TRANSFORMATION OF RHODODENDRON WITH GENES FOR ABIOTIC STRESS TOLERANCE

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Abstract

An Agrobacterium tumefaciens - mediated gene transfer technique was established for large flowering evergreen Rhododendron hybrids and used for the transfer of target genes. The transformation protocol was based on pre-cultured leaves taken from in vitro shoots. Transformation rates between one and two percent were obtained. Two different strategies have been followed. In an unspecific approach rol genes from Agrobacterium rhizogenes were used. Fourteen rol-transgenic rhododendron lines were created. Eleven lines have been transformed with the rolABC gene combination under the control of the native promotor, and the remaining 3 lines with the 35S-rolB construct. Severe growth retardation was only observed in two of the 35S-rolB- transgenic lines. The other lines showed morphological alterations and often strong root systems. Even on lime enriched culture media in vitro rooting was significantly enhanced in some lines. We assume that an improved rooting performance could be obtained that may contribute to a better adaptation of Rhododendron to calcareous soils. In a second approach a gene coding for the enzyme ferric-chelate reductase (Fro2) was introduced in 30 different lines. This enzyme is also in Rhododendron responsible for a better iron uptake under low iron stress soil conditions and should be increased by using a constitutive promotor. Transfer and expression of rol genes as well as the Fro2 gene in different rhododendron rootstock genotypes was proved by molecular analysis of DNA and RNA.

1. Introduction

Lime-induced iron chlorosis (Fe-chlorosis) is the most important nutritional disorder in Rhododendron species growing in nature in humus soils with low pH. Iron chlorosis does not only impair the ornamental value of an evergreen rhododendron plant but is also often accompanied by a drastic reduction of growth and flowering. In addition, the growth of the root system may be also directly impaired by an excess of bicarbonate anions released from the lime salts. Lime stress is not only one of the most serious difficulties in the cultivation of Ericaceous species but is generally recognized as a problem in many other horticultural and agricultural crops such as apple, citrus, grape, soybean or dryland rice (Vose, 1982).

Conventional breeding projects concerning lime tolerance in Rhododendron have been performed at the Institute for Ornamental Plant Breeding (Ahrensburg, Germany) during about twenty years. Rootstock genotypes that showed enhanced root development on a peat-free sandy loam of pH 5.8-6.0 were selected (Preil et al., 1994). However, bicarbonate concentrations exceeding 600 mg/l, which is a quite common concentration in calcareous soils, inhibited plant growth completely and induced strong iron chlorosis symptoms (Chaanin et al., 1996). Additional breeding efforts are therefore necessary to
develop lime tolerant rootstocks or cultivars growing on own roots. The improvement of lime tolerance in rhododendrons by breeding is a time-consuming approach because the polygenic inheritance of the trait makes it difficult to select the desired genotypes in early generations. Molecular marker techniques may enhance the efficiency of selection (Dunemann et al., 1999).

In addition to conventional breeding a gene transfer approach is followed for improving lime stress tolerance of rhododendrons. Abiotic environmental stresses such as drought, salinity and extremes of temperature are generally difficult targets for transformation due to the complex metabolic pathways involved in the traits. Nothing is known about the physiology of lime tolerance in *Rhododendron*, and only few reports are dealing with the physiology of lime-induced iron deficiency in other plant species (for review, see Bergmann, 1992). Therefore, two completely different approaches are followed for rhododendron transformation. First the root system itself should be improved. In the second approach we try to optimize the iron nutrition of the rhododendron plant. As the iron availability on lime enriched soils is a limiting growth factor, genes involved in iron uptake and metabolism may be used to create transgenic plants that are more tolerant to Fe-deficiency.

Genetic transformation of *Rhododendron* has been described (Ueno et al., 1996; Pavingerova et al., 1997; Knapp et al., 1999; Dunemann et al., 2000). In these reports the GUS reporter gene was used to evaluate *Agrobacterium tumefaciens*- or bombardment-mediated transformation procedures, respectively. To our knowledge, the transfer of target genes has not been published for *Rhododendron*. In the present paper we describe a successful transformation of evergreen rhododendron hybrids with rolABC genes from *Agrobacterium rhizogenes* and the *Fro2* gene from *Arabidopsis thaliana* that is involved in iron uptake. New morphological features and alterations in lime stress reactions are presented.

