Effect of the Number of rol Genes Integrations on Phenotypic Variation in Hairy Root-Derived Hypericum perforatum L. Plants

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The extent of phenotypic variation of St. John’s wort (Hypericum perforatum L.) plants transformed with wild agropine ATCC 15834 Agrobacterium rhizogenes plasmid was evaluated with respect to the number of rol genes integrations. The transfer of T1-DNA to plant explants during each transformation event was incomplete with different rolA, rolB, and rolC copy numbers. Along with typical features representing the hairy root syndrome, an altered size, number and density of dark and translucent glands, changes in ability to synthesize secondary metabolites, and reduced fertility were observed. The highest copy number of transferred rol genes resulted in weak expression of transgenic character and comparable quantitative parameters with the controls. Only 1 out of 11 transgenic clones was able to produce seed progeny and not more than 4 out of its 35 offsprings were positive for rolC gene integration. Sterility of the clones was due to retarded development of both gametophytes.

Key words: St. John’s Wort, Transgenes Copy Number, Secondary Metabolites

Introduction

Hypericum perforatum L. is a medicinal plant used in folk medicine since ancient times. The extract is employed for the treatment of mild to moderate depression. H. perforatum possesses specialized structures, translucent and dark glands which are the sites for storage of phloroglucinols – hyperforin and its derivates and naphthodianthrones, especially hypericin and pseudohypericin, respectively (Briskin and Gawienowski, 2001; Soelberg et al., 2007). These two groups of biologically active compounds are of great interest due to their anticancer, antidepressive, and antiviral effects (Kubin et al., 2005; Beerhues, 2006).

Agrobacterium rhizogenes rol genes located in T-DNA are responsible for the altered phenotype of transgenic plants (Nilsson and Ols, 1997) including reduced apical dominance, dwarfing, shorter internodes, increased branching, higher rooting potential, smaller wrinkled leaves, reduced fertility, altered onset of flowering, and flower morphology (Casanova et al., 2005; Christey, 2001). Integration of these genes can also affect the production of secondary metabolites as they may function as potential activators of the secondary metabolism in transformed cells (Bulgakov, 2008). The extent of the hairy root syndrome depends not only on the species and individual genotype but also on unique transformation events. The expression of transgene/s can be influenced by the site of integration, also called position effect (Kooter et al., 1999). Several T-DNA copies can be integrated into the plant genome, even from different bacterial cells (De Block and Debrouwer, 1991). However, a higher copy number of transgenes increases the risk of transgene silencing (Kooter et al., 1999). The hairy root phenotype may thus not be inherited; the progeny of regenerants often does not express the T-DNA genes or does not display the parent phenotype even though the T-DNA is present in their genome (reviewed in Yin et al., 2004).
Although genetic transformation of several plant species became a routine, the possibilities of genetic modification via Agrobacterium-mediated transformation in H. perforatum are still limited. According to Franklin et al. (2008) the main problem hindering transformation of this species is its strong recalcitrance to Agrobacterium infection. In spite of this there are few reports on successful A. rhizogenes-mediated transformation of H. perforatum (Di Guardo et al., 2003; Vinterhalter et al., 2006) including our recent paper on agrobacterial transformation of two other Hypericum species (Komarovská et al., 2009). However, the effect of transgenes on the phenotype of the transgenic Hypericum plants was not assessed in detail so far.

The aim of the present work was to evaluate the impact of the number of rol genes integrations on the phenotype of transgenic H. perforatum. For such purpose (i) integration of A. rhizogenes T-DNA, its copy number and expression, (ii) the extent of the hairy root syndrome at phenotype level including secondary metabolite production in relationship to the rol gene copy number, and (iii) transgenic plant seed formation and transmission of the T-DNA into the offspring were studied.

