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The Use of *Agrobacterium rhizogenes* and its *rol*-Genes for Quality Improvement in Ornamentals

B. Christensen[1)] and R. Müller[2)]
([1)]AgroTech A/S, Taastrup, Denmark and [2)]University of Copenhagen, Faculty of Life Sciences, Taastrup, Denmark)

Summary

Agrobacterium rhizogenes and its *rol*-(root loci)-genes offer the opportunity to introduce new genes for traits contributing to quality improvement in floriculture. Several ornamental plant species have successfully been transformed with *rol*-genes causing changed root morphology termed root inducing (Ri)-phenotype. The typical Ri-phenotype is a compact plant with a characteristic leaf morphology and improved rooting ability. The Ri-phenotype is due to the effect of the four *rol*-genes *rol*A, *rol*B, *rol*C and *rol*D. These *rol*-genes are contributing to different morphological effects in the plant. The *rol*-genes can be introduced all together into the plants by natural transformation using wildtype *A. rhizogenes*, which presents a transformation method without applying recombinant DNA technology. The *rol*-genes can also be transferred to a plant as single genes or in different combination by recombinant DNA technology. This makes transformation with *A. rhizogenes* and its *rol*-genes a promising method in molecular breeding for creating new diversity in ornamental species. Therefore, the aim of this review paper is to review the uses of *A. rhizogenes* and its *rol*-genes for quality improvement in ornamentals.

Key words. ornamental plants – Ri-plasmid – transformation

Introduction

Breeding is important in floriculture industry to increase product range and to improve production and post-harvest performance. New cultivars are continuously being developed by breeders in response to consumers' demands of new and better products such as altered plant morphology, new flower colours, improved display life and other novel traits. Additionally, growers are looking for better production performance such as shorter production time and resistance to plant diseases and pests.

Traditionally, classic breeding has been used to develop new cultivars with desirable traits, but the development in genetic engineering is offering new possibilities for extending the available gene pool, since a gene for a desirable trait, which has not been found within a species' gene pool, can be transferred into a plant from another gene source. Genetic engineering offers not only the possibility to transfer genes between different plant species, but also genes from other kingdoms e.g. bacterial and viral genes.

The genes of the bacterium *Agrobacterium rhizogenes* have shown to have potential to be used in floriculture for plant improvement (Christey 2001). *A. rhizogenes* is a soilborne, plant pathogenic bacterium responsible for the development of hairy roots at the site of infection on a range of dicotyledonous plant species (Tepfer 1990). The development of hairy roots is due to a natural transformation process where infection of wound sites is followed by transfer, integration and expression of a piece of DNA, the T-DNA, which originates on the large Ri-(root-inducing) plasmid (Chilton et al. 1982). *A. rhizogenes* carries on its T-DNA four *rol*-(root loci) genes, which are main determinants for the development of hairy roots. The four *rol*-genes are termed *rol*A, *rol*B, *rol*C and *rol*D, respectively (White et al. 1985; Slightom et al. 1986) and the function of the *rol*-genes is not yet understood.

Plants regenerated from hairy roots exhibit a modified appearance termed Ri-(root inducing) phenotype. The typical Ri-phenotype is a highly branched, dwarf and compact plant with atypical leaf morphology and increased rooting ability and abundant root production. 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 wildtype *A. rhizogenes* strains in several ornamental plant species such as *Kalanchoe blossfeldiana* (Christensen et al. 2008), scented geranium (*Pelargonium fragrans*, *P. odoratissimus*, *P. quercifolia*, *P. graveoles*) (Pellegrineschi and Davolio-Mariani 1996; Saxena et al. 2007), *Antirrhinum majus* (Hoshino and Mii 1998), *Datura arborea* (Giovannini et al. 1997), gentian species (*Gentiana scabra*, *G. triflora x G. scabra*,) (Suginuma and Akihama 1995; Hosokawa et al. 1997) and lisianthus (*Eustoma grandiflorum*) (Handa 1992b). In these species, the Ri-plants displayed various degrees of the typical Ri-phenotype. Since the function of the *rol*-genes is not totally understood, the focus of this review will be on the practical application in floriculture rather than gene functions.

