Plant Cell Rep (2008) 27:1485–1495
DOI 10.1007/s00299-008-0575-0

GENETIC TRANSFORMATION AND HYBRIDIZATION

Transformation of *Kalanchoe blossfeldiana* with *rol*-genes is useful in molecular breeding towards compact growth

Brian Christensen · Sridevy Sriskandarajah · Margrethe Serek · Renate Müller

Received: 19 February 2008 / Revised: 21 May 2008 / Accepted: 12 June 2008 / Published online: 3 July 2008

Abstract Dwarf genotypes of the economically important flowering potted plant *Kalanchoe blossfeldiana* were developed by molecular breeding. Root inducing (Ri)-lines were regenerated by applying CPPU to the hairy roots, which were produced by inoculating leaf explants with a wild-type *Agrobacterium rhizogenes* strain ATCC15834. Amplification by polymerase chain reaction (PCR) and Southern blot analysis confirmed the presence of T-DNA in the Ri-lines. Six Ri-lines were characterised in a greenhouse trial revealing that several morphological traits changed with respect to ornamental value such as plant height, number of lateral shoots, leaf size, leaf number, flower size and number of flowers. The Ri-lines differed in their degree of Ri-phenotype, and the internodes of the Ri-lines were clearly shorter, giving a compact growth habit compared to control plants. Time to anthesis was the same in Ri-line 331 as in control plants and delayed by only 3 days in Ri-line 306 as compared to control plants. A compact plant without delayed flowering can be assumed to be valuable for further breeding.

Keywords Hairy roots · Ornamental plants · Ri-plasmid

Communicated by P. Kumar.

B. Christensen (✉) · S. Sriskandarajah · R. Müller
Department of Agricultural Sciences, Crop Science, University of Copenhagen, Faculty of Life Sciences, Højbakkegård Allé 21, 2630 Taastrup, Denmark
e-mail: bch@life.ku.dk

M. Serek
Institute for Ornamental and Woody Plant Science, Leibniz University of Hannover, Faculty of Natural Sciences, Herrenhaeuser Str.2, 30419 Hannover, Germany

Abbreviations

BM	Basic medium
Cef	Cefotaxine sodium
CPPU	*N*-(2-Chloro-4-pyridyl)-*N*′-phenylurea
DIG	Digoxigenin
GA	Gibberellic acid
GMO	Genetic modified organism
ORF	Open reading frame
PCR	Polymerase chain reaction
rol	Root loci
Ri	Root inducing
T-DNA	Transfer DNA
T_L-DNA	Left T-DNA
T_R-DNA	Right T-DNA
TDZ	*N*-Phenyl-N^1-1-thiadiazol-5-ylurea (thidiazuron)

Introduction

Kalanchoe blossfeldiana is a popular flowering potted indoor and garden plant around the globe and represents one of the economically most important potted plants in Europe. The production was estimated to be 60 million plants with a turnover of 38 million euros in the Netherlands in 2002. The Danish production of *K. blossfeldiana* was estimated to be 27 millions plants with a turnover of around 16 million euros in 2002 (Flora-Dania Marketing 2003) and the production increased to 40 million plants in 2006 (Rasmussen 2007).

Plant quality is imperative for production of potted plants. One important qualitative criterion is that the plants should be compact, but most potted plants like *K. blossfeldiana* have an elongated natural growth habit, which

necessitates growth control through the application of chemical growth retardants (Rademacher 2000). During recent years, growth retardants have increasingly been criticised because they are hazardous to human health and the environment (Fujimoto et al. 1997; Andersen et al. 2002). Some growth retardants, such as paclobutrazol and daminozide, can no longer be used in many countries and more of these retardants are likely to be banned in the near future.

An alternate strategy to the application of chemical growth retardants could be producing compact genotypes by inserting the *rol*-genes from the naturally occurring soil-born bacterium *Agrobacterium rhizogenes* into the plants. *A. rhizogenes* causes the growth of hairy-roots at the site of infection on various plant species by a natural transformation phenomenon due to the insertion of four root loci termed *rol*-genes. Regenerated plants from these transformed (hairy) roots exhibit modified characteristics such as atypical leaf morphology, short internodes, highly branched aerial parts, changes in flower size and improved rooting ability. The change in morphology of the regenerated plants is called the Ri-(root inducing) phenotype and these changes could be useful in creating new phenotypes in ornamental plant species. Ri-plants, which are plants with Ri-phenotype, have been created successfully using wild-type *A. rhizogenes* strains in ornamental plant species, such as scented geranium (*Pelargonium fragrans*, *P. odoratissimus* and *P. quercifolia*) (Pellegrineschi and Davolio-Mariani 1996), *Angelonia salicariifolia* (Koike et al. 2003) and *Datura arborea* (Giovannini et al. 1997).

The wild-type *A. rhizogenes* strain ATCC15834 has been used in transformation studies to produce Ri-plants (Jaziri et al. 1994; Pellegrineschi et al. 1994) and this strain contains an agropine type plasmid pRi15834. This plasmid contains two T-DNA regions, called the T_R-DNA and T_L-DNA (Jouanin 1984; Meyer et al. 2000) and these two T-DNAs are separated from each other by about 24 Kb of non-transferred DNA (Durand-Tardif et al. 1985). The T_L-DNA has a length of about 20 Kb and it contains at least 18 open reading frames (ORF) (Slightom et al. 1986). ORF 10, 11 12 and 15 coincided with *rol*A, *rol*B, *rol*C and *rol*D, respectively. These *rol*-genes are the main determinants of the Ri-phenotype (White et al. 1985; Slightom et al. 1986; Meyer et al. 2000). The T_R-DNA contains two ORFs called *aux1* and *aux2*, which are involved in auxin biosynthesis (Camilleri and Jouanin 1991).

The advantage of *A. rhizogenes* mediated transformation is that it enables the development of transgenic plants via a marker-free selection through the use of hairy root morphology as the primary indicator of transformation. Antibiotic resistance markers are commonly used in creation of genetically modified organisms (GMOs), but releasing GMOs containing antibiotic resistance markers will be banned in 2008 in the European Union (European Union 2001).

The objective of the present study is to introduce dwarfism into the economically important ornamental plant species *K. blossfeldiana*. A successful genetic transformation was achieved using a natural method without applying recombinant DNA technology by introducing *rol*-genes from wild-type *A. rhizogenes* strain ATCC15834 into *K. blossfeldiana*. The presence of the transgenes and copy number were documented, and finally the obtained phenotypes were characterised under greenhouse conditions.

Materials and methods

Plant material

Shoot cultures of *Kalanchoe blossfeldiana* Poelln. 'Molly' (Knud Jepsen A/S, Hinnerup, Denmark) were established in vitro by nodal cuttings from greenhouse grown plants. The nodal cuttings were sterilised by immersion in 1.5% (v/v) NaOCl (VWR, Copenhagen, Denmark) and 0.03% (v/v) Tween 20 (Merck, La Jolla, USA) for 20 min and rinsed three times in sterile water. Five nodal cuttings (10 mm) were placed in each clear plastic container (5 × 6 × 9 cm, Sakata Ornamentals Europe, Marslev, Denmark) containing 80 ml of BM (see below) without hormones. The explants were cultured in a growth room at 24°C and 16 h-photoperiod of 45 μmol m^{-2} s^{-1} (PAR) provided by cool-white fluorescent tubes (Philips, Amsterdam, The Netherlands).

