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BRIEF COMMUNICATION

Improvement of *Arabidopsis thaliana* seed transformation efficiency

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Abstract

Agrobacterium tumefaciens induced transgenosis by treatment of germinating *Arabidopsis thaliana* seed embryos has been achieved with different *Agrobacterium* strains including the strain LBA4404, which was ineffective in seed transformation experiments of the other authors. The frequency of transgenosis was increased several times by application of acetosyringone to the growing *A. tumefaciens* suspension cultures. The DNA demethylating agent 5-azacytidine partly restored the distorted Mendelian segregation ratios in the offspring of transgenic plants.

Key words: acetosyringone, *Agrobacterium tumefaciens*, 5-azacytidine

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A very useful *Arabidopsis thaliana* seed transformation method was developed by Feldmann and Marks (1987). This method became the methodological basis of the project of *Arabidopsis thaliana* gene tagging, which is part of Multinational Cooperation *Arabidopsis thaliana* Genome Research Project. The goal of *Arabidopsis thaliana* T-DNA gene tagging is to saturate the genetic map, *i.e.* to obtain mutations of all genes which can be detected and to clone the mutated genes with utilization of their molecular label by the T-DNA sequence. The method of transgenosis by *A. tumefaciens* for T-DNA tagging should avoid somaclonal variability and it should be as effective as possible.

The drawback of the seed transformation method is its low efficiency and different yields of transformants in different replications. As comes out from the paper of Feldmann (1991), approximately one mutation is recovered from about ten seeds treated. Feldmann (1991) has stated that only the C58C1 Rif strain, containing the

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Abbreviations: AS - acetosyringone, 5-AC - 5-azacytidine, Km^r - kanamycin resistant, Km^s - kanamycin sensitive

cointegrate 3850:1003 Ti plasmid generated transformants. The use of LBA 4404 strains containing six different Ti plasmid binary vectors failed to produce a single transformant among 18 treatments.

In this paper we present data showing that *Arabidopsis thaliana* seed transformation can be achieved by different *Agrobacterium* strains, also by *A. tumefaciens* strain LBA4404 with different binary vectors and that its frequency can be increased considerably by acetosyringone (AS; 3',5'-dimethoxy-4'-hydroxy-acetophenone; *Aldrich*, Germany) in *A. tumefaciens* cultivation medium.

AS, which induces *vir* region genes of the *Ti* plasmids (Engström *et al.* 1987), has been first shown to increase considerably the efficiency of *Arabidopsis* leaf explant transformations (Sheikholeslam and Weeks 1987), but it has never been used before in *Arabidopsis* seed transformation protocols.

Agrobacterium strains used were cultivated in suspension shaken culture in Langley and Kado (1972) medium overnight. The 20 μM AS dissolved in water, was sterilized by ultrafiltration and added in some of the *A. tumefaciens* media in order to test its effects on transformation efficiency. Seeds of *Arabidopsis thaliana* (L.), cv. Dijon, were shaken in 50 cm^3 PG_0 liquid medium (Negrutiu *et al.* 1975) for 16 h at 25 °C in the dark. Infection consisted of adding 3 cm^3 of an overnight culture of *A. tumefaciens* to the seeds in 50 cm^3 of PG_0. After 24 h co-cultivation (shaken at 25 °C in dark) the seeds (T_1) were washed three times with distilled water, sown on vermiculite pre-soaked with a complete nutrient solution (without antibiotics) and cultivated in a greenhouse. The transgenosis of *Arabidopsis* seeds was carried out with three *A. tumefaciens* strains:

1. A. tumefaciens (pAL4404)(pCB1334). This strain has chromosomal background of the Ach5, but it carries the pAL4404 helper plasmid without T-DNA (Hoekema *et al.* 1983) instead of the original Ti plasmid. The vector plasmid pCB1334 (Vlasák and Ondřej 1992), which is derived from pGA472 of An *et al.* (1985), carries the gene 4 from pTiC58 T-DNA.

2. A. tumefaciens (pAL4404)(pKA 1-1). The pKA1-1 plasmid with kanamycin resistance gene has been derived by Angelis *et al.* (1992) from the pGA472 plasmid of An *et al.* (1985).