Agrobacterium rhizogenes transformed plants

Several ornamental and field crop species have been transformed with *A. rhizogenes* and Ri-plants have been produced through shoot regeneration as listed in Table 1. The leaf morphology of the Ri-plants is often changed e.g. wrinkled, thicker, and reduced in size with decreased petiole length (Table 1). In addition, leaves are often darker or paler green. Changes in flower physiology have been observed in Ri-plants, like earlier or delayed flowering or inhibition of flowering. In some species, increased number of flowers, reduced flower size, change in flower shape and reduced fertility and seed set have been recorded. Conversion of biennial to annual flowering has also been observed in a few species. Changes in root physiology and morphology are common in Ri-plants such as increased rooting ability and root mass production. The hairy roots lack geotropism and they are 100 to 1000 times more sensitive to exogenous auxin than normal roots (Shen et al. 1988, 1990). In tuber and tap root forming species, the ability to form these root structures is often lacking and fibrous roots are produced instead (Table 1).

The degree of Ri-phenotypic expression differs among plant species ranging from normal phenotype to strong Ri-phenotype (Table 1). Different strains of *A. rhizogenes* are causing different Ri-phenotype effects within a plant species due to the interaction between the bacterium genes and plant genes (Handa 1992b; Jaziri et al. 1994; Giovannini et al. 1996; Otani et al. 1996). Furthermore, the specific Ri-phenotype in a plant depends on the actual transformation event, since the degree of phenotypic expression can vary among Ri-lines within a species (Aoki et al. 1997; Han et al. 1997; Christensen et al. 2008). The different phenotypes obtained might be due to differences in the number of integrated copies of T-DNA, the insertion site of the T-DNA or even dependent on the length of T-DNA inserted (Tepfer 1984). Furthermore, the difference in Ri-phenotype might be due to the fact that T-DNA-gene products may not function equally in all host species (Porter 1991). However, the difference in the degree of Ri-phenotype expression is offering a possibility for selecting Ri-lines with desirable plant morphology. The Ri-phenotype is stable through vegetative propagation (Pellegrineschi et al. 1994), sexually transmitted to offspring in a Mendelian fashion and dominantly inherited (Tepfer 1984). However, normal looking lateral (revertant) shoots have sometimes been observed in Ri-phenotype plants, which is due to transcriptional inactivation of T-DNA genes and not re-arrangement of the T-DNA (Sinkar et al. 1988b).

Practical application of hairy roots

The ability of hairy roots of hormone-autonomous growth *in vitro* have made hairy roots a widely used tool for production of secondary metabolites, investigation of gene function and the study of root biology (Doran 1997). However, this review will focus on the application in floriculture.

Improvement of the ornamental quality

Some of the Ri-phenotypic characteristics listed in Table 1 are usually regarded undesirable, but at the same time these altered features can have potential for quality improvement of ornamental plants. For instance, in the horticultural industry alterations such as dwarfing, earlier flowering, wrinkled leaves, changed leaf and flower shape, increased branching may appeal to customers and producers (Christey 1997). *A. rhizogenes* transformation has been used for improving the quality of ornamental plants in a number of species. The following examples illustrate the possibilities of the use of Ri-plants in quality improvement of ornamental plants.

Scented *Pelargonium* species are appreciated for their pleasant odour, but they are unattractive due to long internodes causing an elongated and chaotic growth habit. The regenerated Ri-plants had a more compact growth due to reduced plant height with increased number of leaves and branches. In addition to the improved ornamental quality other associated benefits included improved rooting of cuttings and increased production of essential oils (Pellegrineschi and Davolio-Mariani 1996).

Ri-plants were produced in *Angelonia salicariifolia* (willowleaf angelonia), which is a perennial plant native to South America and cultivated as potted plant or for garden use. The Ri-plants exhibited a dwarf phenotype with shorter internodes and smaller leaves, but without leaf wrinkling. Roots grew faster and more vigorously from stem cuttings of the Ri-plants than from control plants. The number, shape and size of the flowers was not altered, but pollen fertility was reduced in the Ri-plants (Koike et al. 2003).

In *Nierembergia scoparia* (broom cupflower), Ri-plants were generated with the aim to produce dwarf plants for potted and cover plants. These Ri-plants had short internodes and small, narrow, curly leaves. Moreover, the plants were fully fertile and dwarfism was obtained in the progeny (Godo et al. 1997).