### 2. Materials and methods

#### 2.1. Plant materials

For rol gene transformation, two rhododendron genotypes (RD2-2, RD2-30) were used. In addition, three rhododendron elite rootstock genotypes (Rh 10, Rh 33 and Rh 37) were selected for Fro2 gene transformation. All genotypes are hybrids representing the genetic background of three rhododendron species *R. caucasicum*, *R. ponticum* and *R. fortunei* (Subgenus *Hymenanthes*). For Fro2 transformation also the cultivar and proven rootstock 'Cunningham's White' was chosen. Shoot-tip explants were cultured *in vitro* on a modified Anderson's medium (Anderson, 1984) and adventitious shoot regeneration was induced on the same medium to obtain starting material for the transformation experiments.

#### 2.2. Genes and bacterial strains

**rol genes**: *Agrobacterium tumefaciens* strain GV3101 harboring the binary vectors pPCV002-rolABC1 and pPCV-CaMVrolB (Spena et al., 1987) was used for transformation. The vectors contain the *nptII* gene for kanamycin resistance driven by the pNOS promotor and the ORFs 10, 11 and 12 corresponding to *rolA*, *B* and *C* from Ri plasmid A4 of *Agrobacterium rhizogenes*. The rolABC construct contains the native promotor of *A. rhizogenes*, and the rolB construct the constitutive 35S promotor. Fro2 gene: A 6.2 Kb Xbal-fragment containing the entire Fro2 coding region was released from the genomic clone j1Z4 (Robinson et al., 1999), subcloned in the expression vector pRT108 (Töpfer et al., 1993) and finally cloned in the binary vector system pLH9000 (Hausmann et al., 1999) allowing a kanamycin - based selection scheme. The marker gene as well as the target gene are driven by the 35S promotor. *A. tumefaciens* strains G5V2260 and GV3101 were transformed with the binary vector pLH9000-Fro2.
2.3. Plant tissue culture and transformation procedure

Leaves taken from the micropropagated shoots were cultured before co-cultivation with *A. tumefaciens* on a medium (97.4) containing the standard components used for adventitious shoot regeneration but differing in phytohormone composition. The medium 97.4 was supplemented with 0.2 mg/l 2,4-D, 1 mg/l Zeatin and 1 mg/l thidiazuron (TDZ). During a pre-culture of about 6 to 8 weeks a strong shoot regeneration was induced on the leaves without a pronounced callus phase. The dense clusters consisting of regenerated shoots and shoot primordia were divided and co-cultured with the Agrobacteria strains. After a co-cultivation phase without antibiotics of 5 to 7 days a subculture on a selection medium containing 100 mg/l kanamycin, 100 mg/l cefotaxime and 150 mg/l timentin was performed. Explants were subcultured after 4 and 8 weeks on the same medium. Then explants with surviving shoots were placed on a standard shoot regeneration medium (74.56) with antibiotics to allow an early *in vitro* propagation of the shoots from different transformation events. After rooting on a hormone-free medium the transgenic plants were transferred into a greenhouse.

2.4. Evaluation of rooting potential *in vitro*

Rooting ability and root growth under lime stress conditions were investigated in *rolABC*-transgenic lines and control genotypes RD2-2 and RD2-30 only. Adventitious shoots were placed on the standard rooting medium supplemented with 10 and 20mM NaHCO₃ and varying pH values. The percentage of rooted shoots was evaluated after three months of culture.

2.5. Molecular analysis

DNA analysis: For Southern hybridizations and PCR analysis of *rol-* and *Fro2-*transformants DNA was isolated from young leaves using a CTAB protocol (Dunemann et al., 1999). PCR analysis with gene-specific primers designed for nptII, rolA, rolB, rolC and Fro2 was repeated by analysing additional plants from the same transgenic line. About 10 µg of DNA was digested with EcoRI and HindIII, blotted on Hybond N+ nylon membrane (Amersham Pharmacia) and hybridized with a nptII - and rolA fragment or a Fro2 probe, respectively. Radioactive DNA hybridizations were performed according to standard procedures. RNA analysis: Total RNA was isolated from very young leaves with the RNasea plant kit (Qiagen, Hilden, Germany). RNA expression studies were done on the basis of RT-PCR using the One-Step RT-PCR kit from Qiagen.

