Brief communication

Inactivation of a transgene due to transposition of insertion sequence (IS136) of *Agrobacterium tumefaciens*

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Agrobacterium strains harbour insertion sequences, which are known to transpose into genomes as well as into Ti plasmids. In this study we report the inactivation of a transgene due to transposition of the *A. tumefaciens* insertion sequence IS136. The transposition was discovered following transformation of plant tissues, although the fidelity of the binary vector was confirmed following transformation into *Agrobacterium*. Such transpositions are rare but can occur and it is thus important to check the fidelity of the binary vector at different times of *Agrobacterium* growth in order to avoid failure in achieving transgene expression.

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Transgenic plants are routinely developed by *Agrobacterium*-mediated transformation protocols. In several instances, the transgene transferred to the host plant is found to be inactive. Inactivation of the transgene has been attributed to incomplete transfers (Deroles and Gardner 1988; Hobbs *et al.* 1990; Zhang *et al.* 2008), structural reorganisations (Meyer 1999, 2000) and epigenetic modifications (Matzke and Matzke 1998; Matzke *et al.* 1999, 2000 Kooter *et al.* 1999; Mourrain *et al.* 2007). We report here an instance of transgene inactivation due to the transposition of an *A. tumefaciens* insertion sequence (IS136) into the transgene.

In an attempt to assess the activity of a fusion protein in transformed calli of cotton (*Gossypium hirsutum* Coker 310FR), we carried out *Agrobacterium*-mediated transformation of cotyledonary leaves of 7-day-old seedlings. A modified *cry1Ac* gene (Perlak *et al.* 1990) from *Bacillus thuringiensis* was fused in frame to the 3′ end of the s*oyabean trypsin inhibitor* (Kunitz type) gene *(SBTI)* isolated from *Glycine max* (Song *et al.* 1993). The fusion gene was placed under the transcriptional control of the double enhancer version of the cauliflower mosaic virus 35S (35Sde) promoter and cloned in the binary vector pPZP200 (Hajdukiewicz *et al.* 1994) (figure 1) carrying the *nptII* gene as a selection marker. The binary vector was transformed into *A. tumefaciens* strain GV3101, which was used to transform the cotyledonary leaves. The transformed explants were washed with Augmentin (250 mg l^{-1}) and placed on Murashige and Skoog (MS) T1 medium (1x MS salts, 1x B5 vitamins, 3% glucose, 0.2% phytagel, hormones (2,4-D [100 μg l^{-1}], kinetin [500 μg l^{-1}], pH 5.8) with kanamycin at a concentration of 50 mg l^{-1} to induce the formation of callus (Chaudhary *et al.* 2003). The medium also contained Augmentin (250 mg l^{-1}). After about a month, total protein was isolated from 20 independent calli to assess the level of expressed fusion protein by enzyme-linked immunosorbent assay (ELISA) using antibodies against Cry1Ac (Cry1Ac/Ab

Keywords. *Agrobacterium tumefaciens*; insertion sequence; transgene inactivation; transposition

Abbreviations used: ELISA, enyme-linked immunosorbent assay; IS, insertion sequence; RT-PCR, reverse transcriptase polymerase chain reaction; SBTI, soyabean trypsin inhibitor (Kunitz type); MS, Murashige and Skoog; 2,4-D, *2*,4-dichlorophenoxyacetic acid

