Biomass Distribution in *Kalanchoe blossfeldiana* Transformed with *rol*-genes of *Agrobacterium rhizogenes*

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Abstract. *Kalanchoe blossfeldiana* transformed with *Agrobacterium rhizogenes* exhibited marked alterations in morphology and biomass distribution. Plants termed root-inducing (Ri) lines were regenerated from hairy roots produced by inoculating leaf explants with *Agrobacterium rhizogenes* wild-type strain ATCC15834. Six Ri lines were characterized in a greenhouse trial and all Ri lines had reduced dry weights of main shoot, lateral shoots, leaves, and flowers compared with control plants. The reduction in dry weights of these organs correlated with reduced plant height, shoot length, leaf area, and number of flowers per plant. Furthermore, an altered distribution of dry matter was evident in the Ri plants, where the greater part of dry matter was allocated into leaves and secondly into flowers, whereas the majority of dry matter in control plants was allocated into flowers and secondly into leaves. Furthermore, a higher percentage of dry matter was allocated into the main shoot of the Ri lines in comparison with that of control plants. Increased dry matter in leaves and in the main shoot in the Ri lines appeared to be at the expense of dry matter allocated into flowers. Moreover, an increased number of vegetative lateral shoots was recorded in the Ri lines, whereas the number of reproductive lateral shoots was decreased. Possible mechanisms behind the altered resource distribution are discussed.

Transforming crop plants with the root loci genes, termed *rol*-genes of *Agrobacterium rhizogenes*, have been used to create new genotypes with desired characteristics (Christensen et al., 2008; Handa, 1992; Hosokawa et al., 1997; Pellegrineschi and Davolio-Mariani, 1996). Plants transformed with these genes exhibit varying degrees of altered phenotype termed the Ri (root-inducing) phenotype. The typical Ri phenotype exhibits reduced plant height and apical dominance giving the plant a compact and bushy appearance (Handa, 1992; Hosokawa et al., 1997; Pellegrineschi and Davolio-Mariani, 1996). Furthermore, the *rol* genes

have been shown to improve postharvest performance (Christensen and Müller, 2009a). These characteristics are very valuable in ornamental plants. The aim of the present study was to further characterize plants obtained in an earlier study (Christensen et al., 2008). The focus of the investigation was to elucidate alteration in biomass distribution between vegetative and reproductive shoots, because changes in resource allocation in flowering plants potentially influence ornamental value.

The source of the *rol* genes is the soil-borne bacterium *A. rhizogenes*, and this bacterium is the causative agent of the hairy root disease characterized by the formation of a large number of roots at the site of infection. During disease inception, a piece of DNA, the T-DNA, which is situated at the large Ri plasmid, is transferred from the bacteria to the plant cell nucleus and integrated into the plant nuclear genome (Chilton et al., 1982). Expression of T-DNA genes, especially *rol* genes, is responsible for the disease symptoms (White et al., 1985). Plants containing

Ri T-DNA can be generated from the hairy roots and these plants exhibit the typical Ri phenotype (Christensen et al., 2008; Handa, 1992; Hosokawa et al., 1997; Pellegrineschi and Davolio-Mariani, 1996). The *rol* genes are the main determinants of the Ri phenotype (Slightom et al., 1986; White et al., 1985).

The wild-type *A. rhizogenes* strain ATCC15834 has been used in molecular breeding studies to produce Ri plants (Christensen et al., 2008; Jaziri et al., 1994; Pellegrineschi et al., 1994) and this strain contains the agropine-type plasmid pRi15834. This plasmid contains two T-DNA regions, termed T_R-DNA and T_L-DNA (Jouanin, 1984; Meyer et al., 2000), which are separated from each other by ≈24 Kb of nontransferred DNA and transferred independently into the plant (Durand-Tardif et al., 1985). The T_L-DNA has a length of ≈20 Kb and it contains at least 18 open reading frames (ORF); and ORF 10, 11, 12, and 15 coincided with *rolA*, *rolB*, *rolC*, and *rolD*, respectively (Slightom et al., 1986; White et al., 1985). The *rol* genes can be transferred to plants either as single genes by recombinant DNA technology or together with the whole T-DNA by natural transformation using wild-type *A. rhizogenes* strains (Schmullig et al., 1988). The advantage of using wild-type strains is that transformed plants can be produced without the use of recombinant DNA technology and the natural transformation processes do not fall directly under the definition of genetically modified organism by the European Union (European Union, 2001). Furthermore, by using wild-type strains, no marker genes are needed for selection, because transformed plants can be selected based on the hairy root morphology (Christensen et al., 2008; Giovannini et al., 1997).

