Plant Cell Rep (2012) 31:13–25
DOI 10.1007/s00299-011-1167-y

REVIEW

Minimizing the unpredictability of transgene expression in plants: the role of genetic insulators

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Received: 4 September 2011 / Accepted: 27 September 2011 / Published online: 11 October 2011

Abstract The genetic transformation of plants has become a necessary tool for fundamental plant biology research, as well as the generation of engineered plants exhibiting improved agronomic and industrial traits. However, this technology is significantly hindered by the fact that transgene expression is often highly variable amongst independent transgenic lines. Two of the major contributing factors to this type of inconsistency are inappropriate enhancer-promoter interactions and chromosomal position effects, which frequently result in mis-expression or silencing of the transgene, respectively. Since the precise, often tissue-specific, expression of the transgene(s) of interest is often a necessity for the successful generation of transgenic plants, these undesirable side effects have the potential to pose a major challenge for the genetic engineering of these organisms. In this review, we discuss strategies for improving foreign gene expression in plants via the inclusion of enhancer-blocking insulators, which function to impede enhancer-promoter communication, and barrier insulators, which block the spread of heterochromatin, in transgenic constructs. While a complete understanding of these elements remains elusive, recent studies regarding their use in genetically engineered plants indicate that they hold great promise for the improvement of transgene expression, and thus the future of plant biotechnology.

Keywords Boundary element · Enhancer-blocking insulator · Enhancer-promoter interference · Plant biotechnology · Chromosomal position effects · Tissue-specific transgene expression

Abbreviations

35S	*35S cauliflower mosaic virus* promoter/enhancer
EXOB	1-kb *Eco*RI/*Sal*I fragment from bacteriophage lambda
MAR	Matrix attachment region
TBS	Transformation booster sequence

Introduction

The use of transgenic plant technology is a vital tool for the improvement of agronomic traits (Lanfranco 2003), the manufacture of valuable proteins for commercial applications (Twyman et al. 2003), and the elucidation of gene function. Unfortunately, while the genetic transformation of plants is becoming a rather straightforward procedure, in practice it often results in plants characterized by transgene mis-expression, silencing and plant-to-plant variability (reviewed by Butaye et al. 2005). These inconsistencies in transgene expression are a major drawback to plant biotechnology and result in the need to screen large numbers of transformants to identify individual lines with the desired expression patterns/levels, and can also confuse the interpretation of resulting phenotypes (Bhat and Srinivasan 2002).

Communicated by R. Reski.

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One source of this unpredictability in terms of transgene expression can be attributed to the incidence of interference within the transgenic constructs themselves. In the past, the majority of plant biotechnological research has been directed toward the improvement of a single trait; however, since it is often the case that crops in field conditions must cope with a number of challenges, the adoption of a more comprehensive approach designed to enhance the performance of multiple traits simultaneously using transformation constructs that bear several transcriptional units is becoming the norm. This is often achieved using a strong, constitutive promoter/enhancer to direct the expression of a selectable marker gene in combination with tissue-, organ- or developmental stage-specific promoters to drive the expression of transgenes in precise temporal and/or spatial patterns. Unfortunately, this approach can be problematic due to the position- and orientation-independent ability of enhancers (which are often contained within promoters) to trigger enhancer-promoter interference, which can influence both the strength and the specificity of transgene expression (reviewed by Singer et al. 2011a).

Another instigator of inter-individual variability during plant transformation experiments is the occurrence of epigenetic chromosomal position effects, which can arise in response to the site within the genome into which the foreign transgenic DNA has integrated (Matzke and Matzke 1998). Due to the random nature of transgene insertion in the majority of higher eukaryotes (Hohn and Puchta 2003), transgenic DNA may integrate into regions of the genome that are transcriptionally repressed (heterochromatin), which can result in transgene silencing. Since much of a plant's genome can be in the form of heterochromatin at any one time (Wang et al. 2006), the chance that foreign DNA will integrate in or near these regions, and consequently be silenced, is relatively high. Additionally, transgenes may be incorporated near endogenous regulatory elements, such as transcriptional enhancers or silencers, which could cause mis-expression (reviewed by Francis and Spiker 2005). These types of position effects often result in the production of transgenic lines displaying a bimodal pattern of reporter gene expression, which is evidenced by a large number of individuals exhibiting high and low levels of expression with only a small number near the mean (e.g. Brouwer et al. 2002; De Bolle et al. 2003).

While various mechanisms exist within eukaryotic genomes to preclude inappropriate enhancer-mediated activation of nearby promoters and chromosomal position effects (reviewed by Kadauke and Blobel 2009), transgenic constructs lack this ability and thus require supplementary means with which to minimize such disturbances. In metazoan systems, one of the main strategies used to curtail these two types of transgenic interference is the design of constructs bearing genetic insulators (e.g. Steinwaerder and Lieber 2000; Ye et al. 2003), which are present naturally in the genomes of a wide range of eukaryotic organisms and function to shield genes from outside signals, thus preventing inappropriate activation or silencing of expression. These regulatory elements have been characterized extensively in animals, the most well-studied of which include the *gypsy* retrotransposon element (Geyer et al. 1986) and scs/scs′ paired elements (Kellum and Schedl 1992) from *Drosophila*, as well as the cHS4 insulator from the chicken *β-globin* locus (Chung et al. 1993). Insulators are typically classified into two groups based on their function. These include enhancer-blocking insulators, which hinder enhancer-promoter communication when situated between the two, and barrier insulators, which protect against the spread of adjacent heterochromatin, thus impeding chromosomal position effects (Fig. 1a, b). While some insulators serve exclusively as either enhancer-blocking or barrier elements, others are able to perform both functions (reviewed by Gaszner and Felsenfeld 2006).

Due to the growing number of reports revealing the unpredictable nature of transgene expression in plants, it is becoming evident that one of the most important technical feats with regards to plant biotechnology in the future is the development of tactics to mitigate transgenic interference. The successful application of these strategies could potentially increase the proportion of transgenic lines displaying a stable, desirable phenotype, which is of the utmost importance for the development of safe and effective genetically engineered plants. Thus, the identification and characterization of genetic insulators that function in plant species, and their inclusion in transgenic constructs, is likely to be imperative for improving transgenic technology. In this paper, we review the current knowledge regarding the use of both enhancer-blocking and barrier insulators to minimize the unpredictability of transgene expression currently observed in plant transformation experiments.

The use of enhancer-blocking insulators to reduce enhancer-promoter interference in transgenic plants

Enhancer-mediated activation of target promoters is an important mechanism of transcriptional regulation in eukaryotes (Dorsett 1999; Ptashne 1986). This phenomenon is independent of the orientation of the enhancer, can occur over very large distances (e.g. Jack et al. 1991; Weterings et al. 1995), and can even operate across separate chromosomes (Morris et al. 1998). While many questions remain unanswered regarding the molecular means by which enhancers promote transcription, there is evidence that in a transgenic context in plants, enhancers initiate transcription autonomously in the correct

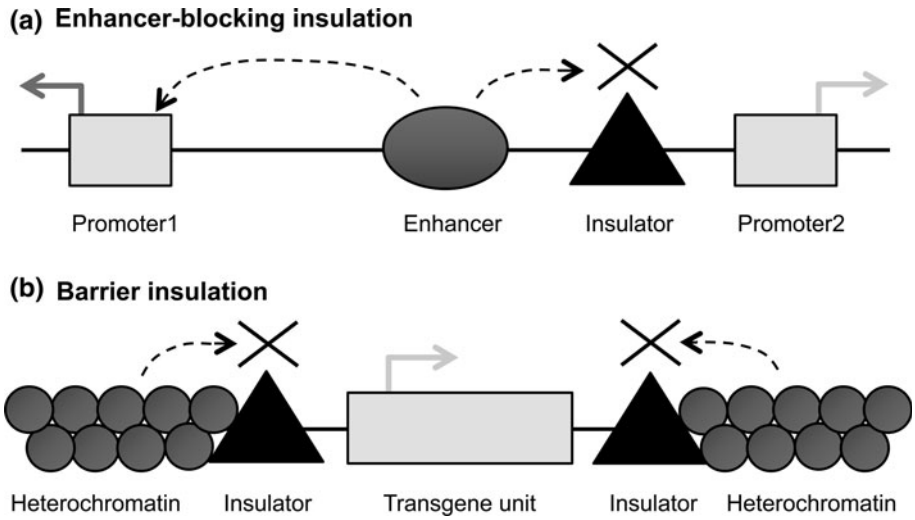

Fig. 1 Diagrammatic representation of enhancer-blocking and barrier insulator function in a transgenic context. The hypothetical role of an enhancer-blocking insulator is depicted in (**a**), while that of a barrier insulator is shown in (**b**). In the absence of enhancer-blocking insulators, enhancers interact with nearby promoters to activate their transcription in an enhancer-specific manner (depicted by a *dark grey arrow*). In the presence of an intervening enhancer-blocking insulator, communication between enhancer and promoter is impeded and transcription of the promoter takes place according to its own inherent specificity (depicted by a *light grey arrow*) (**a**). Barrier insulators that flank a transgene prevent the spread of transcriptionally silenced heterochromatin (indicated by *small grey circles*) into the transgene, thus allowing it to remain transcriptionally active (indicated by *light grey arrow*) and capable of generating its desired product (**b**)

