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New Rhododendron Cultivars through Genetic Engineering

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ABSTRACT

The aim of our study was to review the results of genetic engineering of rhododendrons which have been published in the scientific literature so far. Genetic engineering has great potential to improve rhododendrons, but current protocols are complicated and time-consuming. Assessing each study revealed more and more factors that have a significant impact on the efficiency of genetic transformation. Much work still needs to be done in order to optimize the process, but gene transfer experiments that have been carried out thus far have already proved to be successful. Rhododendrons with a more efficient root system, allowing for higher iron uptake under low iron stress soil conditions have been obtained.

Keywords: *Azalea* sp., *Agrobacterium tumefaciens*, genetic transformation, microprojectile bombardment, *Rhododendron* sp.
Abbreviations: ***chs***, chalcone synthases; ***Fro*2**, ferric-chelate reductase; **GFP**, green fluorescent protein; **GUS**, β-glucuronidase; ***hpt***, hygromycin phosphotransferase; ***npt*II**, neomycin phosphotransferase

CONTENTS

INTRODUCTION

Rhododendrons have become increasingly popular ornamental plants in horticulture. Despite great morphological and physiological variety, existing genotypes do not meet all customers' expectations. Therefore old genotypes are improved and new genotypes are created which fulfill the actual trend of horticultural art. Breeders prefer plants that are resistant to diseases, pests and harmful environmental conditions such as low temperatures, drought or soil salinity (Muras and Klein 1998; Reiley 2000). Cultivation of such plants is easier and cheaper so their popularity in horticulture is higher.

Conventional breeding is difficult and durable because of numerous limitations (e.g. high level of heterozygotic character, pre-zygotic or post-zygotic barriers, poor reproduction or inappropriate morphological architecture of descendant plants (Muras and Klein 1998).

The past few decades has seen a rapid development of molecular genetics, creating new possibilities for plants, including numerous ornamentals, modification by genetic engineering (reviewed extensively in Teixeira da Silva 2006).

Genetic engineering of rhododendrons was initiated at the end of 20th century in the Czech Republic, Japan and Belgium (Pavingerová *et al*. 1995; Ueno *et al*. 1996; Mertens *et al*. 1997). So far two main procedures of gene transfer have been employed for rhododendrons: *Agrobacterium tumefaciens*- or *A. rhizogenes*-mediated transformation and microprojectile bombardment (**Table 1**). Both techniques encountered the same problem – *in vitro* regeneration, which resulted in a decrease in the number of transgenic plants. Therefore conditions for improving *in vitro* regeneration of each rhododendron genotype ought to be studied. Optimization of regeneration parameters helps, but does not guarantee success because of other limiting elements like infection of *Agrobacterium* and antibiotics, that are used for transgenic cell selection. During *Agrobacterium*-mediated transformation it is necessary to check the susceptibility of various genotypes to different strains (Pavingerová *et al*. 1997). Total efficiency of genetic transformation of rhododendrons depends on numerous factors: plant genotype, explant type, transformation procedure and composition of selection and regeneration media.

This review covers the results of genetic engineering of rhododendrons which have been published thus far.

INTRODUCED GENES

Genes used in genetic transformation can be isolated from different organisms. So far there is no information about gene transfer between various rhododendron species.

During initial studies on the genetic transformation of *Rhododendron* sp. only selection and marker genes were introduced in order to establish an efficient procedure for gene transfer. In particular, genes coding for antibiotic resistance, such as *npt*II (neomycin phosphotransferase) or *hpt* (hygromycin phosphotransferase) were employed. Expression of these genes in plant tissues enables the selection of genetically modified cells which are resistant to antibiotics or herbicides contained in the medium. Generally the *uid*A gene (coding for GUS or β-glucuronidase) was used as a reporter, whose expression allowed to identify transformants during a histochemical test (Pavingerová *et al*. 1995; Ueno *et al*. 1996; Mertens *et al*. 1997; Pavingerová *et al*.

