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STRUCTURAL ANALYSIS OF TRANSGENE REARRANGEMENTS AND EFFECTS ON EXPRESSION IN TRANSGENIC MAIZE PLANTS GENERATED BY PARTICLE BOMBARDMENT

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ABSTRACT - We cotransformed maize embryogenic callus with two plasmids, one carrying the linked markers *bar* and *gusA*, and the other carrying the *Bacillus thuringiensis cry1Ab* gene. Molecular analysis of regenerated transgenic plants revealed a diverse range of transgene structures, including intact, truncated and internally-rearranged copies, and copies interspersed with genomic DNA. We found rearrangements associated with both functional and nonfunctional transgenes. We also found that rearrangements could affect the selected marker gene *bar* as well as the nonselected transgenes. Rearrangements often caused loss of transgene expression, but some rearranged transgenes were expressed normally. Conversely, while intact transgenes were often expressed, we also detected some nonexpressed copies, probably reflecting epigenetic silencing mechanisms. All the transgenic lines we studied contained multiple transgenes, generally comprising a mixed array of intact and rearranged copies. Rearrangement is rarely given serious consideration as a factor contributing to transgene silencing and variable transgene expression, but our data show that undetected rearrangements may have a major effect on transgene expression. This has significant implications, especially in studies attempting to correlate transgene copy number and expression levels.

KEY WORDS: Particle bombardment; Transgene rearrangement; Transgene expression; Long PCR

INTRODUCTION

Many factors affect the expression of foreign genes in transgenic plants (MEYER, 1998). One such factor is the tendency for exogenous DNA to undergo recombination and rearrangement. This phenomenon has been observed in many diverse systems and may reflect a combination of events occurring prior to, during or after the integration of exogenous DNA into the host genome (MAYERHOFER *et al.*, 1991; KONCZ *et al.*, 1994). The resulting integrated transgenes may be functional or non-functional in terms of expression.

Little is known of the precise causes and mechanisms of transgene rearrangement, although recent studies involving the characterisation of genomic-transgene and transgene-transgene junctions in plants have provided some indications (GHEYSEN *et al.*, 1991; MAYERHOFER *et al.*, 1991; GORBUNOVA and LEVY, 1997; SALOMON and PUCHTA 1998; KOHLI *et al.*, 1999). In animal systems, exogenous DNA usually undergoes recombination prior to integration to form larger arrays. The structure of these arrays depends on the amount, conformation and complexity of the exogenous DNA. Small amounts of homogeneous supercoiled plasmid DNA favour homologous recombination, resulting in the generation of tandem, head-to-tail arrays (MACFARLANE and WILSON, 1996). Alternatively, larger amounts of more heterogeneous DNA (especially linear DNA) favour illegitimate recombination and the generation of random arrays, because the high concentration of free DNA ends stimulates the production of DNA ligase (BISHOP and SMITH, 1989). This mechanism may predominate in plants (DE GROOT *et al.*, 1994; TSUKAMOTO and IKEDA, 1998).

The activation of DNA repair enzymes, including nucleases, ligases and components of the homologous recombination machinery, is a general response to cellular injury and the presence of exogenous DNA, reflecting a defence mechanism to protect genomic integrity (HUNOLD *et al.*, 1994). However, the effect of exposing exogenous DNA to this enzyme mix is extensive degradation, often resulting in the incorporation of truncated or otherwise rearranged sequences into transgene arrays. The mechanism of DNA integration in transgenic plants is also poorly understood, but is thought to reflect illegitimate recombination between exogenous DNA and breaks in the endogenous chromosomes (GHEYSEN *et al.*, 1991; MAYERHOFER *et al.*, 1991; KONCZ *et al.*, 1994; TINLAND, 1996). Indeed, a link between

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double-strand break repair, illegitimate recombination and the integration of foreign DNA in plants has been established by studies of genomic DNA-transgene junctions (GHEYSEN *et al.*, 1991; MAYERHOFER *et al.*, 1991; SALOMON and PUCHTA, 1998; KOHLI *et al.*, 1999). A recent model suggests that a given primary integration event may trigger further rounds of integration, resulting in complex rearrangements at the transgenic locus (KOHLI *et al.*, 1998). This may occur through the recruitment of further exogenous DNA to repair complexes assembling at the sites of chromosome breaks, and may also result in the deletion and rearrangement of genomic DNA at the site of integration, and the presence of genomic DNA sequences interspersed between transgene copies (MU CHEN *et al.*, 1995; TAKANO *et al.*, 1997; KOHLI *et al.*, 1998; NACRY *et al.*, 1998; PAWLOWSKI and SOMERS, 1998). Transgene recombination junctions in plants are often characterised by microhomology between the recombining DNA ends, suggesting an initial interaction between overlapping single-stranded tails. Such junctions have been observed in diverse systems including rice (TAKANO *et al.*, 1997; KOHLI *et al.*, 1999) and *Arabidopsis thaliana* (SAWASAKI *et al.*, 1998). Secondary structures are believed to play a role in these recombination events, resulting in transgene recombination hotspots such as the CaMV 35S promoter (KOHLI *et al.*, 1999). In a minority of cases, junctions show no homology at all, suggesting occasional illegitimate recombination by adventitious ligation of unrelated fragments. Recombination junctions are also characteristically AT-rich and include putative matrix attachment regions with consensus binding sites for DNA topoisomerase I (SAWASAKI *et al.*, 1998; KOHLI *et al.*, 1999). Since topoisomerases introduce single strand nicks into DNA, it is possible these may generate many of the free ends that take part in integrative recombination events.

