GENETIC TRANSFORMATION AND HYBRIDIZATION

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Agrobacterium-mediated genetic transformation of the desiccation tolerant resurrection plant *Ramonda myconi* (L.) Rchb.

Abstract In this paper we describe the first procedure for *Agrobacterium tumefaciens*-mediated genetic transformation of the desiccation tolerant plant *Ramonda myconi* (L.) Rchb. Previously, we reported the establishment of a reliable and effective tissue culture system based on the integrated optimisation of antioxidant and growth regulator composition and the stabilisation of the pH of the culture media by means of a potassium phosphate buffer. This efficient plant regeneration via callus phase provided a basis for the optimisation of the genetic transformation in *R. myconi*. For gene delivery, both a standard (method A) and a modified protocol (method B) have been applied. Since the latter has previously resulted in successful transformation of another resurrection plant, *Craterostigma plantagineum*, an identical protocol was utilized in transformation of *R. myconi*, as this method may prove general for dicotyledonous resurrection plants. On this basis, physical and biochemical key variables in transformation were evaluated such as mechanical microwounding of plant explants and *in vitro* preinduction of *vir* genes. While the physical enhancement of bacterial penetration was proved to be essential for successful genetic transformation of *R.*
myconi, an additional two-fold increase in the transformation frequency was obtained when the above physical and biochemical treatments were applied in combination. All R_0 and R_1 transgenic plants were fertile, and no morphological abnormalities were observed on the whole-plant level.

Keywords *Agrobacterium tumefaciens* · Desiccation tolerance · Genetic transformation · *Ramonda myconi* · Resurrection plant

Abbreviations GM medium: Germination medium (Toldi et al. 1994) · RA medium:*Ramonda* medium (Tóth et al. 2004) · MSI medium: Modified MS medium for bacterial infection (Borsics et al. 2002) · CPY medium: Culture medium for *Agrobacterium* · BAP: 6-Benzyladenine · NAA: 1-Naphtaleneacetic acid · *sgm* explants: Leaf segment explants · *int* explants: Intact, but microwounded explants

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Introduction

Environmental factors that impose water-deficit stress, such as drought, salinity and temperature extremes, place major limits on plant productivity (Boyer 1982). To overcome these limitations and improve production efficiency in the face of a burgeoning world population, more stress tolerant crops must be developed (Khush 1999). Traditional breeding strategies that have attempted to utilize genetic variation arising from varietal germplasm, interspecific or intergeneric hybridisation, induced mutations and somaclonal variation of cell and tissue cultures have met with only limited success owing to the genetic complexity of stress responses (Flowers and Yeo 1995). In contrast with traditional breeding and marker assisted selection programs, the direct introduction of a small number of genes by genetic engineering seems to be a more attractive and rapid approach to improve stress tolerance. Products of these genes protect, either directly or indirectly, against osmotic

stresses. Engineered over-expression of biosynthetic enzymes for osmoprotectants, scavengers of reactive oxygen species, stress-induced or late embryogenesis abundant proteins are among the approaches reported (for review see Cushman and Bohnert 2000). Although the above resistance genes can be identified and isolated from any living organisms in theory, there are special desiccation-tolerant systems like seeds and resurrection plants, in which the targeted gene isolations and differential screening of dehydration-associated genes have resulted in numerous hits (for review see Ingram and Bartels 1996). Since the physiological role of proteins encoded by these genes are largely unknown, their functional analysis must be completed to allow their utilization in molecular breeding programs. The most frequently applied approach to improve our knowledge on gene functions in plant systems through genetic transformation is the use of sense/antisense gene constructs (Birch 1997). For this purpose, *in vitro* plant regeneration protocols had been established in resurrection plants such as *Craterostigma plantagineum* (Furini et al. 1994; Toldi et al. 2002), *Ramonda myconi* (Tóth et al. 2004) and *Haberlea rhodopensis* (Djilianov et al. 2005) of which only *C. plantagineum* was transformed successfully until now (Furini et al. 1994; Toldi et al. 2002). Here we describe the first genetic transformation procedure for *R. myconi* as the continuation of our previous work on its tissue culture system (Tóth et al. 2004). Additionally, the similarity in genetic transformation between the two resurrection plants *C. plantagineum* and *R. myconi* as a consequence of their common physiological specialisation was investigated and discussed.

