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Transgenosis in the Genus *Hypericum*: Transgenic St. John's Wort Plants

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ABSTRACT

Modification of the plant genome through genetic transformation represents a powerful tool for studying the gene function via its inactivation or overexpression *in planta* with an aim to improve production capabilities of the plants. Ability of many representatives of the genus *Hypericum* to produce several important biologically active secondary metabolites makes these plants an important target for such research. Amongst the *Hypericum* species, the first successfully transformed one through both the most frequently used techniques, *Agrobacterium*-mediated transformation and particle bombardment was *H. perforatum* L. *Agrobacterium*-mediated transformation was recently successfully applied also for *H. tetrapterum* Fries. and *H. tomentosum* L. Despite several groups reported successful regeneration of transformed *H. perforatum* plants, the low transformation efficacy hinder more extensive application of transgenosis in this species. This review summarizes current state of *Hypericum* transgenic research, especially performed on *H. perforatum*, including approaches to transform different organs and verify transgenic nature of hairy roots and hairy-root regenerated plants as well as their morphological, physiological, and biochemical properties under *in vitro* and *ex vitro* conditions. Flowering and seed production of transgenic plants and future prospects of transformation research of the genus are also discussed.

Keywords: *Hypericum perforatum*, hairy root phenotype, morphological characteristics, secondary metabolites

CONTENTS

INTRODUCTION

The species from the genus *Hypericum* are well known for centuries as valuable medicinal plants. The modern scientific research is concentrated on hypericins and hyperforins which possess several pharmacological activities (Kubin *et al.* 2005; Medina *et al.* 2006). However, the biosynthesis of these valuable metabolites is poorly understood. So far only two genes with proposed function in hypericin biosynthetic pathway were isolated and cloned, the *hyp-1* gene coding for the phenolic coupling protein (Bais *et al.* 2003) and the octaketide synthase gene (*HpPKS2*) (Karppinen and Hohtola 2008; Karppinen *et al.* 2008). With regard to hyperforin biosynthesis only one gene, *HpPKS1*, coding for another polyketide synthase (Karppinen and Hohtola 2008) was reported. All the genes were isolated from the most intensively studied species of the genus, *H. perforatum* but the function of these candidate genes *in planta* remains unknown so far. Only crystallographic structure of Hyp-1 protein was published (Michalska *et al*. 2010).

A possible way to study the role of candidate genes *in vivo* is genetic modification of the plant genome. Transgenic research of plants has gained a great interest in the past decades. Plant genetic transformation offers opportunity to introduce new qualities into the plants, e.g. herbicide resistance, resistance to fungal infection and abiotic stress, modification of habitus and physiological properties. Medicinal plants are usually transformed in order to increase their secondary metabolites production. Apart from the common understanding of genetic modification as a means of introducing a new quality, it is also the way to study the gene function and regulation via its inactivation or overexpression *in planta*. The progress of plant biotechnology offers plenty of approaches more or less applicable for a desired purpose or targeted tissue, such as transfer of DNA by polyethylene glycol, electroporation, microinjection or more commonly used particle bombardment and *Agrobacterium*-mediated transformation.

The first steps towards application of transformation procedures to few *Hypericum* species have been encountered, until now only *Agrobacterium*- and biolistic-mediated transformation methods have been successfully applied. *Agrobacterium*-mediated transformation is based on its unique ability to transfer part of its large Ti or Ri plasmid into wounded plant cells where it can be integrated into plant's genome and expressed. Recent reviews on the topic

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Invited Mini-Review

Table 1 Transformation protocol used for *Agrobacterium*-mediated transformation of *H. perforatum* L.[a]

	Protocol used in the work of			
	Di Guardo *et al.* 2003	**Vinterhalter *et al.* 2006**	**Franklin *et al.* 2007**	**Santarém *et al.* 2008**
Agrobacterium species	*A. rhizogenes*	*A. rhizogenes*	*A. tumefaciens* *A. rhizogenes*	*A. rhizogenes*
Plasmid used	pRi 15834 pRi 11325	pA4M70GUS	pCAMBIA1301	pRi 2659
Recombinant sequences	none	*uidA* sequence under 70S (2x 35S CaMV) promoter	*hpt* and *gusA* driven by CaMV 35S promoter	none
Explants used	internodes leaf fragments root segments (without apex)	shoots comprising 3-4 internodes	leaf blade petiole stem segments root segments organogenic nodules	shoots
Inoculation	soaking of gently wounded explants in bacterial suspension for 20 min	wounding with a needle dipped in bacterial suspension at the 1st node above the culture media	infected with bacterial suspension for 5, 10, 20 or 30 min	infected with bacterial suspension
Scored after	30 days	the first hairy roots were visible after 2 weeks	data not published	data not published
The highest percentage of transformed explants	0 (of 40) internodes 25 (5 of 20) leaves [a] 13 (2 of 15) root segments [a]	21 (37 of 178)	0 (of at least 30 in each experiment)	0 (data not published)

[a] results obtained with pRi 15834

can be found elsewhere (e.g. Veena and Taylor 2007; Gelvin 2009; Permyakova *et al.* 2009). On the other hand, particle bombardment belongs to direct DNA transfer methods. Physical principles are exploited to introduce DNA into the plant cell and then factors common to all plants (i.e. DNA repair mechanisms) are relied on to enable stable transgene expression. For more on the topic see Altpeter *et al.* (2005).

