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Rare instances of Cre-mediated deletion product maintained in transgenic wheat

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Abstract

Previously, we described a Cre-*lox* based strategy to convert a complex multi-copy integration pattern to a single-copy transgene (Srivastava *et al.*, 1999). When a *lox*-containing transgenic line of wheat was crossed with a *cre*-expressing line, extra copies of the transgene were deleted by site-specific recombination. This process included the removal of a *lox*-flanked selection marker gene, *bar*. Three out of six F_1 plants were chimeric for the resolved and the complex loci because both completely resolved and incompletely resolved patterns were found in the F_2 population. From one F_1 plant, 4 out of 20 F_2 progeny showed not only incomplete resolution of the complex integration pattern, but also the presence of a circular *loxP-bar-nos3′* fragment, which we refer to as the *bar circle*. This *bar circle* was detected in subsequent generations, and was associated with the presence of both the *lox* transgene and the *cre* locus. We hypothesize that the *cre* gene in these *bar circle* plants must have undergone a genetic or epigenetic change that altered the spatial and/or temporal pattern of *cre* expression. Late expression might excise the DNA incompletely, and late in development. What is surprising is that the DNA is not degraded, but remains in the cells as an extra-chromosomal circular molecule.

Introduction

The Cre-*lox* site-specific recombination system is an attractive tool for the removal of unneeded transgenic DNA from plants (Dale and Ow, 1991; Russell *et al.*, 1992). Transgenes flanked by *lox* recombination sites can be deleted from the plant genome by the Cre recombinase. Cre activity can be introduced into a plant by a variety of methods. The simplest method is to cross in a *cre* gene from a donor plant. Expressed from somewhat constitutive promoters, such as the CaMV 35S RNA promoter, or the maize ubiquitin promoter, germ line transmission of the deletion event is often found in the F_2 population (Dale and Ow, 1991; Srivastava *et al.*, 1999). For the introduction of Cre activity in the absence of a sexual cross, the *cre* gene can be introduced transiently (Gleave *et al.*, 1999) or the Cre protein can be delivered by *Agrobacterium* into the plant cell (Vergunst *et al.*, 2000). Alternatively, a repressed *cre* gene can be co-introduced with the trait gene of interest, and subsequently be induced to produce recombinase activity (Sugita *et al.*, 2000; Zuo *et al.*, 2001). A deletion event loops out the intervening DNA, and since molecular analysis can confirm the loss of the DNA, an assumption has been that the extra-chromosomal DNA must be degraded or diluted to an undetectable level upon cell division.

Previously, we described a set of four plants each harboring a complex multi-copy insertion of the transformation plasmid pVS11 (Srivastava *et al.*, 1999). These four pVS11-transformed lines were each crossed to a *cre*-expressing line to yield 6 F_1 plants (lines A through F). The presence of *cre* in the F_1 hybrid should promote the *lox511* × *lox511*-mediated deletion of extra copies of pVS11 (Figure 1A), as well as that of the *loxP*-flanked *bar-nos3′* fragment. A total of 72 F_2 plants were analyzed by Southern blotting, and all but 10 plants showed incomplete resolution