Expression of *rolA* in transgenic plants results in an aberrant phenotype characterized by dwarf or semidwarf plants with reduced internodes, whereas the *rolB* and the *rolC* gene seem to be important in hairy root formation. Plants transformed with *rolB* alone exhibit often an altered morphology such as reduced apical dominance and wrinkled leaves. *rolC* has a cytokinin-like effect in plants, and plants transformed with *rolC* alone show reduced height and apical dominance, increased number of lateral shoots, earlier flowering, reduced flower size, and reduced pollen production. Little is known about the function of *rolD*, although some reports on early flowering are available (Christensen and Müller, 2009b). Despite the fact that many reports on Ri plants exist, the function of the *rol* genes is far from elucidated and it has been implicated that *rol* genes are altering hormone homeostasis of Ri plants (Prinsen et al., 1994). Because Ri plants have changed morphology, physiology, and hormone homeostasis, it can be hypothesized that *rol* genes have an impact on biomass distribution. Therefore, the objective of this study was to analyze the influence of *rol* genes on resource distribution in Ri lines of *Kalanchoe blossfeldiana*

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transformed with *A. rhizogenes* strain ATCC15834.

Materials and Methods

Plant material. *Kalanchoe blossfeldiana* 'Molly' control plants and Ri lines 306, 312, 317, 319, 324, and 331 with varying degrees of Ri phenotypic expression developed from transformation with *rol* genes of wild-type *A. rhizogenes* strain ATCC15834 (Christensen

et al., 2008) were used. The plants were propagated by cuttings, which were rooted in peat (Pindstrup II; Pindstrup Mosebrug A/S, Ryomgaard, Denmark) under clear plastic in a propagation room at 20 °C. Light was provided by SON-T high-pressure sodium lamps (Philips, Amsterdam, The Netherlands) for a 16-h photoperiod and with an intensity of 145 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (photosynthetically active radiation) at the plant surface. After 14 d, 32 rooted cuttings of each Ri line and control plants were

transplanted into plastic pots (11 cm) with peat (Pindstrup II) and transferred to the greenhouse. Four weeks after transferring to the greenhouse, flower induction was started by short-day treatment (8 h light) at 22/18 °C day/night. The plants were irrigated twice a week with standard fertilizer (Brun Komplet; Garta A/S, Copenhagen, Denmark) with an electrical conductivity of 1.2 $\text{mS}\cdot\text{cm}^{-1}$.

The experiment was evaluated 100 d after the start of the short-day treatment when all plants flowered. Data on number and length of vegetative and reproductive lateral shoots and number of flowers were recorded; leaf area was measured using a leaf area meter (LI-3100 Area Meter; LI-Cor Biosciences, Lincoln, NE). Flowers, main shoot, lateral shoots, and leaves were dried separately at 85 °C for 5 d for dry weight measurements and the leaf area ratio ($\text{cm}^2\cdot\text{g}^{-1}$ plant dry weight) (Poorter and Remkes, 1990) and specific leaf area (SLA) ($\text{cm}^2\cdot\text{g}^{-1}$ leaf dry weight) (Garnier et al., 2001) were calculated. The experiment was carried out as a block experiment consisting

Fig. 1. *K. blossfeldiana* 'Molly' control plants and root-inducing lines transformed with *A. rhizogenes* strain ATCC15834. Bar = 8 cm.

Fig. 2. Plant height and lateral shoot development in *K. blossfeldiana* 'Molly' control plants and root-inducing lines transformed with *A. rhizogenes* strain ATCC15834. (A) Mean length of vegetative lateral shoots (cm). (B) Mean length of reproductive lateral shoots (cm). (C) Mean number of total shoots. (D) Mean number of vegetative lateral shoots. (E) Mean number of reproductive lateral shoots. 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 $\pm$ SD ($n = 32$).

of eight blocks with one replicate per block and the experiment was repeated four times.

Statistical analysis. The data obtained were subjected to analysis of variance using the general linear models (Mardia et al., 1980) (PROC GLM) procedure in the Statistical Analysis System (SAS 9.1) for Windows (SAS Institute, Cary, NC). Multiple comparisons among means were performed using Duncan's multiple range test with the level of significance at $P = 0.05$ (Duncan, 1955).