The nature of each transgene rearrangement determines whether or not that specific transgene is expressed. Some rearrangements cause truncation of the coding region or deletions in the promoter, while others cause terminal deletions or remove intron sequences that do not affect transgene expression. Traditionally, transgene organisation has been characterised by genomic Southern blot analysis and sequencing across recombination junctions, while expression has been determined by western blot or assays for protein activity. Long PCR has recently emerged as a useful technique for structural transgene analysis because it allows more detailed characterisation of transgene rearrangements. Our

analysis using a combination of these techniques allowed us to carry out detailed characterisation of transgene rearrangements in maize plants, and confirmed that structural transgene integrity may not be a reliable indicator of transgene activity. The presence of both intact and rearranged copies at multi-copy transgenic loci, with no way to verify which transgene copies are expressed, suggests that studies attempting to show a relationship between transgene copy number and protein expression in transgenic plants should be interpreted with caution.

MATERIALS AND METHODS

Plant Material, Transformation and Regeneration of Transgenic Plants

The material used for transformation was maize embryogenic callus (*Zea mays* L. cv. Hill; ARMSTRONG and GREEN, 1985). The callus was maintained on MCN6 medium (4 g l⁻¹ Chu N6 basal salts, 1 x Erickson's vitamins, 1 mg l⁻¹ thiamin HCl, 1 mg l⁻¹ 2,4-dihydroxyphenylacetic acid (2,4-D), 20 g l⁻¹ sucrose, 2.88 g l⁻¹ L-proline, 0.1 g l⁻¹ Casamino acids). We cotransformed callus with two plasmids. The selectable plasmid pAHC25 carried the *gusA* and *bar* markers (CHRISENSEN and QUAIL, 1996) while the nonselectable plasmid pUBB carried the *cry1Ab* gene (SARDANA *et al.*, 1996). All three genes were driven by the maize ubiquitin-1 (Ubi-1) promoter. A 1:3 molar ratio of selectable to nonselectable plasmid DNA was used for cotransformation. In each procedure, a total of 10 µg plasmid DNA was mixed with 2.5 mg gold particles (0.95 µm diameter). The preparation of DNA-coated gold particles was as previously described (McCABE and CHRISTOU, 1993). The callus was bombarded twice, with a four-hour interval between bombardments. Four hours prior to the first bombardment, the callus was transferred to MCN6 medium supplemented with 36 g l⁻¹ mannitol and 36 g l⁻¹ sorbitol, providing an osmoticum concentration of 0.4 M (VAIN *et al.*, 1993). The callus was maintained on this medium for the duration of the bombardment procedure and for 16 h afterwards in darkness at 24°C, then transferred to fresh medium and incubated under the same conditions for three days. The callus was then transferred to selection medium PMCN6 (MCN6 supplemented with 3 mg l⁻¹ glufosinate ammonium, the ammonium salt of phosphinothricin; PPT) and maintained in darkness at 24°C. Callus was subcultured on the same medium at two week intervals. After 10 weeks, PPT-resistant colonies were transferred to shoot regeneration medium MCSR1 (MS basal medium supplemented with 0.25 mg l⁻¹ 2,4-D, 10 mg l⁻¹ BAP and 3 mg l⁻¹ PPT). Two weeks later, green shoots developing from these cultures were transferred to MCSR2 medium (MS basal medium supplemented with 3 mg l⁻¹ PPT) for shoot development. After a further two weeks, plants with fully developed shoot systems were transferred to rooting medium MCRR (MS basal medium supplemented with 10 g l⁻¹ sucrose and 3 mg l⁻¹ PPT). Rooted plantlets were then transferred to the glasshouse and grown to maturity.

Genomic Southern Blot Analysis

Aliquots of genomic DNA (20 µg) from randomly selected transgenic plants were digested with a fivefold excess of the appropriate restriction enzyme(s). To analyse pAHC25 integration

patterns, genomic DNA samples were digested separately with *KpnI* or *HindIII*. *KpnI* has a single restriction site in pAHC25, while *HindIII* releases the entire *gusA* expression cassette (4.2 kbp) leaving the entire *bar* expression cassette and vector backbone (5.5 kbp). To analyse pUBB integration patterns, DNA samples were digested separately with the enzymes *EcoRI* or *HindIII*. *EcoRI* has a single restriction site in pUBB, while *HindIII* excises the entire *cryIAb* expression cassette (4.1 kbp). Restriction fragments were fractionated by 0.8% agarose gel electrophoresis, and transferred onto Hybond-N⁺ membranes (Amersham) according to the method of WARREN and HERSHBERGER (1994). Sequences containing the *bar* gene were detected using a 0.5-kbp *BamHI-SphI* fragment of pAHC25, containing most of the *bar* coding region. Similarly, sequences containing the *gusA* gene were detected using a 1.8-kbp *BamHI-SacI* fragment of pAHC25, containing most of the *gusA* coding region. Finally, sequences containing the *cryIAb* gene were detected using a 1.8-kbp *BamHI-EcoRI* fragment of pUBB, corresponding to the *cryIAb* coding region. All probes were labelled with α -[³²P]-dCTP (3,000 Ci mmol⁻¹; Amersham) using the Prime-a-Gene labelling system (Promega) according to the manufacturer's instructions. Hybridisation, washing and exposure conditions were as previously described (WARREN and HERSHBERGER, 1994).

β -Glucuronidase (GUS) and Phosphinothricin Acetyltransferase (PAT) Assays

Histochemical GUS assays were carried out as described by JEFFERSON *et al.* (1987). PAT activity was determined using the thin-layer chromatography (TLC) method (DE BLOCK *et al.*, 1987) with 30 μ g protein extracts. Small leaf sections were ground to a fine powder under liquid nitrogen. When the nitrogen had evaporated, 1 ml of protein extraction buffer (100 mM Tris.HCl pH 8.1, 100 mM 2-mercaptoethanol) was added to the powder. The extracts were then centrifuged at 12,000 x g, 4°C for 10 minutes. Protein concentration was determined by the dye binding method (BRADFORD, 1976).