3. Results

3.1. *rol* gene transformation

Fourteen *rol*-transgenic rhododendron lines were created following a modified leaf transformation protocol. Eleven lines have been transformed with the *rolABC* construct and the remaining 3 lines contain the 35S-*rolB* construct. During the selection of transgenic shoots using a kanamycin concentration of 100 mg/l a parallel micropropagation of the transgenic shoots was performed. A total of more than 400 plants have been raised in a greenhouse.

In general the transgenic rhododendron plants exhibit an altered phenotype typical for *rol*-transgenic plants (Figure 1), although pronounced differences were found between lines. Six of the *rolABC* lines show increased branching, shorter internodes and smaller, slightly wrinkled leaves. Three *rolABC* lines do not show the compact habitus but have an increased apical dominance and suppressed lateral shoot development. The remaining two *rolABC* lines are phenotypically normal but show a slower growth. Two of the 35S-*rolB* transformed lines are extremely dwarfish. Molecular characterization by Southern hy-
bridization analysis, rol gene-specific PCR (Figure 2a) and RT-PCR has clearly shown that almost all plants are genetically modified. However, a few normal looking "escapes" that did not contain the foreign genes have also been found indicating that the original transgenic shoot could be a chimera. Most of the lines are transformed with one or two copies of the different rol gene combinations (hybridization patterns not shown). Interestingly, the rolABC lines exhibiting an increase in apical dominance did not show the rolC fragment.

The presence of a combination of rolA, B and C genes enhanced adventitious root formation on micropropagated rhododendron shoots. The percentage of shoots developing roots in vitro on a hormone-free rooting medium was clearly enhanced in most transgenic lines. In four lines 100 % of the shoots showed a fast and strong root development (data not shown). After application of different levels of lime stress to unrooted in vitro shoots, a pronounced increase in the percentages of rooted shoots was found in some of the rolABC lines (Figure 3). For example, in transgenic line 8/3-6 about 30 % of the explants formed normal roots on medium S3 whereas in the control genotype RD2-30 the rooting capacity was less than 10 %. In general, it was observed that the rolABC transgenic plants formed remarkably strong root systems already during the in vitro rooting phase and also later on during pot culture in the greenhouse. Presently we are investigating the rooting behaviour and the Fe-chlorosis susceptibility in a greenhouse experiment on lime enriched soils.

3.2. Fro2 gene transformation

To improve the iron uptake of a lime stressed rhododendron plant a Fro2 gene construct was cloned in the binary vector pLH9000. Using the modified leaf transformation procedure 30 different Fro2- transgenic lines have been produced and transferred into soil after an in vitro propagation phase. All plants are phenotypically normal and it seems that they are growing very fast and healthy. Growing on a lime enriched soil, some lines appear to have a darker leaf colour compared with the non-transgenic controls. Southern hybridizations with a Fro2 - specific DNA probe proved the transgenic character of the plants (Figure 2b). The presence and expression of the new gene was also confirmed by gene-specific PCR and RT-PCR (not shown). Presently further molecular analyses and enzymatic assays of ferric-chelate reductase activity are performed.

4. Discussion

A transformation method has been developed for large flowering evergreen Rhododendron hybrids and used for the transfer of target genes such as rolABC and Fro2. This "modified leaf transformation" was more efficient than a conventional leaf disk protocol tested in the past. Dependent on the genotype and the transformation experiment, transformation rates between one and two percent were obtained. Preculture of the leaf explants was necessary to induce a strong and probably direct adventitious shoot regeneration prior to the co-culture with the Agrobacteria. Although the transformation was directed to complex meristematic tissues, the frequency of chimeric plants seems to be low. This may be due to the rapid regeneration process and the continuous kanamycin selection applied during micropropagation of transgenic shoots. However, chimeric plant production remains a common problem in transformation procedures even when regeneration occurs via direct shoot regeneration. Some chimerism was also found after transformation of rhododendron stem explants (Pavingerova et al., 1997).