spatiotemporal pattern and exploit at least two different modes of action to exert their activation function. Short-range activation is thought to occur between enhancers and promoters that are in relatively close proximity (<1 kb), whereby the two regulatory elements interact directly without the need for any facilitating mechanisms. Conversely, long-range activation appears to involve transcription initiation at both the enhancer and target promoter. This long-range transcriptional activation resembles the scanning-based mechanisms often seen in animals, such as facilitated tracking, whereby RNA polymerase II and a bound enhancer track along the DNA from enhancer to target promoter to ultimately form a loop (Singer et al. 2010a). The migration of RNA polymerase II along the intervening DNA, and the consequential synthesis of intergenic RNA, has been proposed to supply enhancer-bound proteins to the target promoter and/or 'open' the nucleosomal structure of the associated DNA through the action of histone acetyltransferases (Zhu et al. 2007). This type of crosstalk can cause serious impediments within genetically engineered plants due to the presence of multiple enhancers and promoters within the transgenic construct and/or transgene insertion near endogenous enhancer elements within the genome; both of which can result in transgene mis-expression.

Possibly the most potent enhancer in terms of eliciting enhancer-promoter interference is that contained within the strong, constitutive *cauliflower mosaic virus* (*CaMV*) *35S* promoter (Odell et al. 1988), which is one of the most commonly used promoters for the positive selection of transgenic from non-transgenic lines. Unfortunately, the use of this promoter within transgenic constructs often results in both a loss of specificity and an increase in the level of expression induced by other promoters included within the construct (e.g. Hily et al. 2009; Jagannath et al. 2001; Zheng et al. 2007). For example, this promoter/enhancer has been shown to constitutively activate nearby vascular tissue-specific *AAP2*, root-specific *LRP1*, stamen- and carpel-specific *AGIP*, tapetum-specific *TA29* and *A9*, seed-specific *napin*, embryogenesis-specific *PAB5*, carpel-specific *AGL5*, and petal- and stamen-specific *PI* promoters, resulting in an expression pattern that is indistinguishable from that of the *35S* promoter (Gudynaite-Savitch et al. 2009; Hily et al. 2009; Jagannath et al. 2001; Singer et al. 2010b, 2011b; Yang et al. 2010; Yoo et al. 2005; Zheng et al. 2007). Although the *35S* enhancer is particularly detrimental in terms of eliciting inappropriate enhancer-promoter crosstalk, this phenomenon is not restricted to this specific enhancer, but instead appears to be a rather common feature of these regulatory elements (Gudynaite-Savitch et al. 2009; Liu et al. 2008).

Several approaches have been proposed to prevent such interactions within transgenic constructs, including the use of promoters that contain only weak enhancers and/or are less sensitive to enhancer-mediated interference, as well as the insertion of a spacer DNA fragment between enhancer and promoter. However, these strategies have been found to be rather capricious, and their effectiveness can vary from construct to construct (Gudynaite-Savitch et al. 2009). Inappropriate enhancer-promoter interactions can

also be minimized through the use of enhancer-blocking insulators, which impede communication when situated between an enhancer and promoter. While a relative wealth of knowledge has been acquired concerning the function of these elements in both endogenous and artificial systems in metazoans (e.g. Chung et al. 1993; Geyer et al. 1986; Kellum and Schedl 1992), interest in this field has only recently emerged with regards to plant species. As a result, reports of sequences from various backgrounds exhibiting enhancer-blocking function in plants are only now beginning to accumulate (reviewed by Singer et al. 2011a; Table 1), which may facilitate the development of novel means by which to reduce enhancer-promoter interference in transgenic plants bearing composite vectors in the future.

Enhancer-blocking insulators in transgenic plants

In the first published reports in which enhancer-blocking activities were observed in plants, the phenomenon was attributed to the length of the spacer sequence situated between the enhancer and promoter (Jagannath et al. 2001; van der Geest and Hall 1997). In one report, both 0.9-kb and 1.2-kb genomic fragments derived from the *β-phaseolin* gene, as well as a 1.3 kb 3′ matrix attachment region (MAR), from *Phaseolus vulgaris* were shown to reduce the constitutive *35S* enhancer-mediated activation of reporter gene expression in transgenic tobacco (van der Geest and Hall 1997). In a separate report, Jagannath et al. (2001) found that a 5-kb sequence consisting of coding regions of *acetolactate synthase* from Arabidopsis and *topoisomerase*

Table 1 Examples of enhancer-blocking and barrier insulators that have been shown to reduce enhancer-promoter interference and chromosomal position effects, respectively, in plants

Insulator	Origin[a]	Transformant[b]	References
Enhancer-blocking			
β-phaseolin 3′ MAR	*P. vulgaris*	Tobacco	van der Geest and Hall (1997)
β-phaseolin genomic	*P. vulgaris*	Tobacco	van der Geest and Hall (1997)
β-phaseolin coding	*P. vulgaris*	Tobacco	van der Geest and Hall (1997)
3-kb *topoisomerase*/2-kb *acetolactate synthase*	Pea and Arabidopsis	*Brassica juncea*	Jagannath et al. (2001)
BEAD-1	Human	Arabidopsis	Gudynaite-Savitch et al. (2009)
BEAD-1C	Human	Arabidopsis	Gudynaite-Savitch et al. (2009)
UAS_{rpg}	*A. gossypii*	Arabidopsis	Gudynaite-Savitch et al. (2009)
TBS	Petunia	Arabidopsis	Hily et al. (2009)
		Tobacco	Singer et al. (2011b)
λ EXOB	*λ*	Arabidopsis	Singer et al. (2010b)
Barrier			
Rb7 3′ MAR	Tobacco	Tobacco cell culture	Allen et al. (1996)
		Tobacco	Han et al. (1997)
		Poplar	
		Rice	Vain et al. (1999)
		Theobroma cacao	Maximova et al. (2003)
TM2 MAR	Tobacco	Rice	Xue et al. (2005)
P1-SAR/MAR	Soybean	Tobacco callus	Breyne et al. (1992)
		Barley callus	Petersen et al. (2002)
Gmhsp 17.6L MAR	Soybean	Tobacco	Schöffl et al. (1993)
β-phaseolin 5′ and 3′ MARs	*P. vulgaris*	Tobacco	van der Geest et al. (1994)
Plastocyanin 3′ MAR	Pea	Tobacco	Li et al. (2001)
Adh1 5′ MAR	Maize	Maize callus	Brouwer et al. (2002)
ARS-1 SAR/MAR	*S. cerevisiae*	Tobacco cell culture	Allen et al. (1993)
		Rice	Vain et al. (1999)
Lysozyme A MAR	Chicken	Tobacco	Mlynárová et al. (1994)
		Rice	Oh et al. (2005)
Arylsulfatase insulator	Sea urchin	Tobacco cell culture	Nagaya et al. (2001)
Gypsy	*Drosophila*	Arabidopsis	She et al. (2010)

[a] Denotes indicates organism from which the insulator was derived

[b] Denotes transgenic plant in which the insulating sequence was tested

from pea was able to lessen *35S* enhancer-mediated interference with expression from a tapetum-specific promoter when inserted between the two in transgenic *Brassica juncea*. In both instances, the authors concluded that the *35S* enhancer is only capable of acting at a relatively close range and that virtually any sequence could be utilized as a spacer providing the length was sufficient to block enhancer-promoter communication. However, there is evidence that the *35S* enhancer can exert its effects over distances as large as 78 kb (Ren et al. 2004). Furthermore, previous enhancer-blocking assays in plants using this promoter have shown that it can override a length of 2–4 kb (Hily et al. 2009; Singer et al. 2010b, 2011b). These results insinuate that spacer sequences such as those described above may not necessarily block interference solely as a result of their length, and that instead they may possess inherent, as of yet unidentified, properties that minimize enhancer-promoter interactions.

