The Supervirulence Plasmid pToK47 from Agrobacterium tumefaciens A281 Improves Transformation Efficiency of Hevea brasiliensis

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Abstract: Problem statement: The present study investigates the ability of the pTok47 supervirulence plasmid from Agrobacterium tumefaciens A281 to enhance genetic transformation in Hevea brasiliensis. Approach: Hevea anther callus was transformed via Agrobacterium-mediated genetic transformation using two strains of Agrobacterium (GV2260 and GV3850) harboring the human serum albumin cDNA and the supervirulent plasmid pToK47 from Agrobacterium tumefaciens A281. The transformed callus was selected using kanamycin as the selection agent. Results: The Agrobacterium strain GV2260 benefited from the presence of the supervirulence plasmid in giving a higher frequency of 7.4% transformed callus, 344.8% embryogenesis and 11.6% plantlet production compared to the corresponding strain on its own giving 0.9% transformed callus, 204.5% embryogenesis and 4.4% plantlet production. Similarly, Agrobacterium strain GV3850 conferred a higher frequency using the supervirulent plasmid, resulting in 3.5% transformed callus, 138.5% embryogenesis and 3.5% plantlet production compared to the corresponding strain on its own giving 0.7% transformed callus, 137.5% embryogenesis and 9.0% plantlet production. These findings were confirmed by the Wilcoxon Signed Rank Test that compared the effectiveness of the supervirulence plasmid in increasing the rate of genetic transformation in the calli surviving in kanamycin growth medium for GV2260 (p<0.001) and for GV3850 (p<0.05). Conclusion: This study showed that both Agrobacterium strains benefited from the presence of the supervirulence plasmid in giving a higher frequency of transformed callus, embryos and plantlets. These results suggest that a highly virulent binary vector system might prove especially useful in generating high frequency transformation of Hevea.

Key words: Hevea brasiliensis, Agrobacterium tumefaciens, supervirulence, callus, embryos, human serum albumin, neomycinphosphotransferase II

INTRODUCTION

The main challenge with genetic transformation of tree species is achieving high transformation efficiency for desired clones or cultivars. In this respect, it is important to have an efficient tissue culture system to obtain plantlets from cells containing the transgene. In the case of Hevea brasiliensis (rubber tree), the highly embryogenic clone GL1 is routinely used as a vehicle for genetic transformation.

Agrobacterium tumefaciens harbors a tumor-inducing plasmid (Ti plasmid) and the region of T-DNA within this plasmid encodes for the expression of the plant growth regulator genes for auxin and cytokinin, which leads to the formation of tumors in plant cells[1,2]. The Ti plasmid also harbors another important segment, the virulence (vir) region and this encodes most of the functions necessary for T-DNA transfer to plant cells[3].

In A. tumefaciens A281 (the carrier of the plasmid pTiBo542) the tumors formed after infection with plant cells were larger, were early-appearing and the tumorigenesis applied to a wide range of plants, compared to other A. tumefaciens strains[4]. For the above reason, the plasmid pToK47 was constructed by subcloning a 15.8 kb fragment of pTiBo542 that carries the entire virB, virG and virC operons. When mobilized into Agrobacterium strains A348, A281, A208 and T37...
all the resulting strains displayed larger tumor formation compared with corresponding wild type strains [4].

Several publications have shown that transformation of previously recalcitrant species of monocotyledons (rice, corn and maize) can be accomplished through the use of disarmed Agrobacterium tumefaciens strains to which additional copies of certain virulence genes were added [3,6] and also showed an improved procedure for production of white spruce using Agrobacterium containing the virulence regions from pToK47 [7,8].

In the present investigation, an efficient transformation protocol for Hevea was developed using the supervirulence plasmid pToK47 containing virB, virC and virG genes from Agrobacterium tumefaciens A281.

MATERIALS AND METHODS

Agrobacterium strains and plasmid vectors: The binary vector pLGMR.HSA was constructed by inserting the expression cassette containing the 35S CaMV promoter, the multicloning sites and the CaMV Poly A tail from pJIT62 (Guerineau, France) containing the 35SCaMV promoter, the multicloning sites and the binary vector pLGMR.HSA was constructed by electroporating into electrocompetent cells of virC super-virulence plasmid pToK47 containing GV2260 [9].

In the present investigation, an efficient transformation protocol for Hevea was developed using the supervirulence plasmid pToK47 containing virB, virC and virG genes from Agrobacterium tumefaciens A281.

