

Abstract. Expression of the rolC gene under the constitutive CaMV 35S promoter led to several advantageous alterations in transgenic carnation (Dianthus caryophyllus L. ‘White Sim’). The rolC-transgenic carnation plants exhibited increased axillary budbreak and development when grown under standard commercial greenhouse conditions. Carnation with rolC generated up to 48% more stem cuttings per mother plant than nontransformed plants. Stem cuttings from rolC plants exhibited better rooting ability, with up to five times higher root dry weight than controls. The improved rooting of rolC-transgenic stem cuttings was also apparent when the cuttings were treated with IBA. During the flowering season, rolC-transgenic plants produced up to three times more flowering stems than control plants. It should be noted that the latter alterations, namely increased flowering and rooting, are of major importance to the carnation industry. Chemical name used: indole-3-butyric acid (IBA).

As one of the major contributors to the cut-flower market and a commercial leader in terms of number of stems sold worldwide, carnation (Dianthus caryophyllus) is an important target for the breeding of new cultivars with novel characteristics (Jensen and Baker, 1995). Different carnation types have been developed as a result of hybridization (Holly and Baker, 1991) and they can be divided into two main groups, standard (midi and mignon carnation) and spray (micro and dianthi carnation); a minor group is formed by the pot carnations.

Carnation is propagated vegetatively and is affected detrimentally by inbreeding (Galbally and Galbally, 1997). Hence, controlled breeding is rather complicated and limited because selection of a desired trait in the siblings is performed on the genetic background of the two parents, and because of the high genetic variability among offspring. Furthermore, crosses within and between related species are limited by a rather small available gene pool for new traits (Holly and Baker, 1991).

Genetic engineering has already become an important and useful tool in basic plant research, as well as in the applied introduction of novel traits into many commercially important species (Potrykus et al., 1998). Genetic engineering of cut flowers, however, is rather limited, as their transformation is considered routine in only a few laboratories (Zuker et al., 1998).

New traits in cut flowers include not only yield improvement and resistance to insects or disease, they also consist of new colors and novel plant morphology (Mol et al., 1995; Zuker et al., 1998). In fact, the latter are of great importance for the cut-flower market, where plant architecture and flower color are the main features determining consumer interest. Nevertheless, agronomic traits, such as yield, remain highly important for breeders and growers, as more vigorous and better performing plants can lower the time and cost required for growth and breeding.

Among the different genes affecting plant morphology, e.g., homeotic (Meyerowitz, 1997), Agrobacterium (Gaudin et al., 1994), phytochrome (Keller et al., 1989) and gibberellin (Coles et al., 1992) genes, the rol genes of Agrobacterium rhizogenes (Riker et al.) Conn have been the most widely and successfully employed (Fladung et al., 1997; Nilsson et al., 1996; Schmulling et al., 1993; Scorza et al., 1994; van Altvorst et al., 1992). Although its precise mode of action is still unknown (Faiss et al., 1996; Nilsson and Olsson, 1997), rolC has attracted the most attention through its expression in transgenic plants, either under its own promoter or under the control of a cauliflower mosaic virus (CaMV) 35S promoter, leading to a series of morphological alterations. These include reduced apical dominance, altered leaf morphology, reduced seed production, reduced internode length, male sterility, small flowers and early flowering, bushy and compact phenotype, and even stem fasciation (Kurioka et al., 1992; Nilsson et al., 1993, 1996; Oono et al., 1993; Schmulling et al., 1988).

Nevertheless, studies on the rolC gene and its effects on plant development have been performed mostly in model herbaceous plants, e.g., tobacco (Nicotiana tabacum L.) (Schmulling et al., 1988), Irish potato (Solanum tuberosum L.) (Schmulling et al., 1993), and tomato (Lycopersicon esculentum Mill) (van Altvorst et al., 1992), or tree fruits and timber species (Bell et al., 1999; Fladung et al., 1997; Nilsson et al., 1996). Studies on the use of the rolC gene in cut flowers are rather limited, the only report being in a woody ornamental, rose (Rosa hybrida L. ‘Madame G. Delbard’) (Souq et al., 1996). These transgenic rose plants, expressing the rolC gene under its native promoter, exhibited an array of phenotypic alterations, most of which were disadvantageous horticulturally. They included a dramatically reduced root

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system, wrinkled leaves, high sensitivity to diseases, and desiccation of stems.