More recently, both the 2-kb *transformation booster sequence* (*TBS*) from *Petunia hybrida* and a 1-kb *Eco*RI/*Sal*I fragment (*EXOB*) from bacteriophage lambda were shown to reduce *35S*-mediated activation of flower-specific promoters in vegetative tissues when situated between the two in plant species (Hily et al. 2009; Singer et al. 2010b, 2011b). The fact that a 4-kb fragment from bacteriophage lambda was not able to elicit this same effect under identical conditions implied that the enhancer-blocking ability of both the *TBS* and *EXOB* fragments was not simply a consequence of their length. Additionally, since neither the *TBS* nor the *EXOB* fragment was found to exhibit silencing activity, they likely function as true enhancer-blocking insulators.

As is the case for a proportion of metazoan enhancer-blocking insulators, such as the enhancer-blocking insulators within the imprinted control region of the mouse *Igf2/H19* locus, the mouse *SP-10* insulator, and the upstream insulator of the human *apoB* locus (Abhyankar et al. 2007; Bell and Felsenfeld 2000; Hark et al. 2000), the *TBS* element has been found to display some degree of polarity in that it is more effective in the forward orientation (Singer et al. 2011b). While the mechanism behind this phenomenon is unknown at present, it has been suggested that it may occur in composite elements that contain both an insulator sequence and a transcriptional enhancer. This is believed to be the case for the enhancer-blocking insulator situated upstream of the human *apoB* locus (Antes et al. 2001). As enhancer-blocking insulators function solely when situated between an enhancer and promoter, the orientation of a compound insulator-enhancer element in which the insulator was proximal to the target promoter would theoretically block both internal and external enhancers, while a reversed orientation would block the external enhancer but not that included within the element itself (reviewed by West et al. 2002). Intriguingly, the *TBS* element has been found to initiate transcription of a downstream reporter gene autonomously (Singer et al. 2011b), which seems to be characteristic of enhancers in artificial systems within plant species (Singer et al. 2010a) and raises the possibility that the *TBS* fragment is a composite element containing an internal enhancer upstream of the insulator, which could explain its polarity.

The forward-oriented *TBS* fragment has been shown to be effective for reducing enhancer-promoter interference in both Arabidopsis and *Nicotiana tabacum*, and does not appear to be promoter-specific, which suggests that it may be exploited in a broad range of transgenic plants (Singer et al. 2011b). Similarly, several heterologous sequences exhibiting enhancer-blocking activity in other organisms have recently been tested in plants (Gudynaite-Savitch et al. 2009), but the majority of these sequences were not effective or had an altered function in plant cells. For example, the Fab7PRE enhancer-blocking insulator from *Drosophila* (Barges et al. 2000) exhibited a silencing, rather than an insulating, function in transgenic plants (Gudynaite-Savitch et al. 2009). Nonetheless, a number of these sequences, such as the UAS_{rpg} insulator from *Ashbya gossypii* (Bi and Broach 2006), as well as the BEAD-1 and BEAD-1C insulators from the human T-cell receptor α/δ locus (Zhong and Krangel 1997), were found to reduce inappropriate enhancer-promoter interactions in Arabidopsis (Gudynaite-Savitch et al. 2009).

Possible mechanisms behind enhancer-blocking insulation in plants

Several models, which are by no means mutually exclusive, have been proposed to explain the mechanism behind enhancer-blocking insulator function in animal systems. The binding of protein factors appears to be a requirement for enhancer-blocking insulator activity despite the fact that there is little conservation of sequence identity among them. Indeed, several proteins that bind DNA have been found to be sufficient to impede enhancer-promoter interactions in animals (Bell et al. 1999; Gaszner et al. 1999; Parkhurst et al. 1988) and it has been hypothesized that the binding of these proteins to an enhancer-blocking insulator separates chromatin into topologically distinct domains through the clustering of bound proteins at the nuclear periphery, forming loops of DNA across which enhancer-promoter interactions cannot occur (reviewed by Gaszner and Felsenfeld 2006).

One type of DNA sequence that is involved in chromatin loop formation and localization at the nuclear periphery are MARs, which are non-transcribed, AT-rich sequences that bind a nuclear network of non-histone proteins termed the nuclear matrix (reviewed by Allen et al. 2000). While the

exact in vivo role of these sequences remains a mystery, it has been proposed that they may play an important role in establishing the correct expression patterns of endogenous genes (Lauber et al. 1997; Liu et al. 1997). A small number of these elements have been shown to possess enhancer-blocking properties in animals (Nabirochkin et al. 1998; Stief et al. 1989), which suggests that the functions of MARs and enhancer-blocking insulators may be related. Interestingly, an analysis of the enhancer-blocking capabilities of three MAR-containing sequences in transgenic Arabidopsis indicated that only the *TBS* fragment from petunia, but not the *ADH1* 5′ MAR from *Zea mays* (Avramova and Bennetzen 1993) or the *Rb7* 3′ MAR from *N. tabacum* (Conkling et al. 1990), was able to reduce *35S*-mediated constitutive activation of a stamen- and carpel-specific promoter (Hily et al. 2009). Assays of the enhancer-blocking capabilities of MARs in metazoans have resulted in outcomes that were equally dependent on the MAR tested (e.g. Kellum and Schedl 1992; Stief et al. 1989). In line with this data, there is a growing body of evidence suggesting that the ability of elements to bind the nuclear matrix is not necessarily related to their enhancer-blocking function (e.g. Xu et al. 2004).

Another type of enhancer-blocking insulator exhibiting a possible dependence upon loop formation is that found within the 5′ untranslated region of the *gypsy* retrotransposon from *Drosophila*, which is one of the most extensively studied enhancer-blocking insulators in invertebrate systems (e.g. Scott and Geyer 1995; Spana et al. 1988). Its sequence includes 12 direct repeats of a binding site for the zinc-finger DNA-binding protein Suppressor of Hairy-wing [Su(Hw)] (Harrison et al. 1993; Parkhurst et al. 1988); the binding of which are essential for insulator function and instigate the formation of higher order interactions involving various other proteins and RNA (Georgiev and Gerasimova 1989; Gerasimova et al. 1995). These interactions, in turn, allow the subsequent formation of 'insulator bodies', which have been proposed to organize the chromatin into loops, resulting in the generation of distinct domains that separate enhancer and promoter, thus preventing their communication (reviewed by Gaszner and Felsenfeld 2006). However, recent evidence indicates that the formation of 'insulator bodies' may not be a requirement for insulator function (Golovnin et al. 2008) and instead, the binding of Su(Hw) proteins may simply create a physical blockage of an activating signal, such as histone modification or intergenic transcription that is initiated at the enhancer and progresses toward the target promoter (Wallace and Felsenfeld 2007).

The scs/scs′ paired elements which flank the *Hsp70* locus in *Drosophila* are another well-studied insulator system that provide further support for the importance of DNA-binding proteins (Kellum and Schedl 1991, 1992). The scs and scs′ elements bind Zw5 (Gaszner et al. 1999) and BEAF32 A and B proteins (Zhao et al. 1995), respectively, and the interaction between the two stabilizes loop formation in vivo (Blanton et al. 2003). Yet another example of a protein involved in enhancer-blocking insulator function is the highly conserved and ubiquitously expressed CTCF factor (Filippova et al. 1996), which binds diverse sequences that have been identified in the majority of insulators analyzed to date in vertebrates (Kim et al. 2007). While the exact molecular basis of CTCF-mediated insulator activity remains unclear, a model similar to that proposed for Su(Hw) in *Drosophila* has been developed in which CTCF molecules interact with one another to form clusters and thereby generate loops (Yusufzai et al. 2004). Interestingly, CTCF-binding sites have been found to function in *Drosophila* (Namciu et al. 1998), and CTCF-like proteins have also been identified in invertebrates (Barges et al. 2000; Moon et al. 2005), which indicates that this factor may be involved in enhancer-blocking activity in a range of organisms.