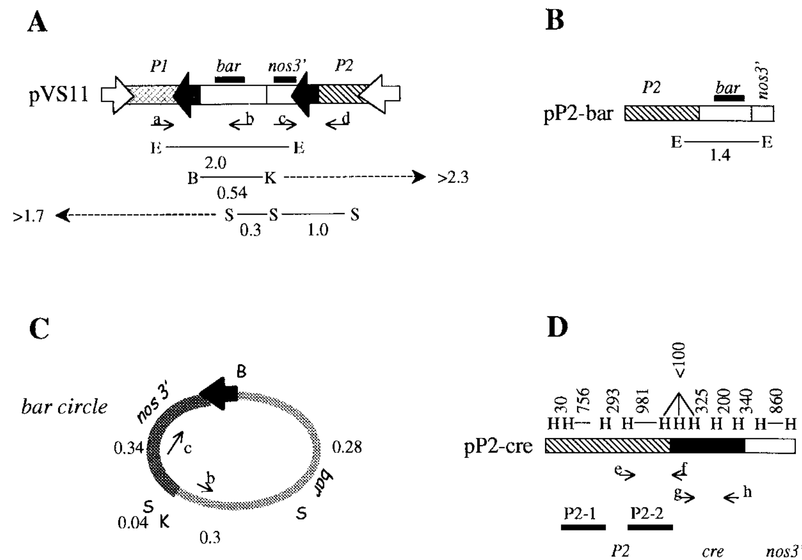

Figure 1. DNA constructs. A. pVS11. B. pP2-bar. C. *bar circle*. D. pP2-cre. Solid arrowhead, *loxP*; open arrowhead, *lox511*; *P1*, rice actin promoter; *P2*, maize ubiquitin promoter; *bar*, phosphinothricin acetyl transferase gene; *cre*, Cre recombinase gene; *nos3′*, transcription terminator of nopaline synthase gene; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; H, *Hpa*II; S, *Sal*I. Fragment sizes between cleavage sites given in kb (A, B and C) or bp (D). Each of the *lox* sites is 34 bp long. Dashed arrows represent transgene-host DNA junction fragments. Thick bars represent DNA probes. Not shown is the pUC18 backbone. PCR primers (a–h) are shown as small arrows.

of the multi-copy locus of the original pVS11 parent. Among these 10 plants, 4 were derived from a single F_1 plant (line B) resulting from a cross between a Cre line, Cre37, and a pVS11 line, VS10.1. These 4 plants were unusual in that they retained the Cre-mediated excision product, as what appeared to be a circular *loxP-bar-nos3′* DNA. This '*bar circle*' was detected in subsequent generations. Segregation analyses suggest that the *bar circle* is associated with the presence of both a particular *cre* locus and the complex pVS11 integration locus. Therefore, it is possible that the *bar circle* may be generated *de novo* in each generation, and possibly at a late stage of plant development such that dilution of the excised DNA could not take place. What is unprecedented is that the excised DNA persists. This suggests that extra-chromosomal DNA, or at least the excision products of site-specific recombination, may not be subjected to cellular degradation to the extent that we commonly presume to occur.

Materials and methods

Biological materials

Transgenic wheat lines VS10.1 and Cre37 have been described previously (Srivastava *et al.*, 1999). VS10.1 contains multiple copies of pVS11 at a single integration locus. Cre37 contains two constructs, pP2-cre and pP2-bar, each of which is integrated as a genetically unlinked locus. Schematic maps of the DNA constructs are shown in Figure 1.

Molecular analyses

Wheat genomic DNA (10 μg) isolated by the CTAB method was cleaved with restriction enzymes and subjected to Southern hybridization with ^{32}P-labeled DNA. PCR primers sequences, as indicated in Figure 1, are a (5′-CAGCATTGTTCATCGGTA-3′), b (5′-CCGGAAACCCACGTCATGCC-3′), c (5′-ATTAGAGTCCCGCAATTATAC-3′), d (5′-AGGCTGGCATTATCTACTCG-3′), e (5′-TCTACTTCTGTTCATGTTTGTG-3′), f (5′-ACGGTCAGTAAATTGGACAT-3′), g (5′-ATGTCCAATTTACTGACCGT-3′) and h (5′-CTAATCGCCATCTTCCAGCA-3′).

Results and discussion

Detection of *bar* circle

We had previously pollinated a wheat transgenic line, VS10.1 line, with a *cre*-expressing line, Cre37. VS10.1 harbors multiple copies of pVS11 (Figure 1A) at a single locus (*vs10.1* locus); Cre37 contains pP2-cre and pP2-bar as two segregating loci (Figure 1B,

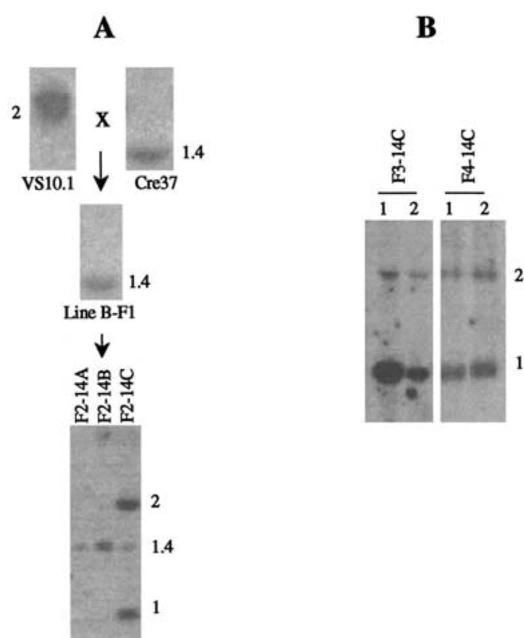

Figure 2. Discovery of *bar circles*. A. Genomic DNA from VS10.1, Cre37, and their progeny lines B-F1 and F2-14 cleaved with *Eco*RI and hybridized with a *bar* DNA probe. B. Genomic DNA of F_3 and F_4 plants derived from F_2-14C cleaved with *Eco*RI and hybridized with *bar* DNA.

