Agrobacterium rhizogenes-Mediated Transformation of *Hypericum tomentosum* L. and *Hypericum tetrapterum* Fries.

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This is the first evidence on successful *Agrobacterium rhizogenes*-mediated genetic transformation of two species from the genus *Hypericum*, *H. tomentosum* L. and *H. tetrapterum* Fries. Hairy root cultures were induced from root segments of both *Hypericum* species by two agropine wild-type strains of *A. rhizogenes*, ATCC 15834 and A4. The transgenic character of the hairy root cultures was proved by PCR amplification of the *rolABCD* genes. In some *H. tetrapterum* transgenic lines *aux* genes were detected as well.

Key words: Hairy Roots, *Hypericum* spp., *rol* Genes

Introduction

Plant transformation is an important experimental tool in plant genetics, developmental biology and physiology. *Agrobacterium*-mediated transformation represents an effective and universal tool for genetic modification of a broad spectrum of plant species. Introduction of foreign genes into the host or gene inactivation enables to study the function of plant genes *in situ*. Availability of such a transformation system would be very useful for the study of genes involved in biosynthetic pathways of valuable secondary metabolites synthesized by medicinal plants. Some representatives of the genus *Hypericum*, especially *H. perforatum* L. used for centuries in traditional medicine for its antidepressive properties, are recently recognized mainly due to the activities of naphthodianthrones and acylphloroglucinols. Among them anticancer and antiviral effects with multiple modes of action belong to the most studied (Kubin *et al.*, 2005; Beerhues, 2006).

The aim of the present work was to develop and optimize a reliable protocol for *Agrobacterium*-mediated transformation of two hypericin-producing species of the genus *Hypericum*, *H. tomentosum* and *H. tetrapterum*, belonging to phylogenetically distant sections of this extensive genus. The availability of a reproducible transformation protocol within the genus *Hypericum* would be helpful in the study of some regulatory aspects of biosynthesis of important secondary metabolites.

Material and Methods

Plant material

Aseptic, 4- to 8-week-old, *in vitro* grown seed-derived plants of *H. tomentosum* L., *H. tetrapterum* Fries., and *Nicotiana tabacum* were used for the experiments. The plant material was cultivated on basal medium containing MS salts and vitamins (Murashige and Skoog, 1962), 30 g l^{-1} sucrose, and 8 g l^{-1} agar; the pH value was adjusted to 5.7 before autoclaving. The plantlets were kept in a culture room at (23 ± 1) °C, a 16/8 h photoperiod, and fluorescent irradiance of 28 μE m^{-2} s^{-1}.

Bacterial strains and culture conditions

For transformation bacterial suspensions of two wild-type agropine strains of *Agrobacterium rhizogenes*, ATCC 15834 and A4 (American Type Cultures Collection), were used. A bacterial suspension was prepared according to the modified protocol of Di Guardo *et al.* (2003). It was initiated from a single cell bacterial colony and cultured on a rotary shaker (120 rpm) in 10 ml liquid NB (nutrient broth, Micropoli) medium at 28 °C. After 24 h, 1 ml of the culture broth was transferred into 9 ml of fresh liquid NB medium, cultivated overnight, and used for transformation of *Hypericum* explants.

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Transformation procedure

H. tomentosum and *H. tetrapterum* root (approx. 1.5 cm long) and leaf (half of leaves) segments were wounded with a scalpel and soaked together with *N. tabacum* leaf segments in a bacterial suspension diluted with sterile distilled water (1:19). Leaf segments of *N. tabacum* were used as positive control of the bacterial virulence and also as a natural additional source of acetosyringone. After 20 min the explants were briefly dried and cultured on MS medium for 2 d. On the third day the explants were transferred onto MS media supplemented with 100 mg l^{-1} cefotaxime (MS-Cx). The root explants were kept in the dark, whereas the leaf explants under light conditions in the culture room. The number of explants developing roots with the hairy root phenotype (transformation efficiency) was evaluated 30 d after co-culture. Putative hairy roots were selected 4–6 weeks after transformation. Fast-growing roots (about 30–40 mm long) with the hairy root phenotype were excised from the explants, cultivated separately on hormone-free MS-Cx medium, and subcultured after 3–4 weeks.

Since this transformation procedure was unsuccessful for leaf explants, several modifications were done: pre-culture of the explants on regeneration medium [MS medium supplemented with zeatin (10 μM) and indole-3-acetic acid (1 μM)] for 5, 10 or 15 d before the transformation procedure, addition of active charcoal into the regeneration medium, pre-treatment of the explants at 4 °C for 4 d before the transformation procedure.