Basic medium

The basic medium (BM) consisted of Murashige and Skoog macro- and microelements (Murashige and Skoog 1962), Staba vitamins, (Staba 1969), 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, 3 g l^{-1} Gelrite (Duchefa, Haarlem, The Netherlands) and 0.5 g l^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES) (Duchefa). The pH was adjusted to 6.3 before autoclaving at 121°C and 103.5 kPa. Filter-sterilised hormones and/or antibiotica were added after autoclaving.

Bacterial strain and production of transformed roots

Agrobacterium rhizogenes strain ATCC15834 (LGC Promochem, Boras, Sweden) was used for induction of hairy roots. This strain was cultured in liquid MYA medium (Tepfer and Cassedelbart 1987) and shaken at 300 rpm for 48 h in darkness at 28°C. In order to obtain transformed (hairy) roots, leaves were excised (approximately 2 × 2 cm) from in vitro raised shoot cultures and inoculated

with *A. rhizogenes* suspension ($OD_{600} = 0.6$) and shaken at 80 rpm for 30 min. In the control treatment, leaves were shaken in MYA medium. After co-cultivation, the leaves were blotted onto sterile filter paper and further co-cultivated with *A. rhizogenes* on half-strength BM containing 15 g l^{-1} glucose and 20 mg l^{-1} acetosyrigone (Sigma-Aldrich, Steinheim, Germany) in Petri dishes (100 × 20 mm) for 2 days in darkness at 24°C. After co-cultivation, the explants were rinsed in sterile water containing 500 mg l^{-1} cefotaxine sodium (Cef) (Duchefa), blotted onto sterile filter paper and cultured on half strength BM containing 500 mg l^{-1} Cef and 100 mg l^{-1} timentin (ticarcillin disodium and potassium clavulanate mixture 15:1, Duchefa). The explants were transferred to fresh medium containing antibiotics every second week to eliminate *A. rhizogenes*.

Plant regeneration

A suitable medium for regeneration of adventitious shoots from roots was developed by the following procedure: excised root tips (approximately 20 mm) of non-transformed plants were cultured in Petri dishes (100 × 20 mm) on BM containing 0, 0.5, 1.5 or 5 mg l^{-1} *N*-(2-Chloro-4-pyridyl)-*N*′-phenylurea (CPPU) (Duchefa) or *N*-phenyl-N^1-1,2,3,-thiadiazol-5-ylurea (TDZ) (Duchefa). Each treatment consisted of five Petri dishes containing 20 root tips, and the experiment was repeated twice. Explants were cultured in the growth room (see above). The number of green nodular structures and shoots were evaluated after 8 weeks.

In order to obtain adventitious shoots from the putative transformed (hairy) roots, root tips (approximately 20 mm) from both transformed and control roots were excised and cultured on BM containing 500 mg l^{-1} Cef, 100 mg l^{-1} timentin for 8 weeks and, based on the results from the above described experiment, CPPU at 1.5 mg l^{-1}. Finally, regenerated shoots were excised and cultured on BM without hormones and the shoots were transferred to fresh BM every fourth week. The shoots rooted on BM without hormones within 4 weeks.

Phenotypic analysis

Morphological characterisation was carried out on Ri-lines 306, 312, 317, 319, 324, 331 and control plants in the greenhouse. After rooting, 32 plantlets of each line and of control plants were acclimatised in a propagation room and grown under clear plastic at 20°C and 16 h-photoperiod of 145 μmol m^{-2} s^{-1} (PAR) at plant surface provided by SON-T high-pressure sodium lamps (Philips). The plantlets were grown in peat (Pindstrup II, Pindstrup Mosebrug A/S, Ryomgaard, Denmark) sterilised by autoclaving. The plastic cover was removed after 10 days and the plants were transferred to the greenhouse after 14 days when they were transplanted into plastic pots (11 cm) with peat (Pindstrup II). Four weeks after entering the greenhouse, flower induction was started by short day treatment (8 h light) at 22°C/18°C day/night. The plants were irrigated twice a week with standard fertiliser (Brun Komplet, Garta A/S, Copenhagen, Denmark), with an electrical conductivity of 1.2 ms cm^{-1}.

The number of days until anthesis was recorded continuously and the experiment was evaluated 100 days after the start of short day treatment. Data on number of shoots, internodes on the main shoot, leaves, flowers and inflorescences were recorded as well as plant height, internode length and flower diameter were also recorded. Leaf area was measured using a leaf area meter (LI-3100 Area Meter, LI-Cor Biosciences, Lincoln, NE, USA). Flowers, main shoot, side shoots and leaves were oven dried at 85°C for 5 days for dry weight measurements. The experiment was carried out as a block experiment, consisting of eight blocks with one replicate per block and the experiment was repeated four times.

Pollen production

In Ri-lines 306, 319 and 331 pollen production was examined visually.

PCR analysis

Polymerase chain reaction (PCR) was used to confirm integration of T-DNA into the plant genome. DNA was isolated with DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) from shoots of Ri-lines and control plants grown in the greenhouse. A specific primer set 2 (Table 1) amplified the *rol*C gene on the T_L-DNA, and primer set 5 was used for amplifying fragments of the T_R-DNA. The following temperature programme was applied for amplification in the DNA thermal cycler (Primus 25 advanced, Peqlab Biotechnologie, Erlangen, Germany): 95°C for 3 min, followed by 35 cycles 95°C for 30 s (denaturation), primer set depending temperature (Table 1) for 30 s (annealing) and 72°C for 1 min (elongation), with a final 5 min elongation at 72°C. The amplified fragments sequences were fractionated in 1% TAE agarose gel.

Southern blot analysis

The number of inserts of T_L- and T_R-DNA in the transformants was analysed by Southern blotting in Ri-lines 306, 312, 319, 324 and 331. DNA from shoots of the Ri-lines and control plants were isolated using the small-scale DNA extraction protocol for Agavaceae (Keb-Llanes et al.

Table 1 Specific primer pairs for PCR, RT-PCR and Southern blot probes used in analysing plants transformed with *A. rhizogenes* strain ATCC15834

Primer set	Gene	Primer sequences	Used in	Amplified fragment length (bp)	Annealing temperature (°C)
1	*rol*A	5′-ACGGTGAGTGTGGTTGTAGG-3′	RT-PCR	403	55.0
		5′-GCCACGTGCGTATTAATCCC-3′			
2	*rol*C	5′-CTAGACACATGAAGAACGCC-3′	PCR	272	56.0
		5′-GAAAAAACCTGATGCTTGCC-3′			
3	*rol*C	5′-TGTGACAAGCAGCGATGAGC-3′	RT-PCR	480	55.0
		5′-AAACTTGCACTCGCCATGCC-3′			
4	*rol*D	5′-AACTTTTCCAGCCCCAAAC-3′	RT-PCR	402	55.0
		5′-GCACTATCAAACCTCGATATCC-3′			
5	T_R-DNA	5′-AAAGCATAGGGGAACGCCAG-3′	PCR, T_R-DNA-probe	393	53.5
		5′-CTTCACGCTTTTGACTTTCCAC-3′			
6	T_L-DNA	5′-GCATTCAATAACGACCGTACAG-3′	T_L-DNA-probe	400	53.5
		5′-GGGAAATGGAGAGAGGGTAG-3′			

