Evidence of Frequent Integration of Non-T-DNA Vector Backbone Sequences in Transgenic Strawberry Plant

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We have studied the occurrence of the integration of non-T-DNA sequences in transgenic strawberry plants obtained through Agrobacterium inoculation. DNA from these plants was subjected to PCR amplification of the sequence of the gene trfA, which is located outside the T-DNA. The percentage of trfA-positive plants varied from 40% to 90%, with a mean of 65.7%. Backbone sequences were confirmed by Southern blot analysis.

[Key words: Agrobacterium tumefaciens, Fragaria x ananassa, T-DNA transfer, genetic transformation]

Agrobacterium-mediated transformation is the most commonly used method to transform dicot and monocot plants. In this method, the genes of interest are cloned into a binary vector between repeats of two 25-bp imperfect borders, the right (RB) and left (LB) T-DNA borders. The proteins VirD1 and VirD2, encoded by Vir genes located in the Ti plasmid, recognize the 25-bp repeat sequences of RB and LB and nick them between their third and fourth bases. After nicking, VirD2 is covalently linked to the 5’ end of the single-stranded T-DNA generated and acts as the pilot protein directing this molecule into a plant cell (1–3). LB, which is not essential for T-DNA transfer, helps define the left end of the T-strand. Thus, Agrobacterium-mediated transformation has long been considered as a clean technology compared with direct transformations, such as particle bombardment or electroporation, because only the sequences between the RB and LB T-DNA borders are introduced into plant genomes. However, detailed studies of transgene integration patterns in several species have shown that vector backbone sequences outside the T-DNA are also frequently inserted into plant genomes (4–7). This problem in the cointegration of vector backbone sequences has been reported. In this study, we determined the presence of genes outside the T-DNA in strawberry plants transformed with binary plasmids derived from pBIN19, and found that vector backbone sequences are integrated at a high frequency in this species when using these vectors.

Transgenic strawberry plants (Fragaria x ananassa Duch.), cv. Chandler, were obtained by leaf disk inoculation with Agrobacterium tumefaciens strain LBA4404 following the protocol of Barceló et al. (14). Three independent transformation experiments with A. tumefaciens harboring different plasmids were performed. In the first one, a binary vector derived from the plasmid pBINPLUS (15) was used. In the second one, a binary vector derived from the plasmid pGUSINT (16) was employed. These plasmids are binary vectors derived from the plasmid pBIN19 (17) and contain the gene nptII for kanamycin resistance in the T-DNA. In the third one, transgenic plants were obtained through co-transformation with both plasmids. Ten to 15 independent transgenic lines from each transformation experiment, and control untransformed plants were analyzed. Kanamycin-resistant plants were transferred to a greenhouse and propagated vegetatively by runners, because cultivated strawberry plants are not propagated by seeds. Plants from the first two transformation experiments were kept in a greenhouse for four years prior to molecular analyse.

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Young leaves from the control and transgenic plants growing in the greenhouse were used as DNA sources. Two hundred milligrams of leaf tissue was washed twice with a washing buffer (100 mM sodium acetate buffer [pH 5] containing 20 mM EDTA, 0.2 M sorbitol and 2% PVP [MW 40,000] and 1% β-mercaptoethanol which were added just before use) (18). After washing, DNA was extracted using the DNeasy plant minikit (Qiagen, Valencia, CA, USA), following the manufacturer’s instructions. The transgenic nature of plants was confirmed by the PCR amplification of a 900-bp fragment of the gene nos-nptII. Additionally, PCR amplification of a sequence of the VirD1 gene from the Ti plasmid was evaluated in the same samples to rule out the possibility of Agrobacterium contamination (19). To screen for transgenic plants that integrated vector backbone sequences, a 381-bp sequence in the gene trfA was PCR-amplified. This gene is located outside the T-DNA (20). PCR was carried out in a 50-µl reaction mixture containing plant DNA (20 ng), 0.25 µM of each primer, 0.2 mM of each dNTP, 1 mM MgCl₂, 2 U of Taq polymerase and 1× Taq buffer. A standard PCR program was 2 min at 95°C followed by 30 cycles of 94°C for 90 s, 52°C for 90 s and 72°C for 2 min, and a final extension phase of 10 min at 72°C.