The purpose of this study was to determine the potential of the rolC gene for improving horticultural traits in carnation. The availability of an efficient and reproducible procedure for the genetic transformation of carnation plants (Zuker et al., 1999) enabled production of transgenic carnation carrying the rolC gene under the CaMV 35S promoter. These transgenic carnations exhibit enhanced yield of stem cuttings and flowering stems, as well as improved rooting ability. To the best of our knowledge, this is the first time that genetically engineered, improved agronomic traits have been generated and analyzed quantitatively in carnation plants.

**Materials and Methods**

**Gene construct and plant genetic transformation.** A 1.46-kb XbaI fragment from plasmid pUC18-CaMV35S-rolC (Tzfira et al., 1997), carrying the *A. rhizogenes* rolC gene driven by a CaMV 35S RNA promoter, was inserted into the XbaI-digested pCGN1559 (McBride and Summerfelt, 1990) binary plasmid to create the binary plasmid pAMrolC. This plasmid contains the nptII selection gene (coding for neomycin phosphotransferase II (NPTII); Beck et al., 1982) also driven by a CaMV 35S RNA promoter, was inserted into the CaMV 35S promoter. These transgenic carnations enabled production of transgenic carnation carrying the rolC gene under the CaMV 35S promoter. These transgenic carnations were transferred to A. rhizogenes strain AGLO (Lazo et al., 1991) and used to transform ‘White Sim’ carnation as described previously (Zuker et al., 1999). The plasmid pCGN7001 is a control plasmid essentially identical to pCGN1559, but carrying the uidA gene coding for β-glucuronidase (GUS) driven by a mannopine synthetase (MAS) promoter and the nptII gene driven by a 35S promoter. Eighteen independent pAMrolC transformants were obtained, of which three randomly chosen rolC-transgenic lines (R-1, R-2, and R-3) were studied in more detail. The transgenic origin of the uidA-expressing control lines was confirmed previously. These plants were phenotypically and morphologically indistinguishable from nontransformed plants (Zuker et al., 1999).

**DNA and RNA blot analyses.** Total DNA and RNA were extracted from leaves of greenhouse-grown plants, as described previously (Zuker et al., 1999). DNA (10 mg) was digested with HindIII and electrophoresed through a 1% agarose gel using Tris-borate buffer (containing 1.3 M Tris, 0.7 M boric acid, and 24.5 mM EDTA, pH 8.4). HindIII is a unique restriction site within the T-DNA fragment, 5′ to rolC. Gels were stained with ethidium bromide and photographed under ultraviolet light. Total RNA (10 mg) was electrophoresed through a 1.2% formaldehyde gel (Vishnevetsky et al., 1996). DNA and RNA were transferred to nylon membranes (Hybond N; Amersham, Little Chalfont, United Kingdom) by capillary blotting as described by Maniatis et al. (1982). The 0.76-kb SalI fragment from plasmid pUC18-CaMV35S-rolC, representing the 3′ prime end of the rolC gene, served as a probe for that gene. Probe-labeling, prehybridization and hybridization of DNA and RNA blots were performed as described by Zuker et al. (1999) and by Vishnevetsky et al. (1996), respectively. The blots were visualized by exposure to Agfa Couix PR2 film (Agfa Co., Newark, Delaware) at −70°C for 5 to 20 h with an intensifying screen.

**Acclimatization of tissue-cultured plants and establishment of plants in the greenhouse.** About 3-cm tall, rooted plantlets were removed from the in vitro culture medium and their roots cleaned of agar. The plantlets were then transferred to plastic planters filled with water-saturated no. 4 perlite (Agrical, Kibbutz Habonim, Israel) and kept for 2 weeks in a greenhouse (natural daylight and daylength) on a heated rooting table (20°C) under intermittent mist (misting cycles of ≈3 min every 30 min, from 8 AM to 5 PM). The plantlets were then transferred to a no. 0.8 volcanic rock (Tof Marom Golan, Marom Golan, Israel) growing medium and grown for an additional 4 weeks in the same greenhouse. Hardened plants were then transferred to a regular greenhouse (natural light and temperature) and decapitated 4 weeks later to allow breaking and elongation of axillary shoots. These plants served as the source for standard stem cuttings (Zuker et al., 1995) used in all subsequent experiments.

