# 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 $T_L$-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 Olsson, 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).

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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 $R_1$ and $R_2$ and leaf-derived $L_1$–$L_9$ were originated from hairy root cultures obtained from one mother *H. perforatum* genotype ($C_M$) by transformation with *Agrobacterium rhizogenes* strain ATCC 15834 (American Type Cultures Collection, Manassas, USA). Control clone $C_L$ was untransformed, *i.e.* regenerated from $C_M$ clone leaf. The clones $R_1$ and $R_2$ and $L_1$–$L_9$ 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 Linsmaier 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 $\mu$mol $m^{-2}$ $s^{-1}$, 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 *aux1*, *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

Table I. Nucleotide sequence of primers used for detection of *rolA, B, C, D* and *aux1, 2* genes in transgenic clones.

| Primer | Nucleotide sequence [nt] | Product lenght [bp] | Tm [°C] | Reference |
|---|---|---|---|---|
| rolA for | 5´-GTTAGGCGTGCAAAGGCCAAG-3´ | 239 | 60 | Zdravković-Korać *et al.* (2003) |
| rolA rev | 5´-TGCGTATTAATCCCGTAGGTC-3´ | | | |
| rolB F1 | 5´-AAAGTCTGCTATCATCCTCCTATG-3´ | 348 | 60 | Zdravković-Korać *et al.* (2003) |
| rolB R1 | 5´-AAAGAAGGTGCAAGCTACCTCTCT-3´ | | | |
| rolC F1 | 5´-AAATGCGAAGTAGGCGCTCCG-3´ | 190 | 60 | Di Guardo *et al.* (2003) |
| rolC R1 | 5´-TACGTCGACTGCCCGACGATGATG-3´ | | | |
| rolD F3 | 5´-CTGAGCGTGTGGCTCATG-3´ | 101 | 60 | Designed according to published *rolD* sequence |
| rolD R3 | 5´-GGAGGTAAAGACGAAGGACAGAG-3´ | | | |
| rolD F1 | 5´-AGCTCTCAACGGCTTCATGTCGAT-3´ | 575 | 60 | Zdravković-Korać *et al.* (2003) |
| rolD R1 | 5´-CTATTCCAACAGGACCTTGCCAAT-3´ | | | |
| aux1 for | 5'-CATAGGATCGCCTCACAGGT-3' | 198 | 61 | Designed according to *aux1* (GenBank acc. no. DQ782955) |
| aux1 rev | 5'-CGTTGCTTGATGTCAGGAGA-3' | | | |
| aux2 for | 5'-AACGATAATAGCCCGCTGTG-3' | 217 | 61 | Designed according to *aux2* (GenBank acc. no. DQ782955) |
| aux2 rev | 5'-CGTCTTGGGTTTGTGGTTCT-3' | | | |

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, $r_{i\ rolA} - r_{i\ rolC}$) 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. Sonications 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

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*/$r_i$) is obtained as the ratio between the normalized quantity of *rolX* gene and the virtual calibrator $r_i$. 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/genes 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.

