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Sequence elements within an *HSP70* promoter counteract transcriptional transgene silencing in *Chlamydomonas*

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Summary

We have shown previously that the *HSP70A (A)* promoter, when fused upstream of other promoters, significantly improves their performance in driving transgene expression in *Chlamydomonas*. Here, we employed the bacterial resistance gene *ble,* driven by the *RBCS2 (R)* promoter or an *AR* promoter fusion, to determine, by which mechanism(s) the *A* promoter may exert its enhancing effect. We observed that transformation rates of *AR-ble* constructs were significantly higher than those of *R-ble* constructs. However, *ble* mRNA levels in pools of transformants generated with either construct type were the same. Co-transformation experiments revealed that the *R-ble* transgene was silenced in 80% of the transformants, whereas this fraction was reduced to 36% in transformants harbouring the *AR-ble* transgene. We conclude that the *A* promoter acts by decreasing the probability that a transgene becomes transcriptionally silenced. We mapped two elements within the *A* promoter that are responsible for this effect. The core of the first element appears to be located between nucleotides −7 and +67 relative to the *HSP70A* transcriptional start site. Its activity is strongly dependent on its spatial setting with respect to the *R* promoter and is increased by upstream sequences (−196 to −8). The second element is independent of the first and is located to the region from −754 to −197. Its activity is spacing-independent and additive to the first element.

Keywords: *Chlamydomonas*, transgene expression, chromatin, *HSP70*, transcriptional gene silencing, synthetic promoters.

Introduction

In most eukaryotic organisms transgenic approaches suffer from the unpredictable expression of introduced transgenes. Expression levels may vary considerably and introduced transgenes frequently become silenced. In plants, the mechanisms underlying silencing have been investigated intensively in the past few years (Fagard and Vaucheret, 2000; Vaucheret and Fagard, 2001). Two types of gene silencing are distinguished: transcriptional gene silencing (TGS), which is characterized by a block in transgene transcription, and post-transcriptional gene silencing (PTGS), which involves the rapid degradation of initially synthesized transcripts.

PTGS is thought to be elicited by double-stranded RNA molecules originating from aberrant transcripts, which are cleaved into pieces of 21–25 nt length (short interfering RNA, siRNA) (Nishikura, 2001; Vaucheret *et al.*, 2001). The siRNAs apparently serve two purposes: Firstly, they act as primers for RNA-dependent RNA polymerases that use the target transcript as a template for a PCR-like amplification. Secondly, they guide endonucleases to homologous transcripts, therefore leading to their degradation. The siRNA molecules may become propagated within the entire organism, resulting in systemic transgene silencing.

TGS of a transgene may be elicited by *cis*- or *trans*-acting elements (Fagard and Vaucheret, 2000). The trigger for *cis*-TGS may originate from the transgene itself, i.e. from arrays of repeated transgene copies (Furner *et al.*, 1998; Mittelsen Scheid *et al.*, 1998), or from differences in the G/C-content of transgenic DNA and genomic DNA at the integration site (Elomaa *et al.*, 1995). In these cases, silenced transgene loci are characterized by methylation and the formation of mitotically- and meiotically-stable, local heterochromatin. Alternatively, *cis*-TGS may be due to heterochromatin already present at or close to the

(random) integration site of the transgene (Iglesias *et al.*, 1997). The spreading of heterochromatin into and out of the transgene locus may result in variegated expression. Trans-TGS of a transgene is induced when a silenced copy of a (*trans*)gene driven by an identical promoter is present at a distant site in the genome. The latter imposes its silent chromatin state either by direct DNA-DNA interactions, or by a diffusible signal (e.g. RNA) to the newly introduced transgene (Kooter *et al.*, 1999).

Approaches to analyse the pathway(s) leading to TGS in plants so far were based on screens designed to identify mutants that relieve silencing of antibiotic resistance genes. The mutants identified were denoted *hog* for homology-dependent gene silencing (Furner *et al.*, 1998), *sil* for silencing (Furner *et al.*, 1998), *som* for somniferous (Mittelsen Scheid *et al.*, 1998), *mom* for mutation in a 'Morpheus molecule' (Amedeo *et al.*, 2000), and *mut* for mutant strains that reactivate transgene expression (Jeong *et al.*, 2002). Several of the *som* loci turned out to be allelic to *ddm1*. The *ddm1* mutant is impaired in TGS and exhibits reduced DNA methylation (Jeddeloh *et al.*, 1999). *DDM1* encodes a protein that is strongly similar to SWI2/SNF2-like chromatin-remodelling proteins. Although the *MOM* gene also codes for a protein with a region related to SWI2/SNF2 proteins, the *mom1* mutant exhibits impaired TGS without affecting methylation (Amedeo *et al.*, 2000). These findings underline the role of chromatin remodelling in TGS.

In *Chlamydomonas*, TGS was found to be involved in the epigenetic silencing of the *aadA* transgene (Cerutti *et al.*, 1997a, 1997b; Jeong *et al.*, 2002). Two mutants, *mut9* and *mut11*, exhibit impaired TGS of the *aadA* gene and a higher sensitivity towards agents inducing double-strand breakages (Jeong *et al.*, 2002). Both genes are thought to be involved in the formation of distinct chromatin structures that are required for TGS and for the repair of DNA damage.

We have shown previously that the *Chlamydomonas HSP70A* promoter is capable of significantly increasing the activities of promoters *RBCS2*, β_2*TUB* and *HSP70B* driving the *HSP70B* and the *aadA* reporter genes (Schroda *et al.*, 1999, 2000). In the present study we set out to dissect the molecular details of the mechanism(s) involved. We show that the *HSP70A* promoter acts by decreasing the probability that a transgene becomes subjected to TGS. We have mapped two sequence motifs within the *HSP70A* promoter that are responsible for this effect.

Results

The A *promoter increases transformation rates of the* ble *resistance gene without affecting* ble *transcript levels*

In Figure 1a, transformation rates for five different constructs of the *ble* gene are given. Truncation of the *R* promoter driving *ble* gene expression from 740 bp (pSP108, Stevens *et al.*, 1996) to 182 bp (pCB845) upstream of the transcriptional start site increased transformation rates approximately 1.5-fold. Truncation of the *R* promoter and insertion of the first *RBCS2* intron into the *ble* coding sequence (pSP115, Lumbreras *et al.*, 1998) resulted in approximately 5.2-fold increased transformation rates. These results agree with those reported previously (Lumbreras *et al.*, 1998), except that in our hands the degree of stimulation of transformation rates was less pronounced.

Insertion of the *A* promoter upstream of the *R* promoter of both intron-containing or intron-less *ble* constructs resulted in an additional, approximately 2-fold increase in the number of zeocin resistant transformants (compare rates for pCB845 with pCB796 and rates for pSP115 with pCB797 in Figure 1a). Expression of the *ble* constructs was also tested in co-transformation experiments with the *ARG7* gene (Debuchy *et al.*, 1989), i.e. in transformants that were initially selected for arginine prototrophy. In these experiments, the rates of co-transformation were 2- to 5-fold higher for *AR-ble* fusions (pCB796 and pCB797) as compared to *R-ble* fusions (pCB845 and pSP115) (Figure 1a).