Material and Methods

Plant material

Shoots of eleven transgenic clones and two non-transformed controls of Hypericum perforatum L., grown in vitro were used for the analyses. The transgenic root-derived clones R1 and R2 and leaf-derived L1–L9 were originated from hairy root cultures obtained from one mother H. perforatum genotype (Cm) by transformation with Agrobacterium rhizogenes strain ATCC 15834 (American Type Cultures Collection, Manassas, USA). Control clone Cc was untransformed, i.e. regenerated from Cm clone leaf. The clones R1 and R2 and L1–L9 were derived from individual hairy roots developed on root and leaf cuttings, respectively. Transgenic plants spontaneously regenerated on hormone-free basal medium. All clones were derived at CRA-FSO, Experimental Unit for Floriculture and Ornamental Species, Sanremo (Imperia), Italy as published earlier by Di Guardo et al. (2003). The mother clone originated from a wild habitat in Sanremo.

Culture conditions

The plant material was cultured on basal medium containing mineral salts according to Lionmaier and Skoog (1965) supplemented with B5 vitamins (Gamborg et al., 1968). 100 mg l⁻¹ myo-inositol, 2 mg l⁻¹ glycine, 30 g l⁻¹ sucrose, and 6 g l⁻¹ agar; pH value was adjusted to 5.6 before autoclaving. The plantlets were kept in a culture room at (23 ± 1) °C, a 16/8-h photoperiod, fluorescent lightening of 33 μmol m⁻² s⁻¹, and 70% relative humidity. They were subcultured in a six-week interval.

Adaptation to ex vitro conditions

Transgenic and control in vitro cultivated plantlets with differentiated roots were transferred to aerated hydroponic vessels placed in a plastic container. Containers were gradually opened during 2–3 weeks to lower the humidity until the plants were able to grow under laboratory conditions. Four weeks after hydroponic cultivation the plants were transferred to autoclaved soil.

Detection of transgenes integrations and their expression

Integration and expression of A. rhizogenes T-DNA genes (rolA, B, C, D and auxI, 2) at the transcript level in the studied transgenic clones were detected by PCR and RT-PCR with gene-specific primers (Table I). Total genomic DNA and RNA were isolated from 100 mg of fresh plant material using the DNeasy Plant Mini Kit (Qiagen, Valencia, USA) and according to Jaakola et al. (2001), respectively. The isolated RNA was treated with DNase I (Invitrogen, Carlsbad, USA) to digest contaminating DNA. Reverse transcription (RT) was performed using 200 U M-MLV reverse transcriptase (Promega, Madison, USA) and 10 mm anchored oligoT primer at 42 °C according to the manufacturer’s instructions. The number of integrated rol genes was measured by quantitative real time PCR (qPCR) and calculated according to Mason et al. (2002). Briefly, the relative amount of the rolA, rolB, and rolC genes was quantified by qPCR and normalized to an endogenous gene standard. Since all the transgenic clones were derived from one maternal genotype, the transformed clones shared, except the integrated T-DNA, the same genes and their copy number; any single-copy gene of Hypericum could be
used as endogenous standard. In this experiment a genomic fragment of the hyp1 gene (the candidate gene of hypericin biosynthesis) was used for template normalization to count the rol/hyp1 ratio. From the normalized relative amount of rol genes in all transgenic clones the theoretical value for one integration of each rol gene (the virtual calibrator, $r_{irolA}$ – $r_{irolC}$) was calculated, and on the base of this value the final number of integrations was estimated. The values of virtual calibrator were 0.55 ($rolA$), 0.67 ($rolB$), and 0.69 ($rolC$). The PCR, RT-PCR, and qPCR reaction/amplification conditions were as described previously in Koperdáková et al. (2009a).

**Evaluation of some quantitative parameters of plant organs**

Plant height, length and number of internodes, axillary shoot number, and length of the longest root were scored on each transgenic and non-transgenic clone. The plants with at least one root longer than 1 cm were scored as rooting and those with at least one flower as flowering. Leaf area and number of dark and translucent glands were determined on two uppermost fully developed leaf pairs. The leaves were photographed under a stereomicroscope with a digital camera (ARTRAY ARTCAM), and the leaf area and length ($l$) and width ($w$) of black nodules were measured by UTHSCSA Image Tool 3.00 and Quick PHOTO Camera 2.1 software. The area of each dark gland was calculated as $\frac{1}{4}\pi lw$. The average area of one dark gland was calculated as quotient of area of all glands on leaf and number of glands on leaf. The overall dark gland area on leaf is a sum of area of all glands on the leaf. Gland density (number $N$ of glands per mm$^2$ of leaf blade) was calculated as quotient of number of glands on leaf and leaf area. From each clone 20 in vitro micropropagated plants were evaluated 6 weeks after subculture.

