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Agrobacterium-mediated transformation of the desiccation-tolerant plant *Craterostigma plantagineum*

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Summary. An efficient procedure for *Agrobacterium tumefaciens*-mediated transformation of the desiccation-tolerant plant *Craterostigma plantagineum* has been developed. Leaf explants were inoculated with *A. tumefaciens* strain GV3101 carrying the gene for kanamycin- or hygromycin-resistance and the ß-glucuronidase reporter gene. Parameters which affected the transformation efficiency were the age of the explant, the degree of wounding and the presence of an antioxidant in the medium. Under optimal conditions, calli originated in more than 80% of leaf explants. Transformed plants were obtained from more than 50% of the cultured calli during regeneration in the presence of a suitable antibiotic. The stable integration of T-DNA was confirmed by Southern blot analysis and its expression by assays for ß-glucuronidase activity.

Abbreviations: GUS = ß-glucuronidase; MUG = 4-methyl-umbelliferyl ß-D-glucuronide; ABA = abscisic acid; NPTII = neomycin phosphotransferase II; CaMV = cauliflower mosaic virus; MSAR = modified MS medium; MS = Murashige and Skoog.

INTRODUCTION

The resurrection plant, *Craterostigma plantagineum* Hochst. (*Scrophulariaceae*) has recently been adopted as a model system to study the molecular mechanisms of desiccation tolerance (Bartels *et al.* 1993). In this species, tolerance to desiccation can be observed in all differentiated tissues, as well as in callus, in which desiccation tolerance is induced by treatment with abscisic acid (ABA) (Bartels *et al.* 1990, 1991). *Craterostigma* genes that are induced in dried leaves, ABA-treated leaves and ABA-treated callus have been isolated and molecularly characterized (Bartels *et al.* 1990). The desiccation-responsive mechanism presumably operates via an endogenous increase in ABA concentration, which leads to the induction of various target genes (Skriver and Mundy 1990; Bray 1991).

To fully understand desiccation tolerance in the *Craterostigma* model-system, an elucidation of the signal-transduction pathway leading from dehydration to target-gene expression is required. This involves the identification and characterization of regulatory genes mediating stress and ABA-induced signals. A prerequisite for these studies is to establish a transformation system for *Craterostigma plantagineum,* in order to characterize the response of the genes to either stress- or ABA-stimuli in the homologous system.

Agrobacterium-mediated transformation is now a routine procedure for introducing genes into many dicot species (reviewed by Potrykus 1991). The aim of the present study was to assess the possibility of transforming and regenerating *Craterostigma* via *Agrobacterium tumefaciens*.

MATERIALS AND METHODS

Plant material. Plants of *Craterostigma plantagineum* were initially obtained from Professor Volk, University of Würzburg (Germany) and then multiplied either using seeds or vegetatively (Bartels *et al.* 1990). Plants used in this study were maintained and propagated in MS basal medium (Murashige and Skoog 1962) in a growth chamber at 24 ±1°C with 16 h/day fluorescent light providing 200 µE /m^2 /s.

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Bacterial strain, plasmids and culture conditions. The following three constructs, all carrying the ß-glucuronidase gene (GUS; Jefferson 1987), were used for transformation: pBI 121, a CaMV 35S promoter-GUS construct (Jefferson *et al.*, 1987) with the NPTII gene as the selectable marker gene; CDeT27-45 construct (Michel *et al.*, 1993), carrying a *Craterostigma* desiccation- and ABA-inducible promoter-GUS and the NPTII gene; and pTgus plasmid (Koncz *et al.* 1994), a promoterless-GUS construct, carrying the hygromycin resistance gene. *Escherichia coli* transformation and isolation of plasmids were as described by Maniatis *et al.* (1982). Constructs were transferred into *Agrobacterium* via electroporation according to Wen-jun and Forde (1989). The *Agrobacterium tumefaciens* strain GV3101 carrying the helper plasmid pMP90 (Koncz and Schell 1986) was used as the host strain for the constructs carrying the NPTII gene, while the strain GV3101 containing the pMP90RK (Koncz and Schell, 1986) as helper plasmid was used for transformation with the pTgus plasmid. The plasmid-bearing agrobacteria were grown overnight at 28°C on a gyratory shaker at 150 rpm in 50 ml YEB medium containing the appropriate antibiotics. The cells were then pelleted by centrifugation at 4°C and 3000 rpm, and resuspended for plant infection in liquid MSAR medium (Koncz *et al.* 1990), supplemented with 0.25 g/l antioxidant mixture (150 mg/l ascorbic acid and 100 mg/l citric acid) to a final density of OD_{550} = 0.5.