Materials and methods

Chemicals

Restriction endonucleases, Taq polymerase and dNTPs were bought from Promega (Madison, WI), while $\alpha[^{32}P]$-dCTP was obtained from Izinta Kft. (Budapest, Hungary). Plant Mini Kit for total DNA extraction was purchased from Qiagen Gmbh (Hilden, Germany). Antibiotics, plant growth regulators and X-GlcA were obtained from Duchefa Biochemie (Haarlem, The Netherlands). All other chemicals were from Reanal Kft. (Budapest, Hungary).

Establishment and maintenance of axenic culture

Seeds of *R. myconi* (L.) Rchb. were obtained from Chiltern Seeds, UK. For the establishment of *in vitro* tissue culture, seeds were surface sterilized in 70% (v/w) ethanol for 1 min and in 30–50% (v/w) Domestos solution (Unilever Hungary Kft.) for 20 min. To remove the remaining disinfectant, the seeds were rinsed five times in sterile deionised water and then germinated on sugar-free GM medium (Toldi et al. 1994) containing half-strength MS elements (Murashige and Skoog 1962), 10 mg/l thiamine, filter sterilized GA_3 (0.1 mg/l) and 8 g/l agar (Plant agar, Duchefa Biochemie

bv.) in plastic Petri dishes. The pH of germination medium was adjusted to 5.8 with 1 M KOH. Germination occurred at $24\pm2°C$ under a 16 h light (approximately 4500 lx)/8 h dark regime. Epicotyls of the 2–3 week-old seedlings were excised and transferred into basal RA medium (Tóth et al. 2004) for micropropagation. Plantlets were grown in Vitro Vent containers (Duchefa Biochemie) at $24\pm2°C$ under a 16 h light (approximately 4500 lx)/8 h dark regime and were subcultured in every 3 weeks.

Bacterial strains

The *A. tumefaciens* strain GV2260 (Deblaere et al. 1985) with the binary vector p35SGUSINT (Vancanneyt et al. 1990) was used for genetic transformation (kindly provided by Dr. Rainer Höfgen MPI of Molecular Plant Physiology, Golm, Germany). The T-DNA region of p35SGUSINT vector contained a p35S-*gus-intron*-t35S transformation cassette in which the β-glucuronidase (*gus*)-coding region is interrupted by the second intron of the potato *st-ls1* gene.

Genetic transformation

Vigorously growing plantlets were selected systematically for at least a year for the purpose of genetic transformation.

Two protocols were followed during the *Agrobacterium*-mediated transformation. Half of the plant material was transformed in a conventional way by using a general protocol developed for *A. tumefaciens* susceptible dicot plants (method A). The other half of the plant material was transformed according to the protocol by which monocot cereals, previously thought to be resistant to *Agrobacterial* infection, could be transformed effectively (method B).

Method A was applied according to Horsch et al. (1988) with slight modifications: About 50 ml of liquid ABM medium (Chilton et al. 1974) supplemented with the proper antibiotics (25 mg/l kanamycin, 100 mg/l rifampicin) was poured into 250 ml Erlenmeyer flasks to propagate transforming *Agrobacteria*. Bacterial cultures were kept in darkness at 28°C overnight on a shaker adjusted to 200 rpm. Healthy-white and dense bacterial cultures were used for transformation. Plant explants were prepared in two ways. Half of the leaves were sectioned by excising their edges that resulted in 5–7 mm × 5–7 mm leaf segments. These leaf segment explants (*sgm* explants) were placed into Petri dishes (10 cm in diameter) containing 10 ml liquid MS medium without further wounding. Subsequently, 1 ml of *Agrobacterium* suspension was added into each Petri dish. The other halves of the leaves were microwounded by sharp scalpel tips (4–5 wounds per leaf) but were not sectioned as the first half. They were applied for transformation as intact, but wounded leaves (*int* explants). The infection was carried out during a 20 min gentle shaking in dim light at 22°C.

Method B was applied according to Hiei et al. (1994) with slight modifications: To propagate transforming *Agrobacteria*, 50 ml of liquid CPY-Infection medium (CPY-I medium,

pH: 5.6) containing 1 g/l yeast extract, 5 g/l triptone, 5 g/l sucrose, 500 mg/l $MgSO_4 \cdot 7H_2O$, 2.0 g/l MES, kanamycin (25 mg/l) and rifampicin (100 mg/l) was poured into 250 ml Erlenmeyer flasks. Bacterial cultures were kept in darkness at $28°C$ overnight on a shaker adjusted to 200 rpm. Healthy-white and dense bacterial cultures were pelleted by centrifugation (10 min, 8000 rpm). These cultures were then resuspended in 40 ml liquid MS-Infection medium (Borsics et al. 2002) supplemented with one-third strength MS macro- and microelements, 10 mg/l thiamine-HCl, 1.0 g/l casein hydrolysate, 10 g/l glucose and 50 µM acetosyringone (MSI medium, pH 5.0). About 40 ml of MSI medium containing the resuspended bacterial cells and the plant explants were added into sterile Petri dishes to carry out bacterial infection.