The enhanced transformation and co-transformation rates observed for *AR-ble* constructs could have been due to elevated *ble* mRNA levels resulting from a higher transcriptional activity of the *AR* promoter as compared to the *R* promoter. To test this possibility, we analysed *ble* transcript levels in pools of zeocin resistant transformants harbouring the various constructs (Figure 1b). Overall, the constructs harbouring the first *RBCS2* intron within the *ble* gene gave rise to approximately 3.8-fold higher *ble* transcript levels (Figure 1b,c, compare pSP115 with pCB845 and pCB797 with pCB796), matching almost exactly the increase observed in transformation rates. Similarly, the truncated Δ-182 *R* promoter in pCB845 led to a slight (approximately 25%) increase in *ble* mRNA levels as compared to the Δ-740 promoter in pSP108. In contrast, and much to our surprise, transformants containing the *ble* gene driven by the *AR* promoter did not show a further increase in *ble* mRNA levels. In contrast, we observed an approximately 10% decrease under all conditions tested, except heat shock (compare pCB796 with pCB845 and pCB797 with pSP115). Transformants harbouring *AR-ble* constructs exhibited high transgene mRNA levels only upon heat shock (Figure 1b,c), but these transcripts, originating from the transcriptional start of the *A* promoter, are unlikely to contribute to Ble protein and zeocin resistance because they contain 6 out-of-frame start codons contributed by the *R* promoter sequences (Goldschmidt-Clermont and Rahire, 1986). Note that, whereas transformants harbouring constructs *AR-HSP70B* and *AR-aadA* showed light-inducible reporter

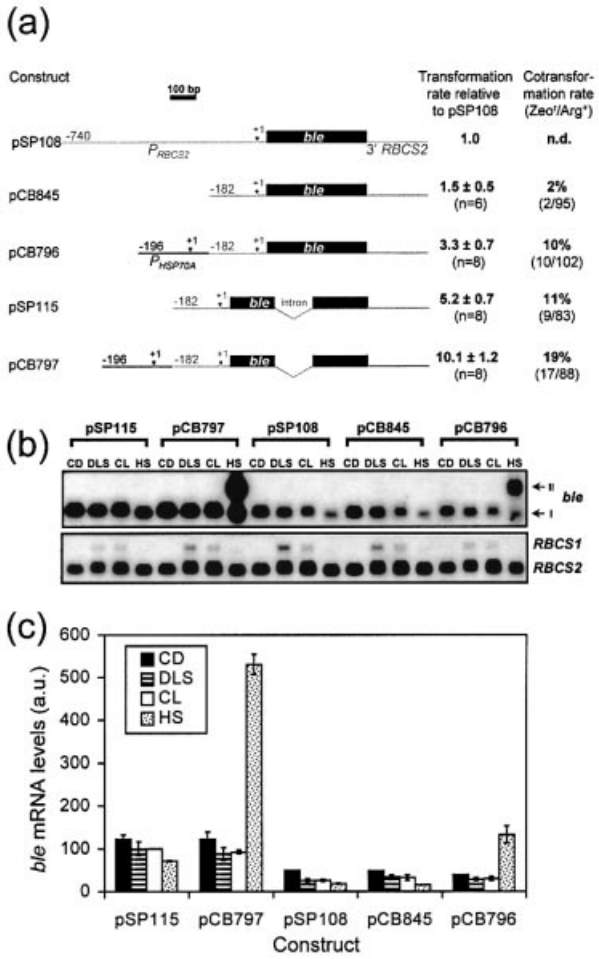

Figure 1. Transformation rates of various *ble* gene constructs and analysis of *ble* mRNA accumulation in transformants.
(a) Constructs used for the generation of zeocin resistant transformants and observed transformation rates. The *ble* gene is indicated as a black box, *RBCS2* sequences are drawn as grey lines, *HSP70A* sequences as black lines. The intron in pSP115 (Lumbreras *et al.*, 1998) and pCB797 is the first intron of the *RBCS2* gene. Transcriptional start sites (+ 1) are marked by arrowheads. Transformation rates relative to those observed with pSP108 (Stevens *et al.*, 1996) are given as the mean and standard error of the mean in n experiments. Co-transformation rates were determined by co-transforming the arginine auxotrophic strain (*arg7*) cw15-302 with plasmids containing the *ARG7* gene (200 ng) and the *ble* gene (1 μg), and are given as the percentage of arginine prototrophic transformants (Arg^+) that are resistant to zeocin (Zeo^r). (b) Expression analysis of pools of zeocin resistant transformants. Shown is a Northern blot of total RNA (10 μg per lane) isolated from pools of 50 zeocin resistant transformants per construct. The blot was hybridized with a probe against the *ble* gene, stripped and rehybridized with the *RBCS2* coding region as a loading control. Cells were grown for 16 h in the dark (CD), then exposed to light (40 μE m^{-1} s^{-1}) for 1 h (DLS), or maintained in continuous light (CL), or heat-shocked for 40 min at 40°C in continuous light (HS). 'I' indicates *ble* transcripts originating from the transcriptional start site of the *RBCS2* promoter, 'II' those originating from the *HSP70A* transcriptional start site. (c) Intensities of the *ble* hybridization signals from two independent experiments were quantitated by phosphor-imaging and corrected for unequal loading by the respective *RBCS2* signal. Values are given in arbitrary units (a.u.) relative to the CL value of pSP115 which was set to 100. Expression levels are presented as the mean and standard error of the mean.

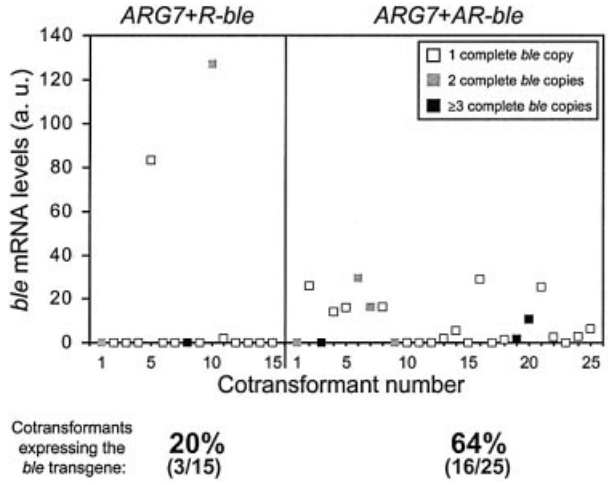

Figure 2. Analysis of *ble* expression in co-transformants.
Chlamydomonas arg7 strain 074 was co-transformed with 100 ng of a plasmid containing the *ARG7* gene and 500 ng of either pSP115 (*R-ble*) or pCB797 (*AR-ble*). For each transformant, the transgene copy number was determined by gel blot analysis of restriction digested total DNA. Clones that contained at least one intact copy of pSP115 or pCB797 were grown in continuous light and total RNA was isolated and subjected to Northern blot analysis using a probe against the coding region of *ble*. Blots were stripped and rehybridized with a probe against the coding region of *RBCS2*. Hybridization signals were quantified, corrected for unequal loading by the *RBCS2* signal and plotted in arbitrary units (a.u.).

gene expression (Schroda *et al.*, 2000), this was not the case for clones containing *AR-ble* (Figure 1b,c).