Until now, the transgenic research was concentrated mainly on *H. perforatum*. Due to its relatively high recalcitrant behavior to *Agrobacterium spp.* bearing plasmids that are the most frequently used transformation vectors, the first information about its successful *Agrobacterium*-mediated transformation was published only few years ago (Di Guardo *et al.* 2003). The aim of this and the later published papers was to document successful induction of hairy roots and regeneration of transgenic plants. Except *H. perforatum*, two other *Hypericum* species were successfully transformed by *A. rhizogenes*, *H. tetrapterum* and *H. tomentosum* (Komarovská *et al.* 2009). Along with this indirect gene transfer, there is also the first report on particle bombardment of *H. perforatum* (Franklin *et al.* 2007).

In this review, the current status of transgenic research in the genus *Hypericum*, characteristics of transgenic *H. perforatum* plants and possible future applications are depicted.

METHODS FOR TRANSFORMATION OF *HYPERICUM* SPECIES

Wild agropine strains of *A. rhizogenes* ATCC 11325 and ATCC 15834 were used in the first successful transformation of *H. perforatum* (Di Guardo *et al.* 2003). Out of leaf, root and stem explants only root and leaf segments exhibited competence to produce hairy roots. Both types of plasmids induced hairy root formation, but those induced by pRi 11325 died soon after the excision from the explants and transfer to media with cefotaxime, an inhibitor of bacterial cell wall synthesis. Transgenic lines were derived only from explants transformed with pRi 15834. It is interesting that only a small part of the segments produced hairy roots, 25% of leaf and 13 % of root cuttings, respectively. While transformation of root segments was efficient on hormone-free medium, transformation of leaf segments was more troublesome and occurred only on medium supplemented with indole acetic acid and zeatin. Nevertheless, 2 hairy root lines derived from roots and 12 from leaf tissues have been isolated and further cultured. These lines spontaneously regenerated shoots and gave rise to the first genetically transformed plants of this species.

The other successful *Agrobacterium*-mediated transformation of the species was reported by Vinterhalter *et al.* (2006). *A. rhizogenes* strain with co-integrated plasmid A4M70GUS harboring β-glucuronidase (GUS) gene construct integrated into the T_L region was used. Shoots comprising 3-4 internodes were inoculated with bacterial suspension at the first node above the culture medium. Hairy root formation was observed on 20.8 % of inoculated shoots and altogether 37 separate hairy root clones were isolated. High potential for spontaneous shoot bud regeneration was registered. Altogether 23 distinct shoot clones were established and 5 of them evaluated for growth characteristics.

On the contrary, Franklin *et al.* (2007) found this transformation technique inefficient. In these experiments several disarmed strains of *A. tumefaciens* and *A. rhizogenes* with plasmid vector pCAMBIA1301 containing the selectable *hpt* gene encoding hygromycin phosphotransferase and the reporter *gusA* gene was used. Various plant parts such as leaf blade, petiole, stem, root segments and organogenic nodules were used. All types of differentiated explants were found to be resistant to *Agrobacterium* infection and T-DNA transfer. However, 1-2% of organogenic nodules showed GUS foci indicating transgene expression. Although these nodules regenerated shoots, none of them was proved to be transgenic.

Infection of adventitious *in vitro* grown shoots of *H. perforatum* with *A. rhizogenes* strain K599 did not lead to hairy roots formation; however, it indicates a possible way of improving hypericin production via elicitation by *Agrobacterium* spp. as plant pathogen (Santarém *et al*. 2008). *Agrobacterium*-mediated transformation procedures used to obtain transgenic *H. perforatum* are summarized in **Table 1**.

Except the extensively used *Agrobacterium*-mediated transformation, also particle-mediated gene transfer was applied. Franklin *et al.* (2007) used synthetically prepared plasmid pCAMBIA1301 for direct DNA transfer by particle bombardment to transform organogenic nodules of *H. perforatum*. The treated nodules formed calli from which shoots were regenerated. Altogether 9 shoots rooted and 7 of them were hardened into garden pots. Only three of them proved to have integrated reporter gene with transformation efficiency 3.1-12.9%. Unfortunately, data on phenotype characterization of these plants are missing.

On the basis of to-date published data, the efficacy of *Agrobacterium*-mediated transformation remains relatively low (**Table 1**). It was documented that ethanol extract of the plant possess antibacterial properties and inhibits growth of *A. tumefaciens* (Milosevic *et al.* 2007). Moreover, recent findings of Franklin *et al.* (2008, 2009) indicate that cell