Sgm and *int* explants, transformed according to method A or method B, were dried shortly on sterile filter paper discs after the infection and were placed onto RA callus induction media (Tóth et al. 2004) for an additional 2 days of co-culture. Subsequently, explants were transferred onto selective callus induction medium (basal RA medium containing 0.2 mg/l BAP and 2.0 mg/l picloram) supplemented with kanamycin (1.0 mg/l) and cefotaxime (400 mg/l). Subculturing took place in every 2 weeks until fast growing, fresh green cell colonies appeared on the browning surface of dying leaf segments. After 4–6 weeks selection, leaf segment explants with surviving calli were transferred onto selective RA regeneration medium supplemented with 0.2 mg/l BAP, 1.0–2.0 mg/l NAA, as well as filter sterilized kanamycin (1.0 mg/l) and cefotaxime (350 mg/l). Putative transgenic plants were then transferred onto selective basal RA medium (contained 1.0 mg/l kanamycin) for micropropagation after the 4-week long regeneration process. Replicates of each independent primary transformant plus appropriate controls were grown in a containment glasshouse under conditions of $20°C$ day and $16°C$ night temperature, with supplementary light to maintain 16-h daylight period. Microplants were planted out in 9.0 cm pots containing a 1:1 mix of sand and perlite and were watered every second day with liquid medium that contained half-strength RA macro- and microelements (pH 6.0). For testing the fertility and for the analysis of progenies, stigmas were pollinated manually by using fine brushes.

PCR analysis

For PCR reactions, total DNA was extracted from the greenhouse-grown wild-type and putative transgenic plants using Qiagen Plant Mini Kit (Qiagen Gmbh, Hilden, Germany), plasmid DNA isolation from *E. coli* was performed using Qiagen Plasmid Mega Kit following the manufacturer's instructions. The PCR analysis was carried out in a Perkin Elmer Cetus thermocycler in 50 µl reaction mixtures containing commercial buffer, 2,5 mM $MgCl_2$, 200 µM dATP, dTTP, dCTP, dGTP, 3 U Taq polymerase, DNA (100 ng total, or 10 pg plasmid), and oligonucleotide primers in 1 µM final concentration. To amplify an internal

*npt*II fragment (781 bp) the following primer pair was used according to Beck et al. (1982):

$(+)5' - AAGATGGATTGCACGCAGGTTC-3'$

$(-)5' - GAAGAACTCGTCAAGAAGGCGA-3'$

Reactions were started with a denaturation at $94°C$ for 4 min, followed by 35 cycles with the following parameters: $94°C$ for 1 min, $55°C$ for 1 min and $72°C$ for 1.5 min. The program was terminated by an extension at $72°C$ for 7 min. Amplification products were analysed by electrophoresis in 1% agarose-ethidium bromide gels.

Southern hybridisations

Purified DNA (10 µg) was digested overnight with *Bam*HI and *Hind*III, as these enzymes cut once within the T-DNA insert, and separated by electrophoresis on 0.8% agarose gels. DNA was blotted onto nitrocellulose (Hybond-C extra, Amersham) membranes. A 1.9 kb long gel-purified *Pst*I fragment from the plasmid pFF19G (Timmermans et al. 1990), representing the *gus* coding sequence, was labelled by the random priming approach (Feinberg and Vogelstein 1983) using $\alpha[^{32}P]$-dCTP. Hybridization and autoradiography were carried out according to standard protocols (Sambrook et al. 1989) and instructions of the manufacturers.

Histochemical GUS analysis

Gus expression was detected by immersing intact leaves of putative transgenic and wild-type plants into the substrate (1 mM X-Glca: 5-bromo-4-chloro-3-indolyl-β-ᴅ-glucuronic acid cyclohexyl-ammonium) that was vacuum infiltrated into the samples. The incubation for enhancement of the specific enzyme reaction was performed as reported by Jefferson (1987) and Jefferson et al. (1987).

Statistical analysis

Students' *t*-tests were performed using MS Excel 2000 (Microsoft Corporation, Seattle, USA). Differences between results are described as being significant where $P \leq 0.05$, and not significant where $P > 0.05$.