Whether CTCF-dependent and/or insulators exhibiting similarities to invertebrate-specific elements (e.g., scs/scs′ and *gypsy*) are present in plants remains unclear. While no [Su(Hw)]- or BEAF-32/Zw5-dependent insulators have been tested for their enhancer-blocking activity in transgenic plants as of yet, two CTCF-dependent insulators from humans (BEAD-1 and BEAD1-C), as well as two *Drosophila* insulators with dCTCF-binding sites (Mcp and Fab8) have been examined. While both BEAD1 fragments appeared to exhibit some enhancer-blocking activity in transgenic Arabidopsis, this was not the case for the *Drosophila* elements (Gudynaite-Savitch et al. 2009). As of yet, no functional equivalents of CTCF-binding sites have been identified in plants; however, a large number of zinc-finger gene families exhibit at least some degree of similarity at the amino acid level with the zinc-finger domains of vertebrate CTCF proteins (Engelbrecht et al. 2004) and may provide a similar function. In any case, the fact that insulators from humans and fungi impart at least partial blocking of *35S*-mediated activation of a nearby promoter in plants (Gudynaite-Savitch et al. 2009) implies that at least a proportion of the insulator machinery in eukaryotes may be evolutionarily conserved (Wallace and Felsenfeld 2007).

Another model of enhancer-blocking insulator action postulates that enhancer-blocking insulators could act as decoy promoters, either by interacting directly with the enhancer or by interfering with communication between an enhancer and promoter. In line with this hypothesis, numerous similarities have been found between enhancer-blocking insulators and promoters, including specific chromatin-modification signatures, localization to particular nuclear regions, and the binding of specific transcription

factors (reviewed by Raab and Kamakaka 2010). In addition, several enhancer-blocking insulators in *Drosophila* have been shown to contain promoter sequences (Bae et al. 2002; Drewell et al. 2002; Geyer 1997). While it is not known whether this is also the case for enhancer-blocking insulators found to function in plants, bioinformatic analysis of the *TBS* element did indicate the presence of putative promoter regions. Furthermore, while the ability of the *TBS* to initiate transcription of a downstream reporter gene could be attributed to its inclusion of an enhancer element(s), it is also possible that this activity results from the presence of a cryptic promoter that could contribute to its activity (Singer et al. 2011b).

There is evidence supporting each of these models in animal systems, which implies that no single model is applicable to all enhancer-blocking insulators and that this class of insulating sequence may utilize diverse mechanisms to carry out a similar function. Furthermore, while these elements may function in a similar manner in plant and animal systems, it is also possible that enhancer-blocking insulators in plant species utilize novel, as of yet uncharacterized, mechanisms.

The use of barrier insulators to reduce chromosomal position effects in plants

Genomic regions can range from highly active (euchromatin) to transcriptionally silenced (heterochromatin) as a result of differential nucleosome arrangements, interactions of non-histone proteins, and histone modifications and variants (Bernstein et al. 2006; Ghirlando and Felsenfeld 2008; Mutskov et al. 2007). Euchromatin is often referred to as being in an 'open' conformation and possesses irregularly spaced nucleosomes that are highly acetylated and methylated at H3K4 and H3K79. Heterochromatin is more condensed than euchromatin due to the positioning of nucleosomes at short, regular intervals, and often exhibits high levels of CpG methylation. Histone modifications that are typical of heterochromatic regions consist of extensive methylation at H3K27 and H3K9, a lack of acetylation, and the presence of heterochromatin protein 1 (HP1). Furthermore, unlike euchromatin, heterochromatin is capable of spreading through the extension of H3K9 methylation, which leads to the HP1-mediated recruitment of further histone methyltransferase activity (reviewed by Gaszner and Felsenfeld 2006).

The distinct positioning of euchromatin and heterochromatin within the nucleus of eukaryotic cells is thought to correlate with particular environments appointed for chromatin activation and repression, respectively (Heard and Bickmore 2007). The presence of these genomic zones of activity/repression often proves to be a hindrance to plant biotechnology, as chromatin-mediated silencing of the introduced transgene can occur if integration takes place within or near a region of heterochromatin. Since the position of transgene insertion in plants is largely a random event, these site-dependent chromosomal position effects can trigger significant variability between individual transformants in terms of transgene expression levels. A related phenomenon, known as position effect variegation, has been suggested to be the consequence of a stochastic spread or retreat of heterochromatin toward or away from the transgene (Volfson et al. 2006) and results in lines bearing heterogeneous levels of expression that vary from cell to cell within the organism. This type of mis-expression is not necessarily evident when amounts of transgenic product are measured quantitatively in a particular tissue as it will simply manifest as a lower than expected average level, and instead is only detectable when expression levels are compared at the cellular level.

One possible strategy to counteract this effect in transgenic plants is to flank a transgene with elements that block the spread of heterochromatin, allowing the foreign gene to be expressed appropriately regardless of its insertion site within the host genome. Barrier insulators, which have been proposed to play a role in genome organization through the arrangement of chromatin fiber into functional domains whereby genes in one domain are protected from the regulatory effects of another (Lunyak 2008), are one such element.

Barrier insulators in transgenic plants

Possibly the most well-studied class of putative barrier elements with potential applications in plant transgenic technology are MARs, which have been suggested to trigger the formation of chromatin loops, thus delimiting the boundaries of discrete chromosomal domains (Bode et al. 2000). These elements have been isolated from a large number of eukaryotes, including a variety of plants (e.g. Avramova et al. 1995; Chinn and Comai 1996; van der Geest et al. 1994), and are commonly used to flank transcription units within transgene constructs in metazoan systems to mitigate undesirable variations in transgene expression (e.g. Phi-Van et al. 1990; Stief et al. 1989). It has been proposed that these elements will be one of the most important tools for generating transgenic plants with stable expression of foreign genes (Tao et al. 2006); however, despite their promise, results have been somewhat ambiguous and their use in transgenic constructs may not be as straightforward as initially anticipated.

In plants, much of the research carried out concerning the use of transgene-flanking MARs as barrier insulators to reduce chromosomal position effects has shown that these elements result in an increase in the level of transgene

expression and/or a reduction in plant-to-plant variability (Table 1). For example, the 3′ MAR associated with the tobacco *Rb7* gene was found to significantly augment the expression of a flanked transgene when compared to controls lacking this MAR (Allen et al. 1996; Cheng et al. 2001; Ülker et al. 1999), and in some instances was also able to lessen variability between transgenic lines through a reduction in transgene silencing (Halweg et al. 2005; Mankin et al. 2003; Verma et al. 2005). Similarly, the presence of chicken lysozyme A MAR elements (Phi-Van and Strätling 1988) flanking transgenes in tobacco and rice has been found to reduce variability by reducing silencing effects (Mlynárová et al. 1994; Oh et al. 2005). However, separate studies found that this same MAR was only able to elicit a boundary effect in transgenic Arabidopsis with a gene silencing mutant background (Butaye et al. 2004). Further inconsistencies regarding the effectiveness of MARs in transgenic plants have also been documented, with some exhibiting no effect on transgene expression (e.g. De Bolle et al. 2003; Petersen et al. 2002), a decrease in transgene expression (Breyne et al. 1992; Torney et al. 2004), or an increase in transformation efficiency (Buising and Benbow 1994; Petersen et al. 2002). While it has been proposed that the occurrence of these contradictory results may be due to the use of different experimental parameters, it is also possible that MARs are simply a heterogeneous group of elements that share only their ability to bind the nuclear matrix (Holmes-Davis and Comai 1998). Alternatively, it has been suggested that while MARs are able to block *cis*-silencing (for example, the spreading of heterochromatin), they do not prevent *trans*-silencing (for example, post-transcriptional silencing), which may explain at least a portion of these inconsistencies (Allen 2009). In any case, it appears that further research will be required to clarify the mode of action of these elements, as well as their role in plant biotechnology.