quantitative ELISA kit, Envirologix, USA), which is one of the fusion partners. We could not detect the expression of Cry1Ac protein in all the calli tested. In order to evaluate whether this was due to a transcriptional or post-transcriptional event, we analysed the levels of the transcript for the fusion protein in the calli by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. RNA isolated from transformed calli was treated with DNAse (DNA-*free*™ kit, Applied Biosystems, USA) and cDNA synthesis carried out from total RNA using MuLV reverse transcriptase and Oligo $d(T)_{16}$ primers (GeneAmp® RNA PCR, Applied Biosystems, USA). The cDNA pool was used to carry out gene-specific amplification using two sets of primers (figure 1). Primer set C(F) and C(R) was designed to amplify a part of the *cry1Ac* transcript while S(F) and S(R) would amplify the *SBTI* part of the transcript. We observed an amplicon of 420 bp, as expected, when a part of the *cry1Ac* transcript was amplified (figure 2A). However, when amplification of the *SBTI* part of the fusion transcript was carried out, we observed an amplicon that was 2 kb in size in addition to the expected amplicon of 654 bp size in all the transformed calli tested (figure 2B). Gene-specific amplification was carried out as a control on RNA (without the reverse transcriptase step), which showed no amplified fragments (figure 2C). Sequencing of the 2 kb fragment revealed the presence of a 1.3 kb insertion sequence showing ~100% identity with the insertion element IS136 (Accession No. X56562). The insertion in all the cases was observed 183 bp downstream to the initiation codon (ATG) of the fusion protein. Interestingly, a microhomology of the four-base pair (TGAA) was observed between the site of insertion in the *SBTI* gene and the 5′ end of the IS136 element. Further, an insertion of 9 bp (GGAAATGAA) was observed at the downstream junction of the IS136 element and the *SBTI* gene. The rest of the amplicon showed 100% similarity with the sequence of the SBT1 fusion partner. IS136 is reported to have originated from the chromosome of *A. tumefaciens* strain A136, which is a derivative of the nopaline strain C58 (Vanderleyden *et al.* 1986). The *A. tumefaciens* strain GV3101 used in the present study is also a derivative of C58 (Pniewski *et al.* 2006) and is thus expected to carry the IS element in its chromosomal DNA. Sequencing of the 654 bp fragment showed a 100% match with the expected sequence of the transcript. It thus seems that the calli were transformed with two kinds of T-DNA, with and without insertion of IS136. Although a normal transcript was observed by RT-PCR analysis in the transformed calli, it was probably present in such small quantities that no appreciable amount of Cry1Ac protein was present for detection by ELISA.

In order to check the presence of two kinds of T-DNA in the *A. tumefaciens* culture, the binary vector was isolated from the *Agrobacterium* culture used for the transformation experiment. The isolated plasmid was re-transformed into *E. coli* strain XL1 Blue. The plasmid DNA was then isolated from independent transformed *E. coli* cells and PCR was subsequently carried out using the S(F) and S(R) primers. It was observed that while ~10% of the clones showed an amplicon of the expected size, the rest showed the presence of the IS element (figure 3). It thus seemed that transposition occurred during growth of the transformed *Agrobacterium* strain. IS elements are reported to have hotspots for integration, although no specific report is available regarding IS136. In order to test the presence of such hotspots, if any, in the fusion gene, we re-transformed *Agrobacterium* with the binary vector pPZP200: *SBTI+Cry1Ac* and allowed it to grow over several generations. Analysis of the binary vector using *SBTI*-specific primers revealed that no transposition had occurred, suggesting that the insertion observed by us was a rare event.

In the normal course of development of transgenics by *Agrobacterium*-mediated transformation, once the fidelity of the binary vector from transformed *Agrobacterium* is tested, one proceeds with plant transformation experiments. The status of the binary vector in *Agrobacterium* during subsequent generations is usually not checked. However, if transpositions such as the one we observed in our study occur, it could inactivate the transgene, which can only be identified after the development of transgenics. It may thus

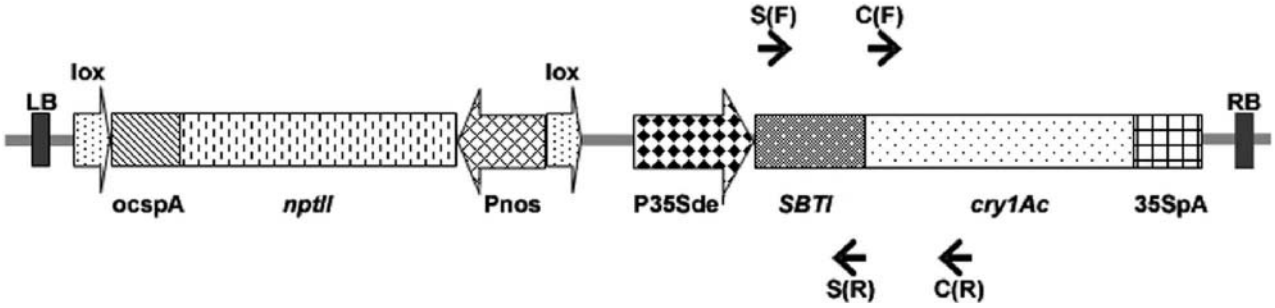

Figure 1. Schematic representation of binary vector pPZP200:*nptII::SBTI+Cry1Ac* construct. The primers S(F) – ATGAAGAGCA CCATCTTCTTTGCTCTC, S(R) – GGCCTCACTGCGAGAAAGGCC, C(F) – ATGGACAACAACCCAAACATCAAC and C(R) – GGCGCTGTTCATGTCGTTGAATTG are indicated.