D). To examine the deletion of the internal *loxP-bar-nos3′* fragment, *Eco*RI-cleaved genomic DNA from F_1 and F_2 plants were hybridized with ^{32}P-labeled *bar* DNA (Figure 1A). As expected, the 2 kb *P1-bar* fragment and the 1.4 kb *P2-bar* fragment were detected from VS10.1 and Cre37 parents, respectively (Figure 2A). F_1 plants with both the *cre* and *vs10.1* loci, such as line B, did not show the 2 kb *P1-bar* fragment and showed only the 1.4 kb band associated with the Cre37 genome. This indicates that the *loxP-bar-nos3′* fragment of pVS11 was deleted, at least in the matured somatic leaf cells.

The F_2 progeny from self-fertilized line B was examined for the amplification of a 0.8 kb *P1-lox-P2* PCR product by primers a + d (Figure 1A). The presence of this product, indicating excision of at least a single *loxP-bar-nos3′* fragment, would also suggest the possibility of resolution of the multiple copies of integrated pVS11 DNA. Twenty B-F_2 plants indeed yielded the 0.8 kb *P1-lox-P2* PCR product. In 16 of them, the Southern hybridization pattern is consistent with the conversion of a multiple-copy locus into a single-copy state along with the deletion of the *loxP-bar-nos3′* fragment. Furthermore, 13 of these F_2 plants were found to have segregated away the *cre* gene. This high 65% (13 of 20) rate of *cre* segregation was unexpected. A high rate of *cre* segregation was also observed from 2 other F_1 lines, at 55%, (11 of 20 from line A) and 50% (3 of 6 from line C). On the other hand, an expected or lower than expected segregation rate was found in the 3 remaining lines, at 25% (2 of 8 from line D), 11% (1 of 9 from line E) and 20% (1 of 5 from line F). Despite the anomalous segregation rates in some of these lines, the important point to consider here is that the absence of *cre* in the F_2 genome implies that DNA excision took place during the F_1 generation, and that the resolved locus in the F_2 plants is due to the germinal transmission of a F_1 excision event.

Of the remaining 4 F_2 plants derived from line B, the Southern hybridization patterns showed that it retained a multi-copy pattern, although some resolution of the original complex pattern was found. Most unexpected is the presence of a prominent 1 kb fragment that hybridized to *bar* DNA, such as in plant F_2-14C (Figure 2A). To rule out the possibility of contamination, this analysis was repeated on the progenies of self-fertilized F_2-14C plants. As before, the DNA of F_3-14C and F_4-14C plants were also found to contain the 2 kb *P1-bar* fragment and the 1 kb fragment (Figure 2B). Since *P2-bar* and *P2-cre* loci in Cre37 are not linked, some F_3 plants that contained 1 kb *bar* fragment did not contain the 1.4 kb *P2-bar* fragment as shown in Figure 2B. The size of the 1 kb fragment is the predicted linear size from *Eco*RI cleavage of a circular *loxP-bar-nos3′* fragment.

Consistent with a circular structure

To determine if the 1 kb fragment could represent *Eco*RI-cleaved form of the circular *loxP-bar-nos3′* fragment, F_3-14C and VS10.1 genomic DNAs were examined using various restriction enzymes. *Sal*I-cleaved VS10.1 DNA is expected to generate a >1.7 kb *P1-bar* transgene-host junction fragment, a 0.3 kb *bar* fragment and a 1 kb *nos-P2* fragment (Figure 1A). The *bar* and *nos3′* probes detected the 0.3 kb and 1 kb bands, respectively (Figure 3A), but the >1.7 kb transgene-host junction band is not obvious, since the VS10.1 transgenic locus contains many copies of integrated pVS11 DNA, as represented by the ca. 7 kb full-length pVS11 band. *Sal*I cleavage of a circular 1.0 kb *loxP-bar-nos3′* fragment is expected to generate the same 0.3 kb *bar* fragment but also a new 0.62 kb *nos3′-loxP-bar* fragment (Figure 1C). The *bar* probe detected the 0.3 kb and 0.62 kb fragments,

