Plant Molecular Biology 8: 291–298, (1987)

Acetosyringone promotes high efficiency transformation of *Arabidopsis thaliana* explants by *Agrobacterium tumefaciens*

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Received 25 September 1986; accepted 9 December 1986

Keywords: acetosyringone, *Agrobacterium tumefaciens, Arabidopsis thaliana*, leaf explants, regeneration, transformation

Abstract

High frequency transformation of *Arabidopsis thaliana* leaf explants has been obtained using a disarmed Ti plasmid containing the coding region of a neomycin phosphotransferase gene (NPT II) as a selectable marker. The rate of transformation ranged from 55 to 63 percent when acetosyringone (AS), a natural wound response molecule, was added to an *Agrobacterium tumefaciens* culture prior to incubation with leaf segments. Without acetosyringone, the transformation rate was approximately 2 to 3 percent. Calli resistant to G418 were regenerated into mature flowering plants in the presence of 10 μg/ml G418. Southern analysis and neomycin phosphotransferase assays confirmed the insertion and expression of the NPT II gene in regenerated *Arabidopsis* plants.

Introduction

Arabidopsis thaliana is a miniature flowering plant with a short life cycle, small genome size (7×10^7 bp), and low amounts of repetitive DNA sequences [17]. Because of these traits and the facility with which mutations in specific biochemical or developmental pathways can be obtained, *Arabidopsis* is being adopted as a model system in a rapidly increasing number of studies of plant genetics and molecular biology [5, 13, 16, 19]. One of the prime factors restricting progress in studies which utilize *Arabidopsis thaliana* has been the lack of an efficient system for genetic transformation. *Arabidopsis* is a dicotyledonous plant and several laboratories have shown that *Agrobacterium tumefaciens* bearing tumor-inducing (Ti) plasmids are able to infect *Arabidopsis* and elicit tumor formation [1]. To date only limited progress has been reported [2, 5] in efforts to transform *Arabidopsis* with disarmed (i.e. non tumor producing) Ti plasmids carrying selectable genetic markers.

In this report we describe experiments which were aimed at testing whether the wound response molecule, acetosyringone [20, 21], might be used to enhance the rate at which *Agrobacterium tumefaciens* containing a Ti plasmid is able to transform *Arabidopsis thaliana.* This approach stems from the recent observations [20, 21] that certain wounded dicotyledonous plants secrete a number of different compounds, including acetosyringone, that elicit the activation of *Agrobacterium* and Ti plasmid genes which are involved in promoting DNA transfer from the bacterium to plant cells. In particular, it appears that neutral or synthetic acetosyringone is capable of triggering the activa-

tion of genes in the virulence region of the Ti plasmid which are necessary to initiate the transfer of T-region DNA from the Ti plasmid to plant chromosomes [10, 22]. If production of wound response molecules by *Arabidopsis* is too low to cause strong activation of the virulence region genes, a pretreatment of *Agrobacterium* with synthetic acetosyringone might be expected to increase the rate at which the bacterium is able to transform *Arabidopsis* plants or tissues. Here we present data that treatment of *A. tumefaciens* with 20 μM acetosyringone prior to incubation with leaf explants leads to a marked increase in the rate of *Arabidopsis* transformation. The high rates of transformation obtained (i.e., approximately ½ to ⅔ of leaf explants) and the relatively simple and efficient procedures for regenerating fertile plants suggest that the present methods for *Arabidopsis* transformation may prove widely applicable for numerous studies of cell, molecular, and developmental biology which can benefit from the availability of an efficient genetic transformation system.

