Matrix attachment regions and regulated transcription increase and stabilize transgene expression

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Received 28 November 2004; revised 31 March 2005; accepted 5 May 2005. *Correspondence (fax 919-515-7959; e-mail george_allen@ncsu.edu) This work was supported in part by a USDA Competitive Research Grant to W.F.T. and G.C.A. R.A. was funded by the Foundation for Science and Technology (FCT), Portugal.

Summary

Transgene silencing has been shown to be associated with strong promoters, but it is not known whether the propensity for silencing is caused by the level of transcription, or some other property of the promoter. If transcriptional activity fosters silencing, then transgenes with inducible promoters may be less susceptible to silencing. To test this idea, a doxycycline-inducible luciferase transgene was transformed into an NT1 tobacco suspension culture cell line that constitutively expressed the tetracycline repressor. The inducible luciferase gene was flanked by tobacco Rb7 matrix attachment regions (MAR) or spacer control sequences in order to test the effects of MARs in conjunction with regulated transcription. Transformed lines were grown under continuous doxycycline (CI), or delayed doxycycline induction (DI) conditions. Delayed induction resulted in higher luciferase expression initially, but continued growth in the presence of doxycycline resulted in a reduction of expression to levels similar to those found in continuously induced lines. In both DI and CI treatments, the Rb7 MAR significantly reduced the percentage of silenced lines and increased transgene expression levels. These data demonstrate that active transcription increases silencing, especially in the absence of the Rb7 MAR. Importantly, the Rb7 MAR lines showed higher expression levels under both CI and DI conditions and avoided silencing that may occur in the absence of active transcription such as what would be expected as a result of condensed chromatin spreading.

Keywords: PTGS, TGS, RNAi, MARs, transgene expression, induction.

Introduction

Plants are routinely engineered to improve agronomic traits, disease resistance or other applications. Most success depends on the predictability and the stability of transgene expression. An increasing number of reports illustrate the advantages of including matrix attachment regions (MARs) in expression constructs for both animal and plant systems (Phi-Van *et al.*, 1990; Allen *et al.*, 1993; Poljak *et al.*, 1994; Petersen *et al.*, 2002; Van der Geest *et al.*, 2004; Goetze *et al.*, 2005; Halweg *et al.*, 2005). MARs are DNA sequences that bind *in vitro* to the nuclear matrix, the insoluble nuclear proteins that remain following the removal of histones (Mirkovitch *et al.*, 1984; Cockerill and Garrard, 1986). Transgenes flanked by MARs are more strongly expressed and resist gene silencing (reviewed in Allen *et al.*, 2000; Bode *et al.*, 2000).

Several models have been proposed to explain how MARs improve transgene expression (reviewed in Allen et al., 2000). For example, the chromatin-opening model proposed that HMGA (high mobility group protein A) acts to displace histone H-1 on the MAR DNA, which creates an open chromatin structure poised for transcription (Kas et al., 1993). It has also been proposed that MARs can serve as boundaries of chromosomal domains, protecting genes within the domain from cooperative spreading of condensed chromatin (Stief et al., 1989; Laemmli et al., 1992) or reducing DNA : DNA pairing interactions within complex loci (Allen et al., 2000). In addition, recent evidence suggests that MARs may act as transcriptional terminators (Mlynarova et al., 2003), reducing transcription into adjacent repetitive sequences or read-through transcription in transgene arrays. Either of these latter events might be expected to lead to formation of dsRNAs. Recent reports

have highlighted the role that such RNAs may play in guiding silencing complexes to the transgenic locus and initiating chromatin changes that lead to transcriptional silencing (reviewed in Matzke and Matzke, 2003; Lippman and Martienssen, 2004).

Our previous work demonstrated that flanking MARs increased transgene expression, even in lines with high transgene copy numbers, when constitutive promoters were used (Allen *et al.*, 1993; Allen *et al.*, 1996; Ulker *et al.*, 1999). Recently we showed that flanking MARs increase the proportion of cells in a clonal transgenic population in which expression is detectable (Halweg *et al.*, 2005). This effect reflects a decrease in the rate of gene silencing in MAR constructs. However, because constitutive promoters were used in these and other previous experiments, it remains unclear whether MARs protect against silencing mediated by transcription of the transgene.

To distinguish between transcription-dependent models and models in which MAR effects are independent of transcription, we have examined MAR effects on silencing events occurring before and after activation of transgene transcription from an inducible promoter. We used tobacco NT1 cells expressing a Tet (tetracycline) repressor protein. These cells were transformed with the luciferase gene under the control of a tetracycline (or doxycycline)-inducible promoter (TripleOp promoter, Gatz et al., 1992). Cells were transformed either in the presence of doxycycline, which allowed transcription during transformation, or in the absence of doxycycline, which prevented transgene transcription during transformation. Thus transformation and initial growth of the transformed cells occurred under continuous induction (CI) or delayed induction (DI) conditions. We compared our results with the MAR construct (MLucM) to a control lacking MARs (XLucX) and to a spacer control in which 1195 bp of non-MAR lambda DNA (Mendu et al., 2001; Halweg et al., 2005) replaced the MAR sequence ($\lambda Luc\lambda$).

The majority of all of the cell lines with the MLucM construct expressed luciferase, while all of the control lines without MARs, with or without a segment of lambda DNA as a spacer control, showed significantly reduced transgene expression. In MLucM and XLucX lines that were transformed and grown without the inducer (DI lines), transgene expression was significantly greater than in the CI lines carrying the same constructs. Our data are consistent with a model in which the process of transcription increases silencing, especially in the absence of the RB7 MAR. In addition, flanking Rb7 MARs protect against silencing by processes independent of transcription from the transgene promoter, a result that has implications for the use of inducible or developmentally regulated promoters in transgenic applications.