2002), but up-scaled to 3 g plant material. Plasmid DNA was isolated by the CTAB miniprep protocol (Delsal et al. 1988). The genomic DNA was digested with the restriction enzymes *Sca*I and *Bam*HI (New England Biolabs, Beverly, MA, USA), and the restriction fragments were separated by electrophoresis in 1% TE agarose gel (25 V, 20 h) and blotted onto a positively charged nylon membrane (Roche Diagnostics, Basel, Switzerland) by rapid downward transfer. The probes were amplified and labelled by PCR using PCR DIG Probe Synthesis Kit (Roche Diagnostics) and pRi15834 as template DNA. Primer set 5 was used for amplifying and labelling T_R-DNA-probe, and primer set 6 (Table 1) was used for the T_L-DNA-probe. The following temperature programme was applied: 95°C for 3 min, followed by 30 cycles 95°C for 30 s (denaturation), 53.5°C for 30 s (annealing) and 72°C for 1 min (elongation), with a final 5 min elongation at 72°C. The labelled probes were purified using DNA gel extraction kit (Millipore Corporation, Billerica, MA, USA). Hybridisation and detection were carried out according to the instruction manual provided by the manufacturer by applying the non-radioactive digoxigenin (DIG) system provided in the DIG Luminescent Detection Kit (Roche Diagnostics). Hybridisation signals were detected using X-ray film (Kodak Biomax MS, Rochester, NY, USA).

Expression analysis

Total RNA was isolated from shoots of the Ri-lines and control plants using RNeasy Plant Mini Kit (Qiagen) and RCL buffer provided by the manufacturer without β-mercaptoethanol, but with added 6.7% v/v polyethylene glycol solution (50% v/v in H_2O, Mw = 20,000, Bioultra, Sigma-Aldrich). Isolated total DNA was treated with deoxyribonuclease I (DNase I amplification grade, Sigma-Aldrich) following the methods described by the manufacturer. The synthesis of cDNA was performed on 1 μg DNase treated total RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The reverse transcription reaction temperature programme was set as follows for cDNA synthesis: 5 min at 25°C (annealing), 30 min at 42°C (extension) and 5 min at 85°C (enzyme inactivation). The first standard cDNA was used as template for RT-PCR reaction.

Statistical analysis

The data obtained were subjected to analysis of variance (ANOVA) using the general linear models (PROC GLM) procedure in the Statistical Analysis System (SAS 9.1) programme package for Windows. Multiple comparisons among means were performed using Duncan's multiple range test with the level of significance $P = 0.05$.

Results

Production of transformed roots

Shoot cultures of *K. blossfeldiana* were easy to establish in vitro and both inoculated and non-inoculated leaf explants (control treatment) from these cultures developed roots (Fig. 1a). Consequently, inoculated leaves developed both transformed and non-transformed roots, but the transformed roots grew much more vigorously than

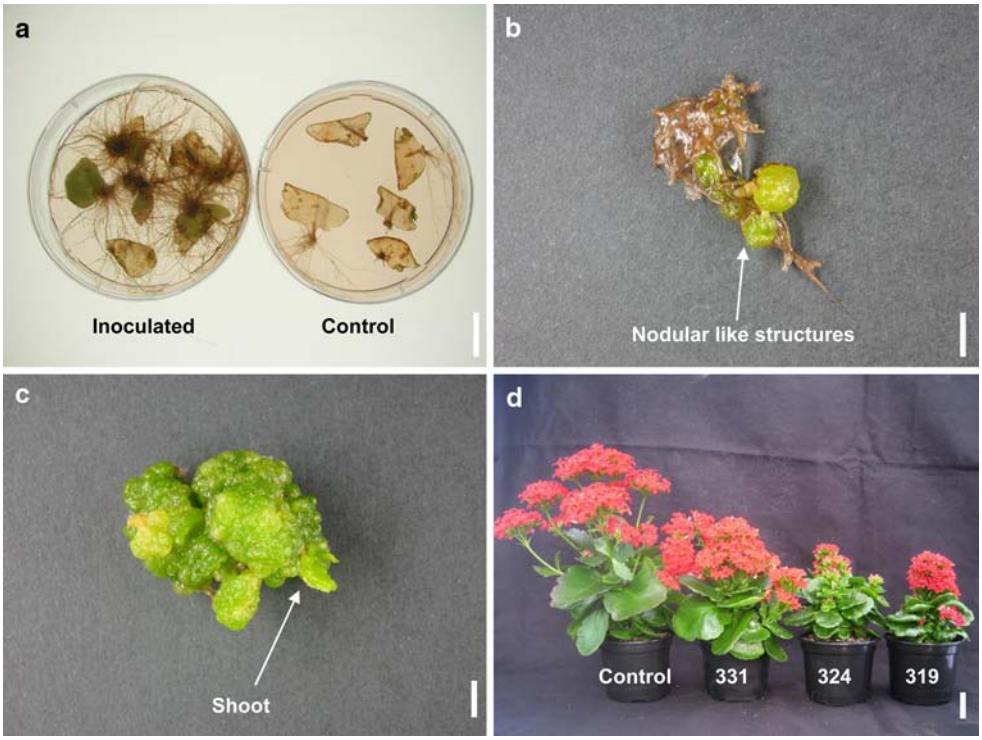

Fig. 1 **a** Development of hairy roots on leaves of *K. blossfeldiana* 'Molly' after inoculation with *A. rhizogenes*. *Bar* 2.5 cm. **b** Development of nodular like structures on roots grown on shoot regeneration medium. *Bar* 2 mm. **c** Shoot initiation on nodular like structures. *Bar* 2 mm. **d** Regenerated flowering control and Ri-plants in the greenhouse. *Bar* 5 cm

non-transformed roots. This made it possible to visually select transformed roots by their vigorous growth.

Shoot regeneration

After 4 weeks on shoot regeneration medium containing CPPU or TDZ, the non-transformed roots developed green nodular like structures (Fig. 1b). Subsequently, shoots were initiated on these nodular like structures (Fig. 1c). The significantly highest number of shoots was obtained at 0.5 and 1.5 mg l^{-1} CPPU or TDZ and the shoot numbers were reduced significantly by increasing the concentration to 5 mg l^{-1} (Table 2). No shoot regeneration was recorded in media without TDZ or CPPU.

Spontaneous shoot regeneration was not observed from the transformed roots, but since CPPU at 1.5 mg l^{-1} resulted in regeneration of the highest number of shoots in non-transformed shoots, this level of CPPU was used for shoot regeneration from transformed roots. From transformed roots, scores of shoots were regenerated using CPPU at 1.5 mg l^{-1}. It was important to excise the regenerated shoots, as soon as, the small shoots were detected visually at about 8 weeks and transfer these shoots to fresh media without hormones; otherwise, uncontrolled callus formed. Uncontrolled callus formation was not observed during regeneration of shoots of non-transformed roots at any CPPU level tested. A total of 64 putative Ri-lines were regenerated.

