effects of convergent transcription might be particularly different in transiently and in stable transformed cells. In transient expression, the presence of the large number of identical promoter copies may reduce simultaneous transcription of the two convergent genes, due to competition of each promoter for possibly limiting transcription factors. In integrated transgenes, only a few gene copies are transcribed but transcription continues for a long time, allowing the establishment of e.g. silencing mechanisms. We expected therefore stronger negative effects of overlapping convergent transcription in transformed cells.

However, reporter gene expression was high in almost all cases suggesting an even further reduced effect of antisense transcription (Table 1). In one transformation experiment, cells were analysed one day after bombardment for transient expression and 3 weeks after bombardment for stable expression (Table 1, lines 3 and 4). Because of the use of the same cells and exactly the same transformation procedure, these data can be directly compared. For the two conditions, absolute expression levels were different but the expression ratios of the two reporter genes were almost the same. In the experiment with TP309 derived callus lines, we followed transgene expression in multiple independent lines for six months, but also during this extended period, no signs for increased expression inactivation could be observed (not shown). The apparent similarity of gene expression in transient and stable transformation experiments was surprising and indicated that gene silencing, which was observed in rice callus cultures with constructs containing inverted repeats within one RNA (Waterhouse et al. 1998) was not induced by convergent transcription units.

Transformation by methods of direct gene transfer usually leads to the integration of several, often rearranged transgene copies. This was verified by a Southern analysis of all transformed callus lines from bombarded TP309 embryos (not shown). It therefore remains unknown from which of these (rearranged) fragments the reporter genes are expressed. The average expression activity could reflect more the properties of the single genes than that of the convergent tandem gene construct. The transgene constructs were flanked by matrix attachment regions. Intact, integrated fragments would therefore be protected to some degree from ″position effects″ caused by chromosomal expression signals (Holmes-Davis and Comai 1998; Vain et al. 1999). Their expression might still be affected by regional chromatin structures or subnuclear localization, but these effects should influence both genes in the same way, i.e. the absolute expression level might vary but the ratio of expression of the two convergent genes should be similar for independent lines. We compared the variation of this ratio in independent, transformed callus lines obtained after bombardment of Oc suspension cells or TP309 embryos. The distribution of the individual expression values for the latter experiment is shown in Figure 4. For the expression ratio, we arbitrarily defined as ″normal″ a ten-fold variation window around the average. In both independent transformation experiments, a larger percentage of the #4 clones was found in this window than for any of the other convergent-gene constructs (Table 2). This indicates that either expression from convergent gene constructs with at least one non-terminated gene is intrinsically more variable or that transformation with the constructs led preferentially to rearranged transgenes. In our experiments, we obtained fewer clones with the constructs #1 to 3 than with #4 (Figure 4, Table 2), #5 and #6 (not shown).

## Discussion

Proper 3′ end processing is an obligatory step in mRNA maturation. Signals specifying these steps are only poorly characterized in plants, but they may contain a reasonably conserved sequence AATAAA, which is however not required in all known signals, and some GT rich elements upstream (Rothnie 1996). The loose conservation of these features allows that also genetically engineered transcription units without terminators produce translatable mRNAs by 3′ end processing of the primary transcript at accidentally available, similar sites; as shown previously (Ingelbrecht et al. 1989) and in this work, this results in only lower expression levels.

The termination signals may contribute to gene expression magnitude and stability in various ways. One feature is their contribution to the sequences at the 3′ end of the RNA; this may influence RNA stability and/or RNA translation (Abler and Green 1996; Fütterer and Hohn 1996). Terminator dependent differences in expression levels have indeed been detected. A gene terminated with sequences from the *Arabidopsis* small subunit of rbcs gene was expressed about 100 fold better in transgenic tobacco than the same gene terminated by sequences from the *Antirrhinum majus* chalcon synthase gene (Ingelbrecht et al. 1989), and the terminator of the potato pinII gene allowed about 60 fold higher expression than that of the T-DNA derived 6b gene (An et al. 1989). So far, only few terminators have been directly compared (see Ali and Taylor (2001) for references) and only few are being used routinely in genetic engineering of plants (Koziel et al. 1996).