Results

Doxycycline regulation of luciferase expression during and after transformation

Doxycycline is only effective as an inducer when the tetracycline repressor protein (TetR) is present. In cells that do not express TetR, or in cells that have been induced with doxycycline, the TripleOp promoter is highly active (Gatz et al., 1992). To ensure that all of the cell lines in our study maintained TetR expression, a translational fusion (TetR-rsGFP) was made between the C terminus of the TetR protein and the N terminus of the soluble-modified red-shifted GFP (rsGFP) (Davis and Vierstra, 1998). Lines expressing the TetRrsGFP fusion were identified by visualization of rsGFP fluorescence under blue light, and a single line was selected for use in all subsequent cotransformations. The cotransformations included a selectable marker plasmid containing hygromycin phosphotransferase and a plasmid containing one of the three configurations of the luciferase (Luc) reporter gene driven by the TripleOp promoter (Figure 1a and 1b). Between 39 and 48 independent transformants were selected on hygromycin for each of the three constructs for both the CI and DI treatments. The experimental protocol for doxycycline induction is shown in Figure 1c. The DI cell lines were induced with doxycycline 38 days after transformation (DAT), and then grown under inducing conditions for the duration of the experiment. The CI cell lines were grown under constant induction for the entire experiment. Both the CI and DI lines were assayed for luciferase activity at 52 and 85 DAT as shown in Figure 1c.

To determine whether the TetR-rsGFP fusion remained functional, luciferase expression was measured in the presence or absence of doxycycline 24 h following transformation (Figure 2). Luciferase activity was measured by counting photon emission per plate of transformed cells in the presence or absence of 100 ng/mL doxycycline. Doxycycline induced luciferase expression by approximately 10–30-fold when compared to the corresponding non-induced cells for each reporter construct (Figure 2).

Delayed transcription results in increased transgene expression levels

To compare the cumulative effect of transcriptional activity on expression from the three constructs, the cell lines were grown under constant or delayed induction. For delayed induction, doxycycline was added to the culture medium 38 DAT. Luciferase activity was then assayed at 52 DAT (14 days



Figure 1 Co-transformation and doxycycline-induction strategy. (a) Constructs for transforming NT1 cells. The first transformation used a binary vector pBIN-TetR-rsGFP for expressing the Tet-rsGFP protein under control of the CaMV 35S promoter and a nopaline synthase polyadenylation signal (NosT). The reporter gene contains a doxycyclineregulatable promoter (TripleOp) controlling the luciferase (Luc) gene with an octopine synthase polyadenylation signal (OcsT). Three versions of the reporter gene are shown, which include a no DNA control (XLucX); a λ DNA spacer-flanked control ($\lambda Luc\lambda$); and an Rb7 MAR-reporter (MLucM). The selection gene used for the cobombardment was hygromycin phosphotransferase (HptII) controlled by the mannopine synthase promoter (MasP) and a nopaline synthase polyadenylation signal (NosT). (b) The first transformation was by Agrobacterium to produce a TetR-rsGFP-expressing NT1 tobacco cell line. The second transformation was the cotransformation with the various reporter genes shown in a cobombarded with the MasP-Hptll selection plasmid in a 4 : 1 molar ratio (reporter plasmid : selection plasmid). (c) A schematic diagram of the treatments used for the delayed induction (DI) lines and the constant induction (CI) cell lines. The horizontal black bars depicts the period of time the DI cell lines were grown in the absence of doxycycline prior to be induced at 38 DAT. The horizontal white bar depicts the period of time the CI and DI cell lines were grown in the presence of doxycycline. The small arrows indicate key times for transformation (day 0), induction (38 DAT), the first luciferase measurement (52 DAT) and the final luciferase measurement (85 DAT).

of induction) and again at 85 DAT (47 days of induction). Luciferase activity was also measured in the CI lines at the 52 and 85 DAT (Figure 3).

Transcriptional activity had a highly significant negative impact (P-value = 0.005) on luciferase expression when the



Figure 2 Doxycycline regulates luciferase expression in cotransformed TetR-rsGFP expressing NT1 cells. The TetR-rsGFP-expressing cells were cotransformed using microprojectile bombardment with three TripleOp-luciferase reporter constructs, MLucM, XLucX, or λ Luc λ either in the presence or absence of 100 ng/mL doxycycline luciferase activity was determined after 24 h growth by counting photons for 2 min (y axis). The black bars show luciferase expression in cells transformed in the presence of doxycycline and the light grey bars show luciferase expression in cells transformed in the absence of doxycycline.

52 DAT data was pooled into two data sets, DI and CI. When each of the construct data sets was analysed separately, the majority of this impact could be attributed to the MlucM and XlucX populations. While this general trend of higher expression levels in DI populations was also seen at the 85 DAT measurement, the effect was reduced compared to 52 DAT, and the populations were no longer significantly different at the P = 0.05 level.

It is also noteworthy that the greatest impact of the DI treatment was seen in the XLucX Lines (Table 1). Luciferase expression was seen in 58% of the DI XLucX lines compared to only 34% of the CI XLucX lines 52 DAT. Statistical analysis showed that luciferase expression and the percentage of expressing DI XLucX lines at 52 DAT was significantly greater than the CI XLucX lines at 52 DAT. This difference indicates that transcriptional activity was a major determinant of expression in the XLucX lines.