In conclusion, our results indicate that factors other than an increase in transcriptional activity of the *R* promoter are responsible for the increase in transformation and co-transformation rates observed for *AR-ble* constructs.

Transgene expression is favoured in constructs containing the A *promoter*

We hypothesized that the *A* promoter may increase the probability of stably integrated *ble* transgenes to become actively expressed. This would lead to increased transformation rates without affecting average *ble* mRNA levels in pools of zeocin resistant transformants. To test this idea, we measured the probability of expression of an integrated transgene. We co-transformed *Chlamydomonas* with the *ARG7* gene and either an *R-ble* construct (pSP115), or an *AR-ble* construct (pCB797). For each construct, 44 arginine prototrophic transformants were analysed by Southern blot for the successful integration of the co-transformed *ble* DNA. 34% (15/44) and 57% (25/44) of the co-transformants were found to contain at least one intact copy of constructs pSP115 and pCB797, respectively (Figure 2). Analysis of *ble* mRNA levels of these clones revealed that only 20% (3/15) of integrated *R-ble* genes gave rise to detectable levels of *ble* transcript, whereas this

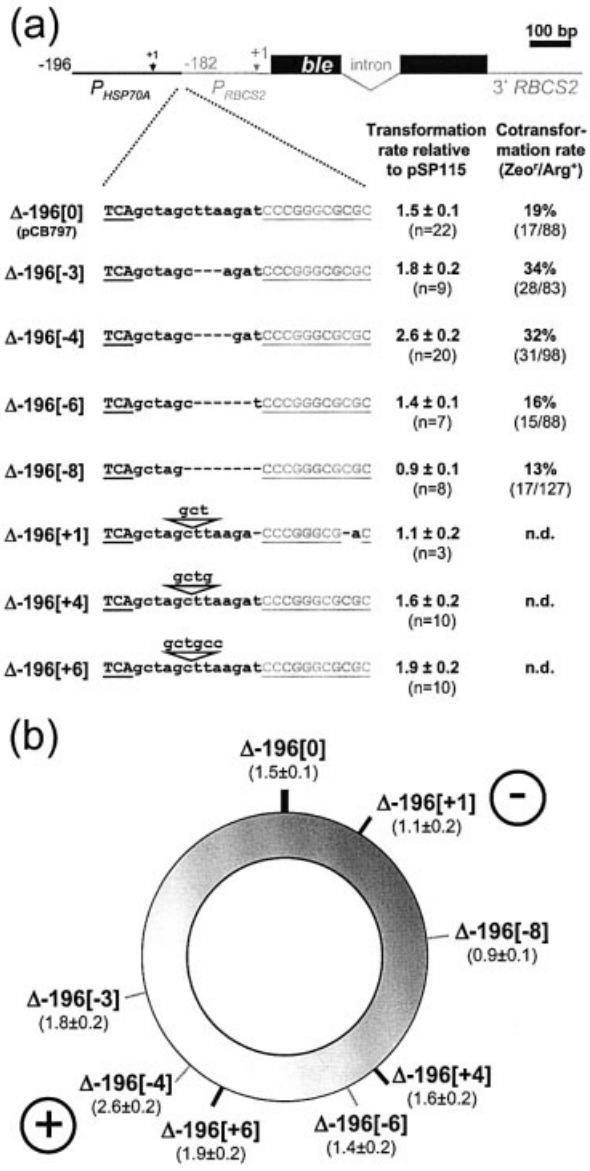

Figure 3. Effect on transformation rates of the relative spatial setting of the *A* promoter versus the *R* promoter in *AR-ble* constructs.
(a) Constructs generated for the analysis of the spacing aspect and transformation rates achieved using these constructs. All constructs were derived from pCB797 described in Figure 1a by deletion (hyphens) or insertion (triangles) of nucleotides in the spacer region (small letters) separating the *A* promoter (black underlined capital letters) and the *R* promoter (grey underlined capital letters). Transformation rates relative to those observed with pSP115 are given as the mean and standard error of the mean in n experiments. Co-transformation rates were determined as described in Figure 1a. (b) Relative spatial setting of *A* and *R* promoters in the constructs described in (a).Based on the assumption that 10.4 nucleotides (the average of nucleosome-free (10.6) and nucleosome-associated (10.2) DNA (Luger *et al.*, 1997) make one helical turn (360°), nucleotide deletions (thin grey spikes) and insertions (thin black spikes) are given as multiples of 34.6° (360 divided by 10.4). The spatial setting of *A* promoter versus *R* promoter in pΔ-196[0] (pCB797) was arbitrarily set at 12 O'clock (thick black spike). The black to white gradient of the circle represents the spatial settings of both promoters inclining from no stimulation (–) to strong stimulation (+) of transformation rates.

was the case for 64% (16/25) of the transformants containing the *ble* gene driven by the *AR* promoter (Figure 2).

Only those co-transformants that exhibited detectable levels of *ble* mRNA were also resistant to zeocin. For neither construct did we observe a correlation between transgene copy number and expression level. It seemed that co-transformants expressing the *ble* gene driven by the *R* promoter alone exhibited a stronger variation in *ble* mRNA levels than those expressing *ble* under the control of the *AR* promoter.

We conclude that, indeed, the presence of the *A* promoter significantly increases the probability for a stably integrated *ble* transgene to become actively expressed. This suggests that the *A* promoter counteracts transcriptional silencing of the *ble* transgene.

Transformation rates depend on the spatial setting of the A *versus the* R *promoter*

We reasoned that specific interactions between both promoters via DNA-bound protein factors might be involved. A productive interaction of such factors is expected to require a proper orientation of their binding sites on the DNA double helix (Cohen and Meselson, 1988). To address this question, we generated a series of seven pCB797-derived constructs with various short nucleotide deletions or insertions in the spacer sequence that separates the *A* and the *R* promoter (Figure 3a). These place the two promoters in several different angles relative to one another (Figure 3b).

Northern blot analysis of pools of zeocin resistant transformants generated with these constructs and with pSP115 revealed that, under continuous light, continuous dark and after light induction, *ble* mRNA levels were the same for all constructs (data not shown). However, significant variations in the transformation rates were observed (Figure 3a). The stimulatory effect of the *A* promoter was high (approximately 1.8- to approximately 2.6-fold higher rates than for pSP115) when the promoter spacing was as in constructs pΔ-196[-3], pΔ-196[+ 6], and pΔ-196[– 4]. No stimulation was observed for constructs pΔ-196[– 8] and pΔ-196[+ 1], whereas pΔ-196[– 6], pΔ-196[0], and pΔ-196[+ 4] showed intermediate stimulatory effects (approximately 1.4- to approximately 1.6-fold increased transformation rates relative to pSP115). The co-transformation rates determined for most of these constructs correlated well with their transformation rates, reaching values of over 30% for pΔ-196[– 3] and pΔ-196[– 4] (Figure 3a).