Results

Since *R. myconi* showed an extreme sensitivity to tissue culture manipulations, first the effects of antibiotics used either as suppressers of the *Agrobacterium* overgrowth or as selective agents were tested on its *in vitro* morphogenesis. As a preliminary experiment, CPY media containing a series of timentin (50, 100, 150, 200, 250 mg/l) and cefotaxime (300, 350, 400, 450, 500 mg/l) were inoculated with

the *Agrobacterium* strain to select those concentrations that inhibit bacterial growth. It was found that selective media should contain at least 150 mg/l timentin or 350 mg/l cefotaxime for that purpose (data not shown). At the same time, besides effective elimination of *Agrobacterium* after transformation, timentin and cefotaxime had to fulfil another important criteria in terms of special requirements of *R. myconi*. Due to a duality of its *in vitro* responses, a rapid and dramatic reaction to negative effects and, on the contrary, an extended reaction to positive effects, those concentrations of timentin and cefotaxime were screened that can give rise to maximum rate of survival during the whole selection procedure. Since higher than 300 mg/l in timentin and 500 mg/l in cefotaxime result in severe tissue necrosis in *R. myconi*, a concentration range of 100–300 mg/l was applied in the case of the former, while another concentration range of 300–500 mg/l was applied in the case of the latter. Figure 1 shows the rate of survival of the nontransformed plant explants in the presence of timentin or cefotaxime calculated both after the first 2 weeks of the incubation (white columns) and after the second 2 weeks of incubation (grey columns). Explants were transferred once into fresh medium that contained the same components as the first media between the two 2-week-long incubation periods. As it can be seen in Fig. 1A, there were no such concentrations of timentin that would satisfy the dual criteria of the evaluation simultaneously. While in the presence of 100 mg/l timentin the survival rates of the explants were nearly 95% both after the second and fourth weeks of incubation. With concentrations over 150 mg/l, which are sufficient enough to protect plant explants against *Agrobacterium*, the survival rates dropped dramatically by 30–40%. At the same time, there was a narrow range of concentration in cefotaxime (350–400 mg/l) that provided proper protection against *Agrobacterium* during the 4-week-long incubation (Fig. 1B) and where the survival rates of plant explants were still high enough (90%). Therefore, 350–400 mg/l cefotaxime was applied to suppress the overgrowth of transforming *Agrobacterium* during the selection procedure.

Besides suppressing the overgrowth of *Agrobacterium*, antibiotics that are often utilized for positive selection of transgenic cell lines and plants have determining importance during a genetic transformation procedure. In our experiments the *npt*II gene, encoding neomycin phosphotransferase, was chosen as selection marker that can inactivate aminoglycoside-type antibiotics by phosphorylation. The application of *npt*II had two reasons. First, it shows moderate tissue toxicity in sub-lethal concentrations, second, there are numerous aminoglycoside-type antibiotics are available with slightly different chemical structures that render different site effects on the morphogenesis of *R. myconi*. Thus, we had a possibility to choose from selective agents (kanamycin, G418, neomycin, paramomycin) that meet the following dual criteria. First, the favoured concentration range of the given antibiotics should allow as high rate of explant survival as it is possible during the whole transformation procedure. However, it should suppress, but not totally inhibit callus development or plant regeneration simultaneously in non-transformed plant explants. The first
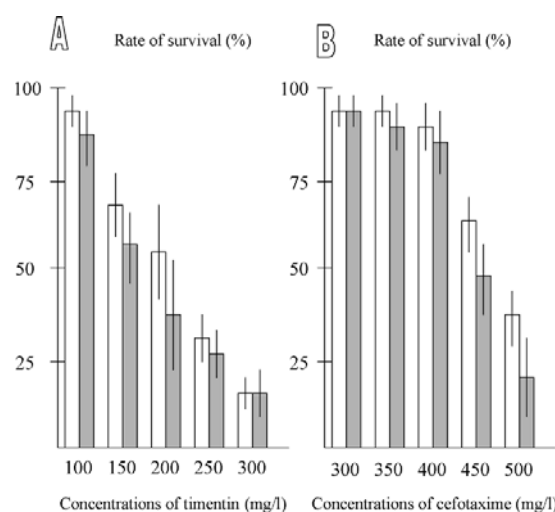
Fig. 1 The influence of inhibitors (**A**, timentin; **B**, cefotaxime) of the *Agrobacterium* overgrowth on the survival rates (%) of nontransformed *R. myconi* explants. Experiments were repeated three times by using 50 explants/treatment in each repetition

information that Table 1 reveals is that the effective concentrations of all the antibiotics examined were unusually low. In the case of kanamycin and G418, the callus development and plant regeneration were suppressed at 1.0 mg/l concentration that was still accompanied with satisfactory survival rates of plant explants (kanamycin: 75%, G418: 60%). In contrast, survival rates dropped below 50% when neomycin (40%) or paramomycin (49%) were applied in concentrations suppressing morphogenesis (1.0 mg/l). On this basis, 1.0 mg/l kanamycin was used for the selection of transgenic cell lines and plantlets in genetic transformation experiments.