Detection of T-DNA genes

Total DNA was extracted from approx. 100 mg of fresh plant material according to Haberer *et al.* (1996). Genomic DNA from the bacterial strains was isolated according to the protocol of Chen and Kuo (1993). Integration of the *rolABCD* and *aux1,2* genes in the genome of the putative hairy root cultures was determined by PCR amplification with gene-specific primers. The presence of bacterial cells in the hairy root cultures was excluded by amplification of the *virC1* gene. The reaction conditions for amplification of the *rolABC* genes were according to Koperdáková *et al.* (2009). The PCR primers (Table I) for amplification of the *rolD*, *aux1,2* and *virC1* genes were designed according to the published sequences of the *rolD* gene of *A. rhizogenes* Ri plasmids, *aux1,2* genes of pRi ATCC 15834 strain of *A. rhizogenes* (DQ782955), and *virC1* gene of pRi or pTi of *Agrobacterium*.

Results and Discussion

In this work we present the successful transformation of two *Hypericum* species, *H. tomentosum* and *H. tetrapterum*. Root and leaf segments of both species were used for transformation experiments with two wild-type agropine strains of *A. rhizogenes* (ATCC 15834 and A4), but only the root segments were capable of transgenic hairy root formation. These segments were sensitive to infection by both *Agrobacterium* strains. The hairy root phenotype of the regenerated roots, such as white hairs, fast growth and branching on hormone-free medium, was observed approx. 3–4 weeks after transformation. The transformation efficiency of *H. tomentosum* varied between 35% for A4 and 44% for 15834 strains, respectively. Higher susceptibility of explants was detected in *H. tetrapterum* transformed with strain 15834 (73%) in contrast to strain A4 (13%).

Table I. Nucleotide sequence of the PCR primers used for detection of T-DNA integration in putative transgenic hairy roots of *Hypericum* spp.

Name	Nucleotide sequence	*T* [°C]	PCR product size [bp]
rolD-for	5'-CTGAGCGTGTGGCTCATG-3'	60	101
rolD-rev	5'-GGAGGTAAAGACGAAGGACAGAG-3'		
aux1-for	5'-CATAGGATCGCCTCACAGGT-3'	61	198
aux1-rev	5'-CGTTGCTTGATGTCAGGAGA-3'		
aux2-for	5'-AACGATAATAGCCCGCTGTG-3'	61	217
aux2-rev	5'-CGTCTTGGGTTTGTGGTTCT-3'		
virC1-for	5'-AATGCGTCTCTCTCGTGCAT-3'	60	425
virC1-rev	5'-AAACCGACCACTAACGCGAT-3'		

The ATCC 15834 strain seems to be more efficient for both *Hypericum* species as the hairy roots were developing more frequently and have emerged earlier after the transformation procedure. The putative hairy root cultures were selected according to the hairy root phenotype of the roots developing on the surface of the infected explants 4–6 weeks after transformation. Several transformed lines (Fig. 1) developed by excision of individual roots growing from the wounded explants were tested for integration of T-DNA genes of *A. rhizogenes* into the plant genome.

Unlike the root segments, leaf explants of *H. tomentosum* and *H. tetrapterum* were not sensitive to the same transformation procedure. All the leaves underwent necrosis within one month. Hairy roots were present only on control leaf segments of *Nicotiana tabacum*. Neither modification of the transformation procedure led to positive results. The leaf explants kept viable for more than 3 weeks only when pre-cultured in the cold, at 4 ºC, but without producing hairy roots. This cold pre-culture was used to suppress the production of secondary metabolites which could have antibacterial effects. Similarly to our results the root segments were proved to be a more suitable explant source also for transformation of *H. perforatum* (Di Guardo *et al.*, 2003).

Since wild-type *Agrobacterium* strains without selectable marker were used for transformation, the introduction of foreign T-DNA genes into the host putative hairy roots was tested by detection of *rolABCD* (localized on T_L-DNA) and *aux1,2* genes (localized on T_R-DNA). Bacterial contamination was excluded after PCR amplification of the *virC1* gene of pRi localized outside the T-DNA and therefore not transferred to the plant genome. Bacterial DNA was used as positive control.

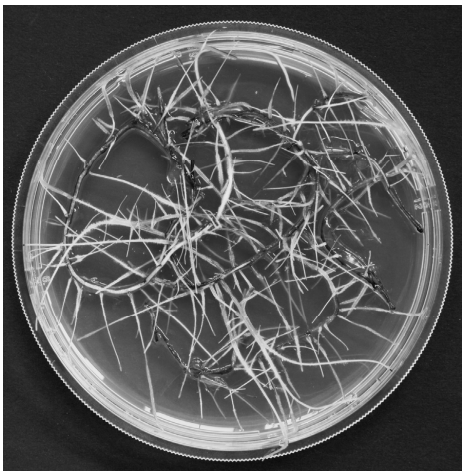

Fig. 1. Hairy root culture obtained by *Agrobacterium*-mediated transformation of *H. tomentosum* with wild *A. rhizogenes* strain A4.