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Invited Mini-Review

Table 1 Examples of genetic transformation of *Rhododendron* sp.

Genotypes	Transformation procedure	Marker genes	Useful traits' genes	Explants	Type and concentration of selecting factor	Efficiency	Reference
Rhododendron: 'America', 'Catawbiense grandiflorum roseum', 'Madame Carvalho', 'Mars', 'Nova Zembla'	*Agrobacterium tumefaciens* LBA 4404	GUS *npt*II	-	Stem segments	kanamycin 20, 50, 100 mg/L	27 of 50 tested shoots contain GUS gene	Pavingerová *et al.* 1995, 1997
Rhododendron 'Percy Wiseman'	*Agrobacterium tumefaciens* LBA 4404	GUS *npt*II	-	Leaf and stem segments	kanamycin 100 mg/L	6 plants of 120 explants tested contain GUS gene (5%)	Ueno *et al.* 1996
Rhododendron simsii 'Hellmut Vogel'	*Agrobacterium tumefaciens* AGLO	GUS *npt*II	-	Leaf segments	kanamycin 10, 20 mg/L vancomycin 400 mg/L	2.9 plants/100 explants contain GUS gene (2.9%)	Mertens *et al.* 1997, 2000
Rhododendron 'Hino-crimson' 'Fuchsia'	Microprojectile bombardment	GUS *hpt*	-	Leaf and stem segments, shoot-tips	hygromycin 2, 4, 8, 16, 32, 64, 128 mg/L	22,2% of explants contain GUS gene	Hsia and Korban 1998
Rododendron ssp.	*Agrobacterium tumefaciens*	GUS	-	Leaf segments	-	1-2 shoots of 100 explants contain GUS gene	Dunemann *et al.* 1999
Rhododendron 'Catawbiense Album' L, 'America', 'Joe Paterno', 'Cunningham's White'	*Agrobacterium tumefaciens*, C58, B6, *Agrobacterium rhizogenes* E8/73	*npt*II GFP	-	Stem segments	Cefotaxime 300 mg/L	3 explants show GFP gene expression	Tripepi *et al.* 1999
Rhododendron 'Catawbiense Album' L.	Microprojectile bombardment	GUS *npt*II *hpt*	-	Leaf segments	kanamycin 50, 100 mg/L hygromycin 2.5, 5 mg/L	Transgenic plants were obtained	Knapp and Brand 1999; Knapp *et al.* 2000
Rhododendron 'Catawbiense Album' L.	Microprojectile bombardment	GUS *npt*II GFP	-	Leaf segments	kanamycin 50, 100 mg/L	Transgenic plants were obtained	Knapp *et al.* 2001
Rhododendron 'Cunningham's White', Rh 10, Rh 33, Rh 37	*Agrobacterium tumefaciens*	-	35SrolB *rol ABC Fro*2	Leaf segments	-	1-2% of transgenic plants obtained	Dunemann *et al.* 2002
Rhododendron 'PJM Hybrid'	Microprojectile bombardment	GUS	-	Leaf segments	-	Transformed explants contain GUS gene	Moore and Tripepi 2003

*Fro*2, ferric-chelate reductase; GFP, green fluorescent protein; GUS, β-glucuronidase; *hpt*, hygromycin phosphotransferase; *npt*II, neomycin phosphotransferase.

1997; Hsia and Korban 1998; Knapp and Brand 1999; Knapp *et al*. 2000; Mertens *et al*. 2000; Knapp *et al*. 2001; Dunemann *et al*. 2002; Moore and Tripepi 2003). Scientists also used green fluorescence protein gene (GFP), isolated from jelly-fish *Aequorea victoria*. Expression of GFP caused green luminescence of the plant tissue following UV irradiation (Tripepi *et al*. 1999; Knapp *et al*. 2001). Hsia and Korban (1998) suggested that macroscopic identification of living, genetically modified tissue would be easier if anthocyanin genes had been used as reporters. So far selective and marker genes were transferred to numerous rhododendron genotypes (**Table 1**).