All putative transgenic plants were subjected to PCR tests and Southern hybridisations in order to confirm the presence of transgenes in their nuclear genomes. Primers designed into the *npt*II gene gave bands, besides the positive control (C+), only in those transformed *R. myconi* lines (Fig. 2E lanes T1, T3, T6) that displayed morphologically normal phenotype on selective media (Fig. 2D). However, no homologous fragments from non-transformed plants (Wt) and from transformed regenerants possessing abnormal phenotype (Fig. 2D) could be amplified (Fig. 2E lanes T2, T4, T5).

Southern hybridisations (Fig. 2F) confirmed the results of PCR analysis showing that the transformation cassette was integrated into the genome of morphologically normal transformants (lane T1, T3, T6) with copy number varying between one and three copies in the lines tested. At the same time, wild-type controls (Wt) and abnormal transformants did not posses the specific hybridisation signal (data not shown). Homologous fragments were also present in the positive controls, which were purified DNA from plasmids applied for transformation (C+).

The frequency of gene delivery and the proper translation of the bacterial T-DNA in the regenerated transgenic plants was estimated by histochemical localisation of the plant-specific GUSINT activity (Fig. 2G). The blue coloration
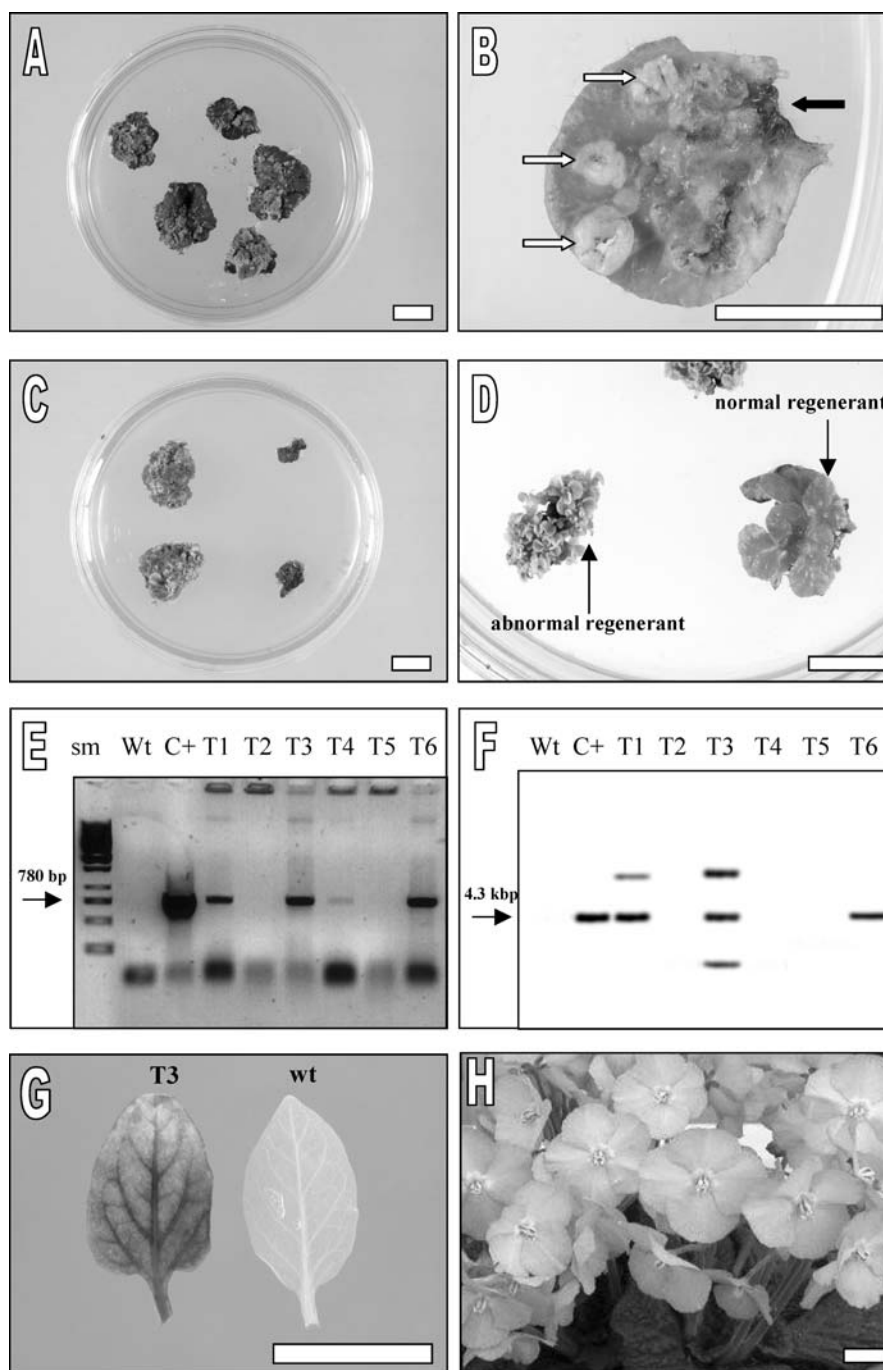
Fig. 2 **A** Leaf segment explants (*sgm* explants) showed low rates of survival during the genetic transformation experiments. **B** Intact leaf explants (*int* explants), in which the starting phase of the callus development can be seen around the areas wounded by scalpel tip under selection pressure (yellow arrows), while conventional cutting resulted in severe tissue necrosis along the edges (black arrow). **C** Intact leaf explants possessed a satisfactory rate of survival in the presence of 1.0 mg/l kanamycin and 400 mg/l cefotaxime during the selection procedure. **D** Morphologically normal and