Western Blot Analysis of Cry1Ab Protein

Protein for western blot analysis was extracted and quantified as described above. SDS-PAGE was carried out using 30 μ g protein per lane, and the fractionated samples were transferred to Hybond-C nitrocellulose filters (Amersham) using the Trans-Blot semi-dry transfer apparatus (Bio-Rad) according to the manufacturer's instructions. Filters were incubated with anti-serum raised against Cry1Ab (S. Bano: National Institute of Excellence in Molecular Biology, Lahore, Pakistan). We used alkaline phosphatase (AP)-conjugated anti-rabbit IgG (Fc) (Promega) as

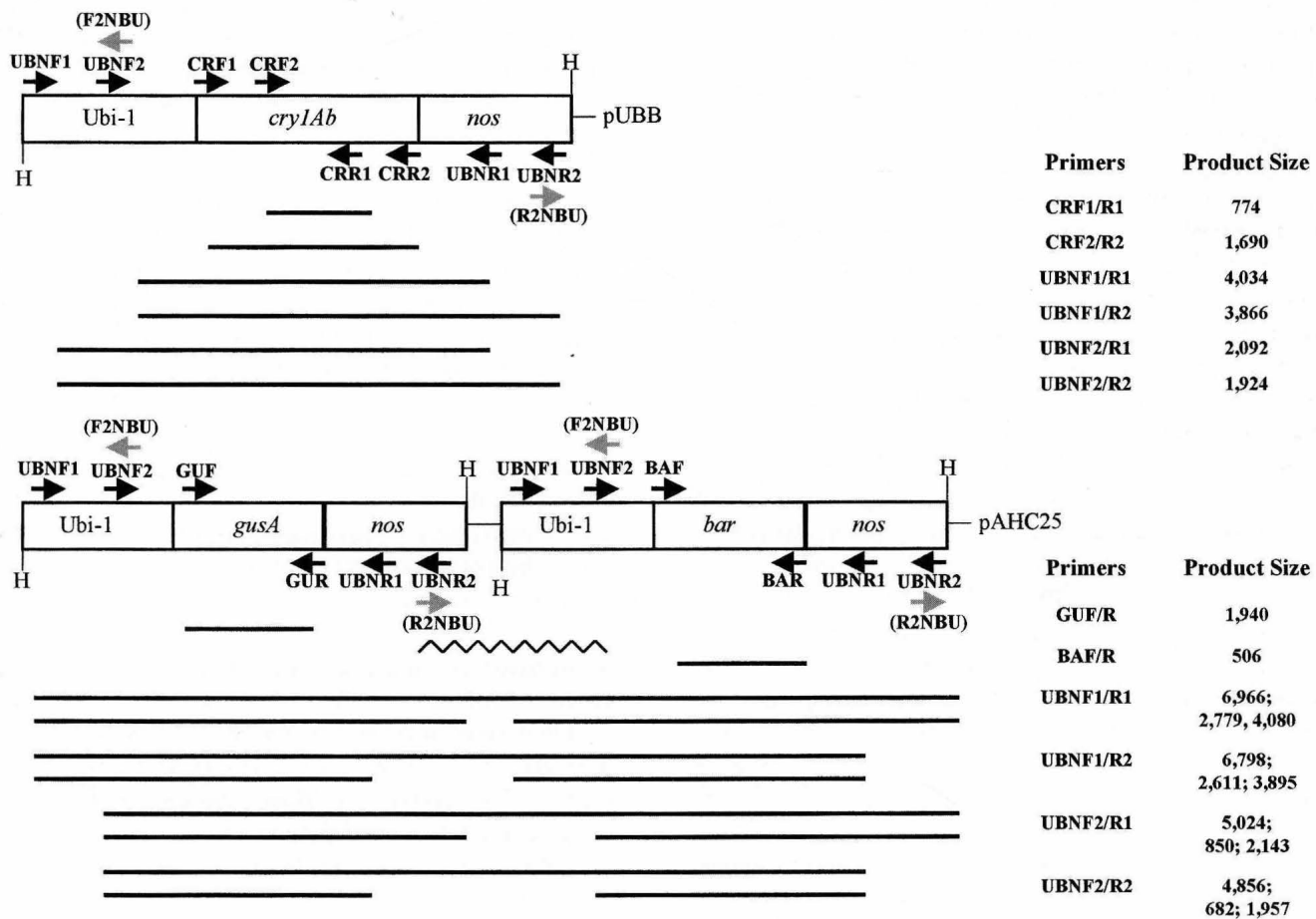


FIGURE 1 - Constructs, primers and products. Primers in black were used for standard L-PCR experiments. Primers in pale grey were used for I-PCR experiments. The orientation and approximate position of each primer with respect to the transforming plasmids (pUBB and pAHC25) is shown. PCR products are shown on the left with the specific primer pairs used and the expected product sizes on the right. H = *HindIII* restriction sites used for Southern blot analysis. Zig-zag line indicates region analysed in detail by sequencing (see Figure 4).

the secondary antibody, followed by AP-detection according to the manufacturer's recommendations.