The transformation of Rhododendron with rol genes from Agrobacterium rhizogenes resulted in 14 lines exhibiting the whole range of morphological characteristics described in the literature. The individual rol genes interact with the metabolism of plant hormones and enhance the sensitivity of transgenic plants or callus tissues towards different phytohormone concentrations (Schmülling et al., 1993). Already the effect of single rol genes on hormonal and developmental characteristics can be drastically and has
been analysed in various plant species (Martin-Tanguy et al., 1996; Fladung et al., 1997; Lemcke et al., 1998). In ornamental plant species especially a modification of plant architecture is of commercial interest. Among the different genes affecting plant morphology single rol genes or rol gene combinations have been most widely and successfully employed, i.e. in Osteospermum (Giovannini et al., 1999), Petunia (Winefield et al., 1999) or Limonium (Mercuri et al., 2001). Improved rooting performance is a specific aim in woody ornamentals or in fruit tree species that are grafted commonly on rootstocks. In Rosa hybrida the rolB gene and especially the combination rolABC enhanced the rooting ability of cuttings in vivo as well as adventitious root formation in vitro (Van der Salm et al., 1997). In addition, the transfer of rol genes can not only improve the rooting ability, but also reduce the size of the transgenic plant. Attempts to develop such dwarfing rootstocks have been described for apple (Zhu et al., 2001) and pear (Bell et al., 1999). Although we are mainly interested in the rooting characteristics of Rhododendron, we are also examining if some of the rol-transgenic lines could be used as dwarfing rootstocks for grafting existing Rhododendron cultivars that have a too vigorous growth. Since flowering of transgenic plants is expected soon we also intend to use the transgenic material in breeding experiments.

The positive effect of a rolABC combination on the rooting ability of transgenic rhododendron plants seemed to be accompanied in most lines by a generally stronger root system not only on standard rooting medium but also on culture medium reflecting heavily lime stress. Whether the rolABC transgenic plants tolerate lime stress in calcareous soils, is presently tested in greenhouse experiments. Because root growth is drastically impaired by lime salts, a stronger and more heavily developed root system may be a general advantage for a rhododendron plant transferred into calcareous soil. In addition, also on normal soil there may be an advantage in tapping fresh sources of mineral nutrients and water due to an increase of the root/shoot-ratio of the plant.

As the iron availability on lime enriched soils is a limiting growth factor, genes involved in iron uptake and metabolism could be used to create transgenic plants that are more tolerant to Fe-deficiency. In the second approach the Fro2 gene for the enzyme ferric-chelate reductase has been introduced in Rhododendron. This enzyme is involved in plant mechanisms to better iron uptake under low iron stress soil conditions (Mori, 2000) and has been discussed for use in transgenic overexpression strategies to enhance iron capture from soil and resistance to iron deficiency stress (Sussman, 1999). To our knowledge a gene transfer of Fro2 has published only for Arabidopsis thaliana frdI mutants (Robinson et al., 1999) but not yet for other plant species. If the constitutive expression of Fro2 is an advantage for an iron-stressed rhododendron plant is presently analysed.

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References

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Figure1: Typical examples for different transgenic rhododendron lines transformed with the rolABC combination; the larger plant is possibly expressing only the genes rolA and B
Figure 2. (A) PCR analysis of 14 rolABC - transgenic plants representing 6 different lines using rol gene-specific PCR primers; Pl: plasmid isolated from GV3101-rolABC, M: 1 kb-ladder; (B) Southern hybridization patterns of 19 Fro2 - transgenic plants representing 11 different lines (1 - 19); M: molecular size standard

Figure 3. In vitro rooting ability of two rolABC-transgenic lines and their original genotypes under increasing lime stress conditions; left diagram: control RD2-2 (white) and rolABC-7/7-1 (grey); right diagram: control RD2-30 (white) and rolABC-8/3-6 (grey)