Phenotypic analysis

At the flowering stage, plant height was reduced significantly ($P < 0.001$) in all six Ri-lines compared to the control plants, and there were significant differences

Table 2 Effects of CPPU and TDZ on shoot regeneration from non-transformed roots of *K. blossfeldiana*

Treatment	Mean number of nodular structures	Mean number of shoots	Mean number of shoots per nodular structure
CPPU			
0	0.0 (±0.0) c[z]	0.0 (±0.0) b[z]	–
0.5	4.2 (±1.9) b	4.8 (±3.2) a	0.9 (±0.3) a b[z]
1.5	9.6 (±4.9) a	6.1 (±3.7) a	0.7 (±0.3) a b c
5	2.1 (±1.6) b c	1.0 (±3.2) b	0.1 (±0.1) c
TDZ			
0.5	4.7 (±2.1) b	5.1 (±3.2) a	1.2 (±0.4) a
1.5	4.1 (±1.6) b	3.1 (±1.9) a b	0.8 (±0.3) a b
5	2.6 (±1.0) b c	1.3 (±0.9) b	0.5 (±0.3) b c

[z] The numbers in a column followed by different letters (a, b, c) are significantly different at $P \leq 0.05$ by Duncan's multiple range test

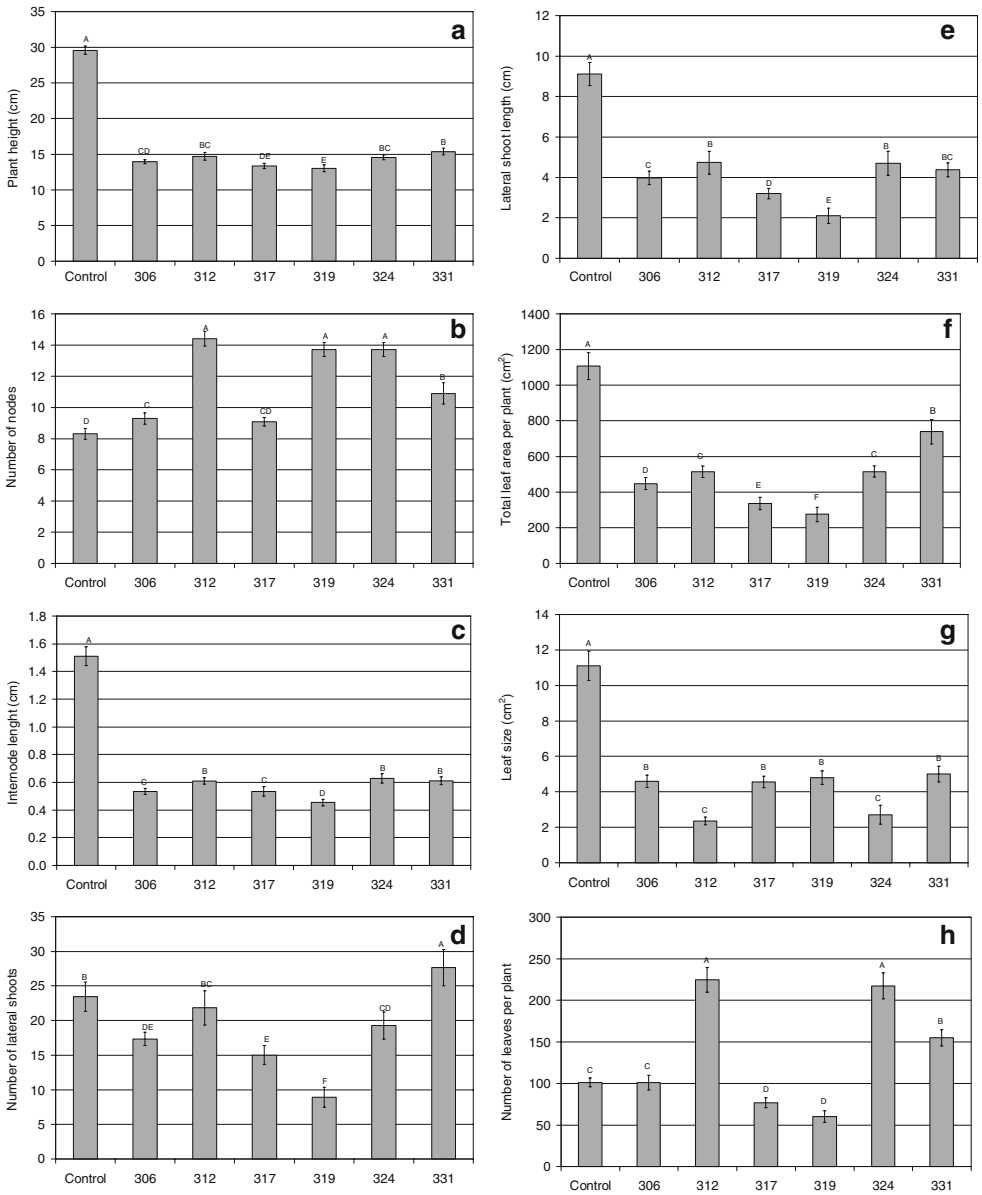

Fig. 2 Vegetative traits of *K. blossfeldiana* 'Molly' control plants and Ri-lines transformed with *A. rhizogenes* strain ATCC15834. **a** Mean plant height (cm). **b** Mean number of nodes per plant. **c** Mean internode length (mm). **d** Mean number of lateral shoots per plant. **e** Mean lateral shoot length (cm). **f** Mean total leaf area per plant (cm^2). **g** Mean leaf size (cm^2). **h** Mean number of leaves per plant. *Bars* marked with different letters (*A*, *B*, *C*, *D*, *E*, *F*) are significantly different at $P \leq 0.05$ by Duncan's multiple range test. *Bars* Mean ± SD ($n = 32$)

among the Ri-lines (Figs. 1d, 2a). The plant height was reduced by 44.4% in line 331 and 51.9% in Ri-line 317 relative to control plants. The number of nodes increased significantly ($P < 0.001$) in the Ri-lines, and the number of nodes ranged from a mean of 9.1 in Ri-line 306 to 14.4 in Ri-line 312 in comparison to the mean of 8.3 nodes in control plants (Fig. 2b). The internode length was significantly lower ($P < 0.001$) in all Ri-lines compared to control plants (Fig. 2c): the internode length was 15 mm in control plants, but the reduction was only 5 mm in line 319, which had the shortest internode length.

The number of lateral shoots per plant in the Ri-lines in comparison to control plants was either at the same level as the case in Ri-line 312, or increased as in Ri-line 331 or reduced as in Ri-lines 306, 317, 319 and 324 (Fig. 2d). The mean lateral shoot length was significantly reduced ($P < 0.001$) in the Ri-lines (Fig. 2e). The reduction ranged from 23.1% in Ri-line 312 to 51.9% in Ri-line 319 relative to that of control plants.