Transformation procedure. Young leaves 5 to 10 mm in length were aseptically removed from the plant and lightly pressed on a sterile folder of fine sand paper to increase the wounded area. The explants were then immersed in the *A. tumefaciens* suspension for 20 min in darkness and co-cultivated for 2 days on solid callus medium without antibiotics (Table1).

Tissue culture media and conditions. MSAR medium (Koncz and Schell 1990) was used as a replacement for regular MS salts. After co-cultivation, the infected explants were subcultured for 10 days on MSAR1 containing 500 mg/l of cefotaxime and 50 mg/l of kanamycin or 15 mg/l of hygromycin (Table 1). These cultures were kept in darkness. Growing calli were maintained by subculturing at 3-week intervals on selective MSAR1. At each subculture step, the cefotaxime was reduced by 100 mg/l, while the antibiotic was maintained as a selectable agent until the plants were transferred to soil. To induce shoot differentiation, embryogenic calli were subcultured on MSAR1a (Table 1).

Shoots were rooted on MS medium supplemented with 2% sucrose and 0.7% agar. Plant hormone concentrations (mg/l) in MSAR1 were: indole-3-acetic acid (IAA), 2.0; 2,4-dichloro-phenoxyacetic acid (2,4-D), 0.5; kinetin, 0.2; N^6-(2-isopentyl) adenosine (9ip), 0.2. In MSAR1a, the plant hormone concentrations (mg/l) were: benzylaminopurin (BAP), 0.5; naphthaleneacetic acid (NAA), 0.1. Both MSAR1 and MSAR1a contained 3% sucrose and were solidified by adding 0.6% Gelrite (Kelco Co.). All MSAR media were supplemented with the antioxidant mixture. The pH was adjusted to 5.8 with KOH before autoclaving. Hormones were filter sterilized and added to the medium. Cultures were maintained at 24 ±1°C with a 16 h photoperiod (200 µE /m^2 /s). Rooted plants were transplanted to jars containing sand and soaked with half strength MS salts for hardening. Plants were then grown to maturity in a phytotron under controlled conditions (Bartels *et al.* 1990).

Determination of GUS expression. Fluorometric GUS assays, using 4-methylumbelliferyl ß-D-glucuronide (MUG) as a substrate (Jefferson, 1987), were carried out on untransformed plants as a control, and plants putatively transformed with pBI 121 CaMV 35S and the CDeT27-45 constructs. In addition to measurements using untreated leaves, assays were also carried out with ABA-treated leaves (floated on a solution of 10^{-4} M ABA for 20 h), leaves incubated with water for 20 h, and dried leaves (placed on a ventilating hood for 20 h).

Southern hybridization analysis. Genomic DNA was extracted from independently transformed and untransformed plants using 2 to 4 g of young leaves according to Doyle and Doyle (1990). About 10 µg of DNA from each plant sample and 100 pg DNA of pTgus plasmid were digested with selected restriction enzymes, separated by eletrophoresis through a 0.8% agarose gel and transferred onto Hybond-N nylon membrane. Hybridizations were performed according to Maniatis *et al.* (1982). The NPTII gene was detected by using the 1.4 Kb *Bam*HI-*Hind*III fragment of the pBin19 vector (Bevan 1984) as a probe; and the GUS gene with the 1.8 Kb *Bam*HI fragment of the pTgus plasmid. DNA fragments were radiolabelled as described by Feinberg and Vogelstein (1983).

RESULTS

A requirement for plant transformation using *Agrobacterium tumefaciens* is the ability to regenerate plants from transformed callus in the presence of selective agents.

Callus development and antibiotic resistance. The frequency of callus formation was influenced by the age of the explant, the degree of wounding and the presence of the antioxidant mixture. Leaf explants longer than 1 cm developed callus only at the base of the petiole, and usually this callus became necrotic within 3 to 4 weeks. Younger leaf explants (<1-cm length) gave a high frequency of callus initiation. Callus development was observed on more than 80% of the cultured leaf explants. *Craterostigma* transformation required extensive wounding of the leaf surface to enhance infection. When explants were not damaged by a scalpel or by treatment with sand paper, they responded poorly to the tissue culture conditions. Because of the high amount of phenolic compounds produced from wounded *Craterostigma* tissue, it was necessary to add the antioxidant mixture to the callus induction and maintainance media as well as to the *Agrobacterium* suspension culture used for leaf inoculation. In the absence of the antioxidant, the explants released phenolic substances whose oxidation products darkened both tissue and medium. In such conditions the explants died within