**Preparation of the extracts and determination of secondary metabolite contents**

Extraction of hypericins and hyperforin from $H. perforatum$ has been optimized by Smelcerovic et al. (2006). The extractions were done using dry plant material (mass 0.39–0.61 g) and 10 ml of methanol by direct sonication. Sonication were performed for 20 min, using a Branson Sonifier B-12 apparatus (Branson, Heusenstamm, Germany) operating at 20 kHz and 60 W. Extracts were finally separated from the residual plant material by filtration (folded filters, grade 597 ½, Schweitzer & Schuell, Dassel, Germany). The residues were washed with 10-ml aliquots of methanol. Filtrates were then combined and methanol was added up to 25 ml. The extracts were stored in the dark at −20 °C until analysis was performed. Extractions were done in duplicate.

The composition of the extracts was analysed using an LC-ESI-MS-MS method slightly modified by Kusari et al. (2009) and Bonkanka et al. (2008). Chromatographic conditions were optimized for separation of hypericin, pseudohypericin, and hyperforin. External calibration was...
performed in the range 0.1–50 μg/ml for pseudohypericin (Alexis Biochemicals, Lausen, Switzerland), hypericin (Sigma, Steinheim, Germany), and hyperforin (Sigma). Standard solutions were stored in the dark at –20 °C. The correlation coefficient for the calibration curves was > 0.989. All procedures were carried out in the dark. The relative standard deviation (RSD) of the analytical method was determined by eighth injections of an extract and was below 6% for all compounds.

**Histological observation of reproductive tissues**

Flower buds and flowers of the transgenic clone R1 and control clone C_M were collected at different developmental stages: small buds without visible petals, buds with yellow petals, buds just before anthesis, fully opened flowers and overblown flowers. The plant material was fixed in Carnoy’s solution for 24 h. After dehydration in butanol and ethanol series and embedding in paraffin, flower sections (7 μm) were cut with a microtome, dried on slides, deparaffinized in toluene, and then stained with alciane blue and safranin. Slides were observed under a light microscope (Olympus C011). Photographs were taken with a C-3020ZOOM digital camera (Olympus).

**Statistical analyses**

The differences between the clones were evaluated by ANOVA or Kruskall-Wallis tests, depending on the result of the Levene test. This was followed by multiple comparisons of mean ranks. The results were evaluated at the 0.05 significance level using Statistica 7 software.

**Results**

In this work plants of eleven transgenic _H. perforatum_ clones, L1–L9 and R1 and R2, initiated from hairy roots developed on leaf (L) and root (R) cuttings were characterized. The phenotypic changes were correlated with the rol genes copy number. Mother genotype (C_M) and non-transgenic clone C_L regenerated from mother clone leaf were used as controls.

**Detection and quantification of T-DNA integrations**

Integration of T-DNA genes was determined by PCR with specific primers. While the rolA, B, C, D genes localized on T_L-DNA were found in all hairy root-derived clones, transfer of aux1 from T_R-DNA was proved only in L9 and aux2 was missing in all samples. Expression of the integrated genes with an exception of the rolD gene was confirmed by RT-PCR. Re-analysis of rolD with another set of PCR primers (rolD-F3/R3), delimiting longer, 575 bp long rolD fragment, did not reveal its transfer. The presence of the shorter 101 bp long rolD PCR product and lack of the

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Table II. Calculated copy number of rolA, B, C genes in transgenic _H. perforatum_ clones. Normalized quantity (Norm) represents ratio of relative quantity of rolX to endogenous reference. The calculated copy number (rolX/r) is obtained as the ratio between the normalized quantity of rolX gene and the virtual calibrator r. The value of virtual calibrator for each gene was calculated according to Mason et al. (2002). The values ± 95% confidence interval are shown. Integer numbers in the interval represent the expected number of integrations (int). The clones/gens with no integer in such interval are marked (*); in such case the copy number is estimated as the nearest integer to the calculated copy number.