A small number of non-MAR elements have also been found to exhibit barrier activity to protect transgenes from chromosomal position effects in plants (Table 1). For example, the 5′ insulator of the sea urchin (*Hemicentrotus pulcherrimus*) *arylsulfatase* (*ars*) gene (Akasaka et al. 1999) was shown to increase reporter gene activity and suppress transgene expression variation in tobacco cells when positioned upstream of the transgene in an orientation-independent manner (Nagaya et al. 2001). Likewise, a study in which various transgenes were flanked by the *gypsy* insulator from *Drosophila* indicated that this element improved the expression levels of reporter genes in Arabidopsis by minimizing those lines with low levels of expression. Variability between individuals was lessened even further when the Su(Hw) protein from *Drosophila* was co-expressed in the same transgenic lines (She et al. 2010). The ability of the *gypsy* insulator to elicit a low level of barrier activity in Arabidopsis in the absence of the Su(Hw) protein suggests that plant-derived factors were providing the insulator function. However, the fact that additional improvements were noted in lines bearing the Su(Hw) protein indicate that this factor was superior to its Arabidopsis counterpart(s) in its capability to provide a barrier function. Since there do not appear to be any Su(Hw) homologues in the Arabidopsis genome, further investigation will be necessary to identify the putative factors required to yield the barrier function of this element in plants.

Possible mechanisms behind barrier insulators in plants

As discussed previously, it appears that the ability of elements to bind the nuclear matrix may not be associated with their function as enhancer-blocking insulators. This may also be the case for MARs and their sporadic capacity to prevent chromosomal position effects in transgenic plants. Indeed, it has been shown that any position effect protection elicited by the chicken lysozyme A barrier element in animals is separable from the MAR itself (Phi-Van and Strätling 1996). Furthermore, the relatively large size of most MARs utilized in insulator assays would permit the concealment of additional independent elements within their lengths, which could yield a broad range of activities (Antes et al. 2001; Holmes-Davis and Comai 1998).

Both enhancer-blocking insulators and barrier insulators appear to require the binding of protein factors. However, while proteins bound to enhancer-blocking insulators seem to provide either a physical blockage of communication or a steric effect through the formation of chromatin loops, those bound to barrier insulators are believed to function through the attenuation of heterochromatic silencing from neighboring genomic regions. Although there are very few common sequence features shared among characterized chromatin barriers, there is increasing evidence for the recruitment of histone acetylase activity by these elements in multiple organisms. For example, the cHS4 insulator from the chicken *β-globin* locus displays both enhancer-blocking (Chung et al. 1997) and barrier (Pikaart et al. 1998) activities, whereas its enhancer-blocking function is effected by the CTCF protein, its barrier activity is independent of this factor (Recillas-Targa et al. 2002) and instead involves proteins that impart cHS4-mediated acetylation and H3K4 methylation of nucleosomes (Huang et al. 2007), as well as protection against DNA methylation (Dickson et al. 2010). It has been proposed that these cHS4-mediated histone modifications render them resistant to H3K9 methylation and HP1 binding, which halts the spread of heterochromatin formation and maintains a local environment of active chromatin (Huang et al. 2007). Further evidence for this theory comes from boundary

elements of mice, *Drosophila*, and sea urchin, which have recently been found to be characterized by a transition from repressive to active chromatin through methylation of H3K4 and/or histone acetylation (Carabana et al. 2011; D'Apolito et al. 2009; Lin et al. 2011).

Another group of elements that has shown promise as barrier insulators is the RNA polymerase III promoters, such as those contained within tRNA genes in *Saccharomyces cerevisiae*. It appears that both high levels of transcription from the tRNA promoter (RNA polymerase III-dependent) and genes that encode histone acetyltransferases are required for their barrier activity in this organism (Donze and Kamakaka 2001). In vertebrates, short interspersed repeats (SINEs), which are retrotransposon fossils that are abundant in eukaryotic genomes, also contain RNA polymerase III promoters and are either derived from 7SL RNA, 5S rRNA or, most commonly, tRNA (Nishihara et al. 2006). As is the case for tRNA genes in *S. cerevisiae*, these promoters have been implicated in the observed barrier activity of several SINEs (Román et al. 2011), including the Alu SINEs that flank the human *keratin 18* (*K18*) gene (Willoughby et al. 2000). It has been suggested that the barrier function of RNA polymerase III promoters is provided through the creation of a gap in the nucleosome structure, as they are frequently occupied by polymerase III, as well as the transcription factors TFIIIC and TFIIIB (Donze and Kamakaka 2001). Taken together, this implies that at least a proportion of promoters transcribed by RNA polymerase III may possess an inherent, conserved mechanism for barrier activity in eukaryotes and is a promising avenue of pursuit for future studies in plants.

Conclusions

There is an imminent need for effective tools with which to mitigate genetic interference within transgenic plants due to ever-increasing reports of transgene mis-expression resulting from inappropriate enhancer-promoter interactions and integration site-dependent chromatin position effects. While several sequences exhibiting either enhancer-blocking or barrier activity in transgenic plants have been identified to date (e.g. Gudynaite-Savitch et al. 2009; Hily et al. 2009; Singer et al. 2010b; She et al. 2010), little is known concerning their molecular mechanisms. Preferentially, transgenic constructs would contain enhancer-blocking insulators between transcriptional units to prevent inappropriate enhancer-promoter interactions, as well as flanking elements exhibiting both enhancer-blocking and barrier activities to minimize interference by nearby endogenous enhancers and chromatin-mediated silencing, respectively. Insulators with such mutual enhancer-blocking/barrier functions are relatively common in metazoan systems, and include the 5′ cHS4 from chicken, as well as the Idefix (Brasset et al. 2010), *gypsy* (Kurshakova et al. 2007) and scs/scs′ (Kellum and Schedl 1992) insulators from *Drosophila*. Unfortunately, studies concerning plant-acting insulators have focused solely on one characteristic or the other; a problem that should be remedied in short order. Interestingly, the *β-phaseolin* 3′ MAR from *P. vulgaris* has been found in separate studies to exhibit potential enhancer-blocking activity (van der Geest and Hall 1997) and to contribute to barrier function (van der Geest et al. 1994) in plants, which suggests that this sequence may be an ideal candidate for further testing regarding its dual functionality.

Thus, the identification of additional DNA sequences exhibiting highly efficient and consistent activities as enhancer-blocking and/or barrier insulators is a priority. This, along with the further characterization and optimization of known insulators that function in a broad range of plant species, such as the *TBS* fragment from petunia and the *gypsy*-element from *Drosophila* (Gudynaite-Savitch et al. 2009; Nagaya et al. 2001; She et al. 2010; Singer et al. 2011b), has the potential to be of tremendous value in the future of biotechnological applications in plants.

Acknowledgments The authors wish to thank Sara Villani for her invaluable assistance. Support was provided by state, federal, and institutional funds appropriated to the New York State Agricultural Experiment Station, Cornell University.

References

Abhyankar MM, Urekar C, Reddi PP (2007) A novel CpG-free vertebrate insulator silences the testis-specific *SP-10* gene in somatic tissues: role for TDP-43 in insulator function. J Biol Chem 282:36143–36154

Akasaka K, Nishimura A, Takata K, Mitsunaga K, Mibuka F, Ueda H, Hirose S, Tsutsui K, Shimada H (1999) Upstream element of the sea urchin arylsulfatase gene serves as an insulator. Cell Mol Biol (Noisy-le-grand) 45:555–565

Allen GC (2009) The role of nuclear matrix attachment regions in plants. Plant Cell Monogr 14:101–129

Allen GC, Hall GE, Childs LC, Weissinger AK, Spiker S, Thompson WF (1993) Scaffold attachment regions increase reporter gene expression in stably transformed plant cells. Plant Cell 5:603–613

Allen GC, Hall GE Jr, Michalowski S, Newman W, Spiker S, Weissinger AK, Thompson WF (1996) High-level transgene expression in plant cells: effects of a strong scaffold attachment region from tobacco. Plant Cell 8:899–913

Allen GC, Spiker S, Thompson WF (2000) Use of matrix attachment regions (MARs) to minimize transgene silencing. Plant Mol Biol 43:361–376