Methods

Transformation and regeneration

Seeds of *Arabidopsis thaliana*, biotype Wassilewskija, were surface sterilized and germinated on medium containing Murashige and Skoog (M.S.) salts [15], 0.8% agar, and 1% sucrose. Leaf explants approximately 3–4 mm square were cut from plants either 14 or 21 days old. They were infected with *Agrobacterium tumefaciens* by dipping them into a late log phase culture of bacteria that had been incubated overnight at 28 °C and 200 rpm in the presence or absence of 20 μM acetosyringone (3′,5′-dimethoxy 4′-hydroxyacetophenone, Aldrich Chemical Co.). For *A. tumefaciens* receiving the acetosyringone treatment, the culture medium (Luria broth) [14] was adjusted to pH 5.6. After 3 days of co-cultivation of bacteria and leaf explants on callus inducing medium (CIM) (M.S. salts supplemented with 1.0 mg/L thiamine, 0.5 mg/L pyridoxin, 0.5 mg/L nicotinic acid, 100 mg/L myoinositol, 5 mg/L IAA, 0.5 mg/L 2,4-D and 0.5 mg/L zeatin), the bulk of the bacteria were removed by rinsing the explants with M.S. medium containing 500 mg/L cefotaxime (Calbiochem). The explants were then cultured for 7 days on solid CIM containing 500 mg/L cefotaxime. After this time the 5–7 mm calli were randomly transferred to either selection medium containing 10 mg/L G418 and 500 mg/L cefotaxime in CIM or non-selective CIM containing only 500 mg/L cefotaxime.

Resistant calli were transferred to shoot inducing medium (SIM) [4] containing M.S. salts, supplemented with 3% sucrose, 1.0 mg/L thiamine, 0.5 mg/L pyridoxin, 0.5 mg/L nicotinic acid, 100 mg/L myoinositol, 0.05 mg/L IAA, 7 mg/L 2-iminopurine, and 10 mg/L G418. Regenerated shoots were rooted and grown to maturity in 80 × 100 mm pyrex jars containing solidified M.S. medium supplemented with the vitamins listed above, 4 mg/L IAA and 10 mg/L G418. Plant materials were grown at 26 °C under a cycle of 16 hours light (7500 lux) and 8 hours dark.

Transformation was carried out with *A. tumefaciens*, strain C58 C1 *rif* [27] containing the cointegrate Ti plasmid vector pGV3850:pAK1003 [23].

Isolation and analysis of DNA

High molecular weight genomic *Arabidopsis* DNA was isolated from 50–200 mg of tissue and purified on CsCl gradients by a rapid 5-hour isolation procedure [25]. DNA was digested with Pst I or Eco RI restriction enzymes. Digested DNA fragments were separated by electrophoresis in a 0.8% agarose gel. After Southern transfer [12], DNA fragments were hybridized with nick translated [12] pAP 1003 plasmid.

Neomycin phosphotransferase assay

Neomycin phosphotransferase (NPT) activity was detected by the *in situ* gel assay of Reiss *et al.* [18]. Proteins in extracts from 150 mg of tissue were first separated by electrophoresis in nondenaturing polyacrylamide gels and the gels were then exposed

to kanamycin sulfate and gamma ^{32}P-labelled ATP. Phosphorylated kanamycin which was produced in those regions of the gel containing NPT activity was transferred by blotting to phosphocellulase paper and detected by autoradiography.

Results

Transformation

To test the possibility that a preculture of *A. tumefaciens* with acetosyringone might increase the rate of *Arabidopsis* transformation, we added synthetic acetosyringone (AS) (20 μM) to an *Agrobacterium* culture approximately 16 hours prior to exposure of leaf segments to the bacteria. The data of Table 1 shows that in Experiments 1 and 2, between 84 and 87% of the explants exposed to *Agrobacterium* were able to produce green calli when cultured on CIM for 10 days before selection on CIM containing the aminoglycoside antibiotic, G418.

When cultured explants were tested for growth on medium containing G418, we found that approximately 2–3% of calli from leaf segments incubated with *A. tumefaciens* not exposed to acetosyringone were resistant to G418. However, if *A. tumefaciens* cells were pretreated with acetosyringone, the rate of explant survival in the presence of G418 increased to 55% and 63% in the two separate experiments. All explants not incubated with *A. tumefaciens* were killed when transferred to medium containing G418.

Table 1. Rates of transformation and viability of *Arabidopsis thaliana* leaf explants incubated with *Agrobacterium tumefaciens* precultured in the presence or absence of acetosyringone.