We show here that terminators from geminiviruses support similar gene expression levels as the most widely used terminator from CaMV. In transient expression experiments all three used geminivirus terminators, each in both possible orientations and in

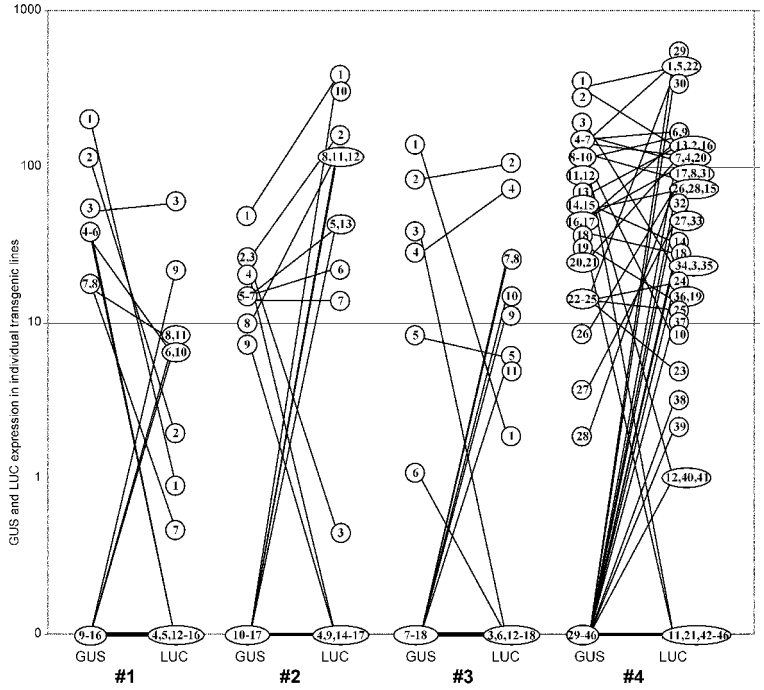


*Figure 4.* Relative reporter activity of independent transgenic TP309 rice suspension cultures. GUS and LUC activity in individual transformed cell lines with convergent reporter gene constructs (#1 to #4, Figure 2) is shown. The corresponding values (with same number) are connected by lines. The average expression value of TP309 lines transformed with single gene constructs #5 and 6 was set to 100%. Values below 0.5% were regarded as background.

*Table 2.* Expression distribution in individual transgenic rice callus lines derived from Oc suspension cells or TP309 immature embryos. For each transformation experiment with constructs #1 to 4, the number of callus lines (and - in brackets - the percentage) expressing none of the reporter genes (-), only GUS (G), only LUC (L) or both (GL) and the total number of lines analysed ($\Sigma$) is given. The average GUS/LUC ratio and the number and percentage of lines with ratios between 3.33-fold lower and 3.33-fold higher (10 fold range) than average is indicated.

| | Reporter expression | | | | | GUS/LUC | Lines in 10x range |
|---|---|---|---|---|---|---|---|
| | – | G | L | GL | $\Sigma$[1] | | |
| #1 | 13 (36) | 5 (14) | 7 (19) | 11 (31) | 36 | 5 | 6 (17%) |
| #2 | 7 (15) | 2 (4) | 11 (24) | 26 (57) | 46 | 0.25 | 15 (33%) |
| #3 | 14 (29) | 8 (17) | 10 (21) | 16 (33) | 48 | 2.2 | 7 (15%) |
| #4 | 5 (7) | 2 (3) | 14 (18) | 55 (72) | 76 | 1 | 36 (47%) |

[1)]For #1 to 3 all obtained clones were tested, for #4 all clones from Figure 4 and about 40% of obtained transgenic Oc lines.