**rolC-transgenic and control plants were crossed with b.l. 12361 (pollen donor) to evaluate the effect of the rolC gene on the flower’s female fertility. Open directed pollination and seed collection and germination were performed as described previously (Zuker et al., 1999).**

**Assessment of developmental traits in the greenhouse.** To evaluate the rooting performance of transgenic plants, stem cuttings were inserted, either directly (nontreated) or following treatment with a commercial rooting powder (Hormoril T-6, Asia Rizel, Ramat Gan, Israel) containing 0.6% (w/w) IBA, in either no. 4 perlite or a rooting medium of 5 parts no. 4 perlite : 1 part no. 2 perlite : 2 parts no. 0.8 volcanic rock : 2 parts peat (v/v). The stem cuttings (50 cuttings per line) were maintained for 3 weeks on heated (20°C) rooting tables. The cuttings which were inserted in perlite only were gently removed and washed of rooting medium. Root systems were dissected, dried at 65°C for 48 h, and weighed. Cuttings with at least one root were counted as rooted cuttings in evaluating the percentage of cuttings developing roots out of total number of cuttings.

Stems cuttings treated with Hormoril and rooted in the peat medium were also transferred to no. 0.8 volcanic rock medium for growth under regular greenhouse conditions (natural light and temperature), to establish mother plants for further clonal propagation. Four weeks after transfer to the greenhouse, plants were decapitated to allow for axillary bud development. Stem cuttings with at least three internodes, originating from the breaking and elongation of the axillary buds, were then harvested from the mother plants.

For yield analyses (Table 1), stem cuttings were harvested from 40 mother plants (for each line), about every 4 weeks. Following five harvest cycles, mean number of stem cuttings per harvest per mother plant was determined. Side shoot analyses (Table 1) were performed with a random sample of 35 stem cuttings (per line), originating from the first harvest cycle of 40 mother plants. Type IV and V stem cuttings were dissected and side shoots, developed from axillary buds, were weighed and their developmental stage ranked according to the number of open leaves (Fig. 1). Plant height at flowering (10 plants per line) was measured from the soil line to the top of the apical flower bud on the flowering stem. The number of petals per flower bud, their length and dry weight (DW) were recorded from a random sample of 20 flowers (per line) at anthesis, during the flowering season (Nov. 1998 to Mar. 1999). The vase life of 25 flowers (per line) with 50-cm-long stems was measured in distilled water, at 20°C, under a 12-h photoperiod provided by cool-white fluorescent lamps (60 µmol·m−2·s−1).

The data were subjected to analyses of variance using SPSS Base 10.0 (SPSS Inc., Chicago, Ill.). Means for rolC-transgenic and control lines were separated using Duncan’s multiple range test, (P ≤ 0.05).
Results

Confirming the Transgenic Nature of Selected Lines. Transgenic plants were generated following two regeneration and selection cycles under high kanamycin pressure as described previously (Zuker et al., 1999). All rolC- and control, uidA-transgenic plants exhibited high resistance to kanamycin, and the latter (Zuker et al., 1999) also exhibited strong GUS expression in all tissues tested. The rolC-transgenic plants (18 independent lines) were screened in vitro for phenotypic alterations, which included autonomous root growth in hormone-free media, axillary budbreak, increased growth rate, and altered internode length. Of 18 lines exhibiting altered morphology, three independent lines (R-1, R-2, and R-3) were hardened and transferred to the greenhouse for further study. Southern blot analysis of HindIII-digested DNA revealed integration of the rolC gene in all three selected lines (Fig. 2A). RNA blot analysis revealed expression of rolC, driven by the constitutive 35S promoter, in these three lines (Fig. 2B), thus confirming their transgenic nature.