Long PCR analysis

Long PCRs (CHENG *et al.*, 1994) were carried out in total reaction volumes of 25 µl, comprising 50 ng genomic DNA, 1x Expand™ PCR buffer-3, 2.25 mM MgCl₂, 50 µM each dNTP, 300 nM each primer and 0.25 µl DNA polymerase mix, prepared according to the supplier's recommendations (Boehringer Mannheim). The thermal profile for each reaction comprised an initial denaturation step at 93°C for 2 min followed by 10 fixed cycles (93°C, 10 s; 70°C, 10 s; 68°C, 4 min) and 20 progressively lengthening cycles (93°C, 10 s; 70°C, 10 s; 68°C for 4 min, increasing by 20 s each cycle) with a final hold at 68°C for 7 min. The sequences of the primers used, and their respective T_m values (in °C), were as follows: BAF, 5'-GGTCTGCACCATCGTCAACC-3' (61.4); BAR, 5'-CCCTGCAGTTACTATCAGATCTCG-3' (62.7); CRF1, 5'-CG-CATTGAAACCGGTTACACTCCCA-3' (74.5); CRR1, 5'-CTTGGGC AGAACACGGAAGCTACC-3' (74.5); CRF2, 5'-TCCCAC-CATCGGGCGCGGATCC ATG-3' (77.1); CRR2, 5'-GCCGGATTG-CAAGTTGCTGCCGCTT-3' (72.4); GUF, 5'-CGACGGCCT-GTGGGCA TTCA-3' (63.45); GUR, 5'-GGGGAGGCTACAGAT-GCTTTGC-3' (63.5); UBNF1, 5'-GACCCGGTCGTGCC CCTCTCTA-GAG-3' (75.9); UBNR1, 5'-TGACACCGCG CGCGATAATTTATCC-3' (75.7); UBNF2, 5'-TCGATGCTCACCCTGTTGTTTGGTG-3' (74.5); UBNR2, 5'-TAATCATCGCAAGACCGGCAACAGG-3' (74.7). The specific primer combinations used, and their expected product sizes, are shown in Fig. 1. PCR products were fractionated by electrophoresis through 0.8% agarose gels containing ethidium bromide, and visualised over a UV transilluminator. For Southern blot analysis of PCR products, 50 µg aliquots were loaded onto 0.8% agarose gels, and following electrophoresis, transferred onto Hybond-N⁺ membranes (Amersham). The membranes were probed in turn using labelled DNA fragments specific for the *gusA*, *bar* and *cry1Ab* genes as described above.

Characterisation of Rearranged Transgenes

Aliquots of genomic DNA (1 µg) from each transgenic maize plant were digested with appropriate restriction enzymes (see Fig. 1) and purified by phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation according to standard procedures (SAMBROOK *et al.*, 1989). Ligation reactions, to recircularise the genomic DNA fragments, were performed at 16°C overnight in a total volume of 250 µl containing 5 units of DNA ligase (Gibco-BRL). The ligation products were purified as described above and dissolved in a final volume of 25 µl, from which 2 µl aliquots were used for inverse polymerase chain reactions (I-PCRs). The reactions were essentially as described above for long PCR, except that the following primers were used: F2NBU (inverse of UBNF2), 5'-CACCAAACAACAGGGTGAG-CATCGA-3'; and R2NBU (inverse of UBNR2), 5'-CCTGTTG CCG-GTCTTGGCATGATTA-3' (see Fig. 1). F2NBU amplifies the Ubi-1 promoter/intron complex and any genomic DNA lying upstream, whereas UBNR2 amplifies the *nos* terminator and any genomic sequences lying downstream. I-PCR products were fractionated by 0.8% agarose gel electrophoresis and isolated using the QIAQuick gel extraction kit (Qiagen). The products were subcloned in the pGEM-T vector (Promega) and 50 µg aliquots of DNA were fractionated by electrophoresis through 0.8% agarose gels and transferred to Hybond-N⁺ nylon membranes (Amersham) for Southern blot hybridisation as described above. A 1.4-kbp *SalI-PstI* fragment of pAHC25 (including part of the Ubi-1

promoter/intron complex) and a 271-bp *SacI-SpBI* fragment of pAHC25 (containing the *nos* terminator) were used as probes to confirm the authenticity of the I-PCR-derived fragments. Some amplification products (500 ng aliquots) were then used for double stranded DNA sequencing (BigDye™ Cycle Sequencing Ready Reaction Kit, PE Applied Biosystems) according to the manufacturer's recommendations. We used the standard SP6 and T7 sequencing primers (Promega) together with F2NBU and R2NBU. The cycling profile comprised an initial denaturation step at 96°C for 1 min, followed by 25 cycles (96°C, 30 s; 50°C, 15 s; 60°C, 4 min).

RESULTS

Bombardment and Recovery of Transgenic Plants

PPT-resistant embryogenic maize callus was observed 10 weeks after particle bombardment. Following regeneration, 30 independent transgenic plants were recovered. The cotransformation frequency for pAHC25 and pUBB was 80%.

Integration Patterns and Transgene Copy Numbers

We analysed randomly selected primary transformants by Southern blot hybridisation to characterise integration patterns for the *bar*, *gusA* and *cry1Ab* genes and estimate transgene copy numbers.

Southern blots hybridised with the *bar*, *gusA* and *cry1Ab* probes showed varying banding patterns, confirming that the transgenic plants represented independent transformation events. Transgene copy number was estimated from copy number reconstructions (JOHNS *et al.*, 1985) and the intensity and number of bands on Southern blots where genomic DNA had been digested with an enzyme cutting once in the transforming plasmid. All seven lines contained multiple copies of all three transgenes per haploid genome (data not shown).

Structural Transgene Analysis by Southern Blot Hybridisation

DNA from the chosen transgenic lines was then digested with *HindIII* to investigate transgene integrity. The enzyme releases diagnostic fragments comprising the Ubi-1 promoter, the coding region and the *nos* terminator, from all three expression units. The expected sizes of the diagnostic fragments were 5.5 kbp (*bar*), 4.2 kbp (*gusA*) and 4.1 kbp (*cry1Ab*). Where rearrangements had occurred, bands of different sizes were generated. These results are summarised in Table 1.

TABLE 1 - Transgene organisation and expression in maize plants. Left column shows characteristics of each of the three transgenes: the presence/absence of normal and truncated fragments on genomic Southern blots, the presence/absence of long PCR products using primers UBNF1/UBNFR1 and the three sets of gene-specific primers, and transgene activity determined by PAT and GUS assay, and western blots to detect *Cry1Ab* protein. L-PCR = long PCR; GS-PCR = gene-specific PCR.