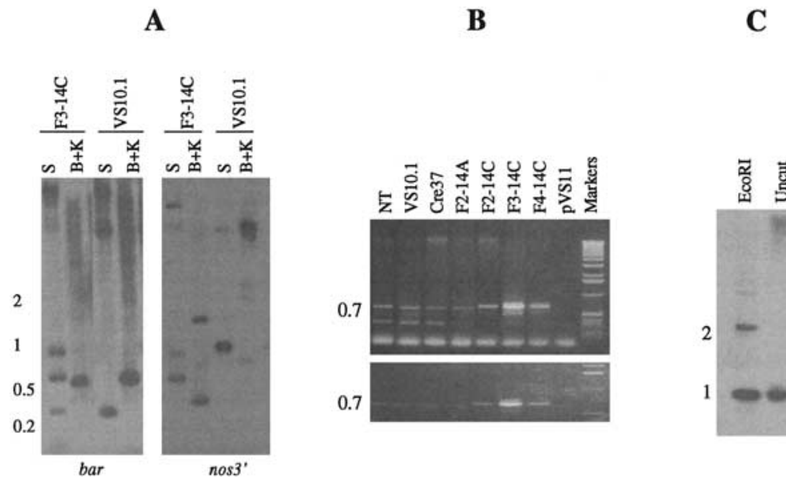

Figure 3. Structure of *bar circle*. A. *Sal*I- or *Bam*HI+*Kpn*I-cleaved genomic DNA of F_3-14C and VS10.1 hybridized with either *bar* or *nos3′* probe; fragment sizes in kb. B. PCR primers b + c on pVS11 DNA, genomic DNA of non-transformed (NT) VS10.1 and Cre37 parents, their resolved progeny F_2-14A and unresolved progeny F_2-14C, and the F_3 and F_4 progeny derived, respectively, from selfed F_2-14C and F_3-14C plants. The kb ladder serves as size markers. The lower panel is a longer run of the same gel (NT, VS10.1, Cre37 and F2-14A lanes) and a *bar circle*-specific band (14C lanes). C. Uncut and *Eco*RI-cleaved genomic DNA of a F_3-14C plant was probed with *bar* DNA. Sizes are given in kb.

whereas the *nos3′* probe detected the same 0.62 kb band. Both probes detected a ca. 1.0 kb band presumed to be the uncut *loxP-bar-nos3′* DNA. The presence of uncut DNA is not surprising since *Sal*I is sensitive to CpG methylation and cuts wheat DNA poorly.

*Bam*HI + *Kpn*I cleavage of VS10.1 DNA should yield a 0.54 kb *Bam*HI-*Kpn*I *bar* fragment and a >2.3 kb *nos-P2* transgene-host junction band. The 0.54 kb fragment was found by the *bar* probe, although a single-copy >2.3 kb transgene-host junction band is not detected by the *nos3′* probe. Instead, hybridization to high-molecular-weight DNA is seen, probably due to multiple copies of pVS11 DNA. With F_3-14C DNA, the same 0.54 kb band is found by the *bar* probe, but the *nos3′* probe detected a unique 0.38 kb fragment (Figure 3A). This 0.38 kb band is expected for a circular *loxP-bar-nos3′* molecule (Figure 1C). The *nos3′* probe also hybridizes to DNA from the *P2-cre-nos3′* locus, hence accounting for the 1.5 kb *Bam*HI-*Kpn*I and the >10 kb *Sal*I-derived bands. In these hybridizations, the presence of high-molecular-weight DNA in F_3-14C also suggests that the original VS10.1 locus did not resolve into a single copy, but compared to VS10.1 DNA, the number of pVS11 copies was somewhat reduced.