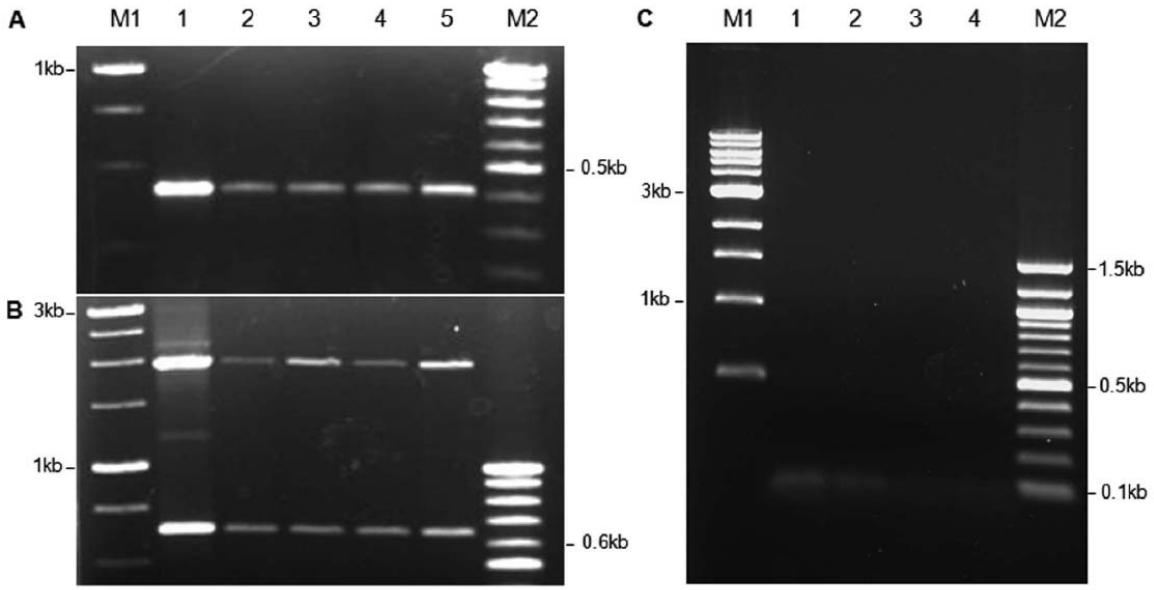

Figure 2. Analysis of transcript of the fusion gene in RNA isolated from five independent calli transformed with the binary vector pPZP200:*nptII::SBTI+Cry1Ac*. Following cDNA synthesis, gene-specific amplifications were carried out using primers specific to the (**A**) *cry1Ac* and (**B**) *SBTI* portions of the transcript. Lanes 1–5 represent profiles from five independent calli, while M1 and M2 are 1 kb and 100 bp size ladders, respectively. (**C**) Gene-specific amplification on RNA without carrying out reverse transcription showed no amplified product, as was also observed in the case of RT-PCR analysis on RNA isolated from untransformed calli (lanes 1, 2: *cry1Ac* and 3, 4: *SBTI*).

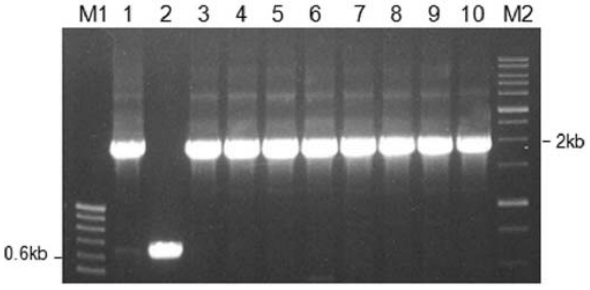

Figure 3. Amplification of a portion of the *SBTI* gene using primers S(F) and S(R) from the binary vector isolated from individual *E. coli* XL1 Blue cells (lanes 1–10). M1 and M2 are 100 bp and 1 kb size ladders, respectively.

be useful to check the fidelity of the binary vector (especially genes within the T-DNA borders) at different times of *Agrobacterium* growth.

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