Antes TJ, Namciu SJ, Fournier REK, Levy-Wilson B (2001) The 5′ boundary of the human apolipoprotein B chromatin domain in intestinal cells. Biochemistry 40:6731–6742

Avramova Z, Bennetzen JL (1993) Isolation of matrices from maize leaf nuclei: identification of matrix-binding site adjacent to the *Adh1* gene. Plant Mol Biol 22:1135–1143

Avramova Z, San Miguel P, Georgieva E, Bennetzen JL (1995) Matrix attachment regions and transcribed sequences within a

long chromosomal continuum containing maize *Adh1*. Plant Cell 7:1667–1680

Bae E, Calhoun VC, Levine M, Lewis EB, Drewell RA (2002) Characterization of the intergenic RNA profile at *abdominal-A* and *Abdominal-B* in the *Drosophila* bithorax complex. Proc Natl Acad Sci USA 99:16847–16852

Barges S, Mihaly J, Galloni M, Hagstrom K, Muller M, Shanower G, Schedl P, Gyurkovics H, Karch F (2000) The *Fab-8* boundary defines the distal limit of the bithorax complex *iab-7* domain and insulates *iab-7* from initiation elements and a PRE in the adjacent *iab-8* domain. Development 127:779–790

Bell AC, Felsenfeld G (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the *Igf2* gene. Nature 405:482–485

Bell AC, West AG, Felsenfeld G (1999) The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. Cell 98:387–396

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125:315–326

Bhat SR, Srinivasan S (2002) Molecular and genetic analyses of transgenic plants: considerations and approaches. Plant Sci 164:673–681

Bi X, Broach JR (2006) UAS_{rpg} can function as a heterochromatin boundary element in yeast. Genes Dev 13:1089–1101

Blanton J, Gaszner M, Schedl P (2003) Protein-protein interactions and the pairing of boundary elements in vivo. Genes Dev 17:664–675

Bode J, Benham C, Knopp A, Mielke C (2000) Transcriptional augmentation: modulation of gene expression by scaffold/matrix-attached regions (S/MAR elements). Crit Rev Eukaryot Gene 10:73–90

Brasset E, Hermant C, Jensen S, Vaury C (2010) The Idefix enhancer-blocking insulator also harbors barrier activity. Gene 450:25–31

Breyne P, van Montagu M, Depicker A, Gheysen G (1992) Characterization of a plant scaffold attachment region in a DNA fragment that normalizes transgene expression in tobacco. Plant Cell 4:463–471

Brouwer C, Bruce W, Maddock S, Avramova Z, Bowen B (2002) Suppression of transgene silencing by matrix attachment regions in maize: a dual role for the maize 5′ *ADH1* matrix attachment region. Plant Cell 14:2251–2264

Buising CM, Benbow RM (1994) Molecular analysis of transgenic plants generated by microprojectile bombardment: effect of petunia transformation booster sequence. Mol Gen Genet 243:71–81

Butaye KMJ, Goderis IJWM, Wouters PFJ, Pues JM-TG, Delauré SL, Broekaert WF, Depicker A, Cammue BPA, De Bolle MFC (2004) Stable high-level transgene expression in *Arabidopsis thaliana* using gene silencing mutants and matrix attachment regions. Plant J 39:440–449

Butaye KMJ, Cammue BPA, Delauré De, Bolle MFC (2005) Approaches to minimize variation of transgene expression in plants. Mol Breed 16:79–91

Carabana J, Watanabe A, Hao B, Krangel MS (2011) A barrier-type insulator forms a boundary between active and inactive chromatin at the murine TCRβ locus. J Immunol 186:3556–3562

Cheng Z, Targolli J, Wu R (2001) Tobacco matrix attachment region sequence increased transgene expression levels in rice plants. Mol Breed 7:317–327

Chinn AM, Comai L (1996) The heat shock cognate 80 gene of tomato is flanked by matrix attachment regions. Plant Mol Biol 32:959–968

Chung JH, Whiteley M, Felsenfeld G (1993) A 5′ element of the chicken β-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. Cell 74:505–514

Chung JH, Bell AC, Felsenfeld G (1997) Characterization of the chicken β-globin insulator. Proc Natl Acad Sci USA 94:575–580

Conkling MA, Cheng C-L, Yamamoto YT, Goodman HM (1990) Isolation of transcriptionally regulated root-specific genes from tobacco. Plant Physiol 93:1203–1211

D'Apolito D, Baiamonte E, Bagliesi M, Di Marzo R, Calzolari R, Ferro L, Franco V, Spinelli G, Maggio A, Acuto S (2009) The sea urchin *sns5* insulator protects retroviral vectors from chromosomal position effects by maintaining active chromatin structure. Mol Ther 17:1434–1441

de Bolle MFC, Butaye KMJ, Coucke WJW, Goderis IJWM, Wouters PFJ, van Boxel N, Broekaert WF, Cammue BPA (2003) Analysis of the influence of promoter elements and a matrix attachment region on the inter-individual variation of transgene expression in populations of *Arabidopsis thaliana*. Plant Sci 165:169–179

Dickson J, Gowher H, Strogantsev R, Gaszner M, Hair A, Felsenfeld G, West AG (2010) VEZF1 elements mediate protection from DNA methylation. PLoS Genet 6:e10000804

Donze D, Kamakaka RT (2001) RNA polymerase III and RNA polymerase II complexes are heterochromatin barriers in *Saccharomyces cerevisiae*. EMBO J 20:520–531

Dorsett D (1999) Distant liaisons: long-range enhancer-promoter interactions in *Drosophila*. Curr Opin Genet Dev 9:505–514

Drewell RA, Bae E, Burr J, Lewis EB (2002) Transcription defines the embryonic domains of cis-regulatory activity at the *Drosophila* bithorax complex. Proc Natl Acad Sci USA 99:16853–16858

Engelbrecht CC, Schoof H, Böhm S (2004) Conservation, diversification and expression of C2H2 zinc finger proteins in the *Arabidopsis thaliana* genome. BMC Genomics 5:1–17

Filippova GN, Fagerlie S, Klenova EM, Myers C, Dehner Y, Goodwin G, Neiman PE, Collins SJ, Lobanenkov VV (1996) An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-*myc* oncogenes. Mol Cell Biol 16:2802–2813

Francis KE, Spiker S (2005) Identification of *Arabidopsis thaliana* transformants without selection reveals a high occurrence of silenced T-DNA integrations. Plant J 41:464–477

Gaszner M, Felsenfeld G (2006) Insulators: exploiting transcriptional and epigenetic mechanisms. Nat Rev Genet 7:703–713

Gaszner M, Vazquez J, Schedl P (1999) The Zw5 protein, a component of the scs chromatin domain boundary, is able to block enhancer-promoter interaction. Genes Dev 13:2098–2107

Georgiev PG, Gerasimova TI (1989) Novel genes influencing the expression of the *yellow* locus and mdg4 (*gypsy*) in *Drosophila melanogaster*. Mol Gen Genet 220:121–126

Gerasimova TI, Gdula DA, Gerasimov DV, Simonova O, Corces VG (1995) A Drosophila protein that imparts directionality on a chromatin insulator is an enhancer of position-effect variegation. Cell 82:587–597

Geyer PK (1997) The role of insulator elements in defining domains of gene expression. Curr Opin Genet Dev 7:242–248

Geyer PK, Spana C, Corces VG (1986) On the molecular mechanism of gypsy-induced mutations at the *yellow* locus of *Drosophila melanogaster*. EMBO J 5:2657–2662

Ghirlando R, Felsenfeld G (2008) Hydrodynamic studies on defined heterochromatin fragments support a 30-nm fiber having six nucleosomes per turn. J Mol Biol 376:1417–1425

Golovnin A, Melnikova L, Volkov I, Kostuchenko M, Galkin AV, Georgiev P (2008) 'Insulator bodies' are aggregates of proteins but not of insulators. EMBO Rep 9:440–445

Gudynaite-Savitch L, Johnson DA, Miki BLA (2009) Strategies to mitigate transgene-promoter interactions. Plant Biotechnol J 7:472–485

Halweg C, Thompson WF, 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

Han K-H, Ma C, Strauss SH (1997) Matrix attachment regions (MARs) enhance transformation frequency and transgene expression in poplar. Transgenic Res 6:415–420

Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM (2000) CTCT mediates methylation-sensitive enhancer-blocking activity at the *H19/Igf2* locus. Nature 405:486–489

Harrison DA, Gdula DA, Coyne RS, Corces VG (1993) A leucine zipper domain of the *suppressor of Hairy-wing* protein mediates its repressive effect on enhancer function. Genes Dev 7:1966–1978

Heard E, Bickmore W (2007) The ins and outs of gene regulation and chromosome territory organisation. Curr Opin Cell Biol 19:311–316

Hily JM, Singer SD, Yang Y, Liu Z (2009) A transformation booster sequence (*TBS*) from *Petunia hybrida* functions as an enhancer-blocking insulator in *Arabidopsis thaliana*. Plant Cell Rep 28:1095–1104

Hohn B, Puchta H (2003) Some like it sticky: targeting of the rice gene *Waxy*. Trends Plant Sci 8:51–53

Holmes-Davis R, Comai L (1998) Nuclear matrix attachment regions and plant gene expression. Trends Plant Sci 3:91–97

Huang S, Xingguo Li, Yusufzai TM, Qiu Y, Felsenfeld G (2007) USF1 recruits histone modification complexes and is critical for maintenance of a chromatin barrier. Mol Cell Biol 27:7991–8002

Jack J, Dorsett D, DeLotto Y, Liu S (1991) Expression of the *cut* locus in the *Drosophila* wing margin is required for cell type specification and is regulated by a distant enhancer. Development 113:735–747

Jagannath A, Bandyopadhyay P, Arumugam N, Gupta V, Kumar P, Pental D (2001) The use of a Spacer DNA fragment insulates the tissue-specific expression of a cytotoxic gene (*barnase*) and allow high-frequency generation of transgenic male sterile lines in *Brassica juncea* L. Mol Breed 8:11–23

Kadauke S, Blobel GA (2009) Chromatin loops in gene regulation. Biochim Biophys Acta 1789:17–25

Kellum R, Schedl P (1991) A position-effect assay for boundaries of higher order chromosomal domains. Cell 64:941–950

Kellum R, Schedl P (1992) A group of scs elements function as domain boundaries in an enhancer-blocking assay. Mol Cell Biol 12:2424–2431

Kim T, Abdullaev Z, Smith A, Ching K, Loukinov D, Green R, Zhang M, Lobanenkov V, Ren B (2007) Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. Cell 128:1231–1245

Kurshakova M, Maksimenko O, Golovnin A, Pulina M, Georgieva S, Georgiev P, Krasnov A (2007) Evolutionarily conserved E(y)2/Sus1 protein is essential for the barrier activity of Su(Hw)-dependent insulators in *Drosophila*. Mol Cell 27:332–338

Lanfranco L (2003) Engineering crops, a deserving venture. Riv Biol 96:31–54

Lauber AH, Barrett TJ, Subramaniam M, Schuchard M, Spelsberg TC (1997) A DNA-binding element for a steroid receptor-binding factor is flanked by dual nuclear matrix DNA attachment sites in the c-*myc* gene promoter. J Biol Chem 272:24657–24665

Li X, Zhu Z, Junwang X, Qian W, Honglin X (2001) Isolation of pea matrix attachment region and study on its function in transgenic tobaccos. Sci China C 44:400–408

Lin N, Li X, Cui K, Chepelev I, Tie F, Liu B, Li G, Harte P, Zhao K, Huang S, Zhou L (2011) A barrier-only boundary element delimits the formation of facultative heterochromatin in *Drosophila melanogaster* and vertebrates. Mol Cell Biol 31:2729–2741

Liu J, Bramblett D, Zhu Q, Lozano M, Kobayashi R, Ross SR, Dudley JP (1997) The matrix attachment region-binding protein SATB1 participates in negative regulation of tissue-specific gene expression. Mol Cell Biol 17:5275–5287

Liu Z, Zhou C, Wu K (2008) Creation and analysis of a novel chimeric promoter for the complete containment of pollen- and seed-mediated gene flow. Plant Cell Rep 27:995–1004

Lunyak VV (2008) Boundaries. Boundaries…Boundaries? Curr Opin Cell Biol 20:281–287

Mankin SL, Allen GC, Phelan T, Spiker S, Thompson WF (2003) Elevation of transgene expression level by flanking matrix attachment regions (MAR) is promoter dependent: a study of the interactions of six promoters with the RB7 3′ MAR. Transgenic Res 12:3–12

Matzke AJ, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. Curr Opin Plant Biol 1:142–148

Maximova S, Miller C, Antúnez de Mayolo G, Pishak S, Young A, Guiltinan MJ (2003) Stable transformation of *Theobroma cacao* L. and influence of matrix attachment regions on GFP expression. Plant Cell Rep 21:872–883

Mlynárová L, Loonen A, Heldens J, Jansen RC, Keizer P, Stiekema WJ, Nap JP (1994) Reduced position effect in mature transgenic plants conferred by the chicken lysozyme matrix-associated region. Plant Cell 6:417–426

Moon H, Filippova G, Loukinov D, Pugacheva E, Chen Q, Smith ST, Munhall A, Grewe B, Bartkuhn M, Arnold R, Burke LJ, Renkawitz-Pohl R, Ohlsson R, Zhou J, Renkawitz R, Lobanenkov V (2005) CTCF is conserved from *Drosophila* to humans and confers enhancer blocking of the *Fab-8* insulator. EMBO Rep 6:165–170

Morris JR, Chen J-I, Geyer PK, Wu C-t (1998) Two modes of transvection: enhancer action in *trans* and bypass of a chromatin insulator in *cis*. Proc Natl Acad Sci USA 95:10740–10745

Mutskov V, Raaka BM, Felsenfeld G, Gershengom MC (2007) The human insulin gene displays transcriptionally active epigenetic marks in islet-derived mesenchymal precursor cells in the absence of insulin expression. Stem Cells 25:3223–3233

Nabirochkin S, Ossokina M, Heidmann T (1998) A nuclear matrix/scaffold attachment region co-localizes with the gypsy retrotransposon insulator sequence. J Biol Chem 273:2473–2479

Nagaya S, Yoshida K, Kato K, Akasaka K, Shinmyo A (2001) An insulator element from the sea urchin *Hemicentrotus pulcherrimus* suppresses variation in transgene expression in cultured tobacco cells. Mol Genet Genomics 265:405–413

Namciu SJ, Blochlinger KB, Fournier REK (1998) Human matrix attachment regions insulate transgene expression from chromosomal position effects in *Drosophila melanogaster*. Mol Cell Biol 18:2382–2391

Nishihara H, Smit AFA, Okada N (2006) Functional noncoding sequences derived from SINEs in the mammalian genome. Genome Res 16:864–874

Odell JT, Knowlton S, Lin W, Mauvais J (1988) Properties of an isolated transcription stimulating sequence derived from the cauliflower mosaic virus 35S promoter. Plant Mol Biol 10:263–272

Oh S-J, Jeong JS, Kim EH, Yi NR, Yi S-I, Jang I-C, Kim YS, Suh S-C, Nahm BH, Kim J-K (2005) Matrix attachment region from the chicken lysozyme locus reduces variability in transgene expression and confers copy number-dependence in transgenic rice plants. Plant Cell Rep 24:145–154