Results

The mean length of vegetative shoots was significantly increased ($P < 0.001$) in the Ri lines except in Ri line 319 (Figs. 1 and 2A), whereas the mean shoot length of reproduc-

tive shoots was significantly reduced ($P < 0.001$) in Ri lines when compared with the values of control plants (Fig. 2B).

The total number of lateral shoots per plant in the Ri lines compared with control plants was either at the same level as in the case of Ri line 312, significantly increased ($P < 0.001$) as in Ri line 331 or reduced as in the other Ri lines (Fig. 2C). However, comparing the number of reproductive and vegetative shoots instead of the total number of shoots gave another picture of shoot development in the Ri lines. In comparison with control plants, the number of vegetative shoots per plant in the Ri lines was significantly increased ($P < 0.001$) except in Ri line 319 (Fig. 2D), whereas the number of reproductive shoots was significantly reduced ($P < 0.001$) except in Ri line 331 (Fig. 2E). The

modification in the number of vegetative and reproductive shoots in the Ri lines resulted in a significant change ($P < 0.001$) in the distribution of reproductive and vegetative shoots (Fig. 3A). All Ri lines produced a higher percentage of vegetative shoots compared with the control plants and the percentage of vegetative shoots was 0.5% in control plants and varied from 15.3% in Ri line 317 to 32.8% in Ri line 324.

The total leaf area per plant was significantly reduced ($P < 0.001$) in the Ri lines compared with control plants (Table 1). The leaf area ratio was significantly increased ($P < 0.001$) in the Ri lines (Table 1). The SLA was the same for most Ri lines compared with that of control plants except for Ri lines 319 and 331. In Ri line 319, the SLA was significantly ($P < 0.001$) lower, whereas the SLA was increased in Ri line 331.

The number of flowers per plant, the flower diameter, dry weight per flower, and the flower diameter and dry weight ratio were significantly increased ($P < 0.001$) in the Ri lines compared with that of control plants (Table 1). Also, the mean dry matter of flowers, lateral shoots, main shoot, and leaves was reduced significantly ($P < 0.001$) in the Ri lines compared with control plants (Table 2). The marked decrease in the amount of dry matter recorded for these organs resulted in a significant reduction ($P < 0.001$) of the total dry matter per plant of the Ri lines (Table 2). The distribution of dry matter of flowers, lateral shoots, main shoot, and leaves relative to the total plant dry weight changed significantly. A significant amount of dry matter ($P < 0.001$) was allocated into the leaves of the Ri lines and the dry matter allocation into leaves in control plants was 34.7%, but it increased to 43.9% in Ri line 306, which had the lowest percentage dry matter distributed into leaves of the Ri lines (Fig. 3B). Ri line 312 had the highest percentage dry matter (55.9%) allocated into leaves. In the Ri lines, the percentage of dry matter allocated into flowers was significantly reduced ($P < 0.001$), whereas the percentage of dry matter distributed into the main shoot was significantly increased ($P < 0.001$) compared with control plants. The percentage of dry matter allocated into flowers of the control plants was 44.3% and varied from 22.3% to 39.6% in the Ri lines. The percentage of dry matter allocated into the main shoot was 8.8% in control plants and varied from 10.0% to 11.1% in

Fig. 3. Lateral shoot and dry matter distribution of *K. blossfeldiana* 'Molly' control plants and root-inducing lines transformed with *A. rhizogenes* strain ATCC15834. (A) Lateral shoot distribution. (B) Dry matter distribution. Bars marked with different letters (A, B, C, D, E) are significantly different at $P \leq 0.05$ by Duncan's multiple range test. Bars: Mean $\pm$ SD (n = 32).

Table 1. Mean leaf area, leaf area ratio, specific leaf area, number of flowers, dry weight per flower, flower diameter, and flower dry weight ratio of *K. blossfeldiana* 'Molly' control plants and root-inducing lines transformed with *A. rhizogenes* strain ATCC15834.