<table>
<thead>
<tr>
<th>Clone</th>
<th>rolA (rirolA = 0.55)</th>
<th>rolB (rirolB = 0.67)</th>
<th>rolC (rirolC = 0.69)</th>
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<tbody>
<tr>
<td></td>
<td>Norm ± SD</td>
<td>rolA/ri</td>
<td>int</td>
</tr>
<tr>
<td>R1</td>
<td>1.85 ± 0.20</td>
<td>3.37 ± 0.71</td>
<td>3–4</td>
</tr>
<tr>
<td>R2</td>
<td>0.58 ± 0.23</td>
<td>1.05 ± 0.83</td>
<td>1</td>
</tr>
<tr>
<td>L1</td>
<td>0.47 ± 0.08</td>
<td>0.85 ± 0.29</td>
<td>1</td>
</tr>
<tr>
<td>L2</td>
<td>0.62 ± 0.05</td>
<td>1.12 ± 0.17</td>
<td>1</td>
</tr>
<tr>
<td>L3</td>
<td>1.10 ± 0.25</td>
<td>2.00 ± 0.89</td>
<td>2</td>
</tr>
<tr>
<td>L4</td>
<td>1.49 ± 0.17</td>
<td>2.70 ± 0.61</td>
<td>3</td>
</tr>
<tr>
<td>L5</td>
<td>1.55 ± 0.22</td>
<td>2.82 ± 0.78</td>
<td>3</td>
</tr>
<tr>
<td>L6</td>
<td>2.64 ± 0.33</td>
<td>4.80 ± 1.17</td>
<td>4–5</td>
</tr>
<tr>
<td>L7</td>
<td>0.72 ± 0.05</td>
<td>1.30 ± 0.17</td>
<td>1*</td>
</tr>
<tr>
<td>L8</td>
<td>0.51 ± 0.05</td>
<td>0.93 ± 0.18</td>
<td>1</td>
</tr>
<tr>
<td>L9</td>
<td>1.64 ± 0.13</td>
<td>2.98 ± 0.48</td>
<td>3</td>
</tr>
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</table>
longer one indicated incomplete transfer of the respective T-DNA region.

The transgenic clones differed in the number of rolA, B, C genes integrations (Table II). The clones R3, L1, L2, L3, L5, and L8 possessed only one or two integrations; in the remaining clones more than three integrations occurred. The highest number of all rol genes was detected in R1 and L9 clones. The clones shared either the same number of integrated rolA, B, C genes (R1, L1, L2, L3, L5, L8, and L9), i.e. the part of the T-DNA covering rolA, B, C genes was completely transferred, or the number of rolA, B, C genes was descending from rolA to rolC indicating an incomplete transfer of this region.

Phenotype of in vitro cultivated transgenic plantlets

The extent of hairy root syndrome expression observed among the transgenic plants varied (Fig. 1). The least pronounced appeared in the clones R1 and L9 with the highest number of rol genes integrations.

As seen from Figs. 2a–f the number of rol genes integrations influenced almost all quantitative characteristics studied with an exception of number of internodes (Fig. 2b). The plants with a higher copy number resembled the controls while those with only one or two integrations expressed the features typical for hairy root syndrome more intensively, including reduced flowering (Fig. 2g). Also formation of roots was reduced in transgenic clones (Fig. 2h).