The mean total leaf area per plant (Fig. 2f) and the mean size of the leaves (Fig. 2g) were significantly reduced ($P < 0.001$) in all Ri-lines compared to control plants. The mean number of leaves per plant differed significantly ($P < 0.001$) among the Ri-lines (Fig. 2h). In the Ri-lines, the mean number of leaves per plant was either increased as in Ri-lines 312, 324 and 331, or decreased as in Ri-lines 317 and 319, or remained at the same level as control plants as the case was for Ri-line 306. The leaves of the Ri-lines were wrinkled, but the Ri-lines differed in their degree of leaf wrinkling. Ri-lines 306 and 331 had only

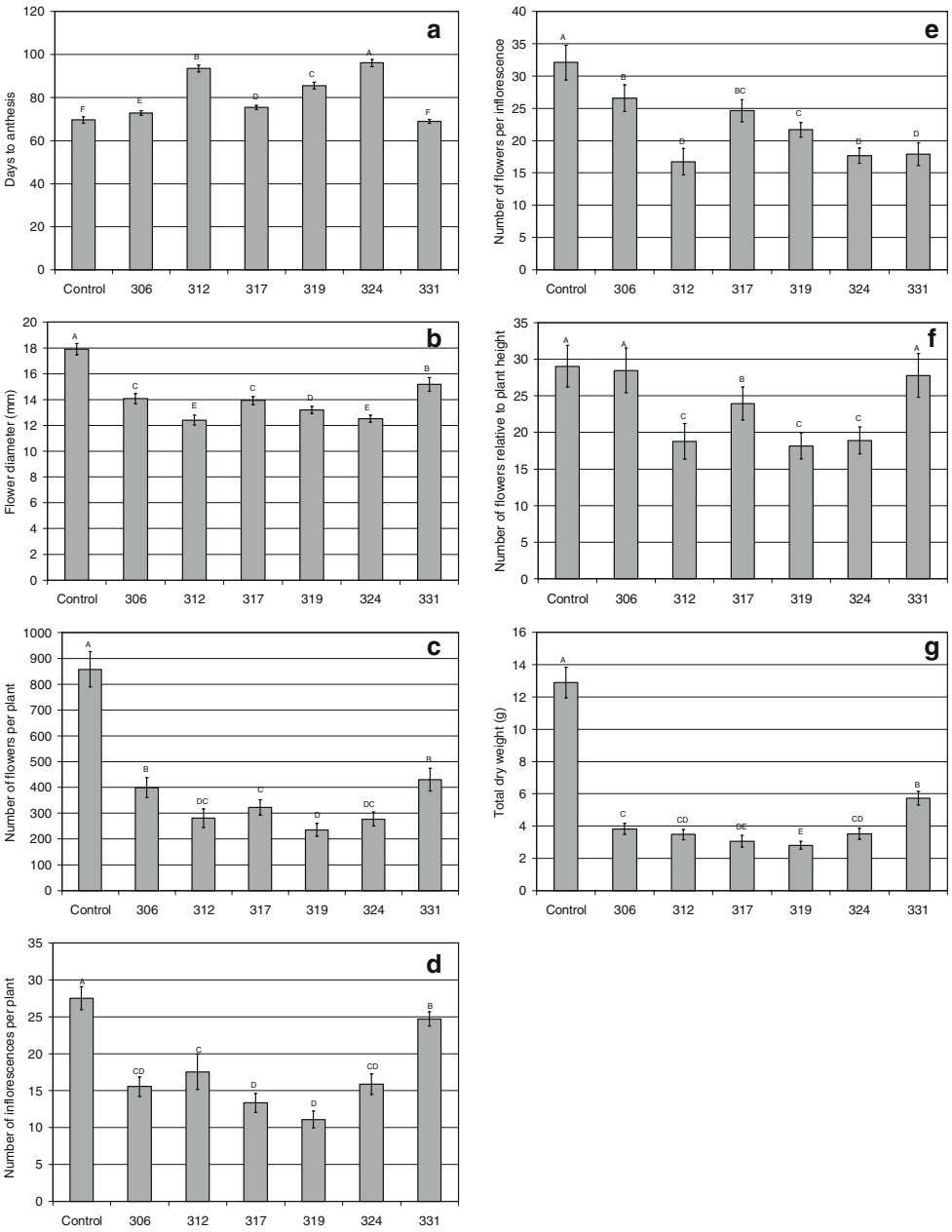

Fig. 3 Generative traits of *K. blossfeldiana* 'Molly' control plants and Ri-lines transformed with *A. rhizogenes* strain ATCC15834. **a** Mean number of days to anthesis from the start of flower induction by short day treatment. **b** Mean flower diameter (mm). **c** Mean number of flowers per plant. **d** Mean number of inflorescences per plant. **e** Mean number of flowers per inflorescence. **f** Mean number of flowers per plant relative to mean plant height. **g** Mean dry weight per plant. *Bars* marked with different letters (*A*, *B*, *C*, *D*, *E*, *F*) are significantly different at $P \leq 0.05$ by Duncan's multiple range test. *Bars* Mean ± SD ($n = 32$)

slightly wrinkled leaves, whereas in the other Ri-lines the wrinkling was more pronounced.

The number of days from the start of the short day treatment to anthesis of the first flower was similar in Ri-line 331 and control plants, but in the other Ri-lines the number of days until flowering was significantly increased ($P < 0.001$) (Fig. 3a). In the Ri-lines with delayed flowering, the delay ranged from 3 days in Ri-line 306 to 27 days in Ri-line 324. The flower diameter was 18 mm in the control plants, but it was reduced significantly in the Ri-lines ($P < 0.001$). Ri-line 331 had the least reduction in flower size as the flower diameter was 15 mm (Fig. 3b). Ri-lines 312 and 324 had the largest reduction in flower diameter. The flower diameter in these two Ri-lines was 12 mm. The total number of flowers per plant was significantly reduced ($P < 0.001$) in the Ri-lines compared to control plants (Fig. 3c). The total number of flowers was 857 per plant in control plants, but this was reduced to 430 in Ri-line 331 and to 399 in Ri-line 306. These two lines had the least reduction. The lowest number of flowers per plant recorded in this study was Ri-line 319, which had 235 flowers.

The inflorescences of the Ri-lines were dense; nearly ball shaped, whereas, the flowers of the control plants were in open cymes (Fig. 1d). The mean number of inflorescences was significantly reduced ($P < 0.001$) in all Ri-lines

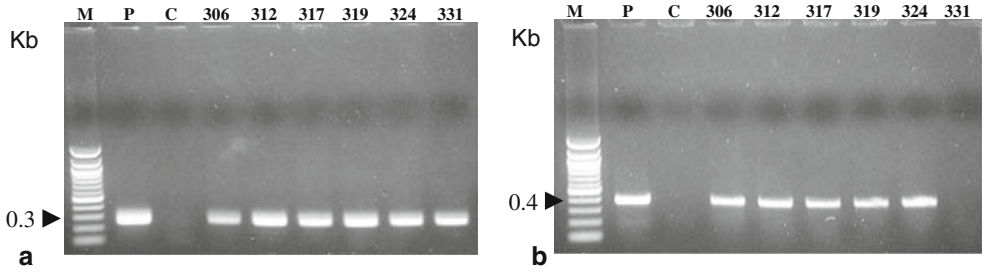

Fig. 4 PCR analysis of Ri-lines of *K. blossfeldiana* 'Molly' transformed with *A. rhizogenes* strain ATCC15834 by amplification of genomic DNA. **a** PCR using primer set 2 for amplifying *rol*C gene on T_L-DNA. *Arrowheads* expected ~0.3 kb fragments. **b** PCR using primer set 6 for amplifying T_R-DNA probe on T_R-DNA. *Arrowheads* expected ~0.4 kb fragments. Lanes: *M* 100 bp DNA ladder; *P* pRi15834; *C* Control plants; *306–331* individual Ri-lines

(Fig. 3d) when compared to control plants. The number of inflorescences was 27.5 in control plants, but ranged from 11.1 in Ri-line 319 to 25.7 in Ri-line 331. The mean number of flowers per inflorescence was also reduced significantly ($P < 0.001$) in the Ri-lines (Fig. 3e). A significant difference ($P < 0.001$) was recorded in the mean number of flowers relative to plant height (Fig. 3f). In Ri-lines 306 and 331 it was at the same level as control plants, whereas, it was reduced in the other Ri-lines. The mean total plant dry weight was reduced significantly ($P < 0.001$) in the Ri-lines compared to control plants (Fig. 3g).