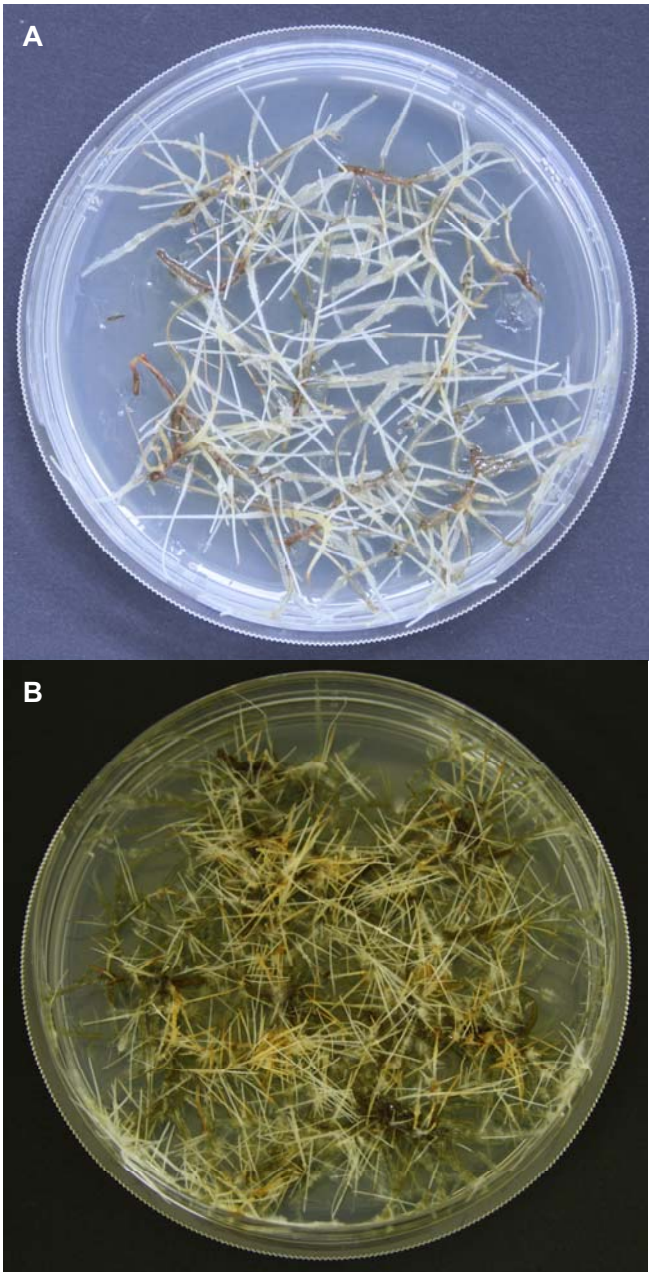

Fig. 1 Hairy root cultures obtained by *A. rhizogenes* ATCC 15834-mediated transformation. (**A**) *H. tomentosum* and (**B**) *H. tetrapterum.*

cultures of *H. perforatum* recognize *Agrobacterium* as a potential pathogen. Evoked defense responses lead to the drastic decrease of *Agrobacterium* viability – only 1% after 12 h of co-cultivation. It was shown that in this time xanthone profile of *H. perforatum* cells has significantly changed. Xanthones may serve as antioxidant system for protection against self-generated reactive oxygen species and also as antimicrobial agent (Franklin *et al.* 2009). Changes in metabolism of plants inoculated with *A. rhizogenes* increased production of phenolic compounds, dramatically decreased polyphenol oxidases and peroxidases activity, decreased levels of flavonoids, but increased the content of hypericin (Santarém *et al.* 2008). These changes as a part of a plant's defense strategy may be a barrier to successful *Agrobacterium*-mediated transformation.

Recently two other species of the genus *Hypericum*, *H. tetrapterum* and *H. tomentosum*, were successfully transformed via *Agrobacterium*-mediated transformation (Komarovská *et al*. 2009). Two wild type *A. rhizogenes* strains ATCC 15834 and A4 were used. Only root segments of both species were sensitive to bacterial infection and gave rise to several hairy root lines (**Fig. 1**). All leaf explants sooner or later underwent necrosis and no hairy root formation was observed. So far no plants were regenerated from the hairy root cultures.

Table 2 Transformation protocol used for *A. rhizogenes*-mediated transformation of *H. tetrapterum* and *H. tomentosum* (Komarovská *et al.* 2009).

		H. tetrapterum		***H. tomentosum***	
Plasmid used		pRi 15834	pRi A4	pRi 15834	pRi A4
Recombinant sequences		none	none	none	none
Explants used		root segments (without apex)			
		leaf segments (halves of leaves)			
Inoculation		infected with bacterial suspension for 20 min			
Scored after		30 days		30 days	
The highest percentage of transformed	leaves	0	0	0	0
	roots	73	13	44	35

The transformation procedure used for transformation of *H. tetrapterum* and *H. tomentosum* (**Table 2**) was almost the same as that for *H. perforatum* in the work of Di Guardo *et al*. (2003). It is noteworthy that in comparison with *H. perforatum*, the transformation efficacy of these two species with the same plasmid pRi 15834 was significantly higher. Successful transformation of these species might be influenced also by their secondary metabolite profile as differences in essential oil composition (Smelcerovic *et al*. 2007; Hosni *et al*. 2008) and also in hypericin and hyperforin content were detected (Smelcerovic, pers. comm.).

HAIRY ROOT CULTURES

Effective transformation via *A. rhizogenes* Ri plasmid leads to production of hairy roots in the site of infection. Hairy roots are fast growing, laterally highly branching, able to grow without the supplement of growth regulators. Such cultures can produce high levels of secondary metabolites synthesized in roots of intact plants (Oksman-Caldentey and Hiltunen 1996).

In the work of Di Guardo *et al.* (2003) transgenic nature of 12 putative hairy root lines of *H. perforatum* was proved by amplification of *rolC* gene fragment which is present within T-DNA of Ri plasmid in *A. rhizogenes*. Absence of the bacterial *virC1* gene which is localized outside the T-DNA and therefore not transferred into the host genome provided proof that hairy roots are free from bacterial contamination (Di Guardo *et al.* 2003). Southern blot with *rolC* gene fragment as a probe revealed only one hybridization signal in the genome suggesting one integration site. There was considerable morphological variability among the hairy root lines. However, all root lines were significantly better elongating and branching than the controls (Di Guardo *et al.* 2003).

Vinterhalter *et al.* (2006) verified the effectiveness of transformation in *H. perforatum* histochemically by GUS assay. Hairy root clones grew well with biomass increase of up to 927% in 35 days. Differences in growth parameters among hairy root clones were not observed (Vintehalter *et al.* 2006).

Transgenic nature of hairy root cultures of *H. tetrapterum* and *H. tomentosum* was confirmed by the presence of *rolABCD* genes (**Fig. 2**). On the other hand the *aux1,2* genes were transferred only into some *H. tetrapterum* hairy root cultures (Komarovská *et al.* 2009).