These results supported our assumption that a proper orientation of sequence elements in *A* and *R* promoter is required to counteract silencing of stably integrated *ble* transgenes. More specifically, the spatial setting of *A* versus *R* in the most efficient constructs (pΔ-196[– 3], pΔ-

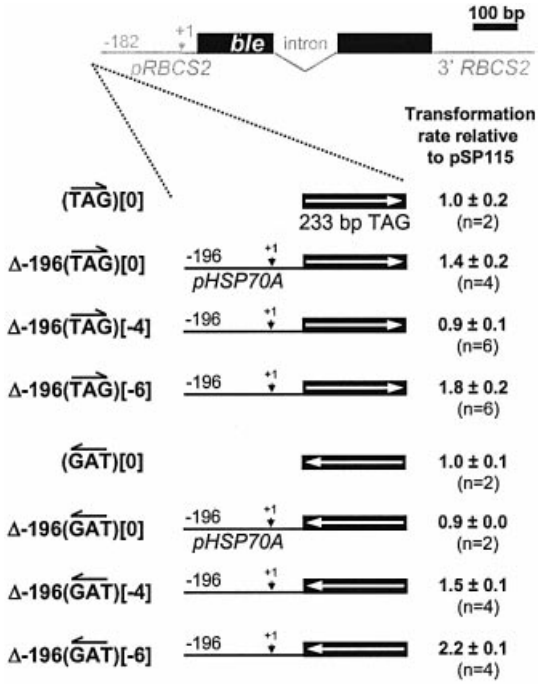

Figure 4. Analysis of the effect of a distantly located *A* promoter on *R-ble* transformation rates.
A 233-bp fragment of bacterial DNA from *Rhodopseudomonas palustris* (TAG) with a similar G/C-content as *Chlamydomonas* genomic DNA is presented as a black box. The white arrow indicates the direction of the sequence (5′ to 3′), other sequence motifs are as described in Figure 1a. The TAG sequence was cloned upstream of the *R-ble* gene, or between the *A* promoter and the *R* promoter that exhibit three different spatial settings. Transformation rates relative to those observed for pSP115 are given as the mean and standard error of the mean.

196[+ 6], and pΔ-196[– 4]) differed by about a half-helical turn relative to that of the least efficient ones (pΔ-196[– 8] and pΔ-196[+ 1]), and by about a quarter helical turn relative to that of the constructs with intermediate efficiencies (pΔ-196[– 6], pΔ-196[0] and pΔ-196[+ 4]) (Figure 3b).

A *promoter sequence motifs may counteract* ble *transgene silencing over a distance of 233 bp*

Cohen and Meselson (1988) have shown that the insertion of a few hundred nucleotides between two interacting elements on the same DNA molecule allowed for an increase in DNA torsional flexibility sufficient to overcome spacing effects. To test whether this was true also for interacting elements on the *A* and *R* promoters, we inserted a 233-bp fragment containing bacterial DNA from *Rhodopseudomonas palustris* (TAG) in both orientations and with three different spacings between the two promoters (Figure 4). As controls, the TAG fragment was also cloned alone in both orientations in front of the *R-ble* gene. The G/C-content of the TAG fragment (60%) is about equivalent to that of *Chlamydomonas* genomic DNA (Harris, 1989).

As shown in Figure 4, the transformation rates of the control constructs p(TAG)[0] and p(GAT)[0] were indistinguishable from that of pSP115, indicating that the TAG sequence was inert. However, constructs that contained the TAG sequence between *A* and *R* promoter, gave rise to significant variations in transformation rates. These were dependent on the orientation of the TAG and on the spatial setting of the two promoters. With TAG in sense orientation, pΔ-196(TAG)[0] gave rise to a approximately 1.4-fold stimulation of transformation rates, whereas no stimulation was seen for the same construct with TAG in antisense orientation (pΔ-196(GAT)[0]). For pΔ-196(TAG)[– 4] we observed the opposite, no stimulation of transformation rates with TAG in sense orientation, but an approximately 1.5-fold increase with TAG in antisense (pΔ-196(GAT)[– 4]). Constructs pΔ-196(TAG)[– 6] and pΔ-196(GAT)[– 6] resulted in approximately 2-fold increased transformation rates nearly independent of the orientation of the TAG (Figure 4). Note that the formerly optimal spacing ([– 4]) in the presence of the TAG resulted only in intermediate stimulation of transformation rates (pΔ-196(GAT)[– 4]), whereas the formerly semioptimal spacing ([– 6]) became optimal.

We conclude that the *A* promoter may exert its effect on counteracting *R-ble* transgene silencing also over a distance of 233 bp. However, the spatial setting of *A* and *R* promoter still is crucial, as is the orientation of the TAG sequence that separates them. Because we are dealing with transgenes that are stably integrated into chromatin, it is very likely that 146 bp of the 233-bp TAG are assembled into a nucleosome (Kornberg, 1977). The latter not only would restrain the torsional flexibility of DNA, but would also represent a sterical hindrance for a productive interaction between factors situated on *A* and *R* promoter. An asymmetric position of such a nucleosome within the TAG may account for the observed dependence of the orientation of the TAG on *A* promoter activity.

Stimulation of transformation rates depends on the length of HSP70A *upstream sequences*

In all *AR-ble* constructs tested to this point, the *AR* promoter fusion contained an *HSP70A* fragment consisting of 196 bp of sequences upstream of the transcriptional start site and 67 bp of 5′ UTR. This region contains the TATA-box, three putative heat-shock elements (HSE I-III), and an inverted CCAAT-box (Figure 5a and Kropat *et al.*, 1995). To determine which of these motifs may be required for counteracting *ble* transgene silencing, we deleted the inverted CCAAT-box and HSE III (Δ-108), or all motifs (Δ-7) (Figure 5a). In addition, we elongated the *A* promoter to 754 bp of sequences upstream of the transcriptional start

site. These constructs were generated with either the high, medium or low efficiency spacings of *A* versus *R* promoter ([– 4], [0], and [– 8], respectively).

As compared to the approximately 2.6-fold, approximately 1.5-fold, and approximately 0.9-fold increases in transformation rates of pΔ-196[– 4], pΔ-196[0], and pΔ-196 [– 8], respectively, their derivatives pΔ-754[– 4], pΔ-754[0], and pΔ-754[– 8] containing the elongated *A* promoter gave rise to significantly increased rates of approximately 3.6-fold, approximately 2.1-fold, and approximately 2.3-fold, respectively (Figure 5a). In contrast, decreasing the length of the *A* promoter region from 196 to 108 nucleotides upstream of the transcriptional start site reduced its stimulatory effect on transformation rates from approximately 2.6-fold to approximately 2.0-fold for the [– 4] spacing and from approximately 1.5-fold to approximately 1.2-fold for the [0] spacing. Surprisingly, constructs pΔ-7 [– 4] and pΔ-7[0] still gave rise to a reproducibly detectable approximately 1.3-fold and a perhaps slight approximately 1.1-fold increase in transformation rates, respectively.