PCR was carried out on the genomic DNA of F_2-14A, F_2-14C, F_3-14C and F_4-14C with outward primers b + c as shown in Figure 1A and C. As control, the same reaction was tested on non-transformed (NT) wheat, VS10.1, Cre37, and pVS11 plasmid DNA. This PCR reaction failed to amplify a fragment from pVS11 DNA, but amplified two background fragments from wheat DNA of both transgenic and non-transgenic plants. One background fragment is about 0.7 kb, similar in size to a slightly larger fragment amplified only from F_2-14C, F_3-14C, and F_4-14C plants (Figure 3B). A clear size difference between the specific band and the background band can be seen in a longer-run gel (Figure 3B, lower panel). The larger size band, presumed to be from a circular *loxP-bar-nos3′* fragment was purified and subjected to an intermolecular Cre-*lox* reaction to see if it contained a functional *loxP* site. Intermolecular recombination between two different sized molecules was expected to form product bands of predicted sizes. The predicted product bands were indeed detected (data not shown). The DNA sequences of both of the ca. 0.7 kb PCR fragments were determined. The larger size 0.7 kb band has a sequence consistent with a circular *loxP-bar-nos3′* fragment, whereas the smaller size 0.7 kb band contains mixed sequences that bear no resemblance to the *loxP-bar-nos3′* DNA (data not shown).

Both the restriction enzyme analysis and the sequence of the 0.7 kb PCR product are consistent with the deduction of a circular *loxP-bar-nos3′* molecule. However, it does not rule out the possibility of a DNA rearrangement consisting of a tandem repeat of linear

loxP-bar-nos3′ fragments. For example, an excised circular *loxP-bar-nos3′* fragment might have reintegrated through Cre-*lox* site-specific integration into another copy of integrated pVS11 DNA. If that were the case, however, the *loxP-bar-nos3′* DNA would be linked to chromosomal DNA. To investigate the latter possibility, uncut DNA was examined alongside with *Eco*RI-cut genomic DNA (Figure 3C). Southern hybridization of F_3-14C DNA showed that the 1 kb *bar* fragment is present even in the uncut genomic DNA. This indicates that at least some of the hybridizing DNA is not physically linked to the genome, but exists extrachromosomally. The migration of the hybridizing DNA in agarose gel appears to be the same for uncut and *Eco*RI-cut DNA. This indicates that the presumptive circular *lox-bar-nos3′* fragment, or the '*bar circle*', is not a supercoiled molecule. Although many extrachromosomal DNA molecules have been reported to be supercoiled (Kinoshita *et al.*, 1985; Kunisada *et al.*, 1986; Sundaresan and Freeling, 1987), DNA circles produced by Cre-*lox* reactions should be relaxed due to the type I topoisomerase activity of Cre (Abremski *et al.*, 1986). Since Cre protein is a site-specific topoisomerase, i.e., it relaxes only *lox*-containing molecules, the observation that the 1 kb DNA appears to be migrating as a relaxed molecule is also consistent with the '*bar circle*' hypothesis.

Amplification in wheat cells?

An intriguing observation of the Southern data is that while F_2 plants contained an equal molar ratio of the 1 kb *bar circle* and the 2 kb *P1-bar* genomic fragments, F_3 and F_4 plants appear to have a higher ratio of the *bar circle* DNA than the genomic locus (Figures 2B and 3C). Two explanations are possible to account for this anomaly. One is that a higher proportion of cells, in this case the leaf cells, underwent Cre-mediated recombination in the F_3 and F_4 generations than in the F_2 generation and hence reducing the number of the 2.0 kb *P1-bar* fragments. Alternatively, or additionally, the *bar circle* may have replicated its DNA, and more copies were produced in the F_3 and F_4 plants. Although we have no data to favor one interpretation over the other, it is interesting to note that high copies of extra-chromosomal DNA in circular and linear forms have been reported in the nucleus of various plant species including wheat (van 't Hof *et al.*, 1982; Kinoshita *et al.*, 1986; Cuzzoni *et al.*, 1990; Buchowicz, 1997). Furthermore, evidence of their amplification has been obtained in bean, rice, pea and wheat (van 't Hof *et al.*, 1983; Kunisada *et al*, 1986; Cuzzoni *et al.*, 1990; Bucholc and Buchowicz, 1995), although their biological significance is not known. The excised *bar circle* would contain native chromatin structure and it is possible to speculate that under some circumstances, it might be recognized by the replication machinery as a substrate.