| Clone | *rolA* ($r_{i\,rolA}$ = 0.55) | | | *rolB* ($r_{i\,rolB}$ = 0.67) | | | *rolC* ($r_{i\,rolC}$ = 0.69) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Norm ± SD | *rolA*/$r_i$ | int | Norm ± SD | *rolB*/$r_i$ | int | Norm ± SD | *rolC*/$r_i$ | int |
| $R_1$ | 1.85 ± 0.20 | 3.37 ± 0.71 | 3–4 | 2.21 ± 0.43 | 3.30 ± 1.27 | 3–4 | 1.81 ± 0.27 | 2.62 ± 0.77 | 2–3 |
| $R_2$ | 0.58 ± 0.23 | 1.05 ± 0.83 | 1 | 0.71 ± 0.04 | 1.06 ± 0.11 | 1 | 0.70 ± 0.05 | 1.02 ± 0.13 | 1 |
| $L_1$ | 0.47 ± 0.08 | 0.85 ± 0.29 | 1 | 0.56 ± 0.03 | 0.84 ± 0.08 | 1* | 0.46 ± 0.11 | 0.66 ± 0.32 | 1* |
| $L_2$ | 0.62 ± 0.05 | 1.12 ± 0.17 | 1 | 0.74 ± 0.04 | 1.11 ± 0.11 | 1 | 0.65 ± 0.06 | 0.95 ± 0.17 | 1 |
| $L_3$ | 1.10 ± 0.25 | 2.00 ± 0.89 | 2 | 0.95 ± 0.17 | 1.41 ± 0.49 | 1 | 0.99 ± 0.16 | 1.43 ± 0.45 | 1 |
| $L_4$ | 1.49 ± 0.17 | 2.70 ± 0.61 | 3 | 1.56 ± 0.21 | 2.33 ± 0.60 | 2 | 1.48 ± 0.19 | 2.15 ± 0.55 | 2 |
| $L_5$ | 1.55 ± 0.22 | 2.82 ± 0.78 | 3 | 1.35 ± 0.18 | 2.02 ± 0.52 | 2 | 1.35 ± 0.19 | 1.96 ± 0.54 | 2 |
| $L_6$ | 2.64 ± 0.33 | 4.80 ± 1.17 | 4–5 | 1.74 ± 0.31 | 2.59 ± 0.91 | 2–3 | 1.25 ± 0.25 | 1.81 ± 0.72 | 2 |
| $L_7$ | 0.72 ± 0.05 | 1.30 ± 0.17 | 1* | 0.95 ± 0.19 | 1.42 ± 0.55 | 1 | 0.89 ± 0.05 | 1.29 ± 0.14 | 1* |
| $L_8$ | 0.51 ± 0.05 | 0.93 ± 0.18 | 1 | 0.66 ± 0.02 | 0.98 ± 0.04 | 1 | 0.69 ± 0.01 | 1.00 ± 0.01 | 1 |
| $L_9$ | 1.64 ± 0.13 | 2.98 ± 0,48 | 3 | 1.97 ± 0.31 | 2.93 ± 0.90 | 3 | 1.80 ± 0.16 | 2.61 ± 0.46 | 3 |

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 $R_1$ 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, $L_1$–$L_9$ and $R_1$ and $R_2$, 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 $L_9$ 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