The spatial setting of *A* versus *R* was again critical: Constructs with the optimal spacing [– 4] yielded transformation rates that were 1.2- to 1.7-fold higher than those with the intermediate [0] or low-efficiency spacing [– 8] (Figure 5a). Interestingly, for the Δ-754 *A* promoter the [– 8] and [0] spacings were equally efficient, suggesting the presence of a spacing-independent activity in the – 754 to – 197 *A* promoter region.

We wondered whether this region acted by increasing the transcriptional activity of the *R* promoter. Clearly, this is not the case, because average *ble* transcript levels in individual zeocin resistant transformants generated with constructs pSP115, pΔ-196[– 4], and pΔ-754[– 4] were essentially the same (Figure 5b). As was the case for the co-transformation experiment in Figure 2, there was a slight reduction in the variation of *ble* mRNA levels in transformants containing *AR-ble* constructs (pΔ-196[– 4] and pΔ-754[– 4]) as compared to those generated with *R-ble (*pSP115). However, a much larger sample size would be required to determine whether this observation is statistically significant.

In conclusion, our results suggest that at least two components in the *HSP70A* upstream region are capable of stimulating *ble* transformation rates. The first is spacing-dependent and is determined by sequences – 196 to + 67. The second component is spacing-independent, is additive to the first, and is located between nucleotides – 754 to – 97. Because both components did not result in higher average transcript levels in transformants, they

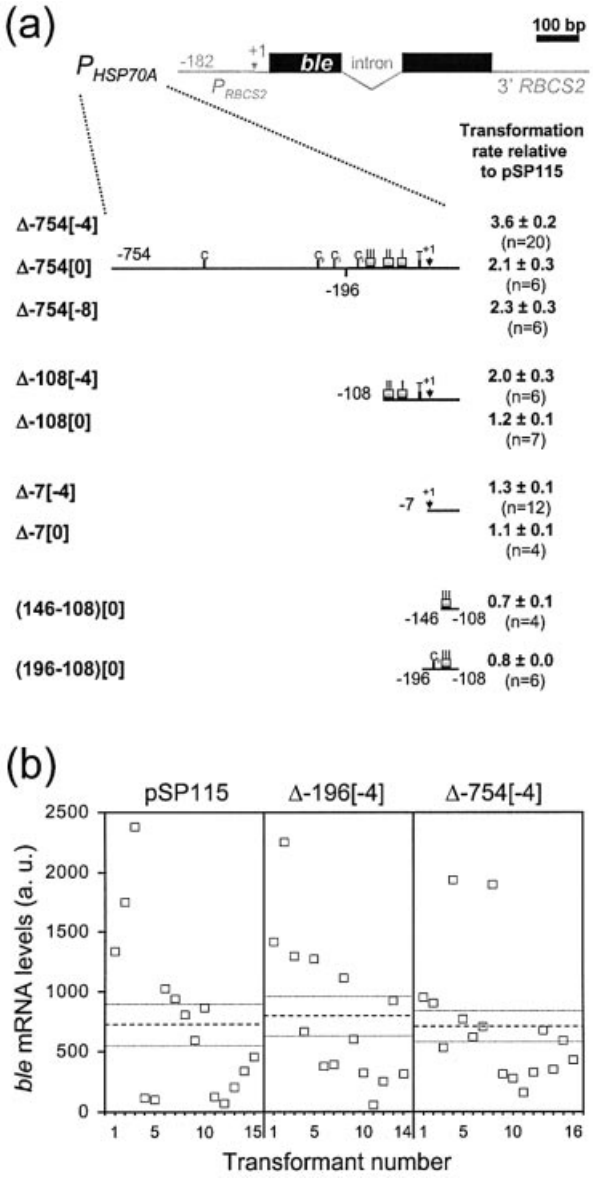

Figure 5. Effect of *A* promoter deletions, isolated *A* promoter elements, and of additional *A* promoter upstream sequences on transformation rates of *AR-ble* constructs.

Transformation rates achieved for *AR-ble* constructs containing 754, 108, and 7 bp of *HSP70A* sequences upstream of the transcriptional start site, or isolated *A* promoter fragments spanning nucleotides – 146 to – 108 and – 196 to – 108. Promoter spacings are given in brackets according to Figure 3. Sequence motifs with homology to transcription factor binding sites upstream of the *HSP70A* transcriptional start site (+ 1) are the TATA-box (T), three putative heat-shock elements (I, II, III), a CCAAT box (C) and three inverted CCAAT-boxes (C_i). Transformation rates relative to those observed with pSP115 are given as the mean and standard error of the mean in n experiments. The co-transformation rate determined for pΔ-754[– 4] was 39% (37 of 95 Arg^+ clones were zeocin resistant). (b) Analysis of *ble* expression in zeocin resistant transformants generated with pSP115, pΔ-196[– 4], and pΔ-754[– 4]. Total RNA of randomly selected transformants was isolated and subjected to Northern blot analysis using a probe directed against the coding region of *ble*. Blots were stripped and rehybridized with a probe against the coding region of *RBCS2*. Hybridization signals were quantified, corrected for unequal loading by the *RBCS2* signal and plotted in arbitrary units (a.u.). Mean mRNA levels (broken lines) were 737 ± 174 for pSP115, 801 ± 162 for pΔ-196[– 4], and 714 ± 130 for pΔ-754[– 4]. Standard errors of the mean are indicated as dotted lines.

clearly do not act as classical enhancers that increase transcription rates. Rather, both elements appear to act on the transcription state by reducing the fraction of transcriptionally silenced *R-ble* transgenes.

Neither isolated elements of the A *promoter nor an A/T-rich fragment are sufficient to counteract* R-ble *transgene silencing*

It has been suggested previously that the −146 to −108 fragment of the *A* promoter contained motifs involved in the control of this promoter by light (Kropat *et al.*, 1995). To test whether these motifs are also involved in counteracting *R-ble* transgene silencing, we cloned the −146 to −108 and the −196 to −108 *A* promoter fragments upstream of the *R* promoter (Figure 5a). The transformation rates observed for these constructs were even below that of pSP115, suggesting that (i) these elements are not involved in counteracting *R-ble* transgene silencing (ii) the spatial setting of these elements relative to the *R* promoter was improper, or (iii) the elements were located too close to the *R* promoter, so that the torsional flexibility of the DNA was restricted and did not allow a productive interaction of DNA-binding proteins.