	Line						
	1	2	3	4	5	6	7
<i>bar</i>							
Normal fragment	+	+	+	+	+	+	+
Truncated fragment	+	—	—	+	+	—	—
L-PCR UBNF1/R1	+	+	—	+	+	—	—
GS-PCR BAF/R	+	+	+	+	+	+	+
PAT activity	+	+	+	+	+	+	+
<i>gusA</i>							
Normal fragment	+	+	+	+	+	+	—
Truncated fragment	+	+	—	—	+	+	+
L-PCR UBNF1/R1	+	+	—	+	+	+	—
GS-PCR GUF/R	+	+	+	+	+	+	+
GUS activity	+	+	—	+	+	—	—
<i>gusA-bar</i> linkage	+	+	—	+	—	—	—
<i>cry1Ab</i>							
Normal fragment	+	+	—	+	—	+	+
Truncated fragment	—	—	+	—	+	—	+
L-PCR UBNF1/R1	+	+	—	+	—	—	—
GS-PCR CRF2/R2	+	+	+	+	+	+	+
<i>Cry1Ab</i> expression	+	+	—	+	—	—	—

(+) and (—) indicate the presence and absence, respectively, of DNA fragments, PCR products, protein expression or enzyme activities as appropriate.

The diagnostic 5.5 kbp *bar* fragment was detected in all the transgenic plants we analysed (Fig. 2A). This was expected, since *bar* was the marker used to select transgenic plants. Additional bands of different sizes were detected in lines 1, 4 and 5, probably reflecting transgene rearrangements. The 4.2-kbp *gusA* fragment was detected in six of seven lines (Fig. 2B, lines 1-6). Additional bands reflecting transgene rearrangements were observed in lines 1, 2, 5, 6 and 7. Notably, line 7 contained *only* truncated *gusA* bands, and not the expected diagnostic fragment. Five of seven lines showed the 4.1 kbp *cry1Ab* fragment (lines 1, 2, 4, 6 and 7 in Fig. 2C). Additional truncated bands were observed in lines 3, 5 and 7, with lines 3 and 5 generating truncated bands only.

Structural Transgene Analysis by Long PCR

To investigate the nature of transgene rearrangements in the selected transgenic lines, we carried out long PCRs with primer combination UBNF1 and

UBNFR1 to identify the regions involved. The expected product sizes are shown in Fig. 1. Amplification products were adjusted to 50 pg and analysed by Southern blot hybridisation to confirm their authenticity. Blotted long PCR products were hybridised with the same three gene-specific probes used on the genomic Southern blots discussed above. The results are summarised in Table 1.

In certain lines, full-length diagnostic products were not generated. The expected 2.8-kbp *bar* amplification product was not detected in lines 3, 6 and 7 (Fig. 3A). Similarly, the expected 4-kbp *gusA* amplification product was not detected in lines 3 and 7 (Fig. 3B) and the 4-kbp *cry1Ab* diagnostic amplification product was not detected in lines 3, 5, 6 and 7 (Fig. 3C). The expected products were generated in the remaining lines, and in positive controls using plasmid DNA as the template. However, to confirm that the lack of products did not simply reflect PCR failure, further experiments were carried out using gene-specific primer sets BAF/BAR,

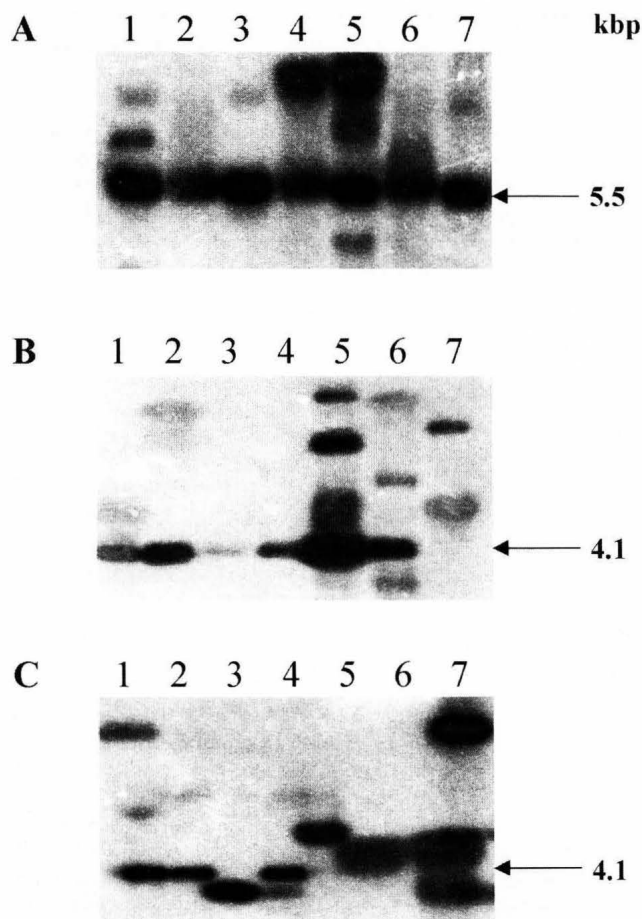


FIGURE 2 - Southern blot analysis of seven transgenic maize plants. Genomic DNA was digested with the restriction enzyme *Hind*III and probed with the coding region of (A) the *bar* gene; (B) the *gusA* gene and (C) the *cry1Ab* gene.

GUF/GUR, CRF1/CRR1 and CRF2/CRR2. These were designed to amplify shorter DNA fragments corresponding to the coding region of the three genes (Figs. 3A-C). Successful amplification with these gene-specific PCR (GS-PCR) primers suggested that external UBNF1 and UBNR1 primer annealing sites had been deleted or disrupted in those lines failing to generate L-PCR products.