So far *Hypericum* hairy roots were not subjected to analysis of secondary metabolite content. As in roots of intact *H. perforatum* plants none or only a very small amount of biologically active compounds - hypericins and acylphloroglucionols were found (Karppinen and Hohtola 2008), accumulation of such compounds in hairy roots is therefore not expected.

Hairy root clones of *H. perforatum* exhibited high potential for spontaneous shoot regeneration on hormone-free medium. Plants regenerated from hairy root cultures were subjected to further evaluation (Di Guardo *et al.* 2003; Vin-

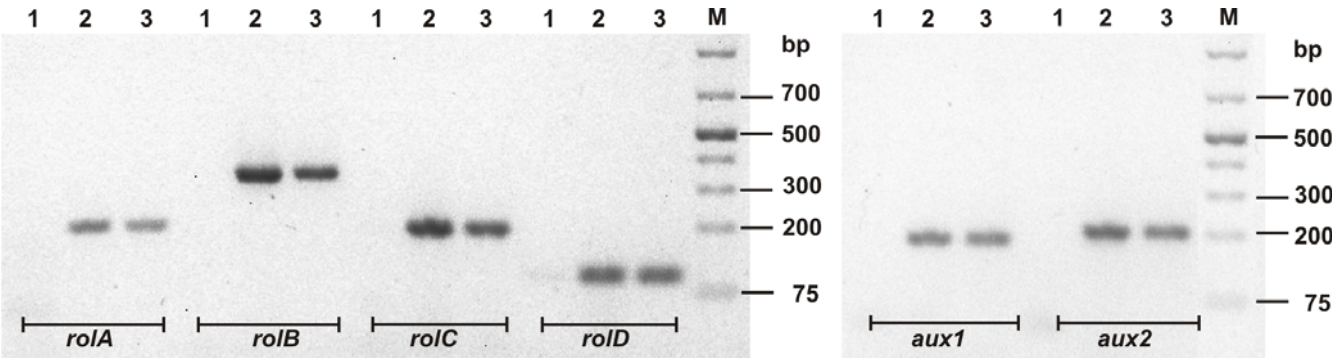

Fig. 2 Detection of *rolABCD* and *aux1,2* genes in hairy roots of *H. tetrapterum* transformed by *A. rhizogenes* strain ATCC 15834. 1 - control (non-transformed roots), 2 - positive control (pRi ATCC 15834), 3 - hairy root culture, M - molecular weight marker.

terhalter *et al.* 2006). As there are no published data on phenotype of transgenic plants derived by particle bombardment, only hairy root-regenerated plant phenotype of *H. perforatum* is discussed in the following part of this pape.

HAIRY ROOT-REGENERATED PLANTS

Morphological and physiological features of transgenic plants cultured *in vitro*

Plants regenerated from hairy roots often display characteristic morphological changes called 'hairy root-phenotype' or 'hairy root-syndrome'. These include small curled leaves, shortened internodes, reduced apical dominance, reduced fertility and plagiotropic roots.

From this point of view it is interesting that regenerated transgenic plants evaluated in the study of Vinterhalter *et al.* (2006) did not show phenotypic changes typical for hairy root-regenerated plant. However some differences in comparison with control plants have been found. While control plants grew poorly, regenerated ones were able to grow on hormone-free medium. After 30 day cultivation transgenic shoots were longer, multiplied better and had higher rooting ability than control. They were also more prone to form flower buds. Differences between individual lines in several growth characteristics – shoot length, multiplication index, percentage of shoots with axillary buds, length of axillary shoot, number of roots, length of the longest root and number of dark glands on leaf – were also recorded and these features were stable in successive subcultures (Vinterhalter *et al.* 2006). No other information on these plants is available.

On the other hand, hairy root syndrome was encountered on all hairy root regenerated plants derived by Di Guardo *et al.* (2003). Plants had typically altered morphology due to *A. rhizogenes* T-DNA gene expression: shorter internodes, increased branching, reduced apical dominance and small leaves. Ploidy level of the plants was not altered, control as well as hairy-root regenerated plants were tetraploid (Di Guardo *et al.* 2003). The same plants were evaluated by Bertoli *et al.* (2008) who selected fast and slow growing lines and analyzed bioactive compounds profile in the whole plant material. Differences were found not only in the content of chlorogenic acid, rutin, hyperoside, quercitrin, quercetin, hypericin and hyperforin but also in their presence/absence indicating specific metabolic profiles of individual hairy root-regenerated plant lines (Bertoli *et al.* 2008).

Two of the lines, the most rapidly growing line B derived from root cutting and the most slowly growing line L derived from leaf segment were selected for a detailed study. Fast growing line B had significantly increased hypericin content in shoots comparing to the other line and control. As hypericins are accumulated in the dark glands of the plant, number and density of the glands on leaves were also determined. The results revealed only slight differences in the number of dark glands per leaf and gland density between the control and transgenic lines. Transgenic lines differed in their ability to form roots. Fast growing line B had abundant while slow growing line L very poor root system. In comparison with control and line B, shoots of line L had significantly reduced ability to form roots (Koperdáková *et al.* 2009b). Clone B plants frequently formed flowers *in vitro* (**Fig. 2**). However, no flowers of line L plants have been registered during more than 5–year cultivation (pers. obs.).

Fig. 3 Transgenic *H. perforatum* clone B plants on hormone-free medium 4 weeks after subculture.