The bacteria were eliminated from the hairy root cultures approx. after 4 subcultures on cefotaxime-containing media. Ten lines of *H. tomentosum* (eight transformed by ATCC 15834 and two by A4) and four lines of *H. tetrapterum* (two transformed by ATCC 15834 and two by A4) were tested for the presence of *rol* and *aux* genes (Figs. 2a, b). The PCR revealed integration of *rol* genes in all transformed cultures. *Aux1,2* genes were not detected in hairy roots of *H. tomentosum*. However, the integration of *aux1,2* genes was proved in both hairy root lines of *H. tetrapterum* transformed by the ATCC 15834 strain and in one line of the same species transformed by the A4 strain. The *rolABCD* genes localized in T_L-DNA are responsible for the hairy root phenotype (Nilsson and Olsson, 1997). Transfer of only

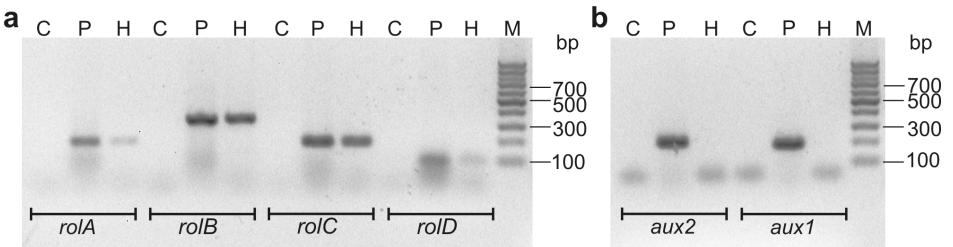

Fig. 2. Detection of integration of the *rolABCD* (a) and *aux1,2* (b) genes in one of the transgenic hairy root lines of *H. tomentosum* transformed by *A. rhizogenes* strain A4. C, control (non-transformed roots); P, positive control (pRi A4); H, hairy root culture; M, molecular weight marker.

T_L-DNA is frequent in hairy roots induced by agropine strains of *A. rhizogenes* (Jouanin *et al.*, 1987). Similarly, in our previous study with transgenic *H. perforatum* plants only genes from T_L-DNA were detected (Koperdáková *et al.*, 2009).

The problems with *Agrobacterium*-mediated transformation of *Hypericum* spp. are probably associated with antibacterial effects of the *H. perforatum* extract which was shown to be effective against Gram-negative bacteria including *Agrobacterium tumefaciens* (Milosevic *et al.*, 2007). Cell cultures of *H. perforatum* recognize *Agrobacterium* as a potential pathogen and rapidly evoke their defence responses, leading to drastic reduction of the *Agrobacterium* viability (Franklin *et al.*, 2008). In our experiments we were able to produce hairy roots only from root explants of *H. tomentosum* and *H. tetrapterum*, whereas the leaf explants were not susceptible to transformation by *Agrobacterium*. Therefore, we propose that the leaves or aerial parts of the plant may contain compounds which affect the transformation process. On the contrary, shoots and leaves of *H. perforatum* were successfully transformed by Vinterhalter *et al.* (2006) and Di Guardo *et al.* (2003), respectively. The wide spectrum of secondary metabolites in *Hypericum* species does not facilitate the search for the active component. Franklin *et al.* (2009) attributed it to the xanthones, as their level was 12-fold increased in a *H. perforatum* cell culture after biotic stress caused by *Agrobacterium* with negative effect on the *Agrobacterium* viability. Simultaneously several newly synthesized xanthones emerged, but an antibacterial effect was detected only with one of them, paxanthone. The efficacy of this compound was lower in comparison with the whole cell extract from the elicited cells. Therefore, a synergistic activity effect with other metabolites in the elicited cells is expected. However, xanthones are accumulated mainly in the roots of *Hypericum* plants (Hölzl and Petersen, 2003) which were proved to be a better source for *Agrobacterium*-mediated transformation in our present and previous studies (Di Guardo *et al.*, 2003). Recently it was found that biotic stress caused by *Agrobacterium* elicited also the production of hypericin in infected shoots of *H. perforatum* (Santarém *et al.*, 2008). Both the species we used contain this photodynamic pigment. In *H. perforatum* hypericins are accumulated only in aerial parts of the plant and are not present in the roots (Košuth *et al.*, 2007). There are also several other compounds present in *Hypericum* plants known to have antibacterial effects, above all hyperforin, which is, however, active mainly against Gram-positive bacteria (Schempp *et al.*, 1999), tannins and proanthocyanidins (Scalbert, 1991), and essential oil (Saroglou *et al.*, 2006) could be mentioned.

In the present study we report on a reliable *A. rhizogenes*-mediated transformation protocol for two hypericin-producing representatives of the genus *Hypericum*. Apart from deriving hairy root cultures which would serve as alternative source for the production of secondary metabolites synthesized and accumulated in roots of *Hypericum* spp., such transformation system represents a universal genetic tool for studying the gene function in plants. Introduction of foreign gene(s) or antisense DNA into the genome of *Hypericum* spp. would help to study the function of genes *in situ*. Especially the gene(s) coding for key enzymes of the biosynthetic pathways are of great interest. Although the biosynthetic pathway(s) leading to the production of hypericins *in planta* is rather hypothetical, there are some candidate genes which may be verified by using this transformation system.

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