abnormal plantlets were differentiated on *int* explants under selection pressure. **E** Amplification of a 781 bp long *npt*II-specific DNA fragment in morphologically normal (T1, T3, T6) and abnormal transformants (T2, T4, T5), and in wild-type *Ramonda myconi* plants (Wt). Purified binary vector p35SGUSINT served as positive control (C+), while the size marker (sm) was *Pst*I digested λ phage DNA. **F** Identification of the integrated T-DNA and estimation of its copy number in the *Ramonda* genome by Southern blot analysis using the *gus* probe. The enclosed gel photograph demonstrates an experiment in which genomic DNA, extracted from putative transgenic (T1–T6) and wild-type plants (Wt), was digested with *Bam*HI that made the estimation of the copy number of the integrated *gus* probe possible (see "Materials and methods" section). Abbreviations are same as in Figure 2E. For positive control, *Bam*HI-digested p35SGUSINT vector was used. **G** Histochemical detection of the expression of the *gusint* reporter gene in a PCR and Southern positive transformant (T3). **H** Fertile and morphologically normal flowers of transgenic *R. myconi* plants grown in the glasshouse. Bars in each panel represent 1 cm

Table 1 Effects of different aminoglycoside-type antibiotics on the survival rates (%) of non-transformed *R. myconi* explants

Selective agents	Applied antibiotic concentrations (mg/l)						
	0.1	0.2	0.5	1.0	2.0	5.0	10.0
Kanamycin	90.1 ± 8.4	87.3 ± 11.0	82.1 ± 14.1	75.1 ± 7.5	36.3 ± 9.1	5.9 ± 2.0	1.7 ± 1.1
G418	92.5 ± 9.1	89.8 ± 7.8	79.2 ± 7.6	60.3 ± 7.1	41.7 ± 6.8	3.7 ± 2.1	2.5 ± 1.9
Neomycin	89.1 ± 9.0	78.5 ± 8.2	63.0 ± 9.9	40.2 ± 8.6	20.9 ± 5.8	7.3 ± 3.8	5.9 ± 2.3
Paramomycin	83.0 ± 6.7	79.9 ± 8.5	67.2 ± 9.4	48.9 ± 9.1	33.5 ± 4.7	4.1 ± 1.9	1.9 ± 1.7

Experiments were repeated three times by using 100 explants/treatment in each repetition ($\pm$SE)

of transgenic *R. myconi* plantlets could be visualized especially in leaf veins marking the actively growing areas (T3).