Variation of dark and translucent glands and secondary metabolite content

Evaluation of size, number, and density of dark glands as a site for hypericin accumulation, and number and density of translucent glands on leaf serving for storage of hyperforin revealed that most of these characters are likely affected by transformation as well as transgenes copy number (Figs. 3a–e). The significantly smaller leaf blade of transgenic clones compared to controls was characterized by a higher dark gland density (Fig. 3d). It was reflected, with an exception of clone L3, in the higher total content of hypericins (hypericin and pseudohypericin) (Fig. 3f, Table III), although a clear correlation between hypericin content and number or size of dark glands was not proved. The majority of the transgenic clones accumulated less hyperforin than controls.
but without unambiguous relationship between the amount of hyperforin and translucent glands number and density.

Histology of gametophytes in transgenic clones and transfer of T-DNA into the seed progeny

Only three transgenic clones, R1, R2 and L2, were successfully adapted to ex vitro conditions. The problems with ex vitro adaptation were mainly due to poor rooting of the plants and a fragile root system. Although all the ex vitro adapted clones flowered, only the clone R2 produced seed progeny. To elucidate the reasons of sterility of transgenic plants, a histological study of pistils and anthers in different stages of development of the clone R1 was performed. The results revealed several anatomical differences in the development of male and female gametophytes compared to the mother non-transgenic clone CM.

The development of both gametophytes in transgenic plants lagged behind the control in all evaluated stages. Finally, mature pollen grains were not observed in transgenic flowers, even after anthesis. The zygotes and early stages of embryo development were visible only in a few ovules of overblown flowers, and endosperm development was not observed in any of the transgenic ovules.

To assess the inheritance and the ability of transgenic Hypericum plants to transmit the foreign T-DNA into the offspring we studied the seed progeny of the only fertile transgenic clone (R2). The screening for the presence of the rolC gene revealed its integration only in 4 out of 35 tested offsprings.

Discussion

Despite several reports on the efficient A. rhizogenes-mediated transformation of H. perforatum (Di Guardo et al., 2003; Vinterhalter et al., 2006), only limited information on the effect of transgenes and their copy number on the phenotype of transgenic H. perforatum plants is available. In our study transgenic clones regenerated from hairy roots derived by A. rhizogenes ATCC 15834 were characterized with respect to the phenotypic alterations related to the transgene copy number. The hairy root syndrome with different extent was observed in all transgenic clones. Contrary to our results all transgenic clones of H. perforatum transformed by A. rhizogenes plasmid A4M70GUS were similar in appearance to the controls (Vinterhalter et al., 2006). However, in the present work the copy number of the rol genes was not studied and so their silencing effect could not be excluded.

In the genome of all transgenic clones analysed in our study the insertion and expression of the rolA, B, C genes from T\textsubscript{1}-DNA was proved. The left part of agropine Ri plasmid T-DNA (T\textsubscript{1}-DNA) is composed of 18 ORFs (open reading frames), where ORFs 10, 11, 12 and 15 correspond to rolA, B, C, D genes (White et al., 1985; Slighton et al., 1986). The physical distance between rolA, B, C and rolD in T\textsubscript{1}-DNA might explain the problems with the transfer of the whole rolD gene in all samples. The rolA, B, C genes copy number was descending in the direction from rolA to rolC in several clones. Similar results with incomplete T\textsubscript{1}-DNA transfer were reported for some of the Coffea arabica hairy roots lines derived by Alpizar et al. (2008). The integration of the T\textsubscript{1}-DNA was confirmed only in clone L2 by the detection of aux1 gene. Incorporation of only T\textsubscript{1}-DNA, essential for hairy root induction, into the plant genome is a well-known feature of transformation with agropine strains of A. rhizogenes (Jouanin et al., 1987), as recently proved e.g. by studies of