The sensitivity of the shoot cuttings of these two transgenic clones (**Fig. 3**) to different exogenous auxins and cytokinins within the concentration range of 0.1 to 5.0 mg/L was also studied (Koperdáková *et al.* 2009a). Morphogenetic response of shoot cuttings to indole-3-acetic acid, indole-3-butyric acid and α-naphthalene acetic acid varied and was concentration-dependent. The number of cuttings of transgenic clones capable of root formation and the onset of rooting on most of media with auxins lagged behind the control. Root-differentiation of transgenic cuttings on the basal medium proceeded much better after regeneration of shoots under illumination. Cuttings of the transgenic clones had higher number of less branching shoots than the untransformed control on basal medium (**Fig. 4**). The different ability of transgenic and control clones to form shoots on media supplemented with benzylaminopurine, dimethylallylaminopurine and kinetin was also registered. An increased amount of cytokinins led to differentiation of shorter shoots with reduced number of leaf pairs (Koperdáková *et al.* 2009a).

The clones were screened for the presence of several *A. rhizogenes* T-DNA genes. Similar copy numbers of *rolABC*

Fig. 4 Comparison of transgenic and control shoot cuttings comprising two leaf pairs used for the auxin and cytokinin bioassay. (**A**) Transgenic clone B, (**B**) transgenic clone L and (**C**) control cuttings.

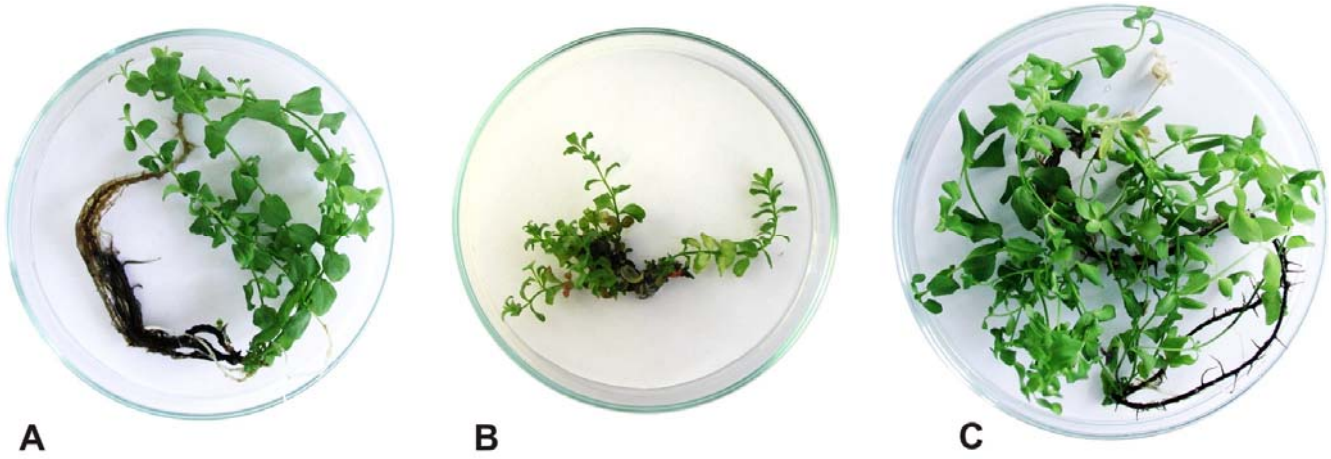

Fig. 5 Shoot cuttings after two 6-week subculture on basal medium. (**A**) Shoot cuttings of transformed clone B, (**B**) transformed clone L and (**C**) control.

insertions with descending tendency towards the *rolC* gene were detected but none of *rolD*, *aux1* and *aux2* genes were integrated into the genome of the both studied clones. The expression of the introduced genes was proven by RT-PCR at the mRNA level (Koperdáková *et al.* 2009a, 2009b). The observed differences between the transgenic clones were therefore attributed to (*i*) unique transformation events comprising different insertion sites and activation of transgene expression and to (*ii*) different origin of transformed cells as clone B was derived from root and clone L from leaf segment.

Phenotype of *ex vitro* cultivated plants

The studied clones were subjected to acclimation to *ex vitro* conditions. Line L plants, possessing very weak root system, were not able to survive in the soil, while line B and control plants were successfully transferred to hotbed. However, there was the difference in the acclimation efficacy – 29% for line B, 68% for control untransformed plants. Therefore phenotype of only one transgenic clone was evaluated in *ex vitro* settings (Koperdáková *et al.* 2009b).

Transgenic plants exhibited bushy dwarf phenotype with considerably smaller leaves and shorter internodes (**Fig. 5**). Number of axillary shoots, however, remained unchanged. Decreased vigor and earlier onset of leaf senescence in transgenic plants was evident. Transgenic plants had increased dark gland density on leaves. However, their number and overall area on leaves and petals was reduced. The flowering was also affected: clone B plants had decreased number and size of flowers (**Fig. 6**). Number of floral whorls was comparable, only number of stamens was slightly reduced (Koperdáková *et al.* 2009b).

Hypericin production in *ex vitro* cultivated plants was also studied. Transgenic plants had significantly reduced hypericin content comparing to control plants as well as to transgenic plants cultivated *in vitro*. The same results were obtained even if individual organs – flowers, leaves and stem, respectively were evaluated (Koperdáková *et al.* 2009b).

Transgenic plants not only started blossom later, set smaller flowers and had reduced number of flowers; but were sterile. No seeds were produced when plants were isolated, open pollinated or after artificial pollination with pollen from control plants. Even control plants pollinated with transgenic pollen did not set seeds. Pollen viability of transgenic plants was reduced (Koperdáková *et al.* 2007).

Histological evaluation of floral buds and flowers (**Fig. 7**) in different stages of development showed that embryo sac and pollen differentiation in transgenic plants lagged behind the controls (Koperdáková *et al.* 2007). Immature pollen grains were still prevalent in anthers of fully developed flowers (**Fig. 8**). Ovules of transgenic plants started to develop later and were smaller than those of control plants (**Fig. 9**). Mature embryo sacs were present only in small part of studied ovules after anthesis. Embryo sac developed in the ovules of transgenic plants, but endosperm differentiation was not confirmed. This may be the reason why transgenic plants did not produce any seeds.