Flanking Rb7 MAR and λ DNA improve stability of expression

Previous studies in both tobacco (Ulker *et al.*, 1999) and rice plants (Vain *et al.*, 1999) demonstrated that transgenes flanked with the tobacco Rb7 MAR sequence showed reduced gene silencing in progeny lines when compared to progeny from non-MAR plants. To determine whether the Rb7 MAR affected



Figure 3 Delayed induction and flanking Rb7 MARs increase transgene expression and stability. Cell lines were transformed with MLucM, λ Luc λ , or XLucX and the individual lines were measured for luciferase activity (relative luciferase activity) 52 or 85 days after transformation. Constant induction (CI, white) cells were grown in the presence of doxycycline for the entire experiment. Delayed induction (DI, grey) cells were grown in the presence of doxycycline from 14 DAT through 85 DAT. The bars for the 52 DAT panels are ordered according to expression levels for each line with the highest expressing lines to the right and silent lines to the left (no bar). The bars for the 85 DAT panels are the same order as that used for 52 DAT to show the change in expression for each individual line. Each graph shows the induction conditions and the assay time (DAT). (a) 52 DAT luciferase expression in CI XLucX cell lines; (b) 85 DAT luciferase expression in CI XLucX cell lines; (c) 52 DAT luciferase expression in DI XLucX cell lines; (d) 85 DAT luciferase expression in DI XLucX cell lines; (e) 52 DAT luciferase expression in CI λ Luc λ cell lines; (f) 85 DAT luciferase expression in CI λ Luc λ cell lines; (g) 52 DAT luciferase expression in DI λ Luc λ cell lines; (h) 85 DAT luciferase expression in DI λ Luc λ cell lines; (i) 52 DAT luciferase expression in CI MLucM cell lines; (j) 85 DAT luciferase expression in CI MLucM cell lines; (k) 52 DAT luciferase expression in DI MLucM cell lines; (I) 85 DAT luciferase expression in DI MLucM cell lines.

gene expression during extended mitotic divisions, luciferase activity was measured at 52 and 85 DAT for both the CI and DI treatments. To control for spacing effects, direct repeats of a 1195-bp fragment derived from bacteriophage λ were included in the same relative position and orientation as the

Rb7 MARs. Importantly, the λ DNA that we used does not bind to the nuclear matrix *in vitro* (Mendu *et al.*, 2001; Halweg *et al.*, 2005) and is similar in size to the Rb7 MAR. Expression stability for the three types of transformants for both the DI and CI treatments was determined by calculating the decrease in percentage of lines showing luciferase expression between the 52 DAT and 85 DAT assay times. A line was scored as not expressing when luciferase activity was the same as non-transformed controls. Table 1 shows that the lowest expression stability was seen in the DI XLucX lines, in which the number of lines that continued to express at 85 DAT decreased by nearly 40%. This loss of expression was reduced in both MLucM and λ Luc λ lines.

It is important to note that while the MLucM and λ Luc λ lines had similar stability between 52 and 85 DAT, 85% of the DI MLucM lines shown in Table 1 showed expression at 52 DAT, compared to only 39% of the DI λ Luc λ lines. Thus, a much higher percentage of the non-MAR, λ Luc λ lines either failed to express initially or were silenced prior to the first assay at 52 DAT.

The Rb7 MAR increases transgene expression levels and the percentage of expressing lines

Statistical comparisons between each of the 12 treatment populations revealed that flanking luciferase with Rb7 MARs resulted in a significant increase in transgene expression levels and a higher percentage of expressing lines at all timepoints and under all induction conditions. A comparison of the DI MLucM lines with either the DI XLucX or DI λ Luc λ lines at the 52 DAT timepoint reveals that the inclusion of the flanking Rb7 MAR significantly increased both the levels of expression and the percentage of expressing cell lines. A similar comparison at 85 DAT shows a significant difference, although not as great as observed for the 52 DAT assay. In contrast to the significant differences seen with the MLucM transformants, when the λ Luc λ and XLucX transformants are compared by the same statistical analysis, no significant difference is seen.

Discussion

We have addressed the question of whether active transcription influences the frequency of transgene silencing in NT1 cells and the effectiveness of using MARs to improve transgene expression. We demonstrate that delayed transcription can preserve transcriptional potential. After transcription initiation, however, silencing ensues so that many lines lose expression when transcription continues beyond 14 days.

Induction treatment	Total number of cell lines	Number of lines expressing 52 DAT (%)	Number of lines expressing 85 DAT (%)	52 DAT expressor lines also expressing at 85 DAT (%)
Delayed induction MLucM	48	41 (85%)	36 (75%)	36/41 (88%)
Delayed induction XLucX	48	28 (58%)	17 (35%)	17/28 (61%)
Delayed induction $\lambda Luc\lambda$	44	17 (39%)	14 (32%)	14/17 (82%)
Constant induction MLucM	43	34 (79%)	32 (76%)	32/34 (94%)
Constant induction XLucX	48	16 (34%)	11 (23%)	11/16 (69%)
Constant induction λLucλ	39	14 (36%)	13 (33%)	13/14 (93%)

Table 1 Total number of transformants and the number and percentage of cell lines expressing luciferase at 52 DAT and 85 DAT

The presence of flanking MAR or λ sequences reduced the negative impact of transcription on expression, but only MARs significantly increased expression levels and the percentage of cell lines exhibiting expression.