After providing evidence of successful genetic transformation, the frequency of the recovery in transgenic plants was evaluated in terms of different explant types and transformation protocols used. The impacts of physical (microwounding) and biochemical treatments (see the recipe of MSI medium: application of low pH, liquid medium supplemented with acetosyringone, aldose-type sugar source and organic nitrogen during the bacterial co-culture) that are thought to enhance the bacterial penetration into target tissues were monitored during the transformation procedure (Table 2). Two types of plant explants were applied as targets of transformation. *Sgm* explants were prepared in a conventional way excising 5–7 mm × 5–7 mm leaf segments by cutting off the edges of leaf blades. At the same time, *int* explants were detached intact leaves where the freshly wounded infection channels were provided by gentle punching of leaf blades with a new, sharp scalpel tip (four to five holes per leaf). Surprisingly, transgenic plants could be recovered on *int* explants only, proving that the physical enhancement (microwounding) of bacterial penetration was essential for the successful genetic transformation of *R. myconi* (Fig. 2B). An additional two-fold increase in the transformation frequency was obtained when the above physical and biochemical treatments were applied in combination. However, it appears that the survival rates of *sgm* explants were too low to produce transgenic plants independently of the transformation protocol applied (Fig. 2A).

Besides microwounding, the non-lethal selection strategy also had a pivotal role in producing transgenic *R. myconi*. Application of kanamycin at sub-lethal concentration not only made high survival rate of *int* explants possible (Fig. 2C) but it also allowed plant regeneration to occur even in non-transgenic explants. In this latter case, only the differentiation of morphologically abnormal plantlets could be obtained, possessing small, bleached leaves and poor root development (Fig. 2D). While these plantlets were non-transgenic according to PCR analyses, Southern hybridisations and GUS staining, the morphologically normal plantlets possessing healthy green, fully expanded leaves all carried the transgenes (Fig. 2 EFG, Table 2). After growing the R_0 generation of transgenic plants, self-pollinated R_1 and different R_0 × wild type crosses were analysed by germinating seeds on growth regulator-free RA medium containing 1.0 mg/l kanamycin. Proge-

nies from selfed primary transformants (R_0) in all cases showed a typical 3:1 (Km^R:Km^S) Mendelian phenotypic segregation when seeds were germinated in the presence of kanamycin (data not shown). In a set of experiments, 21 plantlets from a transgenic × wild type cross were also analysed. The progeny showed a 11:10 Km^R:Km^S segregation, also indicating a monogenic dominant fashion for the transmission of the *npt*II gene. All R_0 and R_1 transgenic plants developed fertile flowers (Fig. 2H), and no morphological abnormalities were observed on the whole plant level.

Discussion

Ramonda species are shade-adapted resurrection plants and are considered homoiochlorophyllous as well, since they preserve more than 80% of the chlorophyll content during dehydration (Markovska et al. 1994). For this reason, these resurrection plants becoming more and more important as experimental models in studying photosynthetic and metabolic processes resulting in an elevated tolerance to desiccation (Augusti et al. 2001; Müller et al. 1997; Quartacci et al. 2002). Exploring the unique physiology of *Ramonda* species will enable these plants to turn into subjects of targeted gene isolations (Pico et al. 2002) in a similar manner to *C. plantagineum* that has become the most extensively studied resurrection plant to date (Ingram and Bartels 1996; Norwood et al. 2003; Scott 2000). It is not an exaggeration to predict that functional analysis of these genes will inevitably follow the isolation work in the near future (Cushman and Bohnert 2000). This requires the development of a method for genetic transformation that allows the application of sense/antisense to unravel the physiological role of proteins encoded by the genes of interest (Birch 1997). Previously, we described methods both for *in vitro* micropropagation and plant regeneration of *R. myconi* (Tóth et al. 2004) that meant an applicable basis for its *Agrobacterium*-mediated genetic transformation. In a similar manner to other resurrection plants, *R. myconi* also possessed an extreme sensitivity during the establishment of its plant regeneration and genetic transformation system. *Craterostigma plantagineum* (Furini et al. 1994; Toldi et al. 2002), *R. myconi* (Tóth et al. 2004) and *H. rhodopensis* (Djilianov et al. 2005) have all been successfully tissue cultured. These tissues all displayed frequent evidence of tissue necrosis, development of hyperhydrated

Table 2 The impacts of explant type, as well as physical (microwounding) and biochemical enhancements (*in vitro* preinduction of *vir* genes) of gene delivery on the efficiency of transformation