PCR determination of Hevea transformants: Primers for HSA and NPTII cDNA analysis in putative kanamycin-resistant callus were as follows: HSA: 5'-atgaagtgggtaacctttatttcc-3' and 5'-ttataagcctagaaggcagcttgac-3' (positions 39-63 and 1869-1848) [4]; NPTII: 5'-gaggctattcggctatgactg-3' and 5'-atcgggagcggcgataccgta-3' (positions 201-222 and 900-879) [10]. The presence of the inserted genes in putative callus and embryoids of Hevea was analyzed using routine PCR techniques [10].

Statistical analysis: In data analysis, variation due to day to day effects were removed by pairing treatments with and without pToK47. As Gaussian distribution of the data was suspect, a nonparametric paired test, the Wilcoxon Matched Pairs Signed Rank Test, was used in statistical evaluation of the effectiveness of the supervirulence plasmid on transformed (i.e., kanamycin-resistant) calli and transformed embryos.

RESULTS

DNA analysis in putative transformants of Hevea: PCR analysis was used to detect the presence of NPTII and HSA in the kanamycin-resistant callus tissues. Genomic DNA from independently transformed and untransformed (control) tissues was subjected to PCR. Fig. 1 shows that the samples from transformed tissues gave the predicted DNA fragment bands of 0.7 kb for NPTII gene (lanes 3-8), Fig. 1a and 1.8 kb for HSA gene (lanes 3-8), Fig. 1b, whereas no amplification was detected in the sample from untransformed tissue.
Fig. 1: PCR analysis of transformed callus tissues of *Hevea* was analyzed. DNA was amplified with specific primers for NPTII and HSA cDNA. (Lane M) 1 kb ladder; (1) pLGMR.HSA (positive control); (2) control plant; (3-8) putative transformants (KAN^r^ callus); Amplified fragments: 1a: NPTII (0.7 kb), 1b: HSA cDNA (1.8 kb)

Table 1: Effect of the supervirulence plasmid in GV2260 on genetic transformation success

|                        | Without pToK47 | With pToK47 | Statistical Significance^a^ |
|------------------------|----------------|-------------|----------------------------|
| Calli cultures         | 2332           | 2332        |                            |
| Transformed calli      | 22             | 174         | p<0.001                    |
| Transformed embryos    | 45             | 600         | p<0.01                     |
| Transformed plantlets  | 2              | 70          | p<0.01                     |

^a^ Wilcoxon’s matched signed rank test

Southern analysis with the DIG HIGH-PRIME labeled NPTII and HSA probe showed a hybridization signal in the six transformed tissues analyzed (lanes 3-8) (Fig. 2a and b). These results show that sustained growth and development of the callus culture on kanamycin medium is a good indicator of successful genetic transformation.

Table 2: Effect of the supervirulence plasmid in GV3850 on genetic transformation success

|                        | Without pToK47 | With pToK47 | Statistical Significance^a^ |
|------------------------|----------------|-------------|----------------------------|
| Calli cultures         | 2332           | 2332        |                            |
| Transformed calli      | 16             | 83          | p<0.05                     |
| Transformed embryos    | 22             | 115         | NS                         |
| Transformed plantlets  | 2              | 4           | NS                         |

^a^ Wilcoxon’s matched signed rank test; NS: Not Significant

While scorings of transformed embryos or plantlets that develop subsequently might also reflect, to some extent, the role of the supervirulence plasmid, these indicators are not as accurate. Individual callus, once transformed with or without the help of the supervirulence plasmid are subsequently multiplied and the final number of transformed calli might not be exactly proportional to the number of original transformants. Moreover, some of transformed calli would be more successful than others in giving rise to multiple embryos and plantlets later. Notwithstanding this, the numbers of embryos and plantlets obtained from callus cultures with the supervirulence plasmid was higher in both GV2260 (Table 1) and GV3850 (Table 2), this advantage is statistically significant in GV2260 (Table 1).
CONCLUSION

Clearly, *Agrobacterium* strains GV2260 and GV3850 containing an additional 15.8 kb fragment carrying extra copies of the virulence regions (*virB*, *virC* and *virG*) from the supervirulence plasmid, pToK47 gave a higher transformation frequency for *Hevea* as compared to controls. The advantage conferred by the supervirulence plasmid was more distinct with the GV2260 *Agrobacterium* strain then with the GV3850 strain. Efficient induction of *Agrobacterium* vir genes thus holds the key for increased rate of transformation in *Hevea* and certainly, increasing the expression of certain vir genes, at least in certain strains of *Agrobacterium* may increase their virulence and host range of infection. Thus, it is important to enhance our knowledge of the function and regulation of vir genes so that it may be possible to construct strains of *Agrobacterium* species that will be capable of transforming plants that are recalcitrant to infection by *Agrobacterium*.

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