The *gusA* and *bar* genes are linked on the plasmid pAHC25 (Fig. 1). Since each expression unit comprises the same Ubi-I promoter and *nos* terminator, the primer set UBNF1/UBNR1 should generate three DNA products when pAHC25 is used as the template, one representing *bar* (2.8 kbp), one representing *gusA* (4.0 kbp), and one representing both (7.0 kbp). Hybridisation with the *bar* probe should

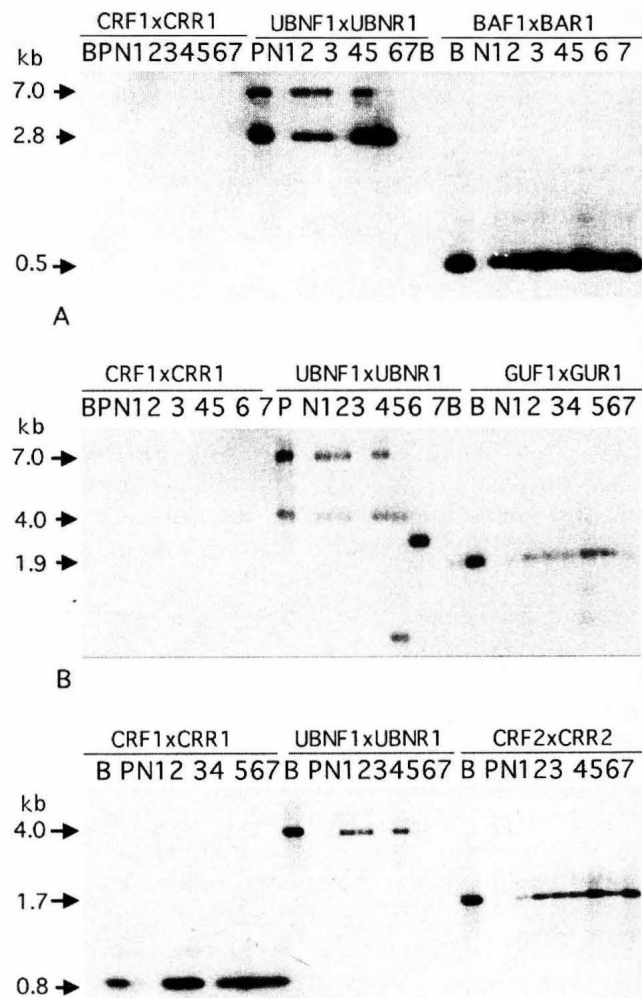


FIGURE 3 - Southern blot analysis of long PCR products from seven transgenic maize plants. (A) Amplification of *bar* with primer combinations CRF1/CRR1, UBNF1/UBNR1 and BAF1/BAR1. P = plasmid pAHC25, B = plasmid pUBB, N = negative control (non-transformed plant). Lanes 1-7 represent independent transgenic plants. The expected product sizes are indicated by arrowheads. (B) Amplification of *gusA* with primer combinations CRF1/CRR1, UBNF1/UBNR1 and GUF1/GUR1. (C) Amplification of *cry1Ab* with primer combinations CRF1/CRR1, UBNF1/UBNR1 and CRF2/CRR2.

identify two of the products (2.8 kbp and 7.0 kbp) and hybridisation with the *gusA* probe should also identify two products (4 kbp and 7.0 kbp). If the same analysis was carried out using genomic DNA from transgenic plants, similar products would be generated only if linkage between the two genes was retained. Such linkage was observed only in lines 1, 2 and 4 (Figs. 3A and B). A single hybridising band in line 5 (Fig. 3A) suggested the loss of linkage between *bar* and *gusA*. In lines 5 and 6, two truncated

gusA bands were detected (Fig. 3B), suggesting that transgene rearrangement involved either deletion or inversion of some of the transgene copies.

Structural Analysis of Transgene Sequences

We used primers annealing to the Ubi-1 intron and *nos* terminator regions to sequence recircularised genomic DNA amplified by inverse polymerase chain reaction (I-PCR). The UBNF1/UBNR1 and UBNF2/UBNR2 primer sets, used for long PCR amplifications in Fig. 3, anneal within these regions. Line 5 was chosen for detailed analysis because it carried the greatest number of transgene rearrangements.

I-PCR products were subcloned in the vector pGEM-T, and Southern blot analysis was carried out to confirm their authenticity and determine if they contained the Ubi-1 promoter/intron complex and the *nos* terminator. Only those clones containing both Ubi-1 and *nos* were selected for sequencing. This strategy was designed to minimise chances of selecting maize genomic sequences cross-hybridising with the recombinant ubiquitin-1 promoter/intron, originally derived from maize (CHRISTENSEN and QUAIL, 1996). Ten inverse PCR products from line 5 were selected for sequencing, and the results were compared to the Ubi-1 and *nos* sequences of the vector.

Four rearranged transgene sequences were detected (Fig. 4). In one case, a genomic sequence identical to a 64-nt sector of the *Z. mays* α -zein gene (THOMPSON *et al.*, 1992) had inserted between the *nos* terminator of *gusA* and the promoter of *bar*. The insertion probably resulted from illegitimate recombination involving 10-nucleotide microhomology at the left junction and three-nucleotide microhomology at the right junction (Fig. 4A). An inversion of the Ubi-1 promoter sequence was also detected (Fig. 4B). Three-nucleotide homology at the inversion breakpoint suggested this rearrangement also involved microhomology-mediated illegitimate recombination. We also observed recombination between the *nos* terminator and the *gusA* gene involving four-nucleotide homology at the junction. The same four nucleotides were involved in a second recombination event between the *nos* terminator and the vector backbone (Figs. 4C, 4D).