We noted that the A/T content (53%) of the *HSP70A* 5′ UTR (the main part of the smallest fragment found active in counteracting *R-ble* transgene silencing) is exceptionally high for *Chlamydomonas* genomic DNA, and that it contains several A/T-rich stretches. Sandhu *et al.* (1998) have reported that randomly synthesized A/T-rich sequences can serve as quantitative enhancers in transgenic tobacco and tomato plants. To test whether A/T-rich sequences were sufficient to counteract *R-ble* transgene silencing, we cloned upstream of the *R* promoter a fragment with an A/T-content of 75% derived from the promoter of the plastidic 16S rRNA gene of *Chlamydomonas* (Dron *et al.*, 1982). The transformation rate of the resulting construct, pMS280, was less than half of that of pSP115 (0.4 ± 0.1, $n = 5$), suggesting that A/T-rich sequences by themselves did not stimulate. Rather, they appear to have a negative influence on the expression of *R-ble* in *Chlamydomonas*.

Discussion

Due to its ease of transformation and its fast generation time (Harris, 1989; Kindle, 1990), *Chlamydomonas* appears as a suitable plant model for statistical studies on the effect of neighbouring DNA sequences on gene expression. The transformation assay presented here allows for a quantitative evaluation of such effects. It uses the bacterial antibiotic resistance gene *ble*, introduced recently as an effective dominant selective marker for the transformation of *Chlamydomonas* (Stevens *et al.*, 1996).

We show that transformation rates with *ble* may be increased by two different means: First, by elevating the transcription rate of the *ble* transgene. This can be achieved by introducing the enhancer-containing first intron of the *RBCS2* gene into the *ble* coding region, or by removing negative regulators from the *RBCS2* promoter (*R*) that drives transgene expression (Lumbreras *et al.*, 1998 and Figure 1). Due to the increase in *ble* mRNA levels, a larger number of transformants now express the Ble protein at a level sufficiently high to neutralize the drug, eventually resulting in higher transformation rates.

However, even in these optimized constructs about 80% of stably integrated *R-ble* transgenes are silent (Figure 2), therefore decreasing the effectiveness of the *ble* gene as a dominant selectable marker. Consequently, decreasing the fraction of silenced *ble* transgenes represents a second means of increasing *ble* transformation rates. This we achieved by fusing the *HSP70A* promoter (*A*) upstream of the *R* promoter driving *ble* gene expression (Figure 1). As shown in Figure 2, the presence of an *A* promoter fragment reduced the fraction of silent *ble* transgenes to about 36%, entailing a 1.5- to 1.9-fold increase in transformation and co-transformation rates of the *AR-ble* construct as compared to *R-ble*. Optimized *AR-ble* constructs resulted in up to 3.5-fold increased transformation and co-transformation rates (Figure 5), suggesting an even lower fraction of silenced *ble* transgenes in transformants. Both means of increasing *ble* transformation rates, i.e. increasing *ble* mRNA levels and reducing the fraction of silent transgenes, act independently but additively (Figure 1).

We have demonstrated previously that the *AR* promoter driving expression of the *aadA* resistance gene yielded increased transformation rates relative to *R-aadA* without increasing *aadA* mRNA levels in pools of resistant transformants (Schroda *et al.*, 2000). In the same study we could also show that transformants containing the *HSP70B* reporter gene driven by its own, the *RBCS2* or the β_2*TUB* promoter were much more likely to express the transgene at high levels when the *A* promoter was present upstream. In view of the results presented here it seems reasonable to assume that the *A* promoter may counteract silencing also in the context of different promoters and transgenes.

Transgene silencing in *Chlamydomonas* has been attributed thus far mostly to TGS (Cerutti *et al.*, 1997a, 1997b; Jeong *et al.*, 2002). Because *R-ble* transgene silencing depends strongly on the nature of the sequences present upstream of the *R* promoter, we will focus our discussion on TGS, although we cannot rule out contributions of PTGS. TGS of the *R-ble* transgene may be triggered (i) by the lower G/C-content of co-introduced vector sequences (50% G/C-content of pBluescript compared to about 65% of genomic DNA) (ii) by the integration into heterochromatic

regions, or (iii) by a tendency of the transgenic *R* promoter to organize into a nucleosome structure that denies access to regulatory sequences by the transcription machinery.

Which sequence elements on the *HSP70A* promoter counteract silencing of *R-ble* transgenes? Our results suggest the presence of at least two independent elements, a proximal and a distal one. The activity of the proximal element is determined by *A* promoter sequences ranging from −196 to +67 relative to the transcriptional start site. The activity strongly depends on the spatial setting of the *A* versus the *R* promoter: whereas optimal spacings resulted in up to 2.6-fold increased transformation rates, unfavourable spacings abolished any stimulatory effect of the *A* promoter (Figures 3 and 4).

Deletion of the Δ-196 *A* promoter to 108 and 7 nucleotides upstream of the transcriptional start site gradually reduced its stimulatory effect (Figure 5a), suggesting that multiple sequence elements (or their binding factors) contribute to the effect. However, whereas the −7 to +67 fragment still increased transformation rates significantly, fragments −146 to −108 and −196 to −108 did not. This suggests that the core of the proximal element capable of counteracting *R-ble* transgene silencing is situated on the −7 to +67 *A* promoter fragment and that the binding of protein factor(s) to this core is only favoured by upstream sequences (− 196 to −108) (see model in Figure 6).

The second *HSP70A* promoter element capable of counteracting *R-ble* silencing is located upstream of position −196. Because this distal element accounts for a more than 2-fold increase in transformation rates even when the *A* promoter is situated in an unfavourable spacing to *R* (Figure 5a), it appears to be spacing-independent and independent of the proximal element (see model in Figure 6).

How may these two *HSP70A* promoter elements counteract *R-ble* transgene silencing? We envisioned the following three mechanisms to be at work: the *A* promoter elements may act as matrix attachment regions (MARs), as transcriptional state enhancers, or by recruiting chromatin remodelling activities.

MARs are sequence elements that serve to anchor chromatin fibres to the nuclear matrix. This results in the organization of chromatin into distinct loops, which are individually regulated (Allen *et al.*, 2000). In plants, MARs have been shown to reduce variation of transgene expression (one extreme of which is transgene silencing) (Mlynárová *et al.*, 1995; Vain *et al.*, 1999). Therefore, the *A* promoter may contain MAR sequences that insulate the *R-ble* transgene from the influence of nearby repressive chromatin. However, plants harbouring transgenes flanked by MARs exhibited at least one of the following traits: transgene expression became copy number-dependent, average expression levels were increased, or the variation in expression was reduced significantly (Allen *et al.*, 2000).