<table>
<thead>
<tr>
<th>Clone</th>
<th>Pseudohypericin</th>
<th>Hypericin</th>
<th>Hyperforin</th>
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<tbody>
<tr>
<td>R1</td>
<td>2.63</td>
<td>0.84</td>
<td>5.68</td>
</tr>
<tr>
<td>R2</td>
<td>2.59</td>
<td>0.47</td>
<td>3.99</td>
</tr>
<tr>
<td>L1</td>
<td>3.82</td>
<td>0.63</td>
<td>5.49</td>
</tr>
<tr>
<td>L2</td>
<td>2.70</td>
<td>0.53</td>
<td>3.88</td>
</tr>
<tr>
<td>L3</td>
<td>1.83</td>
<td>0.34</td>
<td>3.51</td>
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<tr>
<td>L4</td>
<td>3.12</td>
<td>0.50</td>
<td>4.50</td>
</tr>
<tr>
<td>L5</td>
<td>2.17</td>
<td>0.53</td>
<td>1.94</td>
</tr>
<tr>
<td>L6</td>
<td>3.18</td>
<td>0.56</td>
<td>3.44</td>
</tr>
<tr>
<td>L7</td>
<td>2.73</td>
<td>0.44</td>
<td>4.86</td>
</tr>
<tr>
<td>L8</td>
<td>2.82</td>
<td>0.47</td>
<td>3.94</td>
</tr>
<tr>
<td>L9</td>
<td>3.17</td>
<td>0.61</td>
<td>5.72</td>
</tr>
<tr>
<td>C\textsubscript{L}</td>
<td>1.89</td>
<td>0.39</td>
<td>8.07</td>
</tr>
<tr>
<td>C\textsubscript{M}</td>
<td>2.15</td>
<td>0.37</td>
<td>5.24</td>
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Alpizar et al. (2008) and Christensen et al. (2008). Also in our recent experiments with H. tomentosum and H. tetrapterum transformation T_R-DNA was integrated only into the genome of several hairy root lines (Komarovská et al., 2009). Most of the clones possessed one or two copies of the rol genes. The transgenic clones with the highest rol genes copy number, R_1 and L_w, resembled the phenotypic quantitative parameters of the control. This suggests that in the transgenic
clones with higher transgene copy number the silencing of the introduced genes might giving and gave rise to a suppressed hairy root phenotype. Post-transcriptional silencing has been registered frequently in the transgenic lines of eastern white pine with three or more copies but never with only one T-DNA copy (Tang et al., 2007). However in our experiment, a complete switch off of the rol gene expression in the clones R1 and L9 was not observed since the rolA, B, and C gene transcripts were detected and several symptoms of the hairy root regenerated plants noticed as well. The plants with the selfsame number of integrations did not always resemble the same phenotype. It was markedly expressed in the clones L4 and L5 which showed an identical number of rol genes but a different phenotype. One of the reasons is the position effect. Tang et al. (2007) demonstrated by in situ hybridization that foreign genes became silenced if two and more copies of the transgene are introduced in the same chromosome. This may be also the case for the L4-L6 H. perforatum transgenic clones which possessed several T-DNA integrations and had also the intensive hairy root phenotype. However, in these clones incomplete rolA, B, C transfer occurred.

Transgenic plants derived from two different organs of the same mother plant were compared. According to our findings, it seems that the origin of the explants does not influence the habitus of transgenic plants, but may affect rooting of H. perforatum. While both of the root segment-derived clones (R1 and R2) were able to root and grow under ex vitro conditions, only one out of nine leaf explant-derived clones (L4-L6) was capable of rooting and acclimation. Unlike our results plants with introduced rol genes are usually characterized by increased rooting ability which was used for improvement of rooting of woody plants such as apple or cherry tree (Sedira et al., 2001; Gutiérrez-Pesce et al., 1998).

H. perforatum plants are an important source of hypericins which are accumulated in dark glands (Briskin and Gawienowski, 2001). The transformation events in our experiment influenced mainly the size of the dark glands and the total leaf area covered by them. The dark gland density was changed mainly due to the altered leaf size of the transgenic clones. The literature data on correlation between hypericin content and dark gland density are inconsistent. While Southwell and Campbell (1991) and Cirak et al. (2006) reported a close relationship, Čellárová et al. (1994) and Walker et al. (2001) did not find any association. Kirakosyan et al. (2003) observed a markedly different content of hypericin in several lines possessing a comparable number of dark glands on leaf. Neither our results confirmed a close correlation between dark gland number or size and hypericin content, although a certain relationship between the dark gland density on leaf blade and hypericin content was found. The accumulation of hypericin may be affected also by the structure of the dark glands as it was found that the bigger and better developed cells of the peripheral layer of dark glands accumulated higher amounts of hypericin (Kornfeld et al., 2007).