Line	Leaf area per plant (cm ²)	Leaf area ratio (cm ² ·g ⁻¹ plant dry wt)	Specific leaf area (cm ² ·g ⁻¹ leaf dry wt)	Number of flowers per plant	Flower diam (mm)	Dry wt per flower (mg)	Flower diam and flower dry wt ratio (mm·g ⁻¹ flower dry wt)
Control	1108 a ²	86 e	253 b	858 a	17.9 a	6.9 a	2.7 d
306	448 d	118 c	273 ab	399 b	14.1 c	4.0 c	3.7 b
312	515 c	150 a	269 ab	281 cd	12.4 e	2.8 d	4.3 a
317	337 e	112 c	256 b	323 c	13.9 c	3.8 c	3.7 b
319	275 e	97 d	201 c	235 d	13.2 d	4.1 c	3.4 bc
324	515 c	148 a	270 ab	277 cd	12.5 e	2.8 d	4.5 a
331	739 b	130 b	291 a	430 b	15.2 b	4.8 b	3.3 c

²The numbers in a column followed by different letters (a, b, c, d, e) are significantly different at $P \leq 0.05$ by Duncan's multiple range test. Mean $\pm$ SD (n = 32).

Table 2. Mean dry matter of flowers, leaves, lateral shoots and main shoot of *K. blossfeldiana* 'Molly' control plants and root-inducing lines transformed with *A. rhizogenes* strain ATCC15834.

Line	Mean dry matter (g)				
	Flowers	Lateral shoots	Main shoot	Leaves	Total
Control	5.70 ($\pm$ 0.45) a ^c	1.59 ($\pm$ 0.15) a	1.13 ($\pm$ 0.10) a	4.47 ($\pm$ 0.39) a	12.88 ($\pm$ 0.94) a
306	1.51 ($\pm$ 0.17) c	0.26 ($\pm$ 0.04) d	0.42 ($\pm$ 0.04) c	1.64 ($\pm$ 0.14) d	3.83 ($\pm$ 0.35) c
312	0.79 ($\pm$ 0.17) e	0.42 ($\pm$ 0.10) c	0.34 ($\pm$ 0.06) de	1.92 ($\pm$ 0.24) c	3.48 ($\pm$ 0.48) cd
317	1.22 ($\pm$ 0.16) d	0.18 ($\pm$ 0.02) d	0.33 ($\pm$ 0.04) e	1.33 ($\pm$ 0.15) e	3.06 ($\pm$ 0.36) de
319	0.96 ($\pm$ 0.13) e	0.09 ($\pm$ 0.03) e	0.40 ($\pm$ 0.05) cd	1.35 ($\pm$ 0.14) e	2.81 ($\pm$ 0.32) e
324	0.80 ($\pm$ 0.14) e	0.41 ($\pm$ 0.08) c	0.38 ($\pm$ 0.06) cde	1.93 ($\pm$ 0.19) c	3.52 ($\pm$ 0.32) cd
331	1.95 ($\pm$ 0.20) b	0.61 ($\pm$ 0.10) b	0.63 ($\pm$ 0.08) b	2.56 ($\pm$ 0.15) b	5.73 ($\pm$ 0.42) b

^cThe numbers in a column followed by different letters (a, b, c, d, e) are significantly different at $P \leq 0.05$ by Duncan's multiple range test. Mean $\pm$ sd (n = 32).

Ri lines. Compared with control plants, the percentage of dry matter distributed into lateral shoots was either at the same level as in Ri lines 312 and 324 or significantly reduced ($P < 0.001$) like in the other Ri lines. This percentage was 12.3% in control plants, but reduced to 3.2% in Ri line 319, which had the lowest percentage of dry matter distributed into lateral shoots.

Discussion

The present study shows that the transformation of *K. blossfeldiana* with *rol* genes had a marked impact on morphology and dry matter allocation within the plant. An altered distribution of dry matter was evident in the Ri plants, in which the greater part of dry matter was allocated into leaves and secondly into flowers, whereas the majority of dry matter in control plants was allocated into flowers and secondly into leaves. It is interesting that the greater part of dry matter was allocated into leaves in the Ri lines when the leaf area per plant and leaf dry weight per plant were reduced and no change was seen in the SLA for most Ri lines. However, the leaf area ratio was increased, which indicates that the increased allocation of dry matter into leaves is the result of changed morphology and not physiological changes.

The dry weight of flowers per plant was reduced as a result of fewer flowers per plant and lower dry weight per flower. Because the flower size of flowers was reduced in the Ri lines, it was not surprising to find a reduction in dry weight per flower in these lines. However, it is interesting that flower diameter and flower dry weight ratio was increased in the Ri plants. This indicates that less assimilates were allocated into the flowers and this might explain the changed flower physiology reported in *Kalanchoe* Ri lines (Christensen and Müller, 2009a). Christensen and Müller (2009) reported an increased flower longevity and ethylene tolerance in *Kalanchoe* Ri lines. This is supported by results in the orchids *Rhynchostylis retusa* and *Aerides multiflora*, in which a lower amount of sugars and hydrolytic enzyme activity were found in long-lived flowers compared with short-lived flowers (Attri et al., 2007).

