SELECTABLE MARKER FREE TRANSGENIC RAPESEED USING COTRANSFORMATION AND SEGREGATION

Jacob Bade and Brigitte Damm.

MOGEN International, Einsteinweg 97, 2333 CB Leiden, The Netherlands

ABSTRACT

A successful *Agrobacterium*-mediated transformation system was developed for large scale production of transgenic rapeseed (*Brassica napus* cv 'Westar'). Four selectable marker genes were shown to be suitable. Transformation frequencies ranged from 5 to 25 %.
Cotransformation experiments were performed with the *gus* and *npt*II gene separated over two different *Agrobacterium* strains which were applied simultaneously. The *gus* gene was expressed in 30-50 % of the obtained kanamycin-resistant plants. More than 60% of these GUS-positive lines produced S1 seedlings with the desired phenotype: GUS expression without kanamycin resistance.

INTRODUCTION

Cotransformation experiments with a mixture of two *Agrobacterium* strains carrying different T-DNAs have shown that integration of both T-DNAs can take place in the genome of one plant cell (McKnight *et al.*, 1987; Depicker *et al.*, 1985; Ottaviani *et al.*, 1991; De Block *et al.*, 1991). When these integrations occur on different, genetically unlinked loci, both will be transferred independently to the next generation. A progeny plant will contain none, both or only one of the two T-DNAs. In theory a combination of cotransformation and meiotic segregation creates the possibility to produce transgenic lines with a gene of interest, but without a selectable marker.

To investigate whether such a procedure of cotransformation and segregation is feasible for routine production of selectable marker-free transgenic rapeseed, a series of experiments was carried out. Aim was to determine experimental conditions for (a) a high transformation and cotransformation frequency, (b) use of different selectable markers, (c) production of transgenic plants with preferentially a single T-DNA integration and (d) in case of cotransformation, unlinked integration of both T-DNAs in the genome of rapeseed.

EXPERIMENTAL

Establishment of a transformation system

Hypocotyl explants of cultivar 'Westar' were chosen as starting material for transformation experiments, since this explant type was reported being the most suitable (Radke *et al.*, 1988; De Block *et al.*, 1989). Transformation experiments were performed using different binary vectors carrying either the *npt*II (neomycin phosphotransferase), the *hpt* (hygromycin phosphotransferase), the *pat* (phosphinothricin acetyltransferase) or the *als* (acetolactate synthase) gene, all in combination with the *gus*-intron (ß-glucuronidase) reporter gene. Several parameters were investigated with respect to their influence on the transformation frequency and the number of T-DNA insertions.

It was found that a combination of 24 hours liquid cocultivation and a direct transfer to selective medium with kanamycin, hygromycin B or phosphinothricin yielded transgenic plants with frequencies of 10-25 %, calculated as transgenic plants per hypocotyl explant. Selection for presence of the *als* gene with chlorsulfuron resulted in a reduced transformation frequency of about 5 %.

Southern analysis revealed that approximately 30 % of the transgenic plants had a single copy of the T-DNA integrated in their genome. Longer cocultivation periods and increasing concentrations of *Agrobacterium* negatively effected the transformation frequency, but did not change the average number of T-DNA insertions. Extreme reduction of the *Agrobacterium* concentration during explant incubation resulted in very low transformation frequencies. However, data suggest that this treatment yielded proportionally more plants with the favorable single T-DNA insertion.

Cotransformation

In cotransformation experiments two different *Agrobacterium* strains were applied simultaneously, each carrying a different binary vector. One vector contained the selectable marker *npt*II to obtain kanamycin-resistant plants, the other the *gus*-intron reporter gene. A screen for expression of the latter gene in kanamycin-resistant plants was used to identify cotransformation events.
Depending on the experimental conditions a transformation frequency of 5-10% was obtained. A cotransformation frequency of 30-50% was observed, calculated as fraction 'GUS-positive' within the population of kanamycin resistant plants.

Progeny analysis of 30 cotransformants was performed. Per line up to 80 individual seedling were tested for both kanamycin resistance and GUS expression. More than 50 % of the lines showed Mendelian segregation indicative for one or two functional loci of *gus* and *npt*II. Nineteen lines were identified producing at least some seedlings which expressed the *gus* gene, but were sensitive to kanamycin.These segregation data clearly demonstrated that unlinked insertion occurs at sufficient high frequency enabling the recovery of selectable marker-free transgenic rapeseed. Currently progenies of cotransformants are investigated more elaborately at the molecular level to prove abscence of non-functional copies of the selectable marker.

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