For southern blotting, 12 to 15 micrograms of DNA was digested overnight with EcoRI in the presence of 1 mM spermidine, electrophoresed on a 0.8% agarose gel and then transferred to a Hybond N+ membrane. A 381-bp PCR fragment of the gene trfA was used as probe. This probe was labelled with digoxigenin using the DIG High Prime DNA labelling and detection starter kit (Roche, IN, USA). The filter was prehybridized at 42°C in DIG Easy Hyb buffer and hybridized overnight in the same buffer containing the probe. The filter was then washed and the probe detected following the manufacturer’s instructions.

Three sets of transgenic strawberry plants transformed with two different pBIN19-derived plasmids were used in this work. Transgene expression in some of these plants has been evaluated in a previous work (13). In our transformation protocol, regenerated plants were cultivated and proliferated in vitro in the presence of kanamycin for at least 8 months. All the plants were kanamycin-positive when DNA was extracted using the DNeasy plant minikit and subjected to PCR amplification using specific primers for nptII and analyzed. No amplification was observed when using primers for VirD1 from the Ti plasmid, indicating no Agrobacterium contamination (data not shown).

To determine the presence of vector backbone sequences in the transgenic strawberry plants obtained through Agrobacterium infection, we subjected DNA isolated from the control and transgenic plants to PCR analysis using specific primers for trfA amplification. trfA is located outside the T-DNA, and produces two proteins that promote the replication of the binary plasmids (20). No amplification of trfA was observed in DNA from the control non-transformed plants. However, in the first transformation experiment, 10 out of the 15 independent transgenic lines analyzed, 66.6%, showed trfA amplification (Fig. 1A). Of the lines transformed with the pGUSINT-derived plasmid, 90% were trfA-positive (Fig. 1B). Finally, in the cotransformation experiment with both plasmids, only 4 out of the 10 transgenic lines showed trfA amplification (Fig. 1C). Globally, this indicates that 65.7% of transgenic strawberry lines have vector backbone sequences integrated jointly with T-DNA.

The presence of vector backbone sequences in several lines that showed PCR amplification of trfA was confirmed by Southern blotting. As can be observed in Fig. 2, all the lines analyzed were positive, and the number of copies varied from 1 to 3. This result confirms that the binary vector is frequently integrated in transgenic strawberry plants obtained by Agrobacterium-mediated transformation.

For a long time, it has been assumed that genetic transformation by Agrobacterium infection enables the controlled integration of desired genes, i.e., those located in the T-DNA, into plant genomes. However, there is accumulating evidence of frequent failures of this integration system. De Buck et al. (7) screened 125 transgenic Arabidopsis and tobacco plants derived from seven Agrobacterium-mediated transformation experiments and found that 20% to 50% of the plants contained DNA outside the T-DNA. Most of these lines integrated the complete vector. Wenck et al. (6) reported similar percentages of whole binary vector integration in Nicotiana plumbaginifolia and Arabidopsis thaliana transgenic plants. Interestingly, the percentage of transgenic lines containing plasmid DNA was significantly higher in plants obtained through Agrobacterium vacuum infiltration than in lines obtained by root transformation. Kononov et al. (5) transformed tobacco plants with a vector containing the reporter gene gusA outside the T-DNA. Approximately one fifth of the 200 lines obtained showed GUS expression,
and gusA was detected by PCR analysis in 75% of transformants. The high frequency of vector backbone sequence integration and the fact that sometimes this integration occurs independently of the T-DNA have been exploited to obtain marker-free transgenic maize plants using a binary vector with the selectable gene positioned outside the T-DNA (21). At present, it is not clear whether the integration of vector backbone sequences is due to the initiation of T-strand at LB or to T-strand termination failures at LB, and evidence of both mechanisms has been reported (7, 21). However, the inclusion of additional copies of LB in binary vectors efficiently reduces the frequency of vector integration, reinforcing the hypothesis that this process is mainly the result of a reading through mechanism at LB (22).

In this study we have shown that a high percentage of transgenic strawberry plants obtained through Agrobacterium infection contain vector backbone sequences when using binary vectors derived from pBIN19. Thus, molecular analysis to detect non-T-DNA sequences should be performed routinely in transgenic strawberry plants, especially if these plants are going to be considered for field release and future commercialization.

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