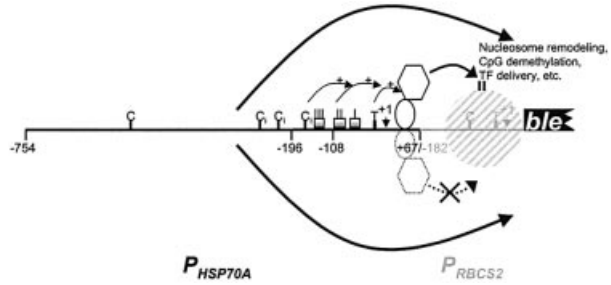

Figure 6. Model for the action of the *A* promoter leading to an activation of the transcriptional state of the *R* promoter.
Sequence motifs on the promoters are as described in Figure 5. For the majority of stably integrated *R-ble* transgenes regulatory sequences on the *R* promoter are not accessible (grey hatched circle), for example due to the unfavourable positioning of a nucleosome. Protein factor(s) (represented as ellipses and hexagons) bind to a first element in the −7 to +67 region of the *A* promoter. Binding is facilitated by factors situated between nucleotides −196 to −8 (arrows marked with +). If adequately spaced (filled lines), these protein factors interact with specific sites on the *R* promoter (shown as two parallel black lines), resulting either in a relief from repressive structures, or in providing access for transcriptions factors (TF). This may be mediated by nucleosome remodelling, CpG demethylation, histone acetylation, etc. Inadequate spacing of *A*-bound factors (dotted lines) do not allow a productive interaction with these sites and therefore do not activate the transcriptional state of the *R* promoter. A second element is located within the *A* promoter between nucleotides −754 to −197. The activity of protein factor(s) bound to this second element is independent of the sterically oriented sites on the *R* promoter (therefore spacing-independent) and independent of the first element (bold filled arrows).

None of these traits were mediated by *A* promoter sequences to the *R-ble* transgene (Figures 2 and 5). In addition, a MAR element should act independent of its spatial setting to a transgene. Therefore, we consider it unlikely that the *A* promoter contains sequences that act as MARs.

Enhancers are sequence elements that bind specific protein factors able to recruit the transcriptional machinery to nearby promoters. Martin (2001) has recently pointed out that enhancers may not only act by increasing the transcription rate of a promoter (as is the case for the enhancer in the first intron of *RBCS2* (Lumbreras *et al.*, 1998)), but may also affect its transcriptional state, leading to a higher probability that a gene becomes expressed. Such enhancers have also been shown to counteract transcriptional silencing of transgenes (Francastel *et al.*, 1999; Walters *et al.*, 1996). Therefore, the *A* promoter may contain transcriptional state-affecting enhancers capable of interacting with nearby promoters.

The third way by which the A promoter elements may counteract silencing is by a modulation of close-by chromatin structures. *Hsp70* promoters from *Drosophila* and *Xenopus* have been shown to be in a constitutively nucleosome-free state immediately accessible to the binding of transcription factors like the heat shock factors or TFIID (Aalfs and Kingston, 2000; Landsberger and Wolffe, 1995; Lis and Wu, 1993). In *Drosophila*, this state is

mediated by the binding of GAGA-factor to $[GA]_n$ repeats, which are present six times in the *hsp70* promoter (Soeller *et al.*, 1993). GAGA-factor acts in concert with the chromatin-remodelling factor NURF which, in an ATP-dependent reaction, modifies the DNA-histone interaction of nucleosome octamers present on and near the *hsp70* promoter, resulting in nucleosome sliding (Hamiche *et al.*, 1999; Tsukiyama *et al.*, 1994).

In *Xenopus*, the constitutively nucleosome-free state requires the presence of two so-called Y-boxes, which contain an inverted CCAAT-box as core motif (Landsberger and Wolffe, 1995). The Y-boxes are recognized by the heterotrimeric NF-Y factor, which is involved in creating a nucleosome-free environment and subsequently modulates transcriptional activity by the recruitment of the p300 histone-acetyltransferase (Li *et al.*, 1998).

In analogy to the *hsp70* promoters of *Drosophila* and *Xenopus*, the *Chlamydomonas HSP70A* promoter may also contain sequence elements capable of recruiting chromatin remodelling factors, which create a nucleosome-free environment on and close to the *A* promoter, thereby relieving repressive chromatin from the downstream *R* promoter. In favour of this hypothesis we have found a repeat of three sequence motifs ATTGG(A/T)G at positions –165, –227 and –270 on the *HSP70A* promoter. As is the case for the Y-boxes on the *Xenopus hsp70* promoter (Li *et al.*, 1998), these motifs contain an inverted CCAAT box and are separated by an integral number of helical turns (four and six), thus all face to the same side of the DNA double-helix. Therefore, if these inverted CCAAT boxes correspond to the distal element they may act, in analogy to the *Xenopus hsp70* promoter, by recruiting chromatin remodelling activities. Alternatively, they may be bound by a CCAAT/enhancer binding protein that affects the transcriptional state of the *R* promoter. The proximal element might as well act as a transcriptional state enhancer or by recruiting chromatin-remodelling activities. In any case, the pronounced spacing dependence of its activity strongly suggests that it directly interacts via bound protein factors with a sterically oriented site on the *R* promoter (see model in Figure 6).

In terms of practical applications, the identification of the *A* promoter elements responsible for counteracting transgene silencing may open up new approaches for the development of transgene constructs that are less prone to TGS in *Chlamydomonas* and perhaps also in other organisms.

Experimental procedures

Algal strains and culture conditions

Chlamydomonas reinhardtii strains 302 (cw_d, *mt+*, *arg7*) and 074 (cw_d, *mt-*, *arg7*) were kindly provided by R. Matagne (University of Liège, Belgium). Strains were grown photomixotrophically in TAP medium (Harris, 1989) on a rotatory shaker at 25°C under continuous irradiation with white light (40 μE m^{-2} s^{-1}). TAP medium was supplemented with 100 mg l^{-1} of arginine when required. Light induction and heat shock were performed as described by Kropat *et al.* (1995) and von Gromoff *et al.* (1989), respectively.