Further research on genetic transformation concerned genes of useful traits, desired by breeders. Ueno *et al*. (1996) tried to introduce *rolC* genes isolated from *Agrobacterium rhizogenes* into rhododendron in order to reduce apical dominance and to shorten the internodes. Dunemann *et al*. (1999, 2002) used 35S-*rolB* and *rolABC* genes that resulted in the rooting of seedlings and in a stronger root system. They created 14 transgenic lines: 11 transformed with the *rolABC* gene combination and 3 transformed with the 35S-*rolB* construct. Growth retardation was observed in two of the 35S-*rolB* transgenic lines. The other lines showed morphological alterations and strong root systems. The authors assumed that the observed improved rooting performance could contribute to a better adaptation of *Rhododendron* to calcareous soils.

In another approach (Dunemann *et al*. 2002) a gene coding for the enzyme ferric-chelate reductase (*Fro*2) was introduced into 30 different lines. This enzyme is responsible for a better iron uptake under low iron-stress soil conditions. Molecular analysis of DNA and RNA confirmed the expression of the *Fro*2 gene in all tested rhododendrons. Measure of ferric-chelate reductase activity in rhododendron lines that were cultured in hydroponics lacking iron revealed a 50% increase of activity of that enzyme. The enhancement of iron uptake efficiency has an affect especially when plants are grown on calcareous soils because lime induced iron chlorosis is one of the most important nutritional disorders in *Rhododendron* species and hybrids.

In 1999, Dunemann *et al*. suggested that genes coding for resistance to pests or diseases should also be taken into consideration. Knapp *et al*. (2000) suggested that the introduction of resistance genes to fungal diseases could limit the use of fungicides, especially during the nursery stage of rhododendrons. Breeders also look for rhododendrons that flower early in spring, repeat flowering during summer or autumn, and have original structure and fragrant flowers.

All these expectations are still not fulfilled in terms of genetic engineering, although research has been continued. In 2001, Pavingerová *et al*. used antisense RNAs of 3′ regions of *chs* (chalcone synthases) genes from *Rhododendron* and *Chrysanthemum* for improvement of flower coloration. Scientists conducted *Agrobacterium*-mediated transformation of *R*. 'Catawbiense Grandiflorum' and obtain transgenic plants. That research is still in progress. In 2007, Heremans and Werbrouck reported that constitutive expression of cell division inhibitor KRP2 in transgenic *Rhododendron simsii* 'Helmut Vogel' results in reduced shoot growth, increased branching and early flower induction. The practical value of such discovery depends on the fact that it will be possible to obtain pot azaleas without pinching and chemical growth reduction.

GENETIC ENGINEERING PROTOCOLS

The most common procedure of gene transfer into rhododendrons was *Agrobacterium*-mediated transformation,

however various strains of *A. tumefaciens* were used: LBA4404 (Pavingerová *et al*. 1995; Ueno *et al*. 1996; Pavingerová *et al*. 1997), AGLO (Mertens *et al*. 1997, 2000). Tripepi *et al*. (1999) applied both wild and genetically modified strains of *A. tumefaciens*: C58 and B6 and *A. rhizogenes* E8/73. The best results were achieved after transformation by genetically modified strain B6. Authors suggested that rhododendron hybrids have different susceptibility to infection caused by distinct strains of *Agrobacterium*. Consequently, bacterial strains have to be carefully selected for hybrid transformation. Mertens *et al*. (1997) revealed that differences in the transformation efficiency by strain AGLO depend on the type of the plasmid by testing three binary plasmids: p35SGUSint, pMOG410 and pCPO201. The highest efficiency was achieved after transformation by strain AGLO which contained pMOG410, followed by AGLO with pCPO201 and p35SGUSint. Transformation resulted in transgenic azaleas that were morphologically identical to untransformed plants. The inheritance of the marker genes in sexually derived progeny was tested and segregation of GUS gene occurred in a Mendelian fashion (Mertens *et al*. 2000).