De novo generation of the bar circle*?*

Primer pairs c + b, a + d, and e + f were used to track the *bar circle* and the *vs10.1* and *cre37* loci, respectively. All F_4 progeny tested from the self-fertilized F_3-14C plant were found to contain both transgenic loci along with the *bar circle*. This indicates that the F_3-14C plant was homozygous for both the *vs10.1* and the *cre37* loci, and that the *bar circle* was either inherited or generated *de novo* in the F_4 progeny. If the *bar circles* were generated *de novo* in the progenies, their presence should be dependent on both the *vs10.1* and the *cre37* loci. To investigate this possibility, homozygous F_4-14C plants were out-crossed with wild-type plants, with F_4-14C serving as the pollen donor. A total of 10 F_1 progeny that contained both the *vs10.1* and the *cre37* loci were analyzed by PCR and Southern blotting to detect the *bar circle*. Only 2, 5F1 and 8F1, of the 10 progeny were found to contain the *bar circle*, and it is not known why 8 of them lacked this molecule. Since the F_4-14C plant was used as pollen donor and chloroplast or mitochondrial transmission through the male line is extremely rare, the detection of the *bar circle* in the F_1 outcross progeny indicates that the *bar circle* is not localized to either organelle.

A total of 31 F_2 progeny from self-fertilized 5F1 were analyzed by PCR for the segregation of the *vs10.1* and *cre37* loci. The *bar circle* in the segregating F_2 population was detected only in plants that contained both *vs10.1* and *cre37* loci (data not shown, see Figure 4). This correlation suggests that the *bar circle* is most likely generated *de novo*. To investigate this hypothesis, the two segregated loci, *vs10.1** (5F2-30) and *cre37** (5F2-31) were reciprocally crossed. Seven F_1 (5F2-30 × 5F2-31) plants were obtained that contained both *vs10.1** and *cre37** loci, and these also contained the *bar circle* as tested by PCR. Of these 7 F_1 plants, 4 were examined by Southern blotting and the presence of the *bar circle* was confirmed (data not shown, see Figure 4). When 5F2-31 (*cre37**) was crossed to the original VS10.1 line, 5 F_1 plants were obtained that contained *vs10.1* and *cre37** loci, all of which tested PCR-positive for the *bar circle*;

Figure 4. The presence of *bar circle* requires *cre37**. An F_4-14C plant backcrossed to wild type yields 5F1 and 8F1 (not shown). Segregated *cre37* and *vs10.1* loci derived from F_4-14C are referred to as *cre37** and *vs10.1**, which were subsequently crossed with VS10.1 and Cre37 lines, respectively. The presence of *bar circle* in the progenies of each of the crosses was determined by PCR and Southern hybridization.

Figure 5. Hypermethylation of *cre* locus. *Hpa*II (H)- or *Msp*I (M)-cleaved genomic DNA of Cre37 and Cre37* were probed with P2-1 or P2-2 DNA (see Figure 1D). Fragment sizes shown in kb.

this was confirmed by Southern blot analysis in 4 of the plants. When 5F2-30 (*vs10.1**) was crossed with the original Cre37 line, 8 F_1 plants containing both *vs10.1** and *cre37* loci were found negative for the *bar circle* by both PCR and Southern analysis (data not shown, see Figure 4). In the Southern blot of *Eco*RI-cut DNA, neither the 1 kb nor the 2 kb band was found. This indicates that while the *cre37* locus was active in deleting the *loxP-bar-nos3′* fragment, a *bar circle* was not maintained. Since the original *cre37* locus is not associated with *bar circles*, the *cre* gene in line B must have changed in activity from its original form in Cre37. Primers g + h (Figure 1D) were used to amplify the *cre*-coding fragment from F_4-14C and Cre37. However, the sequencing of these PCR products failed to identify a genetic change. This leaves the other possibilities such as a genetic change outside of the *cre*-coding region, or/and an epigenetic change such as the methylation of DNA.

Hypermethylation of cre *DNA*

To examine if the epigenetic modification in the *cre* locus is associated with changes in DNA methylation, a Southern blot was done on genomic DNA of Cre37 and Cre37* lines. To detect CpG and CpNpG methylation in the *cre* locus, genomic DNA was cleaved with *Hpa*II or *Msp*I and probed with the upstream (P2-1) or the downstream (P2-2) sequence of promoter *P2* (Figure 1D). Though both enzymes cleave CCGG, *Hpa*II is sensitive to CpG and CpNpG methylation, whereas *Msp*I is sensitive only to CpNpG methylation. With probe P2-1, a 0.75 kb band was detected in *Hpa*II- or *Msp*I-cleaved Cre37 and Cre37* DNA, suggesting a lack of methylation of the two CCGG sites. However, additional higher-size bands were detected in *Hpa*II-cleaved Cre37* DNA (Fig. 5, P2-1), indicating that a proportion of Cre37* cells contained CpG methylation in the adjacent CCGG sites within and possibly beyond the *P2* sequence (see Figure 1D).