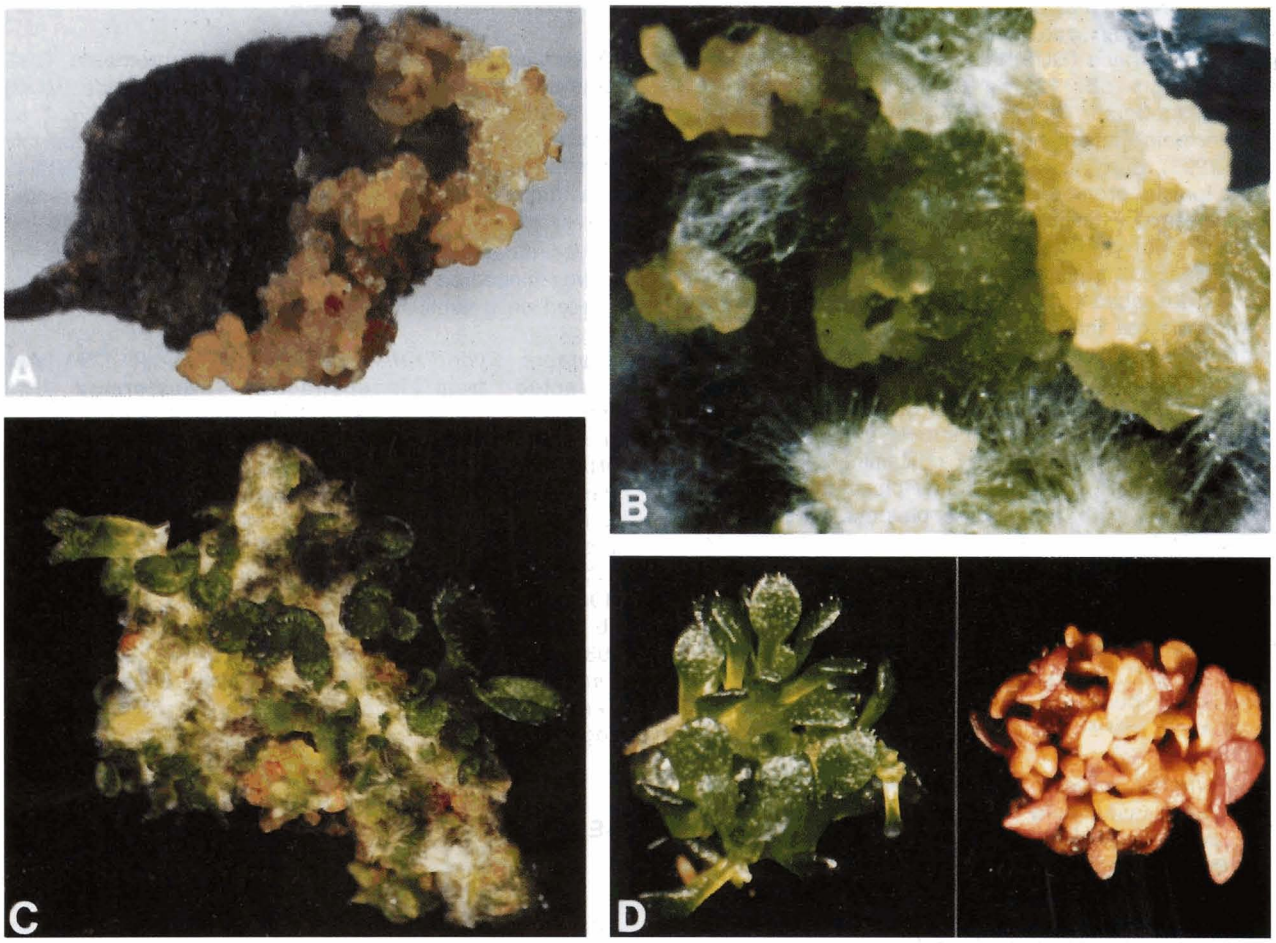

Fig. 1. A. proliferation of callus from an *Agrobacterium* infected explant on kanamycin selection medium. B. One-month old friable embryogenic callus. C. Green shoots developing from somatic embryos. D. Behaviour of explants on kanamycin selection medium: left, transformed shoots; and right, untransformed control.

a few days.