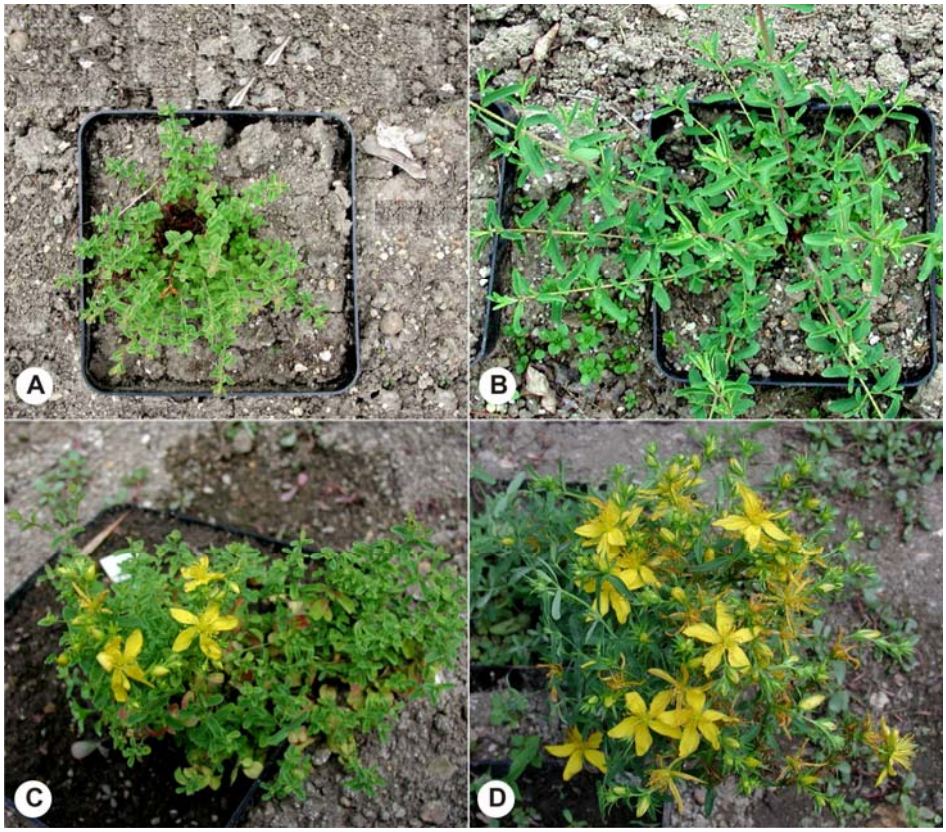

Fig. 6 Habitus of transgenic and control plants grown *ex vitro*. (**A**, **C**) Transgenic clone B and (**B**, **D**) control plants at the (**A-B**) beginning of vegetation period and (**C-D**) in the full blossom.

Fig. 7 Floral parts of transgenic clone B and control plants. (**A**) Sepals, (**B**) petals, (**C**) pistil and (**D**) stamens.

FUTURE PROSPECTS

Although the published papers report on successful transformation of three *Hypericum* species, there is still need for efficient and reliable transformation procedure to deliver foreign genes or modified DNA into the genome of these and other species of this extensive genus. In the studies published till now only relatively low number of explants was transformed and studied. Therefore new approaches or at least optimization of the procedure is required.

High-throughput transformation procedure and recovering of higher number of transgenic plants would enable their wide-screening for desired characters, e.g. content of secondary metabolites or optimization of the metabolite profile. This approach is based on random integration of the *Agrobacterium* genes into plant genome and does not selectively affect targeted genes.

Besides the commonly viewed genetic transformation as a means of bringing new features into the plant, the introduction of foreign gene or gene inactivation represents also a tool for analyzing the function of plant genes. Despite of the tremendous progress in gene targeting, metabolic engineering is rather difficult at current state of knowledge of genetics and biosynthesis of secondary metabolites especially in *Hypericum* spp. with only few recognized and characterized candidate genes.

To elucidate genes of biosynthetic pathway of hypericin and hyperforin and their regulation it is inevitable to develop efficient transformation system for several *Hypericum* species. It would enable testing the function of candidate genes by their overexpression or by their silencing, or knocking down and move forward our knowledge. Recently,

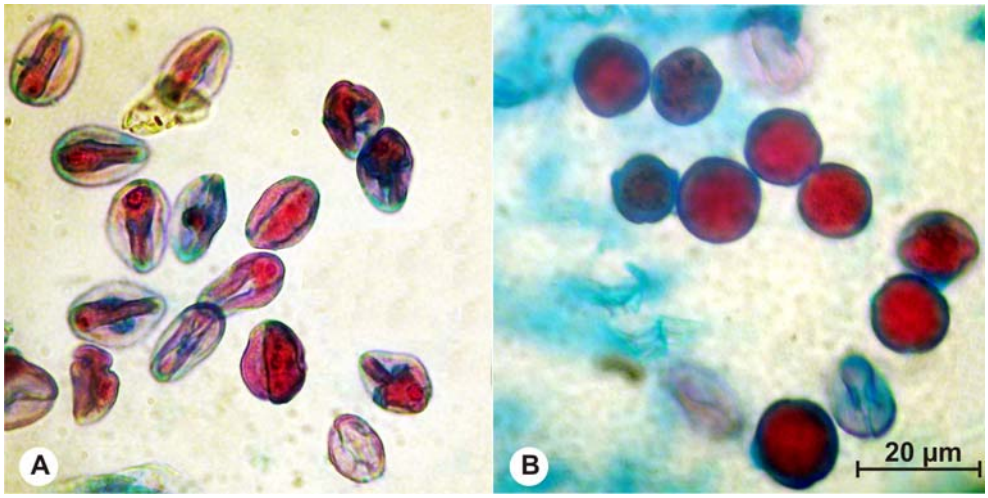

Fig. 8 Pollen grains at the time of anthesis. (**A**) Immature pollen grains of transgenic clone B. (**B**) Mature pollen grains of control plants.