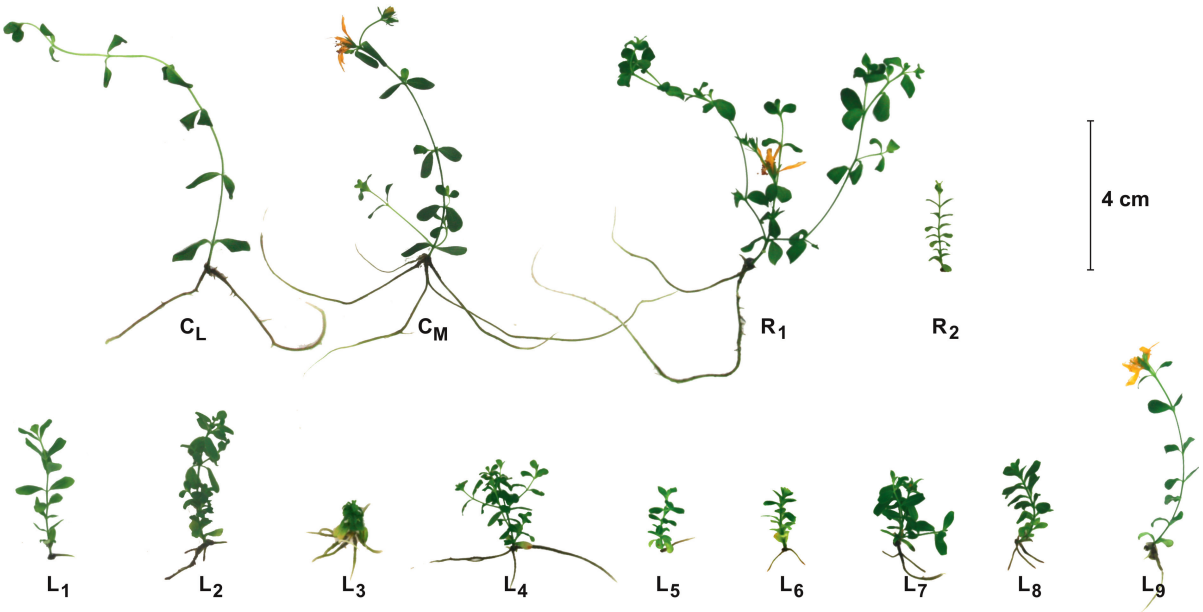


Fig. 1. Phenotypic variation of transgenic clones in comparison with the controls: $C_M$, mother clone; $C_L$, non-transformed clone regenerated from mother clone leaf; $R_1$, $R_2$, transgenic clones derived from root cuttings; $L_1$–$L_9$, transgenic clones derived from leaf cuttings.

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 $R_2$, $L_1$, $L_2$, $L_3$, $L_7$, and $L_8$ 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 $R_1$ and $L_9$ clones. The clones shared either the same number of integrated *rolA*, *B*, *C* genes ($R_2$, $L_1$, $L_2$, $L_7$, $L_8$, and $L_9$), *i.e.* the part of the $T_L$-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 $R_1$ and $L_9$ 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 $L_3$, 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

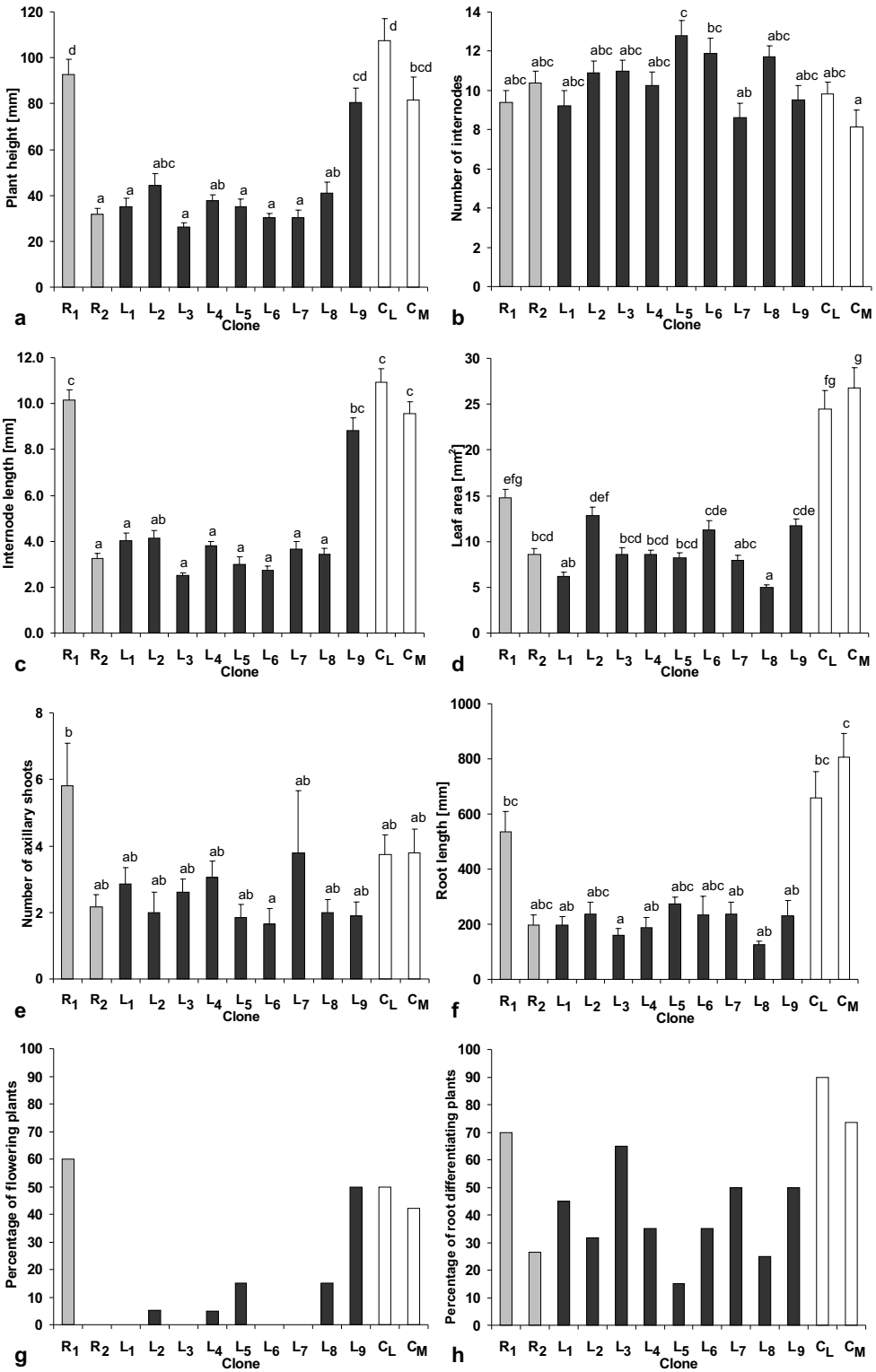
Plant height [mm]
Number of internodes
Internode length [mm]
Leaf area [mm²]
Number of axillary shoots
Root length [mm]
Percentage of flowering plants
Percentage of root differentiating plants
Clone
R1 R2 L1 L2 L3 L4 L5 L6 L7 L8 L9 CL CM
a
b
c
d
e
f
g
h