With probe P2-2, hybridization of *Msp*I-cleaved DNA gave an expected ca. 1 kb band in Cre37, and a slightly larger band (>1 kb) with two minor bands (ca. 1.6 kb and ca. 1.8 kb) in Cre37* (Figure 5). This indicates that CpNpG methylation was undetectable in Cre37, but that at least one *Msp*I site was methylated in Cre37*. The minor ca. 1.6 and ca. 1.8 kb bands detected by probe P2-2, but not by P2-1, indicate that a proportion of Cre37* cells contain CpNpG methylation in 3 or 4 sites in the *cre* sequence (see Figure 1D). Hybridization of *Hpa*II-cleaved Cre37 DNA gave a ca. 1.2 kb band instead of a 1.0 kb band, indicating that one of the sites is CpG-methylated. With Cre37* DNA, a complex P2-2 hybridization pattern was detected on *Hpa*II-cleaved DNA, indicating several *Hpa*II sites in the *P2-cre* and its vicinity are methylated. The varying intensity of bands indicate that different leaf cells of Cre37* may contain different patterns of methylation, as also indicated by the P2-1 hybridization. Overall, the data support the theory of an epigenetic modification in the *cre37** locus. However, it remains to be investigated whether or not this methylation causes differential expression of the *cre* gene.

Concluding remarks

Cre- or FLP-mediated gene deletion is widely used in plant and animal genetic studies and biotechnological applications. When the gene is deleted in actively dividing cells, it is either diluted through cell divisions or degraded by the host enzymes. There is no report, so far, on the maintenance of the deleted DNA in the dividing cells. Ahmad and Golic (1996) have studied FLP-mediated deletion of the *white* gene of *Drosophila*, where a heat shock promoter controlled *FLP* gene. In heat-shocked flies, the *white* gene was deleted in all cells. While, actively dividing cells lost the deleted gene, non-dividing cells retained and expressed the gene, extrachromosomally. The degradation of the circular product is prevented probably due to the presence of native chromatin structure.

In our experiments, the *cre* gene was expressed by the maize ubiquitin promoter, which is active early in plant development (Cornejo *et al*., 1993). Indeed, in most cases the deleted fragments were not detected. It is conceivable that the *cre* gene was epigenetically modified in line B-F1 such that its expression occurs only in non-dividing cells, which leads to the retention of the *bar circle*. However, this hypothesis does not explain why other deletion products from the *vs10.1* locus, such as *lox511-P2-P1* circles, were not detected. One possible explanation is that *loxP* is more recombinogenic *in vivo* as compared to *lox511*. Further, in the present study, DNA was isolated from young leaves that are mitotically active. Therefore, the maintenance of the *bar circles* cannot be explained by the lack of dilution through cell divisions. This invokes the presence of some unknown mechanism in mitotically active cells, which may involve amplification and distribution of the *bar circles*. This mechanism, however, may not be active in rapidly dividing cells such as in shoot apical meristem because *bar circles* are probably not present in germ line cells. Several reports exist on the presence and amplification of extra-chromosomal DNA in certain growth conditions and specific tissue of plants (reviewed by Buchowicz, 1997).

Although we have observed the maintenance of the *bar circle* in 4 of the original 72 plants analyzed and, depending on one's view, it can be considered a rare occurrence, this could nonetheless be a cause for concern depending on how the Cre-*lox* technology is applied. For example, to address the concerns of transgenic DNA in food, suggestions have been made that transgenic DNA can be removed via Cre-*lox* site-specific recombination just before harvest of the edible parts of the plant (Keenan and Stemmer, 2002). Although the idea seems sound, the technical challenges are whether the excision reaction would be near 100% effective, and what would be the fate of the excised DNA. Based on our findings, an expectation would be that a late excision event could generate excision products that may persist in the cells. And, if they should replicate to a higher copy number, it could make the situation worse than if it were left in the genome.

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