Although the dark glands are well known as hypericin accumulation sites, the function of translucent glands as sites of hyperforin accumulation was published only recently (Soelberg et al., 2007). As the translucent gland density on leaves seems to be genetically conserved, the reduced leaf area of transgenic clones leads to a decreased number of these glands. The results of our experiments did not confirm any relationship between hyperforin content and density or number of translucent glands. The capacity of the glands to store hyperforin might be therefore affected or altered by transformation. According to Karppinen and Hohtola (2008) and our preliminary findings (data not shown) the roots are also capable of hyperforin production/accumulation as well as indicating that the translucent glands on the aerial parts might not be the only structures where hyperforin is accumulated.

Genetic transformation by wild-type A. rhizogenes strains may affect the biosynthetic potential as the rol genes act as potential activators of the secondary metabolism (Bulgakov, 2008). Although the hairy root cultures represent a more promising system, there are also several data showing an increased secondary metabolites production in intact transgenic plants as well. For example, Phongprueksapattana et al. (2008) recently reported a significantly higher concentration of mitragynin in transgenic Mitragyna speciosa. However, transgenic plants of H. perforatum analysed in this study did not appear profitable for production of any tested compound even though hypericin and pseudohypericin contents were slightly increased. A more perspective application of transformation within the genus Hypericum would be the use of gene transfer as a means to study the gene func-
tion in situ via introduction of new genes or silencing/inactivation of the candidate genes. Such an approach necessitates a transformation system with high efficacy and reproducibility which is not available for *H. perforatum* yet. Unlike *H. perforatum*, we have recently reported higher efficiency of *Agrobacterium*-mediated transformation in two other *Hypericum* species (Komarovská et al., 2009), which implies the successful use of such approach.

The hairy root-regenerated transgenic plants are usually partially or completely sterile (Casanova et al., 2005; Christey, 2001). Transformation negatively affected the flowering and seed production also in transgenic *H. perforatum*. As found in our previous experiment the floral parts of the transgenic *ex vitro* cultivated *H. perforatum* clone were significantly smaller than the non-transgenic ones (Koperdáková et al., 2009b). Histological observations showed that in transgenic plants the development of both gametophytes was retarded. Moreover, no signs of endosperm presence were encountered. Reduced number of flowers and fertility of the transgenic *H. perforatum* clones along with the problems with acclimation did not enable a deeper study of the T-DNA transmission into the seed progeny. A reduced number of inflorescences per plant was observed, for example, in two hairy root-derived *Helichrysum* clones; this symptom was transmitted into the rolC positive seed progeny (Giovannini et al., 2008). For transgenic rolD-positive plants a higher number of flowers and fruits and earlier onset of flowering is typical (Mauro et al., 1996; Bettini et al., 2003). Successful integration of the whole rolD would therefore positively influence the flowering in *H. perforatum*.

**Conclusion**

This study provides an insight into phenotypic alterations of *H. perforatum* transgenic plants. The level of expression of the hairy root phenotype in the transgenic plants was markedly affected by the copy number of integrated rol genes. The phenotype of transgenic clones with the highest number of rol genes integrations resembled the controls; but the hairy root syndrome was not completely suppressed in these plants. The origin of the explants used for transformation influenced the ability of transgenic clones to adapt to *ex vitro* conditions since better acclimation was observed in clones originating from root segments. T-DNA transfer into the seed progeny as well as seed production ability of transgenic plants was considerably reduced. The future of transgenosis in this medicinal plant or genus can be seen mainly in studying the function of the candidate genes of secondary metabolite pathways. An efficient transformation system should be used as a tool for introducing and/or switching off the genes of interest, which can enable the determination of their function.

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