Another transformation approach used on rhododendrons was microprojectile bombardment, which has been used to estimate the conditions of transient transformation of various rhododendron hybrids. Complete plants were not regenerated from transformed tissues, and only expression of introduced genes was observed. In that way Hsia and Korban (1998) tested efficiency of helium bombardment devices on gene transfer into evergreen azaleas. Moore and Tripepi (2003) on the basis of transient transformation studied various conditions of *in vitro* regeneration of bombarded rhododendron tissues. In 1999, Knapp and Brand used microprojectile bombardment to obtain complete transgenic rhododendrons. The experiment was successful. Molecular characterization of obtained transformants indicated that the transgenes (GUS, GFP, *npt*II and *hpt*) were stably integrated into the *Rhododendron* genome through the vegetative generations (Knapp *et al*. 2000, 2001).

EFFICIENCY OF GENETIC TRANSFORMATION

The opportunities for transgenic rhododendrons are tremendous, but the practical approach is hindered by low *in vitro* regeneration and few shoots that arise from transformed explants. The efficiency of *in vitro* regeneration depends not only on genotype and explant, but also on the composition of the media used for regeneration and selection of transformed plants.

Pavingerová *et al*. (1995), while researching the genetic transformation of the *Rhododendron catawbiense* group, came to the conclusion that susceptibility to infection of *Agrobacterium* and *in vitro* regeneration depends on genotype. They transformed 5 rhododendron genotypes ('America', '*Catawbiense grandiflorum roseum*', 'Madame Carvalho', 'Mars' and 'Nova Zembla') by exactly the same protocol and get various results depending on the genotype tested. Tripepi *et al*. (1999) carried out a study of the genetic transformation of the *catawbiense-hybridum* group and confirmed Pavingerová's results. The best results were obtained with *R*. 'Cunningham White' which was the most susceptible to both wild and genetically-modified *Agrobacterium* strains (C58, B6 and E8/73). So far a complete protocol for rhododendron genetic transformation was worked out by Ueno *et al*. (1996) for hybrids of the *Yakushimanum* group, by Hsia and Korban (1998) for azaleas of the *Kurume* group and by Mertens *et al*. (1997, 2000) for *Rhododendron simsii*. A review on *Kurume* azaleas has recently been written by Okamoto *et al*. (2007).

Iapichino *et al*. (1991) claimed that the biggest influence on rhododendron *in vitro* regeneration was the explant type. Good results were achieved in rhododendron by regeneration from callus (Economou *et al*. 1988), leaf segments (Iapichino *et al*. 1992), ovule culture (Dai *et al*. 1987), and flower buds (Meyer 1982). During genetic transformation of *Rhododendron* sp. leaf segments, stem segments and shoot-tips were most often used (**Table 1**). Shoot *in vitro* regeneration from control plant tissues (i.e. not infected by *Agrobacterium*) is easier than infectd explants. Dunemann *et al*. (1999) observed only *in vitro* regeneration of callus from transformed leaf segments, but they did not form shoots at all, whereas regeneration of callus and shoots from non-transgenic explants reached 90 to 100%. Ultimately, the efficiency of genetic transformation of leaf explants was 1-2%. Meretens *et al*. (1997, 2000) also used leaf explants and obtained a higher efficiency of rhododendron genetic transformation: 2.9%. Pavingerová *et al*. (1995, 1997) identified 27 plants of 50 tested, which showed GUS gene expression after 5-10 mm long stem segments were transformed.