The recorded changes in resource distribution in Ri lines of *K. blossfeldiana* in the present study might be explained by higher cytokinin/auxin ratio, which has been

reported in Ri plants of tobacco (Prinsen et al., 1994). Prinsen et al. (1994) recorded no change in the cytokinin level between the Ri plants and control plants of tobacco, but the Ri plants contained half the content of auxin in the shoot apex and, subsequently, the basipetal auxin gradient was weaker in Ri plants than in control plants. Moreover, transgenic plants with increased cytokinin/auxin ratio such as cytokinin-overproducing plants or auxin-deficient plants display a phenotype similar to the Ri phenotype, e.g., exhibiting dwarfism, loss of apical dominance, and wrinkled leaves (Eklöf et al., 1996; Romano et al., 1991). Transgenic plants overproducing cytokinin in a tissue-specific manner displayed altered mobilization of assimilates suggesting modified distribution patterns by localized overproduction and accumulation of endogenous cytokinin. Local increase in cytokinin created a strong sink for assimilates, which promotes sucrose transport toward that sink (Li et al., 1992).

The cytokinin-like growth alteration of Ri plants has been mainly assigned to the *rolC* gene because plants transformed with only *rolC* display reduced plant height and apical dominance with an increased number of lateral shoots (Schmulling et al., 1988; Zuker et al., 2001). In addition to the cytokinin-like action, it has been suggested that *rolC* may be influencing the source-sink relationship by regulating sugar metabolism and transport (Veena and Taylor, 2007). Further studies on gene expression of the individual incorporated genes in correlation to the observed plant phenotype appear to be useful and will be addressed in a future study.

However, a cytokinin-like effect would be expected to result in an increased lateral branching (Schmulling et al., 1988; Zuker et al., 2001), but lateral branching varied among the Ri lines and the number of lateral shoots per plant was either at the same level or reduced compared with control plants in the present study. This is in contrast to what has been reported in *Pelargonium fragrans*, *P. odoratissimus*, *P. quercifolium*, *P. graveolens* (Pellegrineschi and Davolio-Mariani, 1996), *Antirrhinum majus* (Handa, 1992), and *Gentiana scabra* (Hosokawa et al., 1997), in which Ri lines in these species had an increased number of lateral shoots. However, these reports did not consider the reproductive or vegetative nature of the lateral shoots. The Ri lines of *K. blossfeldiana* in the present study had an increased number of vegetative lateral shoots

causing a change in the shoot distribution to a higher percentage of vegetative shoots compared with control plants. This shows the importance of looking at the distribution of reproductive and vegetative lateral shoots to get the full picture of lateral shoot development in Ri lines. However, the number of reproductive lateral shoots in Ri line 331 was at the same level as that of control plants and Ri line 331 had the least reduction in the number of flowers among the Ri lines. Furthermore, chemical growth retardation using Cycocel Extra (BASF, Ludwigshafen, Germany) and Alar (Cillur A/S, Taastrup, Denmark) also reduced the number of flowers (Christensen and Müller, 2009a; Christensen et al., 2008). Despite the changes in dry weight and shoot distribution, Ri lines 306 and 331 are considered to have commercial potential, because they had improved postharvest quality and a desired reduction in plant height combined with the least Ri phenotypic expression impacting the morphology of other organs (Christensen et al., 2008). In close cooperation with a partner in horticultural industries, the transformants are presently used in a breeding program targeted toward compact plants.

Conclusion

Transformation of *K. blossfeldiana* with *rol* genes had a marked impact on morphology and resource distribution. A distinct reduction in dry weight of the main shoot, leaves, and flowers of the Ri lines was documented and the reduction of dry weight of these organs correlated with the reduction in plant height, leaf area, and number of flowers per plant. These alterations appeared to have been caused by either direct or indirect effects of *rol* genes, but more studies are needed to clarify the specific function and action of the *rol* genes in plants. Nevertheless, the present study will add valuable knowledge to our understanding of the *rol* genes and showed the potential application of these genes in breeding of *K. blossfeldiana* and other ornamentals.

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