Pollen production

Ri-line 306 was male sterile, the pollen production of Ri-line 319 was minimal, whereas, it was only slightly reduced in Ri-line 331 in comparison to control plants.

PCR analysis

In the molecular analysis, 50% of the 64 regenerated putative Ri-lines turned out to be transgenic by confirming the presence of T-DNA with PCR amplification. All Ri-lines, in which the presence of the T-DNA was confirmed, displayed the Ri-phenotype. Plants, where the presence of the T-DNA was not detected, were phenotypically normal.

In the six Ri-lines, which were part of the phenotypic analysis, the presence of T_L-DNA was confirmed in all six Ri-lines (Fig. 4a) and the T_R-DNA was present in all Ri-lines except for Ri-line 331 (Fig. 4b).

Southern blot analysis

Southern blot hybridisation showed integration of the T_L-DNA in the plant genome of all the five analysed Ri-lines (Fig. 5a). Ri-lines 306 and 319 showed one hybridisation band, Ri-line 331 two hybridisation bands and Ri-lines 312 and 324 showed multiple hybridisation bands. Southern blot hybridisation for testing of the integration of T_R-DNA revealed integration of the T_R-DNA in Ri-lines 306, 312 and 324, but not in line 331 (Fig. 5b). Ri-line 306 showed one hybridisation band and Ri-line 324 two hybridisation bands and Ri-line 312 multiple hybridisation bands.

Expression analysis

The RT-PCR products revealed clear bands for *rol*A (403 bp), *rol*C (480 bp) and *rol*D (402 bp) genes (Fig. 6). This further confirms that the *rol*-genes from the Ri-plasmid were integrated into the plant genome of *K. blossfeldiana*, and that they are expressed in the plants. Expression of the *rol*-genes was not detected in the control plants.

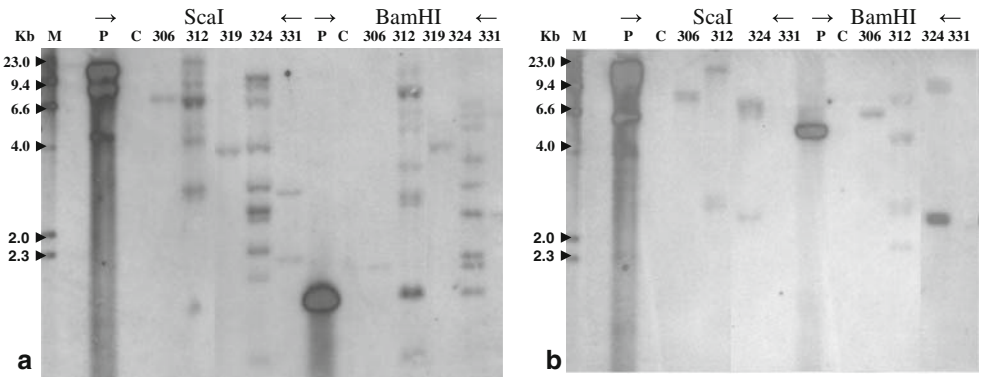

Fig. 5 Southern hybridisation of Ri-lines of *K. blossfeldiana* 'Molly' transformed with *A. rhizogenes* strain ATCC15834. **a** Probe for T_L-DNA. **b** Probe for T_R-DNA. *M* Dig-marker, *P* pRi15834, *C* Control plants; *306–331* individual Ri-lines

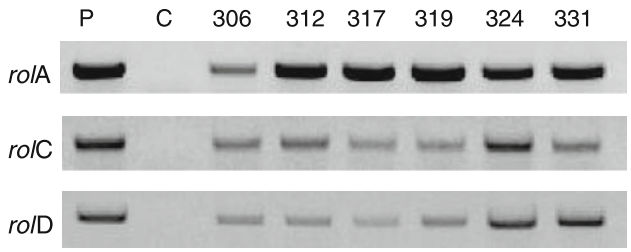

Fig. 6 Expression analysis of *rol*A (primer set 1), *rol*C (primer set 3) and *rol*D (primer set 4) genes by RT-PCR of Ri-lines of *K. blossfeldiana* transformed with *A. rhizogenes* strain ATCC15834. *P*: pRi15834; *C*: Control plants; *306–331* individual Ri-lines

Discussion

In the present study, *K. blossfeldiana* was successfully transformed with genes of *A. rhizogenes*, and it was evident that the obtained Ri-lines exhibited morphological alterations in many traits with relevance to ornamental value. Dwarfism was clearly introduced into the Ri-lines since transformed plants displayed a marked reduction in plant height, lateral shoot length and leaf area. The reduction in leaf area per plant was due to a reduction in the size of the individual leaves. For instance, Ri-lines 312, 324 and 331 had an increased number of leaves, but at the same time a lower leaf area per plant compared to control plants. These results demonstrate that *rol*-genes in *K. blossfeldiana* are not only affecting plant height, but also cause growth retardation of many plant organs, which indicate the potential of these genes in breeding efforts towards compact growth in *K. blossfeldiana*.

In the current study of *K. blossfeldiana*, non-inoculated explants did produce roots, but the putative transformed roots grew much more vigorously than non-transformed roots making visual selection based on the root growth of hairy roots possible. This study shows that it is possible to detect successful transformation events solely based on visible traits avoiding the use of antibiotic resistance genes.

Spontaneous regeneration of shoots from hairy roots grown on hormone free media has been reported in several species (Mugnier 1988; Pellegrineschi and Davolio-Mariani 1996; Chaudhuri et al. 2006). No spontaneous regeneration was observed in *K. blossfeldiana* hairy roots, but Ri-lines were regenerated using CPPU. This corresponds with reports in other plant species where spontaneous regeneration has not been observed and cytokinin like BAP (Jaziri et al. 1994; Koike et al. 2003) or TDZ (Hosokawa et al. 1997) was needed for regeneration. Since CPPU at 1.5 mg l^{-1} induced regeneration of the highest number of shoots in non-transformed shoots, this level of CPPU was used for regeneration of shoots from transformed roots. Profuse shoot regeneration was observed from transformed roots using CPPU. In addition to this, uncontrolled callus developed in transformed roots, but not in control roots. To our knowledge this is the first report on the use of CPPU for shoot regeneration from hairy roots.