Transgene organization patterns and integration position in the genome are thought to affect susceptibility to silencing. Examples include repeated transgene arrays (Assaad et al., 1993; Ye and Signer, 1996; Hamilton et al., 1998; Luff et al., 1999; De Buck et al., 2001; Martienssen, 2003), or integration into genomic regions where transcription results in production of dsRNA with homology to the transgene (Volpe et al., 2002; Danzer and Wallrath, 2004; Lippman and Martienssen, 2004; Schlissel, 2004). In order for RNA-mediated transgene silencing to occur by these mechanisms, posttranscriptional gene silencing (PTGS) must be initiated by production of high levels of transgene mRNA. In our study, the highest percentage of luciferase-expressing lines was found at the 52 DAT measurement in the delayed induction treatment and specifically in the population carrying MAR flanking sequences. At 85 DAT, it is likely that extended growth under inducing conditions resulted in the onset of PTGS in some lines as demonstrated by decreased expression when lines are compared at 52 and 85 DAT. This pattern was particularly evident in the DI XLucX lines, in which the percentage of lines expressing luciferase was nearly 60% at the 52 DAT timepoint, but declined to 35% at 85 DAT. In contrast, the DI MlucM lines showed a much greater stability of expression over time and 85% and 75% of DI MlucM lines expressed luciferase at 52 DAT and 85 DAT, respectively. Clearly, there is a tendency for actively transcribing lines to silence over time, and flanking sequences counteract this tendency.

Our finding that active transcription increases silencing are consistent with those of Que *et al.* (1997), who found that the degree and frequency of silencing of a gene increased when the strength of the promoter increased, and the findings of Vaucheret *et al.* (1997), who showed that active transgene expression was necessary for silencing a transcribed endogenous gene. In a related finding, Vaistij *et al.* (2002) found

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that active transgene transcription was important for initiation and spreading of viral-induced gene silencing from an RNA virus carrying homology to a part of the targeted transgene. Our results extend these findings by demonstrating that the same transgene, under CI or DI conditions, is associated with different silencing frequencies initially, but as induction is continued, the silencing differences are largely eliminated.

Our results also show that MAR-flanked transgenes produce significantly higher levels of luciferase than transgenes without flanking sequences or flanked by λ DNA. It is clear that MARs are positively impacting transgene expression, and insulation from neighbouring chromatin is one possible mechanism to explain these results. Recent studies with yeast have shown that the condensed (silent) chromatin can spread into surrounding regions by a process of RNAi-mediated heterochromatinization (reviewed by Lippman and Martienssen, 2004). Our data show that the Rb7 MARs prevent silencing of the DI transgenes, and one possible mechanism for this effect is that MARs block the spread of silent chromatin into the transgene.

The use of microprojectile bombardment for transformation frequently produces transgene loci with multiple-copy transgene arrays, inverted repeats, or other possible rearrangements (Pawlowski *et al.*, 1998; Ulker *et al.*, 1999; Ascenzi *et al.*, 2003) that are likely to produce dsRNA and initiate RNA-mediated silencing (Wang and Waterhouse, 2000; Martienssen, 2003). In our experiments, it is possible that the Rb7 MAR prevented RNA-mediated silencing *in cis* by preventing transcriptional read though into the regions flanking the transgene. Recently it was found that MARs could prevent transgene RNA transcription from continuing into the flanking DNA (Mlynarova *et al.*, 2003). Other examples of read-through silencing have been shown to occur in tandem genes when the expression of the upstream gene results in the silencing of a downstream gene (Thompson and Myatt, 1997).

We found that when the Rb7 MAR or λ DNA was used to flank a transgene, the expression of the transgene was more stable than in controls without flanking DNA. Both Rb7 MAR

and λ DNA could decrease the likelihood of dsRNA production from complex loci by providing a spacer between transgene arrays, but from the differences in expression levels it is clear that MARs have additional functions over and above the λ spacer.

Our observation of long-term Rb7 MAR protection from mitotic silencing in daughter cells is similar to results of Francastel *et al.* (1999), who used a K562 erythroleukaemia cell system to demonstrate the protective effect of the 5'HS2 enhancer during a 20–24-week test period. Importantly, Ostermeier *et al.* (2003) later demonstrated that the same 5'HS2 enhancer is a MAR that can bind to the nuclear matrix of K562 cells. Thus, transgenes with the 5'HS2 enhancer (MAR) remained active, while mutations of the 5'HS2 were often silenced.

Recently, Butaye *et al.* (2004) showed that a MAR-flanked transgene could be stably expressed at very high levels in *Arabidopsis* mutants deficient in PTGS. In contrast, expression was diminished when the same MAR-flanked transgenes were expressed in the wild-type plants, or when transgenes lacking flanking MARs were expressed in the PTGS mutants. The Butaye *et al.* (2004) results indicate that MARs and PTGS act on different forms of gene silencing and, when combined, a much higher level of transgene expression is possible than when either is used alone. Similarly, our present results show the feasibility of a system in which a combination of MAR-flanked transgenes controlled by inducible promoters may be used to achieve high levels of transgene expression without suppressing PTGS.