Fig. 2. Some quantitative parameters of transgenic and non-transgenic *H. perforatum* clones: (a) plant height; (b) number of internodes; (c) internode length; (d) leaf area; (e) number of axillary shoots; (f) length of the longest root in plant; (g) percentage of flowering plants; (h) percentage of rooting plants. Small letters indicate homogenous groups.

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, $R_1$, $R_2$ and $L_2$, 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 $R_2$ 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 $R_1$ was performed. The results revealed several anatomical differences in the development of male and female gametophytes compared to the mother non-transgenic clone $C_M$.

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 ($R_2$). 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 been excluded.

Table III. Secondary metabolite content (mg/g dry weight) in control and transgenic clones.

| Clone | Pseudohypericin | Hypericin | Hyperforin |
|---|---|---|---|
| $R_1$ | 2.63 | 0.84 | 5.68 |
| $R_2$ | 2.59 | 0.47 | 3.99 |
| $L_1$ | 3.82 | 0.63 | 5.49 |
| $L_2$ | 2.70 | 0.53 | 3.88 |
| $L_3$ | 1.83 | 0.34 | 3.51 |
| $L_4$ | 3.12 | 0.50 | 4.50 |
| $L_5$ | 2.17 | 0.53 | 1.94 |
| $L_6$ | 3.18 | 0.56 | 3.44 |
| $L_7$ | 2.73 | 0.44 | 4.86 |
| $L_8$ | 2.82 | 0.47 | 3.94 |
| $L_9$ | 3.17 | 0.61 | 5.72 |
| $C_L$ | 1.89 | 0.39 | 8.07 |
| $C_M$ | 2.15 | 0.37 | 5.24 |

In the genome of all transgenic clones analysed in our study the insertion and expression of the *rolA*, *B*, *C* genes from $T_L$-DNA was proved. The left part of agropine Ri plasmid T-DNA ($T_L$-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 $T_L$-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 $T_L$-DNA transfer were reported for some of the *Coffea arabica* hairy roots lines derived by Alpizar *et al.* (2008). The integration of the $T_R$-DNA was confirmed only in clone $L_9$ by the detection of *aux1* gene. Incorporation of only $T_L$-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