Functional Transgene Analysis

TLC phosphinothricin acetyltransferase (PAT) assays were carried out to determine the activity of the *bar* gene, while histochemical GUS assays were used to determine the activity of the *gusA* gene. Western blot analysis was carried out to detect

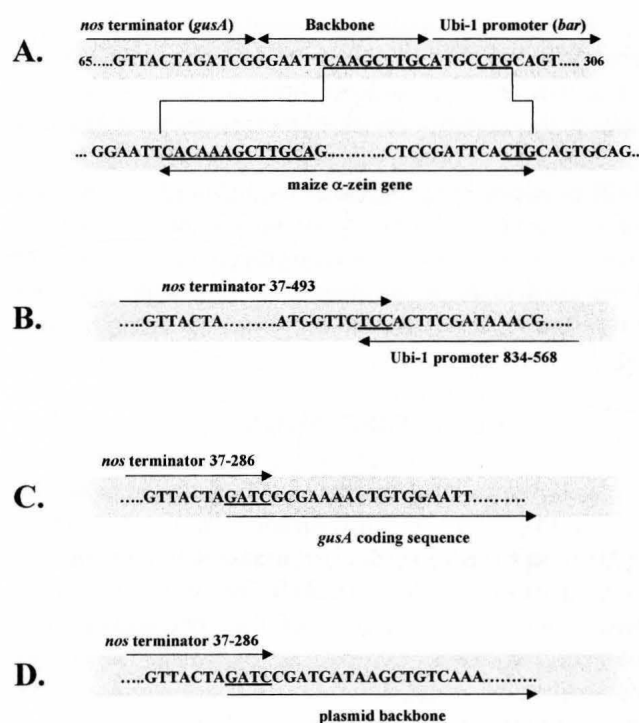


FIGURE 4 - DNA sequence characterisation of rearrangements in line 5. (A) Integration of a genomic fragment homologous to the maize α -zein gene between the *nos* terminator of *gusA* and the Ubi-1 promoter of *bar*. The 19-bp backbone sequence shares 10 nucleotides of homology with the α -zein gene at the left junction (excluding a three-nucleotide insertion, CAA), and three nucleotides of homology at the right junction. (B) Detection of an inversion event: illegitimate recombination has occurred between the *nos* terminator and Ubi-1 promoter involving microhomology at the sequence TCC. (C) Characterisation of a rearrangement involving the *nos* terminator and the *gusA* coding sequence, involving microhomology at the tetranucleotide sequence GATC. (D) The same region is involved in another rearrangement, this time involving the *nos* terminator and part of the vector backbone. Numbers are arbitrary, assigned on the basis of our sequencing reactions.

Cry1Ab protein. The results are summarised in Table 1. All transgenic plants showed PAT activity, as expected, and these plants also released the expected diagnostic *bar* fragment when analysed by Southern blot hybridisation (see Fig. 2A).

Transgenic lines 1, 2, 4 and 5 showed GUS activity. This was also expected, since Southern blots confirmed the presence of the expected diagnostic *gusA* fragment in these lines (Fig. 2B). Lines 3 and 6 also released the expected *gusA* fragment (Fig. 2B) but showed no GUS activity. Line 7 also showed no GUS activity, and the diagnostic *gusA* fragment was absent from the genomic DNA (Fig. 2B).

Western blot analysis showed that only lines 1, 2 and 4 expressed Cry1Ab protein (Table 1). The lack of transgene expression in lines 3 and 5 is likely to reflect the consequences of rearrangement because the expected diagnostic DNA fragment and long PCR products were not detected (Fig. 3C). The lack of transgene expression in lines 6 and 7 may be due to epigenetic silencing mechanisms, as both lines carried the intact diagnostic fragment, although line 7 also carried additional rearranged fragments.

DISCUSSION

It would be desirable to introduce genes of agronomic value into important crop plants such as maize, and to have full and predictable control over their expression. Unfortunately, we still have a relatively poor understanding of the mechanisms that control transgene expression, especially in monocots. Studies in model dicot species such as *Arabidopsis*, tobacco and petunia have identified a number of factors that contribute to transgene silencing and variable transgene expression (MEYER, 1998). In plants carrying single copy transgenes, the position of integration may be the most significant factor. In plants with multicopy transgenic loci, homology-dependent interactions between transgenes are purported to have a significant influence, and many believe that there is a fundamental inverse relationship between transgene copy number and expression level. The evidence from monocot plants is less convincing, with different studies showing positive or negative correlations between transgene copy number and expression level, or indeed no correlation at all (COOLEY *et al.*, 1995; STOCKHAUS *et al.*, 1987; SHIRSAT *et al.*, 1989). These studies show that the relationship between copy number and expression level in monocots is complex, and other influences may be involved.

One factor that is often inadequately addressed in such studies is the presence of transgene rearrangements. It is well known that DNA introduced into plant cells is exposed to a panoply of enzymes stimulated by the transformation process itself and the effect of excess DNA (HUNOLD *et al.*, 1994). The exogenous DNA is therefore exposed to nucleases, ligases and components of the recombination machinery, which not surprisingly results in the integration of truncated and otherwise rearranged copies. Recent studies of plasmid-plasmid junctions in monocots have confirmed the predominance of

microhomology-mediated illegitimate recombination between copies of the transforming plasmid(s) at multicopy transgenic loci, suggesting that rearrangements may occur very frequently (GORBUNOVA and LEVY, 1997; SALOMON and PUCHTA, 1998; KOHLI *et al.*, 1999). These studies have also shown the tendency for certain, recombinogenic sequences to initiate illegitimate recombination events (e.g. a potential cruciform structure in the CaMV 35S promoter; KOHLI *et al.*, 1999). However, in most studies, the structure of transgenic loci is characterised by simple Southern blot experiments, and the full impact of transgene rearrangements upon expression is not realised.