Plasmid constructions

An 856-bp fragment obtained by a partial *Sma*I and a complete *Kpn*I digestion of pSP108 (Stevens *et al.*, 1996), harbouring the Δ-182 *R* promoter, the *ble* gene and the 3′ untranslated region of the *RBCS2* gene, was cloned into *Sma*I-*Kpn*I-digested pCB745 (Schroda *et al.*, 1999), yielding pCB796. pCB845 was generated by removing *A* promoter sequences by digestion of pCB796 with *Spe*I and *Nhe*I and re-ligation. The intron-less *ble* gene was removed from pCB796 by digestion with *Msc*I-*Kpn*I and replaced by a 785-bp *Msc*I-*Kpn*I fragment derived from pSP115 (Lumbreras *et al.*, 1998), which contains the first *RBCS2* intron within the *ble* gene, giving rise to pCB797. Constructs pΔ-196[– 3], pΔ-196[– 4], pΔ-196[– 6], pΔ-196[– 8], pΔ-196[+ 1], pΔ-196[+ 4], and pΔ-196[+ 6] were generated by digestion of pCB797 with *Msc*I-*Nhe*I and insertion of equally digested PCR fragments amplified from pCB797 with the 3′ primer 5′-ATCCTGGCCATTTTAAGATGTTG-3′ and the respective 5′ primers 5′-TTTGCTAGCAGATCCCGGGCGCGCCA-3′, 5′-TTTGCTAGCGATCCCGGGCGCGCCAGA-3′, 5′-TTTGCTAGCTCCCGGGCGCGCCAGAA-3′, 5′-TTTGCTAGCCCGGGCGCGCCAGAAGGA-3′, 5′-TTTGCTAGCGCTTTAAGATCCCGGGCGCGCCA-3′, 5′-TTTGCTAGCGCTGTTAAGATCCCGGGCGCGCCA-3′, and 5′-TTTGCTAGCGCTGCCTTAAGATCCCGGGCGCGCCA-3′. As revealed by sequencing of the PCR product, the 5′ primer for pΔ-196[+ 1] was synthesized with errors at three positions, leading to the product shown in Figure 3a. Cloning of the elongated *A* promoter upstream of pΔ-196[– 4], was initiated by digesting pCB353 (Müller *et al.*, 1992) with *Pst*I-*Xba*I and subcloning of an approximately 1.9 kb fragment containing *HSP70A* 5′ sequences into *Pst*I-*Xba*I-digested pBluescript SK^+ (Stratagene, La Jolla, CA, USA), giving rise to pMS187. pΔ-196[– 4] was digested with *Bst*EII-*Kpn*I and the resulting 1092 bp fragment was cloned into *Bst*EII-*Kpn*I-digested pMS187, giving rise to pΔ-754[– 4] (pMS188). pΔ-196[– 8] and pΔ-196[0] were cleaved with *Nhe*I-*Kpn*I and the resulting approximately 1 kb fragments were cloned into *Nhe*I-*Kpn*I-digested pΔ-754[– 4], yielding pΔ-754[– 8] and pΔ-754[0], respectively. To create pΔ-108[0], the approximately 1 kb fragment of *Nhe*I-*Kpn*I-digested pCB797 was inserted into equally cleaved pCB711 (Schroda *et al.*, 2000). Into *Nhe*I-*Kpn*I-digested pΔ-108[0], an approximately 1 kb *Nhe*I-*Kpn*I fragment of pΔ-196[– 4] was inserted to yield pΔ-108[– 4]. To generate pΔ-7[0], pCB797 was digested with *Spe*I-*Bst*EII, and, after filling-in of the ends with Klenow polymerase, the vector was religated. Digestion of pΔ-7[0] with *Nhe*I and *Nco*I and insertion of the 985 bp *Nco*I-*Kpn*I fragment from pΔ-196[– 4] gave rise to pΔ-7[– 4]. Constructs containing the bacterial TAG sequence from *Rhodopseudomonas palustris* were initiated from pCB586 that contained the 215 bp *Eco*RI-*Xba*I TAG sequence (Kropat *et al.*, 1995) cloned into pBluescript SK^+ (Stratagene). Digestion of pCB586 with *Xba*I released a 233-bp fragment containing the TAG that was cloned into *Spe*I-*Nhe*I-cleaved pCB797, resulting in p(TAG)[0] (5′-3′) or p(GAT)[0] (3′-5′). pΔ-196(TAG)[0], pΔ-196(GAT)[0], pΔ-196(TAG)[– 4], pΔ-196(GAT)[– 4], pΔ-196(TAG)[– 6], and pΔ-196(GAT)[– 6] were constructed accordingly after digestion of pCB797, pΔ-196[– 4], and pΔ-196[– 6] with *Nhe*I.

Construction of plasmids p(146–108)[0] and p(196–108)[0] was initiated by digestion of pCB710 (Schroda *et al.*, 1999) with *Bam*HI-

*Nhe*I and insertion of equally digested PCR fragments amplified from plasmids containing the Δ-146 and Δ-196 *A* promoter deletions (Kropat *et al.*, 1995) with the 3′ primer 5′-AGAGCTAGCCCCGCCCTCATAG-3′ and the 5′ primer 5′-AATAGGATCCACTATAGGGC-3′, yielding pCB835 and pCB836, respectively. Digestion of the latter with *Nhe*I-*Kpn*I and insertion of the approximately 1 kb *Nhe*I-*Kpn*I fragment from pCB797 gave rise to p(146–108)[0] and p(196–108)[0], respectively. To construct pMS280, an A/T-rich region from *Chlamydomonas* chloroplast DNA was amplified with the 3′-primer 5′-ATTTCTAGAACGTTCCCAGTGTTTTTAATTTAAC-3′ and the 5′-primer 5′-GTATCGATCCCGGGAAAACAATTATTATTTTACTGC-3′. After digestion with *Xba*I-*Sma*I, the resulting 115 bp fragment was cloned into *Spe*I-*Eco*47III-digested pΔ-196[+ 6].

Correct cloning was confirmed by restriction analysis and DNA sequencing.

Nuclear transformation of Chlamydomonas

Chlamydomonas nuclear transformation was carried out using the glass beads method (Kindle, 1990). Plasmids were purified by PEG precipitation or anion exchange chromatography (QIAGEN, Hilden, Germany). Because slight variations in transformation rates were observed for different plasmid preparations, experiments were carried out with up to four independent preparations for each construct. Prior to transformation, the plasmid containing the *ARG7* gene was linearized with *Eco*RI, all *ble* constructs were linearized with *Kpn*I. For transformation, cells were grown to 1–2 $\times$ 10^7 cells ml^{-1} and concentrated to 3 $\times$ 10^8 cells ml^{-1}, of which 0.33 ml were vortexed with 1 μg of plasmid DNA and 0.3 g of acid-washed glass beads. Immediately after vortexing, cells were spread onto TAP-agar plates. To select for drug resistance, plates were supplemented with 10–15 μg ml^{-1} zeocin from KAYLA (Toulouse, France) for the experiments in Figure 1, or 1.1 μg ml^{-1} zeocin from Invitrogen (Cergy Pontoise, France) for all other experiments. The antibiotic from Kayla was less efficient and resulted in much greater variations than that from Invitrogen. Plates were first incubated overnight in the dark (leading to approximately 3-fold higher transformation rates) and then transferred to light of about 20 μE m^{-2} s^{-1}. Transformants were counted after approximately 10 days.

Nucleic acid analyses

Total DNA and RNA were prepared as described by Schroda *et al.* (2001) and Kropat *et al.* (1997), respectively. DNA and RNA gel blot analyses were performed using standard methods (Sambrook *et al.*, 1989). Radioactive DNA probes were prepared by the random priming technique (Feinberg and Vogelstein, 1983), using [α-^{32}P]dCTP (Amersham, Braunschweig, Germany). Blots were probed with a 381-bp *Msc*I-*Sal*I fragment from pCB845 containing the *ble* gene and a 370-bp *Sst*II-*Alw*NI fragment from the *RBCS2* coding region that hybridizes to both the *RBCS1* and *RBCS2* mRNAs (Goldschmidt-Clermont and Rahire, 1986). Hybridization and washing of membranes was carried out as described by von Gromoff *et al.* (1989). Signals were quantified by phosphorimaging.

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