Experimental procedures

Plasmid constructs

Figure 1a shows schematic representations of the five plasmids that were used in our study. A binary vector, pBIN-TetR-rsGFP was used for the first transformation using *Agrobacterium* (Figure 1b). pBIN-TetR-rsGFP contains a kanamycin resistance cassette and a tetracycline repressor-GFP translational fusion protein. The tetracycline repressor-soluble modified, red-shifted GFP (TetR-rsGFP) fusion protein was produced according to the following cloning strategy. The rsGFP (Davis and Vierstra, 1998) from plasmid CD3-327 (*Arabidopsis* Biological Resource Center, Columbus, OH), was amplified by polymerase chain reaction (PCR) using Boehringer high fidelity *Taq* with the primers 5'-GCGCGTCGACAAATGAGTAAAGGAGAAGAAC-3' and 5'-GCGCGTCGACCAAATGAGTAAAGGAGAAGAAC-3' and 5'-GCGCGTACCGAGCTCTTATTTGTATAGTTC-3' and cloned into the Sall and Asp718 sites of pBC (Stratagene, La Jolla, CA) to produce pBC-rsGFP, which was verified by sequencing. The TetR (Gatz et al., 1992) lacking the TAA was amplified by PCR from pTet, kindly provided by Christiane Gatz (Albrecht-von-Haller-Institut, Universität Göttingen, Germany) using the primers 5'-GCGCGGATCCAATGTCTA-GATTAGATAAAAGT-3' and 5'-GCGCTTCTCGAGTAGAC-CCACTTTCACATTTAAGTTG-3' and cloned into the BamHI and Xhol sites of pKSII (Stratagene) to produce pKSII-TetR, which was verified by sequencing. The translational fusion gene TetR-rsGFP was then produced by cloning the rsGFP gene into of Xhol/KpnI sites of the pKSII-TetR plasmid, resulting in pKSII-TetR-rsGFP. The TetR-rsGFP gene from pKSII-TetR-rsGFP was then cloned into the BamHI and Acc65I sites of a derivative of pBIN19 (Bevan, 1984), that confers kanamycin resistance in plants and contains a CaMV 35S promoter and a nopaline synthase polyadenylation signal flanking a pUC19 multiple cloning site. The resulting binary vector pBIN-TetR-rsGFP (Figure 1a) was then used to transform NT1 tobacco suspension cells using Agrobacterium tumefaciens strain LBA4404 as described by An (1985).

We then constructed three reporter plasmids (Figure 1a). The first, XLucX, contains a Luc reporter gene without flanking DNA and was produced as follows: The EcoRI/HindIII fragment containing the TripleOp promoter-octopine synthase polyA signal (OcsT) flanking a pUC19 multiple cloning site from pBinHygTX (kindly provided by Christiane Gatz) was ligated into the EcoRI/HindIII sites of pKSII, to produce pKSII-TripleOp-OcsT. The luciferase gene from pLUC (Bonin et al., 1994) was then inserted into the Acc65/Xbal site of pKSII-TripleOp-OcsT to produce XLucX. The second reporter plasmid, which contains the Rb7 MAR (accession number U67919) flanking the luciferase gene, was named MLucM, and was constructed by inserting the 35S-Luc-OcsT fragment from XLucX into the HindIII/EcoRI sites of the pKSII multiple cloning site flanked by the Rb7 MAR (Allen et al., 1996).

The third reporter plasmid contains the reporter gene flanked by spacer sequences and was produced as follows: The 21 231-22 425 region of the bacteriophage λ (accession number NC_001416) was amplified by PCR using the primers 5'-GCGCGTCGACGGCCTTTCCGGCAGGTGCGC-3' (forward) and 5'-TCGTAAGCTTCTGCAGTGCAGCATCCTGAAGCGG-3' (reverse). The λ PCR product was cloned into *Hin*dIII and Sall sites of pKSII (Stratagene, La Jolla, CA) to produce pKSII- λ . The λ Luc λ reporter plasmid was then produced by using PCR to amplify the identical 1195 bp λ fragment from pKSII- λ , using the primers 5'-GCGCGTCGACGGCCTTCCG-GCAGGTGCGC-3' (forward) and 5'-TCGTAGTCTGCA-GTGCAGCGC-3' (forward) and 5'-TCGTACTAGTCTGCA-GTGCAGCATCCTGAAGCGG-3' (reverse). The amplified product was then cloned into the *Eco*RI/Spel sites of pKSII- λ resulting in pKSII- λ mcs λ , where mcs is the pKSII multiple cloning site. The TripleOp-Luciferase-OcsT cassette from pKSII-TripleOp-OcsT was then cloned into the *Eco*RI/*Hin*dIII sites of pKSII- λ mcs λ to produce the spacer control, λ Luc λ (Figure 1a).

Agrobacterium transformation of NT1 cells with pBIN-TetR-rsGFP

The *Nicotiana tabacum* cell line NT1, originally obtained from G. An, Washington State University, was transformed by *A. tumefaciens* strain LBA4404 as described by An (1985), and the microcalli were selected on NT1 agar plates containing 50 mg/L kanamycin (Sigma Chemical, St. Louis). The kanamycin-resistant microcalli were then made into suspension cultures (Allen *et al.*, 1996) and grown in a medium containing (per litre) Murashige and Skoog salts (Gibco Invitrogen, Carlsbad, CA) supplemented with 100 mg inositol, 1 mg thiamine HCl, 180 mg KH₂PO₄, 30 g sucrose and 2 mg 2,4-dichlorophenoxyacetic acid. The pH was adjusted to 5.7 before autoclaving using 1 \bowtie KOH. Cells were subcultured once per week by adding 3 mL of inoculum to 100 mL of fresh medium in 500 mL Erlenmeyer flasks that were then incubated in the dark at 125 r.p.m. at 25 °C.