combination with two different promoters allowed similar expression levels, suggesting that 3′ end formation is similarly efficient and RNA stability and translation is similar to constructs with a CaMV terminator. In stably transformed callus lines, expression constructs, in which the WDV SIR was used to simultaneously terminate two convergent reporter genes, resulted in an very similar distribution of reporter gene activity levels as observed for CaMV 35S terminated single genes. The bidirectional activity of the geminivirus terminators represents a potentially interesting property for applications in genetic engineering. First, as shown here, they can be used to simultaneously terminate two genes with one sequence element. This reduces the number of required sequence elements for the construction of multi-gene cassettes and helps to avoid sequence duplications in transgene constructs. The probability of repeat-induced transgene silencing may therefore be reduced (Fagard and Vaucheret 2000; De Wilde et al. 2000). Second, such terminators could inhibit unwanted antisense transcription from a downstream, convergent

promoter sequence, which may be present in the integration site or be produced by transgene rearrangements. T-DNA has been shown to integrate frequently into transcribed regions (Herman et al. 1990; Koncz et al. 1989), the probability for antisense transcription could therefore be high. In this respect, not all geminivirus terminators might be equally useful, since the v- and cRNA 3′ ends of e.g. the begomovirus ACMV slightly overlap (in contrast to the monopartite mastreviruses) and thus a short region for dsRNA formation might be available. Interestingly, begomoviruses encode a silencing suppressor (Voinnet et al. 1999).

We attempted to mimic a situation of convergent, overlapping transcription by transforming accordingly constructed convergent gene expression cassettes. We expected a strong negative effect of antisense transcription on reporter expression in transiently transfected cells and a probably even stronger effect in transgenic cells. However, similar as reported previously for expression in tobacco cells (Paszty and Lurquin 1990), the effect in transient expression was at most 3 fold and it was rather less pronounced in stable transformants, where effects of silencing could have become visible (Waterhouse et al. 1998). The effects in transient expression were smaller than previously obtained by expression of GUS anti-sense RNA in *trans* (Fütterer et al. 1993). The absence of a strong negative effect in stably transformed cell lines may be related to a problem connected to all transformation experiments involving direct gene transfer methods. Integration of just a single, intact gene copy is a rare event. Much more frequently, and also in our experiments, several intact and/or rearranged copies are present. In rearranged or partial copies, undefined DNA sequences are located closer to the expressed genes than when the complete, convergent expression cassette including the flanking MARs is integrated and therefore stronger variations can be expected. This should particularly affect the ratio of expression of the two reporter genes. The narrower distribution of this expression ratio for the construct #4 thus suggests that expression from this construct is indeed more stable and predominantly defined by the inherent transcription signals. The wider distribution for the other constructs and particularly for #3 without any terminator in contrast suggests that in this case either cellular parameters have a larger influence (e.g. by modulation of antisense RNA effects) or that the genes are expressed predominantly from rearranged transgenes. Since the selectable marker gene used for transformation was the same and independent from the convergent reporter gene cassette, a selection for such rearranged clones would imply that the non-rearranged ones confer a detrimental property to the cell. Double-stranded RNA (reviewed in Kumar and Carmichael (1998)) or stalled transcription complexes (Andera and Wasylyk 1997; Ljungman et al. 1999), which could in our case be the result of the collision of the convergent transcription complexes or the two waves of positive supercoiling preceding these complexes (Uptain et al. 1997), are known triggers for arrest of cell divison in other eukaryotic systems. Overall, the transformation efficiency with constructs #1 to 3 was lower than with #4 to 6. This could be indicative for problems with regeneration of calli harbouring convergent, overlapping transcription units with strong promoters.

According to the findings of efficient induction of gene silencing by double-stranded RNA (Angell and Baulcombe 1997; Fire et al. 1998), constructs with engineered simultaneous sense and anti-sense transcription might be envisaged as efficient tools to silence plant genes. It has been discussed that RNA-RNA interactions might be particularly efficient in such a case because of the close proximity of synthesis of the two RNA molecules (Stam et al. 2000). We tested whether expression from such constructs is particularly prone to silencing, but could not observe any difference between the convergent or single gene constructs in rice callus lines. It is possible that in rice callus establishment of silencing is slower or less efficient, however, silencing by expression of RNAs with double-stranded regions has been reported for rice callus lines (Waterhouse et al. 1998). It is also possible that in the tested gene configurations not much anti-sense RNA is produced but that oppositely transcribing polymerase complexes are arrested at collision sites or displaced from the DNA template.

Transformation experiments at a larger scale and possibly using Agrobacterium mediated gene transfer to generate more single-copy transgenics without possible transcription from truncated or rearranged fragments would be required to clarify this question and also to study the possible negative effect of overlapping transcription on transformation efficiency.

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