Parkhurst SM, Harrison DA, Remington MP, Spana C, Kelley RL, Coyne RS, Corces VG (1988) The *Drosophila su(Hw)* gene, which controls the phenotypic effect of the gypsy transposable

element, encodes a putative DNA-binding protein. Genes Dev 2:1205–1215
Petersen K, Leah R, Knudsen S, Cameron-Mills V (2002) Matrix attachment regions (MARs) enhance transformation frequencies and reduce variance of transgene expression in barley. Plant Mol Biol 49:45–58
Phi-Van L, Strätling WH (1988) The matrix attachment regions of the chicken lysozyme genes co-map with the boundaries of the chromatin domain. EMBO J 7:655–664
Phi-Van L, Strätling WH (1996) Dissection of the ability of the chicken lysozyme gene 5′ matrix attachment to stimulate transgene expression and to dampen position effects. Biochemistry 35:10735–10742
Phi-Van L, von Kries JP, Ostertag W, Strätling WH (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
Pikaart MJ, Recillas-Targa F, Felsenfeld G (1998) Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators. Genes Dev 12:2852–2862
Ptashne M (1986) Gene regulation by proteins acting nearby and at a distance. Nature 322:697–701
Raab JR, Kamakaka RT (2010) Insulators and promoters: closer than we think. Nat Rev Genet 11:439–446
Recillas-Targa F, Pikaart MJ, Burgess-Beusse B, Bell AC, Litt MD, West AG, Gaszner M, Felsenfeld G (2002) Position-effect protection and enhancer blocking by the chicken β-globin insulator are separable activities. Proc Natl Acad Sci USA 99:6883–6888
Ren S, Johnston JS, Shippen DE, McKnight T (2004) TELOMERASE ACTIVATOR1 induces telomerase activity and potentiates responses to auxin in Arabidopsis. Plant Cell 16:2910–2922
Román AC, González-Rico FJ, Moltó E, Hernando H, Neto A, Vicente-Garcia C, Ballestar E, Gómez-Skarmeta JL, Vavrova-Anderson J, White RJ, Montoliu L, Fernández-Salguero PM (2011) Dioxin receptor and SLUG transcription factors regulate the insulator activity of B1 SINE retrotranspososons via an RNA polymerase switch. Genome Res 21:422–432
Schöffl F, Schröder G, Kliem M, Rieping M (1993) An SAR sequence containing 395 bp DNA fragment mediates enhanced, gene-dosage-correlated expression of a chimaeric heat shock gene in transgenic tobacco plants. Transgenic Res 2:93–100
Scott KS, Geyer PK (1995) Effects of the su(Hw) insulator protein on the expression of the divergently transcribed Drosophila *yolk protein* genes. EMBO J 14:6258–6267
She W, Lin W, Zhu Y, Chen Y, Jin W, Yang Y, Han N, Bian H, Zhu M, Wang J (2010) The *gypsy* insulator of *Drosophila melanogaster*, together with its binding protein suppressor of hairy-wing, facilitate high and precise expression of transgenes in *Arabidopsis thaliana*. Genetics 185:1141–1150
Singer SD, Cox KD, Liu Z (2010a) Both the constitutive *cauliflower mosaic virus 35S* and tissue-specific *AGAMOUS* enhancers activate transcription autonomously in *Arabidopsis thaliana*. Plant Mol Biol 74:293–305
Singer SD, Hily J-M, Liu Z (2010b) A 1 kb bacteriophage lambda fragment functions as an insulator to effectively block enhancer-promoter interactions in *Arabidopsis thaliana*. Plant Mol Biol Rep 28:69–76
Singer SD, Cox KD, Liu Z (2011a) Enhancer-promoter interference and its prevention in transgenic plants. Plant Cell Rep 30:723–731
Singer SD, Hily J-M, Cox KD (2011) Analysis of the enhancer-blocking function of the *TBS* element from *Petunia hybrida* in transgenic *Arabidopsis thaliana* and *Nicotiana tabacum*. Plant Cell Rep. doi:10.1007/s00299-011-1109-8 (in press)
Spana C, Harrison DA, Corces VG (1988) The *Drosophila melanogaster suppressor of Hairy-wing* protein binds to specific sequences of the gypsy retrotransposon. Genes Dev 2:1414–1423
Steinwaerder DS, Lieber A (2000) Insulation from viral transcriptional regulatory elements improves inducible transgene expression from adenovirus vectors in vitro and in vivo. Gene Ther 7:556–567
Stief A, Winter DM, Strätling WH, Sippel AE (1989) A nuclear DNA attachment element mediates elevated and position-independent gene activity. Nature 341:343–345
Tao Y, Shang-long Z, Jing-mei L, Da-ming C (2006) Approaches to improve heterogeneous gene expression in transgenic plants. Chin J Agric Biotechnol 3:75–81
Torney F, Partier A, Says-Lesage V, Nadaud I, Barret P, Beckert M (2004) Heritable transgene expression pattern imposed onto maize ubiquitin promoter by maize *adh-1* matrix attachment regions: tissue and developmental specificity in maize transgenic plants. Plant Cell Rep 22:931–938
Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21:570–578
Ülker B, Allen GC, Thompson WF, Spiker S, Weissinger AK (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 JW, Christou P, Allen GC, Thompson WF (1999) Matrix attachment regions increase transgene expression levels and stability in transgenic rice plants and their progeny. Plant J 18:233–242
van der Geest AHM, Hall TC (1997) The β-phaseolin 5′ matrix attachment region acts as an enhancer facilitator. Plant Mol Biol 33:553–557
van der Geest AHM, Hall GE Jr, Spiker S, Hall TC (1994) The β-phaseolin gene is flanked by matrix attachment regions. Plant J 6:413–423
Verma D, Verma M, Dey M, Jain RK, Wu R (2005) Molecular dissection of the tobacco *Rb7* matrix attachment region (MAR): effect of 5′ half on gene expression in rice. Plant Sci 169:704–711
Volfson D, Marciniak J, Blake WJ, Ostroff N, Tsimring LS, Hasty J (2006) Origins of extrinsic variability in eukaryotic gene expression. Nature 439:861–864
Wallace JA, Felsenfeld G (2007) We gather together: insulators and genome organization. Curr Opin Genet Dev 17:400–407
Wang Y, Tang X, Cheng Z, Mueller L, Giovannoni J, Tanksley SD (2006) Euchromatin and pericentromeric heterochromatin: comparative composition in the tomato genome. Genetics 172: 22529–22540
West AG, Gaszner M, Felsenfeld G (2002) Insulators: many functions, many mechanisms. Genes Dev 16:271–288
Weterings K, Schrauwen J, Wullems G, Twell D (1995) Functional dissection of the promoter of the pollen-specific gene *NTP303* reveals a novel pollen-specific, and conserved *cis*-regulatory element. Plant J 8:55–63
Willoughby DA, Vilalta A, Oshima RG (2000) An Alu element from the *K18* gene confers position-independent expression in transgenic mice. J Biol Chem 275:759–768
Xu Q, Li M, Adams J, Cai HN (2004) Nuclear location of a chromatin insulator in *Drosophila melanogaster*. J Cell Sci 117:1025–1032
Xue H, Yang Y-T, Wu C-A, Yang G-D, Zhang M-M, Zheng C-C (2005) TM2, a novel strong matrix attachment region isolated from tobacco increases transgene expression in transgenic rice calli and plants. Theor Appl Genet 110:620–627

Yang Y, Singer SD, Liu Z (2010) Evaluation and comparison of the insulation efficiency of three enhancer-blocking insulators in plants. Plant Cell Tissue Organ 105:405–414

Ye X, Liang M, Meng X, Ren X, Chen H, Li ZY, Ni S, Lieber A, Hu F (2003) Insulation from viral transcriptional regulatory elements enables improvement to hepatoma-specific gene expression from adenovirus vectors. Biochem Biophys Res Commun 307:759–764

Yoo SY, Bomblies K, Yoo SK, Yang JW, Choi MS, Lee JS, Weigel D, Ahn JH (2005) The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene. Planta 221:523–530

Yusufzai TM, Hagami H, Nakatani Y, Felsenfeld G (2004) CTCF tethers an insulator to subnuclear sites, suggesting shared insulator mechanisms across species. Mol Cell 13:291–298

Zhao K, Hart CM, Laemmli UK (1995) Visualization of chromosomal domains with boundary element-associated factor BEAF-32. Cell 81:879–889

Zheng X, Deng W, Luo K, Duan H, Chen Y, McAvoy R, Song S, Pei Y, Li Y (2007) The cauliflower mosaic virus (CaMV) 35S promoter sequence alters the level and patterns of activity of adjacent tissue- and organ-specific gene promoters. Plant Cell Rep 26:1195–1203

Zhong X-P, Krangel MS (1997) An enhancer-blocking element between α and δ gene segments within the human T cell receptor α/δ locus. Proc Natl Acad Sci USA 94:5219–5224

Zhu X, Ling J, Zhang L, Pi W, Wu M, Tuan D (2007) A facilitated tracking and transcription mechanism of long-range enhancer function. Nucleic Acids Res 35:5532–5544