Control leaf-explants were wounded and plated on antibiotic-containing medium without bacterial inoculation. Callus initiation occurred from all young explants 2 weeks later. To determine the sensitivity of this callus to the antibiotic, higher concentrations of kanamycin (100 to 150 mg/l) and hygromycin (30 to 50 mg/l) were tested, although no differences in callus growth were observed. Calli generally turned brown at the base in contact with the medium; nevertheless, these calli could be subcultured and maintained for several months. It was evident that kanamycin and hygromycin inhibited, but did not completely block the formation of new callus tissue. Therefore, identification of transformants could only be performed during the regeneration process.

A large proliferation of callus resistant to the applied antibiotic was observed from those explants that were co-cultivated with *A. tumefaciens*. This callus was produced in clusters at the edge of the infected explants. The tissue exhibited a light green appearance, and was compact with a slightly nodular surface (Fig.1A). The individual callus clusters were subcultured separately 3 weeks after culture initiation in order to maintain the potential single transformation event of each cluster. In this first subculture each callus was divided into two halves: one was subcultured on MSAR1 for callus maintenance and the other was transferred to MSAR1a for plant regeneration. The transformed calli proliferated rapidly in MSAR1 selective medium, on which they were maintained for more than 1 year by subculturing at 3 week intervals.

Regeneration from callus. All calli subcultured on MSAR1a became greener, with a semi-friable consistence. During the second subculture, callus tissue became more friable and hairy, and a large number of embryogenic structures proliferated on its surface (Fig. 1B). On the same medium, the

Table 1. Procedure and media used for *Craterostigma plantagineum* transformation. Time and light conditions required for each step are also reported.

Procedure	Medium	Time	Light
leaf explant infection	MSAR	20'	darkness
co-cultivation	MSAR1 (MSAR + 2.0 mg/l IAA + 0.5 mg/l 2,4-D + 0.2 mg/l kin + 0.2 mg/l 9iP)	2 d	darkness
callus initiation	MSAR1 + 50 mg/l kanamycin[a] (or 15 mg/l hygromycin) + 500 mg/l cefotaxime[b]	10 d	light[c]
callus maintenance	as above	subcult. every 3 wks	light
shoot differentiation	MSAR1a (MSAR + 0.5 mg/l BAP + 0.1 mg/l NAA)	4-5 wks	light
root formation	MS	2-3 wks	light
plant development	MS	2 mths	light
hardening	sand watered with half-strength MS salts	10-15 d	light

[a] Kanamycin and hygromycin were maintained at constant concentration during the culture *in vitro* .
[b] At each subsequent subculture step the cefotaxime was reduced by 100 mg/l.
[c] Light intensity was maintained at 200 µE /m^2 /s.

growth and development of these embryogenic structures led to the formation of multiple green shoots (Fig. 1C). By subculturing, these shoots continuously proliferated on MSAR1a (Fig. 1D, left). The non-inoculated control explants, that grew poorly in callus medium, developed red-bleached shoots in MSAR1a (Fig. 1D, right) that did not develop further.

Green shoots were transferred to MS medium, without hormones and supplemented with kanamycin or hygromycin, for rooting and further development. More than 50% of the subcultured shoots gave rise to plantlets. The continuous regeneration of plants on selective medium was a positive indication that the putative transformants were stable. Rooted plants, with six to eight leaves and with roots 2-3 cm long, were transferred to sterile sand, soaked with half strength MS salt solution for 2 weeks for hardening and then moved to a growth chamber where they grew to maturity.

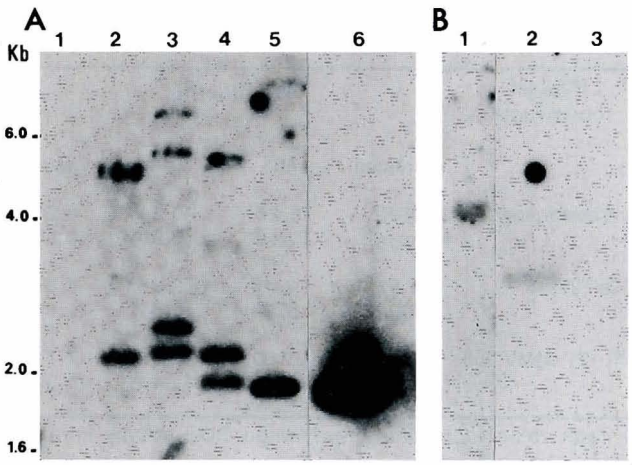

Fig. 2. Southern blot analysis of two transformed plants compared with untransformed plants. **A** and **B** represent digested genomic DNA derived from plants transformed with the pTgus plasmid and CDeT27-45 construct, respectively. **A**, lane 1: DNA from an untransformed plant digested with *Eco*RI. Lanes 2, 3, 4 and 5: transformed plant DNA digested with *Eco*RI, *Hind*III, *Eco*RI-*Hind*III and *Bam*HI respectively. Lane 6: 100 pg pTgus plasmid digested with *Bam*HI. **A**, was probed with the GUS gene. **B**, lanes 1 and 2: transformed plant DNA digested with *Eco*RI-*Hind*III and probed with GUS (lane 1) and NPTII (lane 2) probes. Lane 3: untransformed plant DNA probed with GUS and NPTII gene.