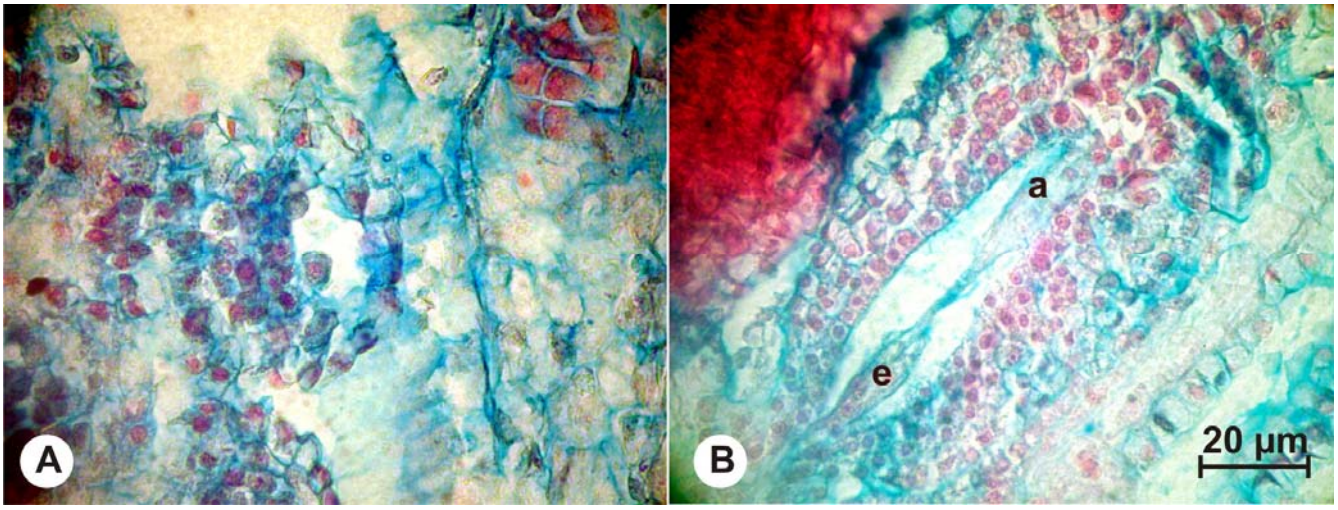

Fig. 9 Development of female gametophyte in floral buds just before anthesis. (**A**) Section of transgenic clone B ovule with functional megaspore. (**B**) Mature embryo sac of control plant with visible egg apparatus (**e**) and antipodal cells (**a**).

the first effective *A. rhizogenes*-mediated transformation of hypericin-producing species, *H. tetrapterum* and *H. tomentosum*, was developed (Komarovská *et al.* 2009). Higher transformation efficacy suggests that these species would be more suitable for study of candidate genes involved in the biosynthetic pathway.

Even though hairy root cultures of *Hypericum* spp. are not the system of choice for production of the most important biologically active compounds, hypericins and hyperforins, because of their lack in intact roots, these cultures may be used for production of other interesting root accumulated secondary metabolites, e.g. xanthones which also have pharmacodynamic effects (Muruganandam *et al.* 2000). However, production of hypericins and hyperforins by these cultures cannot be excluded in the future when the regulation of their biosynthetic pathway in the plant is uncovered.

The lack of aerial parts in hairy root cultures can become an important limitation for attempts to elucidate gene patterns in the absence of systemic signaling pathways. This might be overcome by using of composite plants, consisting of wild-type shoots from which transgenic roots are induced. It was shown that root-produced RNAi signals can silence endogenous genes or transgenes in the aboveground portion of these plants (Veena and Taylor 2007). In *Hypericum* spp. it would enable modification of its biosynthetic abilities without serious impact on plant morphology and physiology resulting from *A. rhizogenes*-mediated transformation.

CONCLUSIONS

Even though *H. perforatum*, a model for the whole *Hypericum* genus, has long been considered to be recalcitrant to *Agrobacterium*-mediated transformation, several reports on successful *Hypericum* transformation have appeared.

Transformation efficacy was influenced by type of explant and also by plasmid type used. Leaf and root cuttings and shoots with 3-4 internodes were shown to be responsive for transformation by *A. rhizogenes* (Di Guardo *et al.* 2003; Vinterhalter *et al.* 2006; Komarovská *et al.* 2009). Till now no report on successful transformation using *A. tumefaciens* has been published. Particle bombardment was applied only to organogenic nodules (Franklin *et al.* 2007). Wild type plasmids as well as binary vectors were used.

Although the rate of transformation of *H. perforatum* was rather low, several groups reported on successful regeneration of transformed plants from hairy root cultures and transformed organogenic nodules. Phenotype of transgenic plants was further characterized only in hairy root-regenerated plants with more or less pronounced hairy root-syndrome (Vinterhalter *et al.* 2006; Koperdáková *et al.* 2009b). Higher transformation efficiency in other two recently transformed *Hypericum* species suggests for enlargement of genetic transformation to other representatives of the genus.

Transgenic research has definitely promising applications in the *Hypericum* genus. The first protocols are available although further improvement is needed. However, the major constraint remains little knowledge of plant's genes, their regulation and expression.