Explant type and transformation protocol	Frequency of transformation								
	Rate of survival (%)[a]	Regeneration rate (%)[a]		PCR positive (%)		Southern positive (%)		GUS positive (%)	
		Norm[b]	Abnorm[b]	Norm[b]	Abnorm[b]	Norm[b]	Abnorm[b]	Norm[b]	Abnorm[b]
sgm									
Method A	11.5±2.1	0.0	8.3±2.8	0.0	0.0	0.0	0.0	0.0	0.0
Method B	13.9±3.5	0.0	7.5±1.8	0.0	0.0	0.0	0.0	0.0	0.0
int									
Method A	65.3±9.4	17.3±3.1	10.3±2.6	13.5±3.0	0.0	10.1±1.9	0.0	10.1±1.9	0.0
Method B	62.8±9.2	30.1±6.9	20.4±4.3	23.7±5.8	0.0	21.4±2.6	0.0	21.4±2.6	0.0

Experiments were repeated three times by using 100 explants/treatment in each repetition (±S.E.). *sgm* explants, leaf segment explants; *int* explants, intact, but microwounded leaf explants; method A, Horsch's protocol (Horsch et al. 1988); method B, protocol described in this paper

[a] Survival and regeneration rates measured under selection pressure

[b] Regenerated plants possessing morphologically normal or abnormal phenotypes

leaves and secretion of polyphenols into culture media under suboptimal conditions. In addition, *R. myconi* and *H. rhodopensis* displayed a sort of duality in their *in vitro* responses probably as a result of slow metabolism and growth. Rapid and dramatic reactions to negative effects were paralleled with extended and moderate reaction to positive effects (Djilianov et al. 2005; Tóth et al. 2004). This made the genetic transformation of *R. myconi* difficult, because most steps in a transformation procedure generate various types of physiological stresses that decrease survival rates of both transgenic and non-transgenic tissues. Indeed, the low survival rates of those plant explants that were made ready for genetic transformation by cutting off all edges of leaf blades (*sgm* explants) impeded the recovery of transgenic plants in our experiments (Fig. 2A, Table 2). At the same time, detached leaves where the *Agrobacterial* penetration was promoted by microwounding (*int* explants) survived the transformation procedure at a satisfactory frequency (Fig. 2C), which, in turn, allowed an efficient production of trangenic *R. myconi* plants (Fig. 2E–G, Table 2). In this latter case, we speculated that the proportion of wounded and non-wounded areas within an *sgm* or *int* explant was the most important factor in determining survival rates. While large wounded surfaces rendered a strong exposure to stresses in the case of *sgm* explants, large non-wounded surfaces might enhance the recovery of minor wounds in *int* explants after the bacterial infection (Fig. 2B).

Other important component of our 'low stress' transformation was the development of a non-lethal selection strategy for *R. myconi*. Most of the toxic drugs that are used as inhibitors of bacterial growth or as selective agents have side effects that can decrease or prevent plant regeneration not only in non-transgenic explants but also in transgenic ones (for review see Oreifig et al. 2004). This fact meant an additional risk knowing the *in vitro* behaviour of *R. myconi*. Therefore, concentrations of both *Agrobacterium* eliminating and selective agents were optimised with the intention to accomplish the essential preconditions of a non-lethal selection strategy. That is, on the one hand, survival rates of plant explants have to be as high as possible in the presence of antibacterial drugs to make successful genetic transformation possible. On the other hand, the optimal concentration of the selective agent has to suppress, but not totally inhibit morphogenesis on non-transformed explants to allow a simple visual pre-selection of transgenic regenerants. In other words, we were in search of such sub-lethal concentrations of the applied selective agent, in which the main distinctive features of transgenic plants might be the green colour, morphologically normal phenotype and a more intense growth, while non-transgenic regenerants are likely to be bleached showing morphological abnormalities and retarded growth. As it can be seen in Fig. 2D the moderate tissue toxicity of kanamycin applied at sub-lethal concentration (1.0 mg/l) made the differentiation of two types of regenerants possible in its presence. Examining all the abnormal regenerants no transgenic one was found among them, while normal regenerants carried the transgenes with no exceptions (Fig. 2E–G, Table 2).

In contrast to *C. plantagineum* where the physical enhancements of bacterial penetration had negative rather than positive effects (Toldi et al. 2002), such treatment (microwounding) had primary importance in the success of genetic transformation of *R. myconi* (Fig. 2B). An additional two-fold increase in the transformation frequency was obtained when the above physical and biochemical treatments (*in vitro* preinduction of *vir* genes) were applied in combination (Table 2). These modifications together with the appropriate tissue culture system resulted in a reliable method for genetic transformation of the physiological model plant *Ramonda myconi*.

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