The procedure of transformation, selection and regeneration of *Craterostigma plantagineum* is summarized in Table 1.

Southern blot analysis. To confirm the transformation of *Craterostigma*, genomic DNA from untransformed plants and plants transformed with the pTgus plasmid and the CDeT27-45 construct was extracted from leaf tissue, digested and hybridized with GUS or NPTII probes. A Southern blot analysis of untransformed plants, two independently transformed plants and the pTgus plasmid is shown in Figure 2.

The GUS gene did not hybridize to DNA from a control *Craterostigma* plant (Fig. 2 A lane 1), but hybridized with DNA from the transgenic plant (Fig. 2 A lanes 2-5). A 1.8 kb *Bam*HI fragment was observed when genomic DNA and pTgus plasmid were digested with *Bam*HI (Fig 2 A lanes 5 and 6 respectively), while larger novel plant-T-DNA hybrid fragments were observed when genomic DNA was digested with *Eco*RI, *Hind*III, and *Eco*RI-*Hind*III (Fig. 2 A lanes 2, 3 and 4 respectively) demonstrating the integration of the T-DNA into the plant genome.

DNA from a plant transformed with the CDeT27-

Table 2. GUS expression in transformed *Craterostigma plantagineum* plants. GUS activity assayed as 4-MUG production is expressed in pmol /mg protein/min[a].

Plasmid constructs	Treatments			
	untreated	ABA	dried	water
untransformed	0.22 (0.18)[b]	0.25 (0.19)	0.34 (0.20)	0.29 (0.20)
CDeT27-45	4.58 (2.90)	17.44 (8.28)	3.26 (1.54)	6.86 (2.60)
pBI 121 CaMV 35S	7.11 (2.32)	7.90 (2.00)	7.45 (1.45)	7.71 (1.99)

[a] Each value represents the average GUS activity from ten untransformed plants, ten independent transformants containing the CDeT27-45 construct or five independent transformants containing the pBI 121 CaMV 35S construct.
[b] (±Standard error).

45 construct showed the expected 4.1 Kb *Eco*RI-*Hind*III (GUS gene plus 27-45 promoter) fragment that hybridized with the GUS gene probe (Fig. 2 B lane 1). The same digested DNA showed a hybridizing fragment with the NPTII probe (Fig.2 B lane 2), while genomic DNA from an untransformed plant (Fig. 2 B lane 3) did not hybridize with labelled GUS or NPTII fragments (Fig. 2B lane 3). These results showed that foreign DNA was integrated into the *Craterostigma* genome.

GUS assay. Leaf tissue from ten untransformed plants, ten plants transformed with the CDeT27-45 construct, and five plants transformed with the pBI 121 CaMV 35S were assayed for GUS enzymatic activity by quantitative fluorometric assays. Results reported in Table 2 show that transformed plants expressed between 9.5- and 69-fold higher GUS activity than untransformed tissue.

CONCLUSIONS

The objective of this study was to develop an efficient system for the stable transformation of *Craterostigma plantagineum.* The transformation protocol reported here is already being used for the *in vivo* assay of promoter sequences, to examine the regulatory mechanisms underlying desiccation-inducible gene expression (Michel *et al.* 1994). Because of the high ploidy level, a genetic approach based on conventional mutagenesis experiments cannot be adopted in *Craterostigma* (Bartels *et al.* 1994). However, the *Agrobacterium*-mediated transformation now available can make antisense RNA studies possible, with the aim of inhibiting the expression of relevant genes activated during desiccation and thus, assessing their contribution to desiccation tolerance. The creation of dominant mutants by T-DNA gene-tagging can also be considered, using the Agrobacterium-mediated transformation system in *Craterostigma*. This technique has become a powerful tool for the isolation of structural and regulatory genes from higher plants (Feldmann 1991; Walden *et al.* 1991; Koncz *et al.* 1992).

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