The TetR-rsGFP expressing cell lines were identified by visualizing the rsGFP expression with a Zeiss (Carl Zeiss, Inc., Thornwood, NY) confocal microscope equipped with a 40x NA 1.2 water-immersion lens, with excitation from an argon/ krypton laser at 465–495 nm and emission recorded with a 515–545-nm band-pass filter. To confirm that the TetR-rsGFP fusion was functional, cell lines transformed with the pBIN-TetR-rsGFP binary vector were re-transformed (An, 1985) with either a β-glucuronidase or luciferase genes driven by the doxycycline-regulatable TripleOp promoter and tested for induction by doxycycline (Gatz *et al.*, 1992).

Co-transformation with the luciferase and hygromycin genes by microprojectile bombardment

A cell line, which expressed TetR-rsGFP and showed tight doxycycline-regulation in preliminary experiments, was selected as the parent line. The parent line was then used in all the cotransformation experiments that were performed as described earlier (Allen *et al.*, 1993; Allen *et al.*, 1996). Microprojectile bombardment was carried out with a DuPont PDS-1000 particle accelerator using a rupture disk value of 1100 psi with the sample positioned 5.5 cm from the launch assembly (Allen *et al.*, 1993; Allen *et al.*, 1996).

The luciferase (Luc) reporter gene driven by the Triple-Op promoter (Gatz et al., 1991), which is derived from the CaMV 355 promoter (Benfey et al., 1989), was used to measure expression, and a hygromycin phosphotransferase gene (HptII) driven by the mannopine synthase promoter (MasP) was used to select for cells that had stably integrated transgene DNA. Co-transformation mixtures contained a 4 : 1 molar ratio of luciferase reporter plasmid to hygromycin phosphotransferase (HptII) selection plasmid, MasP-HptII. Therefore, each 500 ng MAR transformation mixture consisted of 435 ng MLucM and 65 ng MasP-HptII, whereas control mixtures contained 310 ng XLucX, or 435 ng λ Luc λ and 65 ng MasP-Hptll as described earlier (Allen et al., 1996). Doxycycline was used to induce transcription of the luciferase reporter gene during bombardment in some of the lines in order to allow for determination of the effects of active transcription during the transformation process. Lines that were exposed to doxycycline during bombardment were maintained on doxycycline for the duration of the experiment.

After bombardment, the Petri plates were sealed with parafilm and incubated for 24 h in the dark at 25 °C. The cells were then transferred to fresh plates containing medium supplemented with 50 µg/mL hygromycin, with or without 100 ng/mL doxycycline, to select stable transformants grown under inducing or non-inducing conditions. Isolated hygromycin-resistant microcalli began to appear in approximately 2 weeks, and at 18 days they were transferred to fresh plates containing 50 µg/mL hygromycin NT1 medium with, or without, 100 ng/mL doxycycline. For inducing the luciferase in the cell lines transformed in the absence of doxycycline, microcalli grown for 19 days (38 days) were transferred to NT1 media containing 0.8% agar with 100 ng/mL doxycycline and transferred weekly. At 52 and 85 days after transformation (DAT), approximately 40 mg (± 3 mg S.E., based on 10 replications) of cells, grown under inducing or non-inducing conditions, were removed from each microcallus and measured for luciferase activity as described in succeeding discussions.

Luciferase expression

Transient luciferase expression was measured 24 h after the microprojectile bombardment from each plasmid transformation and doxycycline treatment. Transient luciferase activity was measured by transferring the lens paper from two plates of transformed cells from each treatment into a sterile Petri dish containing 400 μ L of 5 mM sodium luciferin (Biosynth, Naperville, IL) with, or without 100 ng/mL doxycycline. The plates were incubated for 10 min to allow substrate penetration. A Hamamatsu photon imager was used to quantify the number of photons produced per 2 min (Hamamatsu Corp., Bridgewater, NJ).

To measure luciferase expression in independent stably transformed cell lines, 40 mg of callus was placed into a black 96-well plate (BD Falcon, San Diego, CA), which was followed by the addition of 200 μ L of 5 mm luciferin solution into each well. The number of photons emitted during a 2min period was measured as described previously. Relative luciferase activity was determined by comparing the number of photons produced per 2 min. The maximum luciferase activity found was 5669 photons per 2 min in the CI MLucM line 23 at 52 DAT. The maximum photon count was set to 6000 photons per 2 min and relative luciferase activity was calculated as a percentage of the luciferase activity for each line with 6000 as 100%. Thus, the relative luciferase activity for 5669, is determined by calculating $(5669/6000) \times 100 =$ 94.5. The photon images were then measured using the public domain IMAGEJ program (Java version of NIH image developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/). Photon intensity was measured as the area under peaks produced by NIH image.

Statistical analysis

All statistical analyses were performed using GRAPHPAD PRISM version 3.0a for Macintosh, GraphPad Software, San Diego California USA, www.graphpad.com. All data sets were tested for normality using the Kolmogorov-Smirnov (KS) test. Only four data sets, which included the MLucM lines with constant induction or delayed induction measured at 52 and 85 days, passed the normality test, and data transformations failed to improve Gaussian approximation. Lack of normality was not unexpected in non-MAR lines because of the large number of silenced lines in these populations. Comparisons of non-normal data sets were made using the distributionfree Mann–Whitney test for unpaired data, and the Wilcoxon matched pairs test for paired data (Rao, 1998). Comparisons of normal data sets were made using standard t tests. Repeated measurements on the same line at 52 and 85 DAT were considered as paired data.