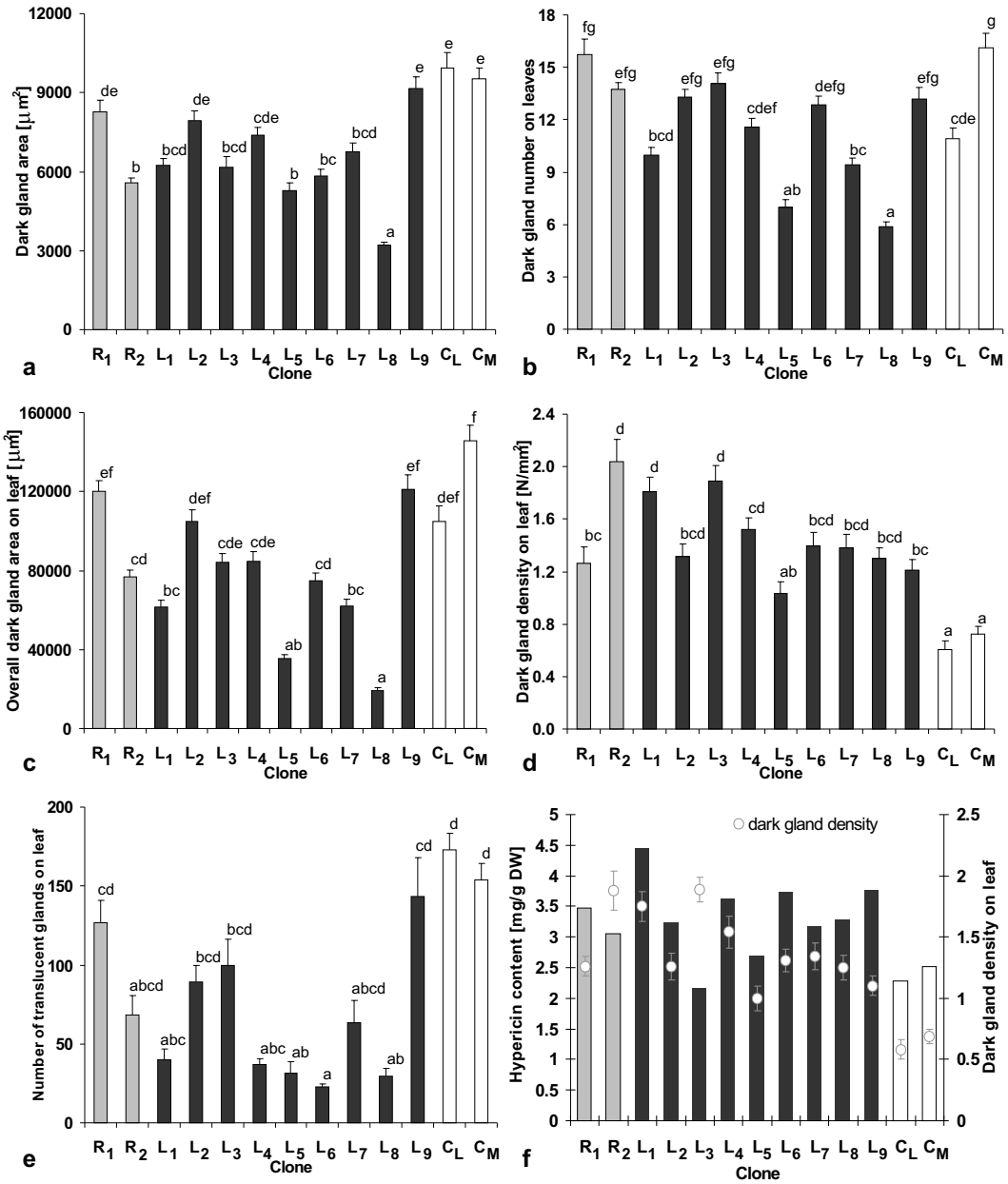


Fig. 3. Some quantitative parameters of dark and translucent glands on leaves: (a) dark gland area; (b) dark gland number on leaves; (c) overall dark gland area on leaf; (d) dark gland density on leaf; (e) number of translucent glands on leaf; (f) relationship between hypericin content and dark gland density. Small letters indicate homogenous groups.

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 $L_9$, 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 $R_1$ and $L_9$ 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 $L_4$ and $L_5$ 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 $L_4$–$L_6$ *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 ($R_1$ and $R_2$) were able to root and grow under *ex vitro* conditions, only one out of nine leaf explant-derived clones ($L_1$–$L_9$) 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; Gutièrrez-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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