Strategies for generating marker-free transgenic banana plants based on the Cre-*lox* site-specific recombination system

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Abstract

Bananas and plantains are an important source of food and income for millions of persons in the world. Their production is constrained by many biotic and abiotic stress factors but their improvement through traditional plant breeding methods is very difficult because they do not produce seeds, are polyploid and have a long generation time. Biotechnological approaches like genetic transformation techniques may therefore have a great impact on banana improvement. In addition, genetic engineering in banana can also be useful to increase food quality and for molecular farming purposes, e.g. to express oral vaccines. In this work a number of issues regarding the generation and use of transgenic banana plants were addressed.

First, a protocol for plant regeneration through somatic embryogenesis in the banana cv. 'Dwarf Cavendish' (*Musa* AAA) was developed. Then, several conditions for *Agrobacterium*-mediated gene transfer based on this regeneration system were studied. The efficiency of the different treatments was assessed by transient GUS expression, number of transformed embryo colonies and regenerated plants. The highest efficiency was obtained when embryogenic cell suspensions were inoculated with *Agrobacterium* for 6h in medium supplemented with 200 µM acetosyringone and 1.0 mM spermidine; under these conditions more than 600 independent transgenic lines were obtained per ~50 mg fresh weight of settled cells. Spermidine showed an enhancing effect, increasing significantly the transient GUS expression, the number of transformed embryo colonies and the number of regenerated plants in comparison with the same treatments without this polyamine.

Secondly, methods to obtain marker-free transgenic banana plants were developed. This research was motivated by the fact that selectable marker genes are no longer needed once a transgenic event is selected, but on the contrary the continued presence of such marker genes has technological drawbacks and raises public concerns. In these experiments, we used the cultivar 'Grande Naine' (*Musa* AAA), which is important in the export market but has been seriously affected by fungus attack. Marker-free transgenic banana plants from this cultivar have been obtained using a Cre/*lox* auto-excision system. We have investigated two strategies to induce the activity of the Cre protein and the recombination of *lox* sites. In the first approach a binary expression vector was used, in which the *cre* recombinase gene under the control of a heat shock promoter as well as a selectable marker gene cassette were placed between two *lox* sites in direct orientation, while the gene of interest was inserted outside of the *lox* sites. Heat shock inducible promoters GmHSP17.6-L and AtHSP18.2, derived from soybean and Arabidopsis respectively were tested. The results showed that a transient heat

shock treatment of primary transgenic embryos is sufficient for inducing *cre* and excising both the *cre* gene and the selectable marker genes. Excision efficiency was determined by PCR and confirmed by Southern hybridization and it reached 59.7 and 40.0 % for the GmHSP17.6-L and HSP18.2 promoters, respectively. In a second approach, an embryo specific promoter was used instead of a heat-shock promoter. The promoter of the embryo globulin gene *REG-2* from rice was chosen for this purpose. The activity of this promoter was first tested in various phases of the transformation protocol using a pREG2::uidA construct. GUS expression was visualized only in formed embryos and not in cell clumps during the selection phase. Subsequently, the *REG-2* promoter was cloned upstream the *cre* gene in an auto-excison vector, resulting in excision of the selectable marker and *cre* genes in mature somatic embryos. PCR analysis on regenerated plants revealed that 34% of them were completely marker-free, results that were thereafter confirmed by Southern blot hybridization. These results demonstrate the feasibility of using developmentally controlled promoters to mediate marker excision in banana.

Finally, as an application of these Cre/*lox* based systems, we obtained marker-free transgenic banana cv. 'Grande Naine' (*Musa* AAA) constitutively expressing the tobacco osmotin gene *ap24*. AP24 has previously been shown to possess antifungal activity in both *in vitro* and *in vivo* assays. We have developed a vector that contains between two *lox* sites in direct orientation the selectable marker gene cassette *pNos-hpt-tNos* and the *cre* recombinase gene driven by the heat shock promoter pHSP18.2 from *Arabidopsis thaliana*. After selection, hygromycin resistant embryos were subjected to heat-shock and thereafter shifted to antibiotic-free regeneration medium. PCR and Southern blot analysis on DNA from regenerated plantlets confirmed the integration of the *ap24* gene and the excision of the selectable marker and *cre* genes in 40% of the regenerants. Then, several lines were screened in an artificial inoculation assay with *Mycosphaerella fijiensis* under greenhouse conditions. *M. fijiensis* infection causes the devastating Black leaf streak or Black Sigatoka leaf spot disease in banana. Several AP24 expressing transgenic lines tested showed enhanced tolerance to the pathogenic fungus.