Transforming bacterium	Primary explant cultures (10 day incubation)		Transferred callus cultures (8 day incubation)				Percent transformation
			Number of calli tested		Number of surviving calli		
	Number of explants tested	Percent of explants forming callus	Selection on G418	No selection	Selection on G418	No selection	
Experiment 1							
None	48	92%	13	21	0	19	-
A. tumefaciens	69	86%	48	7	1	7	2.1%
A. tumefaciens treated with acetosyringone	138	84%	29	10	16	8	55.2%
Experiment 2							
None	58	84%	21	20	0	20	-
A. tumefaciens	74	87%	37	N.D.	1	N.D.	2.7%
A. tumefaciens treated with acetosyringone	140	84%	91	N.D.	58	N.D.	63.7%

N.D. – Not Determined.

Leaf explants from plants 21 days old (experiment 1) or 14 days old (experiment 2) were infected with *Agrobacterium tumefaciens* that had been incubated overnight in the presence or absence of 20 μM acetosyringone. After 3 days of co-cultivation of bacteria and leaf explants on callus inducing medium (CIM), the explants were cultured for 7 days on solid CIM containing 500 mg/L cefotaxime. Calli were then transferred to either selection medium containing 10 mg/L G418 and 500 mg/L cefotaxime in CIM or nonselective CIM containing only 500 mg/L cefotaxime. In experiment 1 surviving calli were scored after 8 days on selection medium. In experiment 2 the surviving calli were scored after an additional 20 days (i.e., 20 days after transfer to shoot inducing medium containing G418).

Regeneration

Transfer of putatively transformed calli to shoot inducing medium (SIM) [4] containing G418 resulted in prolific shoot production and flower formation within 6 weeks. Root regeneration was achieved within 2 weeks after transferring shoots to a rooting medium (Fig. 1). Regenerated plants were transferred to pots in the greenhouse. Seeds were produced by regenerated plants in 21 days and were collected to analyze the inheritance of the resistance trait (Table 2).

Fig. 1. Arabidopsis thaliana regenerated from leaf explants transformed by *A. tumefaciens*. Callus derived from leaf explants exposed to *A. tumefaciens* as described in Methods was grown on CIM containing 10 mg/L G418 and 500 mg/L cefotaxime for 18 days. Resistant calli were transferred to shoot inducing medium (SIM) [4] (see Methods). All calli selected on G418 survived and grew upon transfer to SIM and after 6 weeks 58% (28/48) produced multiple shoots with flowers. Regenerated shoots were rooted within 2 weeks and grown to maturity on solidified M.S. medium (see Methods).

Table 2. Progeny testing for antibiotic resistance in seeds from nontransformed (WS) and transformed (SA2 and SA10) *Arabidopsis thaliana*.

	Kanamycin					
	0 μg/ml		50 μg/ml		100 μg/ml	
	% germ.	survivors/total	% germ.	survivors/total	% germ.	survivors/total
WS	91%	106/106 = 1.0	85%	2/127 = 0.02	77%	0/ 96 = 0.0
SA2	90%	87/ 87 = 1.0	98%	75/ 98 = 0.76	81%	58/ 73 = 0.79
SA10	91%	106/106 = 1.0	93%	95/ 98 = 0.97	93%	80/ 84 = 0.95
	G418					
	0 μg/ml		10 μg/ml		20 μg/ml	
	% germ.	survivors/total	% germ.	survivors/total	% germ.	survivors/total
WS	84%	119/119 = 1.0	69%	13/166 = 0.08	72%	0/140 = 0.0
SA2	89%	25/ 25 = 1.0	83%	30/ 40 = 0.75	81%	36/ 44 = 0.82
SA10	88%	30/ 30 = 1.0	83%	39/ 40 = 0.97	60%	29/ 41 = 0.71

Seeds were collected from self-fertilized plants and germinated in petri dishes containing agar, MS medium, and various concentrations of G418 or kanamycin. In tests for kanamycin resistance, seedlings were scored after 11 days (SA10) or 13 days (WS and SA2) of germination. In tests for G418 resistance, seedlings were scored after 28 days. Progeny were scored for antibiotic resistance by the number of seedlings surviving antibiotic treatment over the total number of germinating seedlings in each treatment.