Acknowledgements

We thank the *Arabidopsis* Biological Resource Center (Columbus, OH) for providing the soluble modified redshifted GFP plasmid and Christiane Gatz for pTET1 and the Tet Operator-TripleOp doxycycline regulatable promoter system. We also thank Kim Sampson and Tuyen Nguyen for help in constructing XLucX, MLucM, and λ Luc λ . We also gratefully acknowledge David Collings for help in screening for TetR-rsGFP expression, and Dominique (Niki) Robertson for her comments on the manuscript.

References

- Allen, G.C., Hall, G.E., Childs, L.C., Weissinger, A.K., Spiker, S. and Thompson, W.F. (1993) Scaffold attachment regions increase reporter gene expression in stably transformed plant cells. *Plant Cell*, **5**, 603–613.
- Allen, G.C., Hall, G., Michalowski, S., Newman, W., Spiker, S., Weissinger, A.K. and Thompson, W.F. (1996) High-level transgene expression in plant cells: effects of a strong scaffold attachment region from tobacco. *Plant Cell*, **8**, 899–913.
- Allen, G.C., Spiker, S. and Thompson, W.F. (2000) Use of matrix attachment regions (MARs) to minimize transgene silencing. *Plant Mol. Biol.* **43**, 361–376.
- An, G. (1985) High efficiency transformation of cultured tobacco cells. *Plant Physiol.* **79**, 568–570.
- Ascenzi, R., Ulker, B., Todd, J.J., Sowinski, D.A., Schimeneck, C.R., Allen, G.C., Weissinger, A.K. and Thompson, W.F. (2003) Analysis of trans–silencing interactions using transcriptional silencers of varying strength and targets with and without flanking nuclear matrix attachment regions. *Trans. Res.* **12**, 305–318.
- Assaad, F.F., Tucker, K.L. and Signer, E.R. (1993) Epigenetic repeatinduced gene silencing (RIGS) in *Arabidopsis. Plant Mol. Biol.* 22, 1067–1085.
- Benfey, P.N., Ren, L. and Chua, N.-H. (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J. 8, 2195–2202.
- Bevan, M. (1984) Binary Agrobacterium vectors for plant transformation. Nucl Acids Res. 12, 8711–8721.
- Bode, J., Benham, C., Knopp, A. and Mielke, C. (2000) Transcriptional augmentation: modulation of gene expression by scaffold/ matrix-attached regions (S/MAR elements). *Crit. Rev. Eukaryot. Gene Expr.* **10**, 73–90.
- Bonin, A.L., Gossen, M. and Bujard, H. (1994) *Photinus pyralis* Luciferase – vectors that contain a modified luc coding sequence allowing convenient transfer into other systems. *Gene*, **141**, 75– 77.
- Butaye, K.M.J., Goderis, I.J.W.M., Wouters, P.F.J., Pues, J.M.-T.G., Delaure, S.L., Broekaert, W.F., Depicker, A., Cammue, B.P.A. and De Bolle, M.F.C. (2004) Stable high-level transgene expression in *Arabidopsis thaliana* using gene silencing mutants and matrix attachment regions. *Plant J.* **39**, 440–449.
- Cockerill, P.N. and Garrard, W.T. (1986) Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell*, **44**, 273–282.
- Danzer, J.R. and Wallrath, L.L. (2004) Mechanisms of HP1-mediated gene silencing in *Drosophila*. *Development*, **131**, 3571–3580.
- Davis, S.J. and Vierstra, R.D. (1998) Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant Mol Biol.* **36**, 521–528.
- De Buck, S., Van Montagu, M. and Depicker, A. (2001) Transgene silencing of invertedly repeated transgenes is released upon deletion of one of the transgenes involved. *Plant Mol. Biol.* **46**, 433–445.