Yield and Morphology of Stem Cuttings from rolC-Transgenic vs. Control Plants. About 50% of all stem cuttings from GUS-transgenic plants were type III (i.e., three internodes) (Fig. 1), and only about 6% were type V. In contrast, up to 97% of the rolC-transgenic cuttings were types IV and V (Fig. 3). The overall yield of cuttings per mother plant was up to 48% higher in rolC-transgenic plants than in control plants (Table 1).

Side shoots of rolC-transgenic stem cuttings exhibited up to 1.5-fold higher DWs than those originating from control plants (Table 1). Relative to control plants, the side shoots from rolC-transgenic plants were well developed (Table 1). In rolC-transgenic plants, the mean number of open leaf pairs on the side shoots at the 5th position (i.e., 5th node from apex) ranged from 1.4 to 2.0. In line R-1, all side shoots at the 5th position had two open pairs of leaves. Furthermore, side shoots at the 4th position in all rolC-transgenes averaged at least one pair of open leaves. In contrast, in control plants, none of the side shoots developing at the 4th or 5th positions had open leaves (Table 1).

Rooting of rolC-Transgenic Carnation Plants. When stem cuttings were not treated with IBA (Hormoril rooting powder), control (GUS-transgenic) cuttings yielded short and undeveloped adventitious roots, whereas rolC-transgenic cuttings developed a denser root system with greater DW than the control cuttings (Figs. 4 and 5). Moreover, the percentage of cuttings developing roots of the total number of cuttings was about two times higher in R-1 and R-3 plants than in controls. In fact, nontreated stem cuttings of line R-1 exhibited a root system and DW similar to those of control plants treated with rooting powder.

Table 1. Yield and properties of stem cuttings of rolC-transgenic (R-1, R-2, and R-3) and control (C) carnation plants.

| Line (no.) | Stem cutting yield (no.) | Dry wt (mg) for positions 4 and 5 | Open pairs of leaves (no.) |
|------------|--------------------------|----------------------------------|---------------------------|
|            |                          |                                  |                           |
|            | | Position 4 | Position 5 |
| R-1        | 4.6 a*                   | 47 a                             | 1.6 a | 2.0 a |
| R-2        | 4.2 ab                   | 44 a                             | 1.6 a | 1.8 ab |
| R-3        | 4.0 b                    | 36 b                             | 1.1 a | 1.4 b |
| C          | 3.1 c                    | 29 c                             | 0 b  | 0 c  |

*Stem cuttings were harvested from 40 mother plants a total of five times at 3- to 5-week intervals. Mean number of stem cuttings per harvest per mother plant is presented.

†Mean weight of side shoot at positions (nodes) 4 and 5, per stem cutting. A random sample of 35 stem cuttings was harvested from 40 mother plants.

‡Mean number of open pairs of leaves per side shoot at positions 4 or 5.

§Mean separation within columns by Duncan’s multiple range test (P ≤ 0.05).
The genic plants, they were grown to flowering in the greenhouse. A shorter period of only 2 weeks was required before rolC-transgenic carnation stem cuttings could be transferred for further development to standard greenhouse conditions. Of approximately 3 weeks was essential for rooting of control carnation stem cuttings, a shorter period of only 2 weeks was required before rolC-transgenic carnation stem cuttings could be transferred for further development to standard greenhouse conditions.

**Plant growth and flower development.** To further assess the growth habits of rolC-transgenic and control (GUS-transgenic) plants, they were grown to flowering in the greenhouse. The rolC-transgenic clones developed a greater number of shoots and a denser root system than control plants (Fig. 5). Furthermore, during the 5-month flowering season, the mean number of flowering stems per mother plant in R-1, R-2, and R-3 rolC-transgenic clones was significantly greater ($P \leq 0.05$) than in control plants: (7.2 ± 1.4, 8.1 ± 1.7, 9.4 ± 1.8 and 3.2 ± 1.0, respectively). The average number of flower buds on flowering stems was the same (4.5 ± 0.5 flowers/stem) in rolC-transgenic and control plants.