Southern analysis

To confirm that survival and growth of explants on medium containing G418 was the result of transfer of the NPT II gene from *Agrobacterium tumefaciens* to *Arabidopsis*, we carried out Southern analyses of DNA from both control plants and putative transformants. The autoradiograph displayed in Fig. 2 confirms the presence in G418-resistant plants of DNA fragments complementary to Ti plasmid DNA (lanes 1–4 and 7–10). The sizes of the DNA fragments generated by Eco RI and Pst I digests correspond in size to those expected from integration into plant cells of the complete T-DNA region of the cointegrate Ti plasmid 3850:pAK1003 [23]. In addition, a number of different border fragments (i.e., restriction enzyme digestion fragments which span the junction between the inserted T-DNA region and the host genomic DNA) are present in the DNA from different transformants. With the restriction enzymes and nick translated probes used in these experiments only right border fragments are detected. No hybridization to restriction enzyme digestion fragments of the expected size is observed with DNA from tissue of control, nontransformed plants (Fig. 2, lanes 5–6 and 11). Note that DNA from both transformed and nontransformed tissues contains an approximate 5.2 kb fragment in both Eco RI and Pst I digestions which serves as an internal marker for DNA load size. Judging from the relative intensity of hybridization to this band of DNA, as well as from the presence of multiple border fragments, it would appear that multiple copies of the T-region DNA may have been integrated into some of the transformed plants (e.g., plants SA2, SA5 and SA11, Fig. 2).

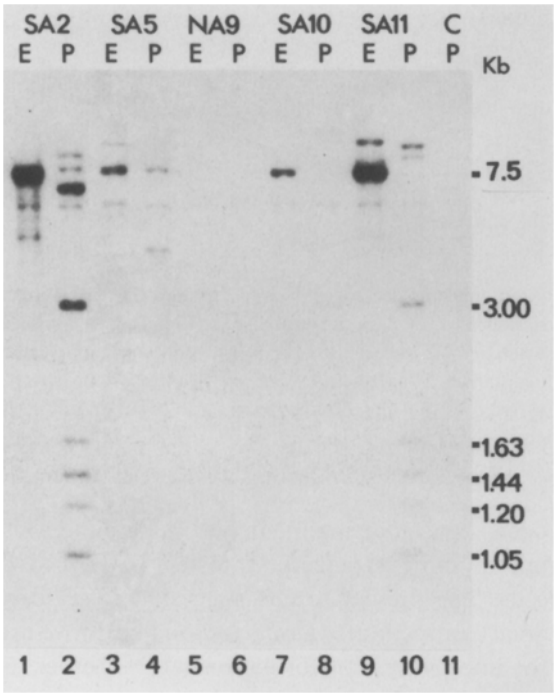

Fig. 2. Southern analysis of DNA from transformed and nontransformed *Arabidopsis* calli and shoots. DNA was isolated from nontransformed tissue (designated as C for control, lane 11), from tissue that was carried through the transformation procedure but was not subjected to selection on G418-containing CIM medium (designated NA for nonselected tissue incubated with *A. tumefaciens* exposed to acetosyringone, lanes 5 and 6), and from tissue growing on CIM medium containing G418 (designated as SA for selected tissues transformed with *A. tumefaciens* exposed to acetosyringone, lanes 1–4 and 7–10). To perform Southern analysis as soon as possible after *Agrobacterium* transformation, DNA was extracted from only 50 to 200 mg of tissue and purified on CsCl gradients by a rapid 5 hour isolation procedure [25]. One third of the DNA from each of the samples (approx. 0.25–1 μg) was digested with either Eco RI (E) or Pst I (P) and loaded in one well of a 0.8% agarose gel. After electrophoresis the DNA was blotted to a nitrocellulose filter [12] and hybridized at 42 °C in 50% formamide, 5 × SSPE to pAK1003 plasmid DNA which was ^{32}P-labelled by nick translation [12].

NPT II assays

Of ten randomly selected plants or calli that were selected as putative transformants by their growth on G418, all proved positive when tested for neomycin phosphotransferase activity by the method of Reiss *et al.* [18]. Figure 3 displays results with representative transformed (lanes 3, 5, 7 and 8) and nontransformed (lanes 1, 2, 4, 6 and 9) tissue samples. The amount of NPT enzyme activity varies significantly between transformants (cf. lane 3 vs. lane 8). Preliminary visual comparisons of Southern analyses with enzyme assay results suggest only a weak correlation between estimated NPT II gene copies and total enzyme activity (data not shown).