3. A. tumefaciens (pGV2260)(GUSint strain). It has the chromosomal background of B6S3, the helper plasmid pGV2260 derived by Deblaere *et al.* (1985) and vector plasmid 35SGUSint (Vancanneyt *et al.* 1990) which is derived from the pBIN19 plasmid of Bevan (1984) and carries intron containing β-glucuronidase (GUS) gene. The vector plasmids of all *A. tumefaciens* strains used carried NPTII gene which confers kanamycin resistance to transgenic plants. The expresion of GUS gene in transgenic plants has been proven by the fluorimetric method of Jefferson *et al.* (1987).

The T_2 seeds were collected in bulk from about 100 plants in each variant, air dried and cleaned. To select kanamycin resistant plants, the T_2 seeds were sown on PG_0 medium (Negrutiu *et al.* 1975) with 50 mg dm^{-3} kanamycin sterilized by ultrafiltration and added after autoclaving.

5-azacytidine (5-AC) is known to remove cytosine methylation in T-DNA,

integrated in plant nuclei (Hepburn *et al.* 1983) and to reactivate silent transgenes. Part of seeds was therefore sown on agar PG_0 medium (Negrutiu *et al.* 1975) with 2 mg dm^{-3} 5-AC (*Sigma*, Germany) dissolved in water and sterilized by ultrafiltration and added after autoclaving. This chemical in the medium does not show any observable effect on control seeds.

The differences between kanamycin sensitive and resistant plants appeared during the second and third week of cultivation, when the cotyledons and vegetation apices in sensitive plants got white and growth was stopped (Table 1). Although the number of T_2 seeds as well as kanamycin resistant plants is different the effect of AS is evident. The efficiency of transformants was markedly increased (almost by one order of magnitude) when *A. tumefaciens* cultures have been cultivated in the presence of AS.

Table 1. Efficiency of *Agrobacterium*-mediated transgenosis of *Arabidopsis thaliana* seeds as affected by 20 μM acetosyringone.

Vector	AS	No. of T_2 seeds	No. of T_2 Km^r plants
pCB1334	-	18600	191 (1.02)*
pKA 1-1	-	20200	151 (0.75)
pKA 1-1	+	27900	1405 (5.03)
35SGUSint	-	167	0
35SGUSint	+	188	6 (3.19)
		67055	

* - percentage of Km^r plants in parentheses

The genetic transmission of kanamycin resistance transgene has been followed in part of T_2 plants into T_3 generation. The resistant T_2 plants were cultivated separately to maturity and their seed separately sown on kanamycin medium. When cultivated on media with 5-AC, from 25 offsprings of transformants carrying pKA1-1 T-DNA, 24 transmitted the kanamycin resistance marker into T_3 offspring and in six T_2 transgenic GUSint plants all transmitted the kanamycin resistance into T_3 progeny.

Several authors have observed suppression of transgenes. One of the mechanisms for inactivation of transgene was proven to be DNA methylation and the demethylating agent 5-AC has been used to reactivate silent transgenes (Pavingerová and Hrouda 1991, Zhu *et al.* 1991).

Pavingerová and Hrouda (1991) have shown progressive distortion of segregation ratios (decrease of proportion of plants showing the transgenic character) in succeeding generations. It has been partly restored by application of 5-AC (2 mg dm^{-3}) into the cultivation medium. Similar effect was found here, in T_3 of the GUSint carrying transgenic lines (Table 2), where the 5-AC application have increased the incidence of Km^r plants approximately 20 times. These results differ from those of Feldmann and Marks (1987), who have observed Mendelian segregation ratios in their offspring of seed transformants in 90 % of cases. The differences can be explained by different *Arabidopsis* lines used or by different

A. tumefaciens strains used or by differences in methods.

The utilization of both AS and 5-AC for increasing the efficiency of transformation can be recommended also in new, more efficient methods of *Arabidopsis thaliana* transgenosis, which by-pass tissue cultures, like the infiltration method of Bechtold *et al.* (1993) and the method of direct inoculation of cut surfaces described by Chang *et al.* (1994).

Table 2. Segregation of kanamycin resistant seedlings in T_3 of 35S GUSint transformants as affected by 5-azacytidine.

Line	Km^r : Km^s with 5-AC	without 5-AC
1	1 : 20	0 : 15
2	2 : 22	1 : 7
3	23 : 24	1 : 15
4	21 : 3	0 : 28
5	13 : 1	0 : 23
6	2 : 29	0 : 32
Total	62 :109	0 :120

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