APPENDIX

Procedure for *Agrobacterium rhizogenes*-mediated transformation of *Hypericum* species (Di Guardo *et al.* 2003; Komarovská *et al.* 2009)

MATERIALS

Culture media

1. Murashige-Skoog (MS) medium (Murashige and Skoog 1962) supplemented with vitamins (Gamborg *et al*. 1968). For 1 L of MS medium 7 g Murashige-Skoog medium including Gamborg B5 vitamins (Duchefa, Haarlem, Netherland), 100 mg *myo*-inositol, 2 mg glycine, 30 g sucrose and 6 g agar (Remi M.B., Jablonec nad Nisou, Czech republic) is added into redistilled water, pH adjusted to 5.6 before autoclaving.
2. MS-Cx medium. MS medium containing 100 mg/L Cefotaxime (Duchefa, Haarlem, Netherland). Cefotaxime stock solution contains 1 mg/1 mL redistilled water. The solution is filtered through 0.45 µm filters and stored at -20ºC.
3. Nutrient broth (Imuna, Šarišské Michaľany, Slovakia)
4. Nutrient agar (Imuna, Šarišské Michaľany, Slovakia)

Bacterial strains

1. *Agrobacterium rhizogenes* wild agropine strain ATCC 15834 (American Type Cultures Collection, Manassas, USA)
2. *Agrobacterium rhizogenes* wild agropine strain A4 (American Type Cultures Collection, Manassas, USA)

Analysis of T-DNA transfer into the hairy roots

1. DNA extraction: DNeasy Plant Mini Kit (Qiagen, Valencia, CA)
2. PCR: The PCR is performed in 30 mL reaction volume containing 1X *Taq*-polymerase reaction buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 mM forward and reverse primer, 1 U *Taq*-polymerase and 30-50 ng of DNA. Gene specific primers for amplification:
   a. 239 bp-long DNA fragment of *rolA*
      rolA-F 5' GTT AGG CGT GCA AAG GCC AAG 3'
      rolA-R 5' TGC GTA TTA ATC CCG TAG GTC 3'
   b. 348 bp-long DNA fragment of *rolB*
      rolB-F 5' AAA GTC TGC TAT CAT CCT CCT ATG 3'
      rolB-R 5' AAA GAA GGT GCA AGC TAC CTC TCT 3'
   c. 190 bp-long DNA fragment of *rolC*
      rolC-F 5' AAA TGC GAA GTA GGC GCT CCG 3'
      rolC-R 5' TAC GTC GAC TGC CCG ACG ATG ATG 3'
   d. 101 bp-long DNA fragment of *rolD*
      rolD-F 5' CTG AGC GTG TGG CTC ATG 3'
      rolD-R 5' GGA GGT AAA GAC GAA GGA CAG AG 3'
   e. 198 bp-long DNA fragment of *aux1*
      aux1-F 5' CAT AGG ATC GCC TCA CAG GT 3'
      aux1-R 5' CGT TGC TTG ATG TCA GGA GA 3'
   f. 217 bp-long DNA fragment of *aux2*
      aux2-F 5' AAC GAT AAT AGC CCG CTG TG 3'
      aux2-R 5' CGT CTT GGG TTT GTG GTT CT 3'
   g. 425 bp-long DNA fragment of *virC1*
      virC1-F 5' AAT GCG TCT CTC TCG TGC AT 3'
      virC1-R 5' AAA CCG ACC ACT AAC GCG AT 3'
3. Gel electrophoresis: 1.6% agarose, gel prepared in 1X TAE buffer stained with ethidium bromide (0.5 µg/mL); TAE buffer: 40 mM Tris acetate, 1 mM EDTA pH=8.3.

METHODS

Preparation of *Agrobacterium rhizogenes* suspension

1. Grow bacteria on nutrient agar for 2 days at 28°C.
2. Prepare bacterial suspension by inoculation of single cell colony in 10 mL of liquid nutrient broth; grow on rotary shaker (orbital shaking, 120 rpm) 24 hrs at 28°C.
3. Subculture the bacterial suspension; use 1 mL of the 24 h culture and 9 mL of fresh nutrient broth, culture overnight.

Transformation of *Hypericum*

1. *In vitro* grown seed-derived plants of *H. perforatum* L., *H. tomentosum* L., *H. tetrapterum* Fries., and *Nicotiana tabacum* are grown for 4- to 8-week on MS medium.
2. Leaf (half of leaf) and root (1 to 2 cm long, without apex) segments of *Hypericum* plants together with leaves of *N. tabacum* as additional source of acetosyringone and positive control of transformation are wounded with scalpel and co-cultured with bacterial suspension (1 mL) in sterile distilled water (19 mL), for 20 min at laboratory temperature.
3. The explants are dried for a moment on sterile filter paper, subsequently transferred onto MS medium and kept for two days in dark at 24°C.
4. The explants are then cultured on MS-Cx medium 30 days at 24ºC; the roots are kept in dark, leaf explants at 16/8 hr photoperiod with fluorescent irradiance of 28 $\mu E/m^2s$.
5. The putative hairy roots are subcultured on RM-Cx medium until the culture is free from any bacteria.

Characterization of transgenic plants by molecular analyses

1. Integration of *A. rhizogenes* T-DNA genes into the host (transgenic nature of the putative hairy root cultures) is tested by PCR amplification of *rol* and *aux* genes. The amplification conditions are: 94°C 5 min; 40 cycles (94°C 30 s; 60°C (*rolABCD*, *virC1*) or 61°C (*aux1,2*) 30 s; 72°C 30 s); 74°C 4 min. Amplification and length of the desired products is analyzed by gel electrophoresis.
2. False positives are tested by PCR amplification of *Agrobacterium vir* genes (the genes not transferred into the host).

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