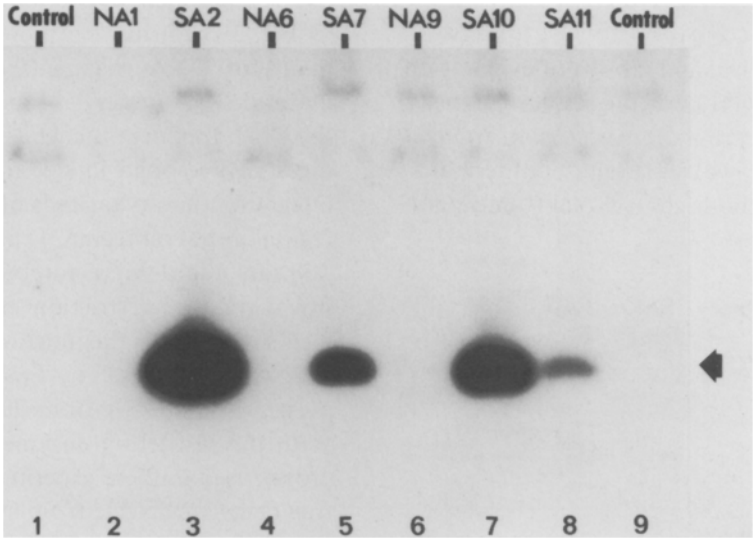

Fig. 3. Assays for neomycin phosphotransferase activity in transformed and nontransformed *Arabidopsis* tissues. Extracts were made from 150 mg of randomly selected control tissues, tissues selected for resistance to G418 (designated SA; see Fig. 2), and nonselected, nontransformed tissues (designated NA). Neomycin phosphotransferase activity was detected by the *in situ* polyacrylamide gel assay of Reiss *et al.* [18]. The arrow designates those regions of the gel which contained NPT II activity and which therefore produced ^{32}P-labelled phosphorylated kanamycin. The radioactive kanamycin was transferred by blotting to phosphocellulose paper and detected by autoradiography [18].

Discussion

Two features of the present transformation procedure may be singled out as potentially important factors which contribute to the high rates of transformation observed. The first is the use of synthetic acetosyringone. Its incorporation into our transformation procedure is based on recent observations that acetosyringone [20] and other natural exudates [21] produced by wounded plant cells are capable of activating genes in the virulence (VIR) region of the Ti plasmid. The activity of these genes apparently contributes to the precise excision of T-DNA from the Ti plasmid at the 25 bp border sequences which reside at both ends of the T-DNA region [10]. This event has been proposed as a key step in the transfer of DNA from *A. tumefaciens* to the genomes of higher plants [10]. The low rate of transformation of *Arabidopsis* by *Agrobacterium tumefaciens* not treated with synthetic acetosyringone suggests that while natural exudates of wounded *Arabidopsis* may be sufficient to elicit some VIR region activity, the addition of exogenous acetosyringone may stimulate the bacterium to more efficiently transfer T-DNA to host cells. The present results suggest that acetosyringone treatment of *Agrobacterium* may prove useful for improving transformation rates in other difficult to transform plant species.

A second factor which may lead to higher transformation rates is the culture conditions to which bacterial cells and leaf explants are subjected during and after transformation. Prior to mixing with fresh leaf explants, *Agrobacterium tumefaciens* is incubated for approximately 16 hours with 20 μM acetosyringone at pH 5.6. The low pH incubation is apparently important for high level activation of the VIR region [21]. After a three day exposure to *A. tumefaciens*, explants are rinsed and transferred to fresh callus inducing medium (CIM) supplemented with cefotaxime to kill any remaining bacteria. Eight days of growth is allowed on this medium prior to subjecting explants to selective medium containing G418. After an additional 8 days, the small surviving calli are transferred to shoot inducing medium (SIM) containing G418.

Based on work of K. Feldmann and M. D. Marks in our laboratory, the relatively short growth period (3 – 7 days) on a callus inducing medium appears to be important to achieving efficient shoot regeneration [6]. This approach using short exposures to CIM to induce competence for regeneration follows from the earlier work of Christianson and Warnick [4] and has allowed the development of procedures for regenerating shoots from 90 to 100% of *Arabidopsis* explants in as little as 21 days [6].