Height of the flowering stems of rolC-transgenic clones was not significantly different from that of control plants, being 85 ± 5, 87 ± 4 and 95 ± 6 vs. 75 ± 5 cm in R-1, R-2, R-3 and control plants, respectively. To time flowering was similar in rolC-transgenic clones and control plants, about 14 weeks after decapitation of mother plants. Vase life of flowers of control and rolC-transgenic clones was also the same (12 ± 2 d). An assessment of flower size during the flowering season revealed that R-1 and R-3 flowers are equal to controls, in all analyzed parameters, i.e., DW, petal number, and size. R-2 flowers (Fig. 5) were significantly smaller ($P \leq 0.05$): 410 ± 54 mg flower DW with 35 ± 3 petals (24 ± 3 mm in length) as compared to 696 ± 53 mg DW with 55 ± 2 petals (33 ± 3 mm in length) in control flowers. Crosses between R-1, R-2, R-3 and nontansformed male b.l. 12361 revealed that introduction of the rolC gene does not affect the flower’s female fertility (data not presented). The effect of the rolC gene on male fertility could not be tested, because ‘White Sim’, the cultivar used in this study to generate transgenic clones, is male-sterile.

**Discussion**

To date, applicability of the rolC gene, alone or in combination with rolA and rolB genes, to cut-flower improvement has been tested only in roses (Souq et al., 1996; van der Salm et al., 1997). In that case, the rolC gene expressed under its native promoter led mainly to negative—from a horticultural point of view—alterations. In contrast, the use of the rolABC gene, similar to rolB, caused a significant improvement in the rooting of rose stem cuttings. To assess whether the rolC gene would exert a beneficial effect on carnation, 35S-rolC-transgenic plants were generated.

Although a slight reduction in apical dominance was observed in rolC-transgenic carnation plants, it did not result in a compact form or low growth rate as has been described previously for different 35S-rolC-transgenic plants (e.g., Bell et al., 1999; Fladung et al., 1997; Nilsson et al., 1996) and which would be considered undesirable in carnation. In fact, reduction in plant apical dominance resulted mainly in enhanced lateral shoot development, leading to a 1.48 times higher yield of stems per mother plant. Furthermore, the proportion of well-developed roots out of total number of cuttings.

(Figs. 4 and 5). The enhanced rooting ability of rolC-transgenic plants vs. controls was also apparent following treatment of the cuttings with IBA. The rolC-transgenic plants developed up to twice the root DW of control cuttings. Moreover, while a period of approximately 3 weeks was essential for rooting of control carnation stem cuttings, a shorter period of only 2 weeks was required before rolC-transgenic carnation stem cuttings could be transferred for further development to standard greenhouse conditions.

![Fig. 3. Distribution of stem-cutting types in rolC-transgenic (R-1, R-2, and R-3) and control (C) plants. Stem cuttings were classified as III, IV or V according to their number of internodes, as depicted in Fig. 1.](image)

![Fig. 4. Adventitious root dry weight and rooting percentages in stem cuttings of rolC-transgenic lines (R-1, R-2, and R-3) and control (C) carnation plants after 30 d on rooting tables, as described in Materials and Methods. Values represent means of 15 stem cuttings per line; vertical lines represent SE. Numbers above the bars represent the rooting percentages, i.e., percentage of cuttings developing roots out of total number of cuttings.](image)
to-type flowers. In the remaining 15 rolC-transgenic lines, flower size was reduced in only one of them (data not presented).

The beneficial effect of the rolC gene in carnation was also reflected in the rooting capacity of stem cuttings. The root system of R-1 transgenic lines was actually quite similar to that of control plants treated with the commercial rooting powder Hormoril. The advantageous effect of rolC was apparent both with and without the addition of auxin, similar to the data reported on rolABC-transgenic rose (van der Salm et al., 1997). It should be noted that rooting of rolB-transgenic roses was negatively affected by auxin (van der Salm et al., 1997).

Based on the research reported to date, it is apparent that successful use of rol genes for plant molecular breeding depends on the plant system used. In carnation, the rolC gene improved rooting and increased the yield of stem cuttings and flowering stems. These are highly important traits to carnation propagators and growers. Improved rooting, for example, can reduce the labor required to produce rooted stem cuttings. The importance of this trait is even more pronounced for commercial cultivars that exhibit very low rooting percentages despite application of auxin (Holly and Baker, 1991). With respect to the yield characteristics, their improvement will obviously lead to higher profitability for the carnation horticultural industry.

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