An important factor which influences the genetic transformation of rhododendrons is the composition and the character of the medium that is used for regeneration and selection. *In vitro* regeneration from transformed rhododendron tissues was generally based on Anderson (1984) medium (Ueno *et al*. 1996; Mertens *et al*. 1997; Hsia and Korban 1998). Pavingerová *et al*. (1995, 1997) applied modified Anderson's medium that contained 8 mg/L 2iP (D-*myo*-inositol-1,4-bisphosphate), 1 mg/L IAA (indole-3-acetic acid) and 2 g/L PVP (polyvinylpirrolidone). These substances reduced the influence of negative phenols on tissue cultivated *in vitro*. Tripepi *et al*. (1999) obtained good results by using WPM medium (McCown and Lloyd 1981) supplemented with 4.9 μM 2iP. Moore and Tripepi (2003) observed higher GUS gene expression in explants that were cultured on sucrose-free medium than on medium containing 0.09, 0.2, 0.4 or 0.6 M sucrose. Higher GUS gene expression was also observed in explants cultured for 6 days in darkness, than in explants cultured in light. Finally, the highest GUS gene expression was noticed in explants that were precultured on regeneration medium for 9-12 days before DNA transformation. Effectiveness of such protocol was confirmed by Dunemann *et al*. (1999) who precultured leaf segments for 1-2 months before co-cultivation with *Agrobacterium*. Ultimately, they achieved 1-2% of stable transgenic rhododendrons.

Selection of transformed tissue after genetic transformation using positive selection systems is based on selective media that contain antibiotics. The most often used antibiotics that have been used in rhododendron transformation experiments are kanamycin and hygromycin. If selection is applied directly after plant tissue and bacteria co-cultivation, it may stunt regeneration. The time period between infection and application of selecting medium differs: 24 hours (Pavingerová *et al*. 1995, 1997), 48 hours (Mertens *et al*. 1997), 4 days (Ueno *et al*. 1996), to 6 weeks (Tripepi *et al*. 1999). Regeneration of transformed tissues is also influenced by the concentration of antibiotics added to medium. A low concentration does not eliminate all non-transgenic cells, although higher concentration of antibiotics decreases shoot regeneration. Pavingerová *et al*. (1997) suggested that 20 mg/L of kanamycin, added to selection medium, generates too many false transformants, whereas 100 mg/L is sufficient for effective selection of transgenic plants. Hsia and Korban (1998) observed growth inhibition of explants that were cultured on medium containing more than 5 mg/L of hygromycin.

EXAMINATION OF PLANTS' TRANSGENIC CHARACTER

A genetic transformation experiment does not end when transformants are obtained. Transformed plants have to be checked in terms of transgene integration, correctness of transcription/translation and gene expression.

The transgenic character of modified rhododendrons was checked generally by a histochemical protocol which allowed for the detection of GUS gene expression. Such a procedure was used for example during detection of transient expression in order to quickly estimate the efficiency of

genetic transformation (Hsia and Korban 1998; Moore and Tripepi 2003). In other studies, besides a histochemical approach, detection of the transgene was carried out by molecular techniques such as PCR and Southern blot (Pavingerová *et al*. 1995; Ueno *et al*. 1996; Pavingerová *et al*. 1997; Knapp *et al*. 1999, 2000) or Northern blot (Mertens *et al*. 2000).

Studies of gene transfer reveal big differences of gene expression in transgenic rhododendrons. High GUS gene expression occurred when explants were examined shortly after genetic transformation, from 70% to 100% (Dunemann *et al*. 1999). Unfortunately, after regeneration only 1-5% of stable transgenic plants were obtained (**Table 1**). That phenomenon has been already explained by Pavingerová *et al*. (1997), whose research indicated chimerism of transgenic rhododendron plants. Initially, such transformants showed high expression of introduced genes, but after vegetative propagation expression decreased. That problem could be overcome either by using higher concentration of selecting antibiotics (e.g. 100 mg/L kanamycin) immediately after transformation or by single-cell origin of transgenic plants.

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