Selection of transformants in our experiments is based on growth of callus from *Agrobacterium* treated explants on the aminoglycoside antibiotic, G418. Resistance to G418 is conferred by expression of the NPT II gene under the control of the promoter number 1 of a back to back dual promoter system isolated from the T-DNA region of an octopine Ti plasmid [23]. This promoter system allows strong expression of the NPT II gene in many of our transformed *Arabidopsis* plants (Fig. 3) and confers resistance in several transformants tested to G418 at concentrations of 20 μg/ml or higher (data not shown). Kanamycin sulfate, an antibiotic closely related to G418, which has been employed successfully as a selective agent for transformants in some plant systems (e.g. [3, 7, 8]), was not satisfactory for use with *Arabidopsis* leaf explants. That is, transformants could be selected at 20 μg/ml of G418, but at levels of kanamycin sufficient to kill nontransformed tissue, all transformed tissues were also killed.

Analysis of the inheritance of the antibiotic resistance trait in seed progeny from two transformed plants, SA2 and SA10 (Table 2), suggests that at least one functional NPT II gene (or two or more closely linked genes) passed through meiosis in the transformed plant, SA2, and that two or more functional genes were segregated among progeny of transformed plant SA10. In contrast to the advantages of selecting transformed leaf explants on G418 (vs. kanamycin), analysis of germination of progeny seeds on medium containing kanamycin is more clear cut than when G418 is used. On kanamycin, nonresistant seedlings soon turn white and die. On medium containing G418, seed germination is often low and nonresistant seedlings die a more gradual death that is sometimes difficult to score early after germination. Nevertheless, the tests with either G418 or kanamycin demonstrate that resistance to high levels of the antibiotics is inherited in what appears to be a normal Mendelian fashion by progeny of both SA2 and SA10. Seeds for analysis of the S_2 population currently are being produced.

The availability of a high efficiency transformation system for *Arabidopsis* will expedite progress in several areas of research. It will allow testing of expression of homologous and heterologous plant genes (both natural and modified) in a system where genetic analysis of progeny is rapid (i.e. 5 – 6 weeks per generation). Because the genome size of *Arabidopsis* is small (approx. 7×10^7 bp), and because gene redundancy is often less than in other higher plants [17], the analysis of transcription and regulation of genes inserted into the *Arabidopsis* genome may be less complicated than in other higher plant systems. The small genome size and the apparent ability of *Agrobacterium* to insert multiple gene copies into cells of leaf explant (Fig. 2) also encourages prospects for the insertional inactivation of particular genes of interest. Such inactivation not only creates useful mutations but also facilitates the ultimate cloning of inactivated gene sequences by virtue of their immediate proximity to Ti plasmid vector sequences. As pointed out by Sommerville *et al.* [5, 19] and Meyerowitz and Pruitt [13] one of the most important advantages of *Arabidopsis* as a model system for molecular and genetic studies is the fact that in an organism, such as *Arabidopsis*, where there is relatively little repeated DNA [17], many genes which code for scorable phenotypes can be identified and isolated using a combination of fine genetic mapping with restriction fragment length polymorphisms (RFLPs), chromosome walking [5, 13], and, finally, complementation by genetic transformation of mutants with cloned copies of wild-type genes. The present transformation procedure, coupled with ongoing research on RFLPs [13] and other molecular techniques in a rapidly growing number of laboratories, offers the possibility that detailed studies of genes involved in key aspects of plant growth and development may be approachable in the near future in *Arabidopsis*.

Acknowledgements

We thank Dr Jeff Velten and Dr Jeff Schell for generously providing the Ti-plasmid vectors and plasmids used for these transformation studies. This work was supported in part by a grant from the National Science Foundation (DCB: 8609623).

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Note added in proof

After this manuscript had been submitted for publication, an article by Lloyd *et al.* appeared (Science 234:464–466 (1986)) which described a similar leaf disc transformation system for *A. thaliana* var. Columbia. This system employed a hygromycin resistance gene as a selectable marker and utilized feeder layers to maximize recovery of transformed plants. With this approach approximately one third of the original explants produced calli and one half or more of these regenerated shoots.