The regenerated Ri-lines displayed a marked reduction in overall plant height, which was caused by a reduction in internode length. The decrease in height occurred despite an increased number of nodes. Similar results have been reported in scented geranium (Pellegrineschi and Davolio-Mariani 1996) and in *Angelonia salicariifolia* (Koike et al. 2003). In *Datura arborea*, the Ri-lines had either the same or reduced plant height compared to control plants, but all Ri-lines had reduced internode length and an increased number of nodes (Giovannini et al. 1997). Ri-lines of *N. scoparia* had the same height as control plants, but the Ri-lines still had shortened internodes and increased number of nodes (Godo et al. 1997). This implies that one of the characteristics of Ri-plants is that the reduced internode length is compensated by an increased number of nodes. However, in the case of *K. blossfeldiana* and other ornamental plant species this does not counteract for the reduced internode length.

A general feature of the *rol*-genes is that they reduce the apical dominance resulting in increased lateral branching (Pellegrineschi and Davolio-Mariani 1996; Hosokawa et al. 1997; Godo et al. 1997). Reduced apical dominance was not observed in the present study, since the number of lateral shoots per plant compared to control plants was either at the same level or reduced. This observation can be explained by differences in the effect of *rol*-genes in different plant species, due to interactions with the plant's own genes and differences in Ri-phenotypic expression depending on the used strain of *A. rhizogenes* (Tepfer 1984; Jaziri et al. 1994).

Delayed flowering (Tepfer 1984; Oksman-Caldentey et al. 1991; Otani et al. 1996) or even inhibition of flowering has been associated with Ri-plants (Damiani and Arcioni 1991; Pellegrineschi et al. 1994; Giovannini et al. 1997). Other molecular methods to create dwarfism in plants, for example producing gibberellic acid (GA) deficient or insensitive dwarf plants by manipulation of GA synthesis and transduction pathways have also been linked to delayed flowering (Fridborg et al. 1999). Delayed flowering is not acceptable in commercial production as it increases production time and thus production costs. In the present study, the Ri-lines of *K. blossfeldiana* showed various degrees of delayed flowering, but interestingly, the time to anthesis from the start of short day treatment was the same for Ri-line 331 as for control plants and only delayed by 3 days in Ri-line 306. A compact plant without delayed flowering can be assumed to be valuable in breeding programmes.

Reduction in the number and size of the flowers was recorded in the *Kalanchoe* Ri-lines. The reduction in the number of flowers was due to less inflorescences and a reduced number of flowers per inflorescence. However, comparing the number of flowers relative to plant height revealed that it was similar for Ri-lines 306 and 331 and control plants, indicating that ornamental value of these plants was not reduced. Furthermore, even though the size of the flowers was reduced, the reduction in flower diameter was only 17% in Ri-line 331, which has to be compared to a reduction in plant height of 44.4% and leaf size of 55% of that recorded for control plants. Consequently, the reduction in flower size was smaller when compared to the growth reduction in other organs. Even though, reduction in the number of flowers and flower size are normally not considered desirable in potted plants, this did not substantially affect the ornamental value of Ri-lines 306 and 331.

Kalanchoe blossfeldiana is an easy to root plant species and 100% rooting is normally obtained in 'Molly' without using any rooting hormones. In the present investigation, stock plants of Ri-lines and control plants have been kept in the greenhouse and propagated by cuttings with 100% rooting and no difference has been observed in rooting performance between Ri-lines and control plants. The stock plants have been renewed by cuttings for six generations without any change in the Ri-phenotype, which demonstrates that the Ri-phenotype is stable through vegetative propagation.

The T_L-DNA/plant border on the left side of the T_L-DNA can vary among plant species, but in most cases the T_L-DNA/plant border on the left side of the T_L-DNA is located upstream from ORF1 (Slightom et al. 1985; Taylor et al. 1985; Ooms et al. 1985; Jouanin et al. 1987). However, in *Nicotiana tabacum* the ORFs 1–7 were not always integrated into the plant genome (Durand-Tardif et al. 1985; Jouanin et al. 1987). The five investigated *K. blossfeldiana* Ri-lines showed hybridisation signals in the Southern hybridisation using the T_L-DNA probe, which started at 238 bp upstream from ORF1, and the T_L-DNA/plant border in these *K. blossfeldiana* Ri-lines will be further upstream from this probe. The results of the Southern blotting documented that the location of T_L-DNA/plant border on the left side of the T_L-DNA in the Ri-lines of *K. blossfeldiana* corresponds to observations in other plant species.

The *rol*-genes are the main determinants of the Ri-phenotype (Meyer et al. 2000), and in *N. glauca*, the difference in Ri-phenotype could not be correlated to presence or absence of T_R-DNA in the plants (Taylor et al. 1985) and tobacco plants transformed with only T_R-DNA were phenotypically normal (Camilleri and Jouanin 1991). On the contrary, the transgenic plants of *Solanum dulcamara* transformed with T_R-DNA showed epinasty (Mcinnes et al. 1991). In the current study, Ri-line 331 had the least typical Ri-phenotype of the studied Ri-lines in terms of reduction in plant height, total leaf area, leaf size, days to flowering, flower size and number of flowers per plant. The Southern hybridisation showed that Ri-line 331 had two inserts of T_L-DNA, but no inserts of T_R-DNA was detected by PCR amplification targeted against the corresponding T_R-DNA sequence or by Southern hybridisation. This indicates that not only the *rol*-genes on the T_L-DNA influence the Ri-phenotype, but also genes on the T_R-DNA, for example the *aux*-genes, may have an effect on the phenotype in the Ri-plants of *K. blossfeldiana*.

Ri-lines 312 and 324 showed the most distinct Ri-phenotype in terms of reduced leaf size, increased number of leaves, delayed flowering and reduced number of flowers. The Southern hybridisation revealed that the Ri-lines differed in the number of T-DNA inserts and Ri-lines 312 and 324 had multiple inserts of both T_L-DNA and T_R-DNA. Moreover, the copy number of T_L-DNA was higher than the copy number of T_R-DNA, but no insert of T_R-DNA was detected in Ri-line 331. Absence of T_R-DNA has also been reported in other species (Taylor et al. 1985; Jouanin et al. 1987; Jaziri et al. 1994). These results indicate that T_L-DNA and T_R-DNA were integrated independently into the plant genome, and that T_L-DNA was integrated at much higher frequency than the T_R-DNA. Furthermore, the present findings imply that a higher number of Ri-T-DNA copies integrated into the plant genome increases the phenotypic effect in the Ri-line.

Conclusion

This study demonstrated successful transformation and regeneration of Ri-lines of the economically important potted ornamental plant *K. blossfeldiana*. The phenotypic analysis revealed that many morphological traits with relevance to ornamental value were changed in the Ri-lines in comparison to control plants. Most striking was the growth retarding effect on all plant organs studied, showing that the genes of *A. rhizogenes* are very effective in producing dwarfism in *K. blossfeldiana*. The Ri-lines had altered flower physiology to various degrees, and some of the Ri-lines had no, or minor delay, in time to anthesis. This makes transformation with *A. rhizogenes* and its *rol*-genes a promising method in molecular breeding for creating new diversity in *K. blossfeldiana* and other ornamental species especially for production of compact plants.

Acknowledgments The study was funded by a PhD grant from the University of Copenhagen, Faculty of Life Sciences and the authors would like to thank Knud Jepsen A/S, Hinnerup, Denmark, for providing plant material for the study.