- Francastel, C., Walters, M.C., Groudine, M. and Martin, D.I. (1999) A functional enhancer suppresses silencing of a transgene and prevents its localization close to centrometric heterochromatin. *Cell*, **99**, 259–269.
- Gatz, C., Kaiser, A. and Wendenburg, R. (1991) Regulation of a modified *CaMV 35S* promoter by the Tn10-encoded Tet repressor in transgenic tobacco. *Mol. General Genet.* **227**, 229–237.
- Gatz, C., Claus, F. and Regina, W. (1992) Stringent repression and homogeneous de-repression by tetracycline of a modified *CaMV* 35S promoter in intact transgenic tobacco plants. *Plant J.* 2, 397– 404.
- Goetze, S., Baer, A., Winkelmann, S., Nehlsen, K., Seibler, J., Maass, K. and Bode, J. (2005) Performance of genomic bordering elements at predefined genomic loci. *Mol. Cell. Biol.* **25**, 2260–2272.
- Halweg, C., Thompson, W.F. and Spiker, S. (2005) The Rb7 matrix attachment region increases the likelihood and magnitude of transgene expression in tobacco cells: a flow cytometric study. *Plant Cell*, **17**, 418–429.
- Hamilton, A.J., Brown, S., Han, Y.H., Ishizuka, M., Lowe, A., Solis, A.G.A. and Grierson, D. (1998) A transgene with repeated DNA causes high frequency, post-transcriptional suppression of ACCoxidase gene expression in tomato. *Plant J.* **15**, 737–746.
- Kas, E., Poljak, L., Adachi, Y. and Laemmli, U.K. (1993) A model for chromatin opening – stimulation of topoisomerase-II and restriction enzyme cleavage of chromatin by distamycin. *EMBO J.* **12**, 115–126.
- Laemmli, U.K., Kas, E., Poljak, L. and Adachi, Y. (1992) Scaffoldassociated regions: cis-acting determinants of chromatin structural loops and functional domains. *Curr. Opin. Genet. Dev.* 2, 275–285.
- Lippman, Z. and Martienssen, R. (2004) The role of RNA interference in heterochromatic silencing. *Nature*, **431**, 364–370.
- Luff, B., Pawlowski, L. and Bender, J. (1999) An inverted repeat triggers cytosine methylation of identical sequences in *Arabidopsis*. *Mol. Cell*, **3**, 505–511.
- Martienssen, R.A. (2003) Maintenance of heterochromatin by RNA interference of tandem repeats. *Nature Genet.* **35**, 213–214.
- Matzke, M. and Matzke, A.J. (2003) RNAi extends its reach. *Science*, **301**, 1060–1061.
- Mendu, N., Massel, M. and Spiker, S. (2001) Increasing loop domain size does not diminish effects of matrix attachment regions on transgene expression in tobacco cells culture. *FEBS Lett.* **496**, 66–67.
- Mirkovitch, J., Mirault, M.-E. and Laemmli, U.K. (1984) Organisation of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. *Cell*, **39**, 223–232.
- Mlynarova, L., Hricova, A., Loonen, A. and Nap, J.P. (2003) The presence of a chromatin boundary appears to shield a transgene in tobacco from RNA silencing. *Plant Cell*, **15**, 2203–2217.
- Ostermeier, G.C., Liu, Z., Martins, R.P., Bharadwaj, R.R., Ellis, J., Draghici, S. and Krawetz, S.A. (2003) Nuclear matrix association of the human beta-globin locus utilizing a novel approach to quantitative real-time PCR. *Nucl Acids Res.* **31**, 3257–3266.
- Pawlowski, W.P., Torbert, K.A., Rines, H.W. and Somers, D.A. (1998) Irregular patterns of transgene silencing in allohexaploid oat. *Plant Mol. Biol.* **38**, 597–607.
- Petersen, K., Leah, R., Knudsen, S. and Cameron-Mills, V. (2002) Matrix attachment regions (MARs) enhance transformation fre-

quencies and reduce variance of transgene expression in barley. *Plant Mol. Biol.* **49**, 45–58.

- Phi-Van, L., von Kries, J.P., Ostertag, W. and Strätling, W.H. (1990) The chicken lysozyme 5' matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes. *Mol. Cell. Biol.* **10**, 2302–2307.
- Poljak, L., Seum, C., Mattioni, T. and Laemmli, U.K. (1994) SARs stimulate but do not confer position independent gene expression. *Nucl Acids Res.* 22, 4386–4394.
- Que, Q.D., Wang, H.Y., English, J.J. and Jorgensen, R.A. (1997) The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence. *Plant Cell*, **9**, 1357–1368.
- Rao, P.V. (1998) *Statistical Research Methods in the Life Sciences*. Pacific Grove, CA. USA: Brooks/Cole Publishing Company.
- Schlissel, M. (2004) The spreading influence of chromatin modification. *Nature Genet*, **36**, 438–440.
- Stief, A., Winter, D.M., Stratling, W.H. and Sippel, A.E. (1989) A nuclear DNA attachment element mediates elevated and positionindependent gene activity. *Nature*, **341**, 343–345.
- Thompson, A.J. and Myatt, S.C. (1997) Tetracycline-dependent activation of an upstream promoter reveals transcriptional interference between tandem genes within t-DNA in tomato. *Plant Mol. Biol.* **34**, 687–692.
- Ulker, B., Allen, G.C., Thompson, W.F., Spiker, S. and Weissinger, A.K. (1999) A tobacco matrix attachment region reduces the loss of transgene expression in the progeny of transgenic tobacco plants. *Plant J.* **18**, 253–263.
- Vain, P., Worland, B., Kohli, A., Snape, J.W., Christou, P., Allen, G.C. and Thompson, W.F. (1999) Matrix attachment regions increase transgene expression levels and stability in transgenic rice plants and their progeny. *Plant J.* **18**, 233–242.
- Vaistij, F.E., Jones, L. and Baulcombe, D.C. (2002) Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell*, **14**, 857–867.
- Van der Geest, A.H.M., Welter, M.E., Woosley, A.T., Pareddy, D.R., Pavelko, S.E.P., Skokut, M. and Ainley, W.M. (2004) A short synthetic MAR positively affects transgene expression in rice and *Arabidopsis. Plant Biotechn J.* 2, 13–26.
- Vaucheret, H., Nussaume, L., Palauqui, J.C., Quillere, I. and Elmayan, T. (1997) A transcriptionally active state is required for posttranscriptional silencing (cosuppression) of nitrate reductase host genes and transgenes. *Plant Cell*, **9**, 1495–1504.
- Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I. and Martienssen, R.A. (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science*, **297**, 1833–1837.
- Wang, M.B. and Waterhouse, P.M. (2000) High-efficiency silencing of a beta-glucuronidase gene in rice is correlated with repetitive transgene structure but is independent of DNA methylation. *Plant Mol. Biol.* **43**, 67–82.
- Ye, F. and Signer, E.R. (1996) RIGS (repeat-induced gene silencing) in *Arabidopsis* is transcriptional and alters chromatin configuration. *Proc. Natl. Acad. Sci., USA*, **93**, 10881–10886.