We transformed maize callus with three genes and selected seven plant lines for detailed analysis of transgene structure and expression. The presence of transgene rearrangements was first suggested by the appearance of truncated bands on genomic Southern blots where diagnostic fragments of a specific size were expected. Such bands were seen in all lines, either in addition to or instead of the expected bands. We carried out further characterisation of rearrangements in these lines by long PCR, as this provided a more precise diagnostic assay. Southern blot analysis of long PCR products followed by sequencing showed that integration of foreign DNA into the maize genome was accompanied by extensive transgene rearrangements, including truncations, internal deletions, insertions of genomic DNA and inversions. In several cases, we found that rearrangements had deleted or disrupted external PCR primer sites (in the promoter and terminator) while leaving the internal gene-specific primers sites intact. The loss of external annealing sites could reflect erosion by exonuclease activity, a phenomenon reported in many plant systems (TAKANO *et al.*, 1997; KOHLI *et al.*, 1998; NACRY *et al.*, 1998). An important issue raised by these results is the distribution of intact and rearranged transgene copies at multicopy loci. We presented strong evidence that all our plants carried both intact and rearranged transgene copies. However, genomic Southern blot analysis was sufficient for the detection of only the most obvious structural aberrations, such as large deletions, while many more subtle types of rearrangement were detected by long PCR. These results suggest that in the majority of studies, most transgene rearrangements go undetected and their potential influence on transgene expression is ignored. For example, multicopy transgenic loci were observed in an earlier study of transgenic maize plants, on this occasion regenerated

from cell suspension cultures transformed by particle bombardment (REGISTER *et al.*, 1994). These loci were also shown to contain rearrangements, but only at the resolution of a standard genomic Southern blot. Perhaps for this reason, the authors of this report concluded that more frequent transgene rearrangements occurred in the nonselected transgenes compared to the selected marker. In contrast, we found that rearrangements were just as likely to affect the selectable marker, probably because long PCR enabled us to detect a broader spectrum of rearrangements.

Our sequencing results highlighted some of the rearrangements foreign DNA might undergo following introduction into maize, but because we only analysed two short regions of the transformation plasmid, we drew no conclusions concerning the existence of recombination hotspots such as those observed by KOHLI *et al.* (1999). We did confirm, however, that rearrangement junctions involved short regions of homology (2-10 bp) between the recombining ends, confirming the predominance of microhomology mediated recombination in transgene rearrangement and integration.

The analysis of transgene function showed that structural integrity was not a reliable indicator of transgene activity. Intact and rearranged copies were easily discriminated by Southern hybridisation and long PCR, but the effects of such rearrangements on transgene expression were unpredictable. In this study, intact transgenes were defined as those generating the expected diagnostic fragment on Southern blots *and* the expected long PCR product(s) using the UBNF1/UBNF2 primer combination. In the case of the *bar* gene, all seven lines carried the diagnostic Southern fragment but only lines 1, 2, 4 and 5 also generated the long PCR product. However, all seven lines expressed *bar*, as expected given the regime of PPT selection, suggesting that the structural aberrations in lines 3, 6 and 7 did not affect transgene expression. The structural alterations were too subtle to detect by Southern blot, and gene-specific PCR showed that the *bar* coding region was not affected. It is therefore likely that these structures reflected exonuclease-dependent nibbling at the ends of the construct, resulting in the loss of at least one external primer site but no loss of sequences critical for transgene expression.

Six of the lines (lines 1-6) carried the expected *gusA* diagnostic fragment, but line 3 lacked the corresponding long PCR product. Interestingly, line 3 showed no GUS activity, suggesting that in this case

a subtle rearrangement detectable by long PCR but not by genomic Southern blot resulted in the loss of transgene expression. There was also no GUS activity in line 7, which lacked both a diagnostic Southern fragment and a long PCR product. In this case, loss of transgene expression probably resulted from the more overt rearrangement detected by Southern blot. However, despite generating both a diagnostic Southern blot fragment and the correct long PCR product, line 6 also showed no GUS activity. It is possible that loss of expression in this line resulted from rearrangements that we could not detect using our assay, but the inactivity could reflect an alternative epigenetic silencing mechanism such as *de novo* DNA methylation or the production of aberrant RNA species (MEYER, 1998).

Three of the lines (1, 2, 4) carried intact *cry1Ab* transgenes and showed Cry1Ab expression. Two lines (6 and 7) generated diagnostic *cry1Ab* fragments on Southern blots but no long PCR products, and in both lines *cry1Ab* was inactive. As was the case for *gusA* in line 3, it is possible that the subtle rearrangements detected by long PCR resulted in the loss of *cry1Ab* expression. In the absence of long PCR data, the natural assumption would be that these plants contained intact transgenes and had undergone some form of silencing. Thus, the influence of epigenetic silencing mechanisms would be exaggerated when rearrangement could be the cause of transgene inactivity. The remaining two lines (3 and 5) lacked both a diagnostic Southern fragment and a long PCR product, and lacked *cry1Ab* expression. It is likely that overt transgene rearrangements were responsible for the lack of expression in these lines.

Our results highlight some of the problems and misconceptions that might cause erroneous conclusions to be drawn from transgene expression studies. Firstly, transgene rearrangements occur very frequently and are likely to be present at most multicopy transgenic loci. Secondly, many such rearrangements are impossible to detect by traditional genomic Southern blot analysis, which could lead to false assumptions of epigenetic transgene silencing. Thirdly, the effects of transgene rearrangements are unpredictable: we described rearrangements associated with both active and inactive transgenes, as well as apparently intact transgene copies that showed no activity. Such phenomena may reflect DNA rearrangements occurring respectively in non-critical regions (e.g. the Ubi-1 intron, where rearrangements would be unlikely to disrupt trans-

gene expression) and essential sequences (e.g. the promoter or coding region, where rearrangements would have more dramatic effects). Finally, it is not straightforward to determine which transgenes at a multicopy transgenic locus are active or inactive. Therefore, studies showing a relationship between transgene copy number and expression level should be examined critically to consider the effects of other molecular factors, such as rearrangements. While our results show that transgene rearrangements occur frequently in transgenic maize plants, such rearrangements do not always abolish transgene expression. The analysis of transgene rearrangements that permit continued stable expression of the transgene product, and their comparison to rearrangements that result in transgene inactivity, could help in the design of better transformation vectors, e.g. by deleting sequences that actively promote recombination. In time, this could allow the more routine recovery of plants with stable and predictable levels of transgene expression.

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