References

Andersen HR, Vinggaard AM, Rasmussen TH, Gjermandsen IM, Bonefeld-Jørgensen EC (2002) Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro. Toxicol Appl Pharmacol 179:1–12

Camilleri C, Jouanin L (1991) The TR-DNA region carrying the auxin synthesis genes of the *Agrobacterium rhizogenes* agropine type plasmid pRiA4: nucleotide sequence analysis and introduction into tobacco plants. Mol Plant Microbe Interact 4:155–162

Chaudhuri KN, Ghosh B, Tepfer D, Jha S (2006) Spontaneous plant regeneration in transformed roots and calli from *Tylophora indica*: changes in morphological phenotype and tylophorine accumulation associated with transformation by *Agrobacterium rhizogenes*. Plant Cell Rep 25:1059–1066

Damiani F, Arcioni S (1991) Transformation of *Medicago arborea* L. with an *Agrobacterium rhizogenes* binary vector carrying the hygromycin resistance gene. Plant Cell Rep 10:300–303

Delsal G, Manifioletti G, Schneider C (1988) A one-tube plasmid DNA mini-preparation suitable for sequencing. Nucleic Acids Res 16:9878

Durand-Tardif M, Broglie R, Slightom J, Tepfer D (1985) Structure and expression of Ri T-DNA from *Agrobacterium rhizogenes* in *Nicotiana tabacum.* Organ and phenotypic specificity. J Mol Biol 186:557–564

European Union (2001) Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC—commission declaration

Flora-Dania Marketing (2003) Temarapport 2003. Salgsudvikling i Danmark and Holland 1995–2002. Flora-Dania Marketing A/S, pp 1–7

Fridborg I, Kuusk S, Moritz T, Sundberg E (1999) The *Arabidopsis* dwarf mutant *shi* exhibits reduced gibberellin responses conferred by overexpression of a new putative zinc finger protein. Plant Cell 11:1019–1031

Fujimoto H, Tetsuo M, Tomonari M, Hamada T, Yakushiji M (1997) Growth-retardant and teratogenic effects of ketone bodies on early embryogenesis in rat whole embryo culture. J Matern Fetal Investig 7:92–98

Giovannini A, Pecchioni N, Rabaglio M, Allavena A (1997) Characterization of ornamental *Datura* plants transformed by *Agrobacterium rhizogenes*. In Vitro Cell Dev Biol Plant 33:101–106

Godo T, Tsujii O, Ishikawa K, Mii M (1997) Fertile transgenic plants of *Nierembergia scoparia* Sendtner obtained by a mikimopine type strain of *Agrobacterium rhizogenes*. Sci Hortic 68:101–111

Hosokawa K, Matsuki R, Oikawa Y, Yamamura S (1997) Genetic transformation of gentian using wild-type *Agrobacterium rhizogenes*. Plant Cell Tissue Organ Cult 51:137–140

Jaziri M, Yoshimatsu K, Homes J, Shimomura K (1994) Traits of transgenic *Atropa belladonna* doubly transformed with different *Agrobacterium rhizogenes* strains. Plant Cell Tissue Organ Cult 38:257–262

Jouanin L (1984) Restriction map of an agropine-type Ri plasmid and its homologies with Ti plasmids. Plasmid 12:91–102

Jouanin L, Guerche P, Pamboukdjian N, Tourneur C, Delbart FC, Tourneur J (1987) Structure of T-DNA in plants regenerated from roots transformed by *Agrobacterium rhizogenes* strain A4. Mol Gen Genet 206:387–392

Keb-Llanes M, González G, Chi-Manzanero B, Infante D (2002) A rapid and simple method for small-scale DNA extration in *Agavaceae* and other tropical plants. Plant Mol Biol Rptr 20:299a–299e

Koike Y, Hoshino Y, Mii M, Nakano M (2003) Horticultural characterization of *Angelonia salicariifolia* plants transformed with wild-type strains of *Agrobacterium rhizogenes*. Plant Cell Rep 21:981–987

Mcinnes E, Morgan AJ, Mulligan BJ, Davey MR (1991) Phenotypic effects of isolated pRiA4 TL-DNA *rol* genes in the presence of intact TR-DNA in transgenic plants of *Solanum dulcamara* L. J Exp Bot 42:1279–1286

Meyer AD, Tempe J, Constantino P (2000) Hairy root: a molecular overview. Functional analysis of *Agrobacterium rhizogenes* T-DNA genes. In: Stacey G, Keen NT (eds) Plant–microbe interactions. APS Press, St. Paul, pp 93–139

Mugnier J (1988) Establishment of new axenic hairy root lines by inoculation with *Agrobacterium rhizogenes*. Plant Cell Rep 7:9–12

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Oksman-Caldentey KM, Kivela O, Hiltunen R (1991) Spontaneous shoot organogenesis and plant regeneration from hairy root cultures of *Hyoscyamus muticus*. Plant Sci 78:129–136

Ooms G, Karp A, Burrell MM, Twell D, Roberts J (1985) Genetic modification of potato development using Ri T-DNA. Theor Appl Genet 70:440–446

Otani M, Shimada T, Kamada H, Teruya H, Mii M (1996) Fertile transgenic plants of *Ipomoea trichocarpa* Ell induced by different strains of *Agrobacterium rhizogenes*. Plant Sci 116:169–175

Pellegrineschi A, Davolio-Mariani O (1996) *Agrobacterium rhizogenes*-mediated transformation of scented geranium. Plant Cell Tissue Organ Cult 47:79–86

Pellegrineschi A, Damon JP, Valtorta N, Paillard N, Tepfer D (1994) Improvement of ornamental characters and fragrance production in lemon-scented geranium through genetic-transformation by *Agrobacterium rhizogenes*. Biotechnology 12:64–68

Rademacher W (2000) Growth retardants: Effects on gibberellin biosynthesis and other metabolic pathways. Annu Rev Plant Physiol Plant Mol Biol 51:501–531

Rasmussen AA (2007) Top 14 over danske kulturer. Gartnertidende 123:14–15

Slightom JL, Jouanin L, Leach F, Drong RF, Tepfer D (1985) Isolation and identification of TL-DNA/Plant junctions in *Convolvulus arvensis* transformed by *Agrobacterium rhizogenes* strain A4. EMBO J 4:3069–3077

Slightom JL, Durand-Tardif M, Jouanin L, Tepfer D (1986) Nucleotide sequence analysis of T_L-DNA of *Agrobacterium rhizogenes* agropine type plasmid. Identification of open reading frames. J Biol Chem 261:108–121

Staba EJ (1969) Plant tissue culture as a technique for the phytochemist. Recent Adv Phytochem 2:75–106

Taylor BH, Amasino RM, White FF, Nester EW, Gordon MP (1985) T-DNA analysis of plants regenerated from hairy root tumors. Mol Gen Genet 201:554–557

Tepfer D (1984) Transformation of several species of higher plants by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. Cell 37:959–967

Tepfer M, Cassedelbart F (1987) *Agrobacterium rhizogenes* as a vector for transforming higher plants. Microbiol Sci 4:24–28

White FF, Taylor BH, Huffman GA, Gordon MP, Nester EW (1985) Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. J Bacteriol 164:33–44