Several species in the genus *Gentiana*, which contains herbaceous perennial plants of ornamental importance, have been transformed with *A. rhizogenes*. The Ri-plants of *G. scabra* had elongated internodes compared to the rosette phenotype of control plants, reduced apical dominance causing vigorous emergence of lateral branches at the base of the stems. The leaves were wrinkled and the root growth increased and most interesting, the Ri-plants exhibited early flowering (Suginuma and Akihama 1995). The time to anthesis is a very important parameter in the commercial production of potted plants and the reduction of this time period is interesting since this reduces the production costs.

Ri-plants of the gentian interspecific hybrid, *G. triflora x G. scabra*, showed varying degrees of Ri-phenotypic expression such as dwarfing, reduced apical dominance, highly branched stems with smaller and elliptically shaped leaves (Hosokawa et al. 1997). Most interesting, the Ri-plants had increased flowering (Hosokawa et al. 1997) and reduced plant height, extensive branching and all these characteristics are very useful horticultural traits in ornamental plants. In another gentian hybrid (*G. cruciata, G. purpurea*), Ri-plants had short internodes with smaller and rolled leaves rather than the typical wrinkled ones in Ri-plants of other plant species (Momcilovic et al. 1997).

In another *Gentianaceae*, *Eustoma grandiflorum* (lisianthus, prairie gentian), which is a popular cut flower; attempts have been made to create a dwarf phenotype for the use as potted plant by *A. rhizogenes* transformation.

Table 1. Ri-phenotype in different plant species transformed with wildtype pRi-plasmids from *A. rhizogenes*.

Plant species	Strains	Regeneration	Phenotype	Reference
Angelonia salicariifolia (Willoweleaf angelonia)	A13 (MAFF02-10266), D6	BAP	Reduced plant height and internode length, increased number of nodes, smaller leaves, reduced pollen fertility, no alteration in flower number, shape or size, increased rooting ability	(Koike et al. 2003)
Anagallis arvensis (Pimpernel)	ATCC31798, A4	Spontaneous	Normal phenotype except inhibited flowering	(Mugnier 1988)
Antirrhinum majus (Snapdragon)	A4	Spontaneous	Reduced apical dominance and internode length, increased branching, reduced leaf size, changed leaf shape, increased number of flowers, some lines with reduced fertility, viable seeds	(Handa 1992a)
	ATCC31798, A4	Spontaneous	Reduced internode length, dark green and wrinkled leaves	(Mugnier 1988)
Astragalus sinicus (Chinese milk vetch)	DC-AR2	Spontaneous	Compact, bushy plants with reduced plant height, leaf size and internode length, prolific, irregularly branched and masses of fine plagiotropic roots with increased root mass	(Cho et al. 1998)
Atropa belladonna (Deadly nightshade)	ATCC15834	BAP	Thick, dark-green wrinkled leaves, reduced internode length	(Jaziri et al. 1994)
	MAFF03-01724	Spontaneous	Lanceolated leaves, reduced leaf size, green-pale coloured leaves	(Jaziri et al. 1994)
	ATCC15834	BAP	Some lines with reduced internode length, wrinkled leaves, thick roots. Some lines looked phenotypically normal	(Aoki et al. 1997)
Brassica oleracea var. *acephala* (Ornamental kale)	A4T	BAP and NAA	Various degrees of increased leaf edge serration, leaf wrinkling, plagiotropic roots, good seed set	(Christey and Sinclair 1992)
Catharanthus roseus (Madagascar periwinkle)	15834	Spontaneous	Reduced apical dominance with highly branched stem, reduced internode length and leaf size, wrinkled leaves in some lines, abundant root system	(Brillanceau et al. 1989)
Cichorium intybus (Belgian endive)	8196	Spontaneous	Wrinkled leaves, conversion from biennial to annual flowering, most lines were infertile	(Sun et al. 1991b)
	A4RSII	Spontaneous	No leaf wrinkling, bolting without flowering, fertile	(Sun et al. 1991b)
Convolvulus arvensis (Morning glory)	A4	Spontaneous	Reduced apical dominance, increased leaf width-to-length ratio, wrinkled leaves, abundant flowering	(Tepfer 1984)
Datura arborea (Angel's trumpet)	NCPPB1855	Spontaneous	Same or reduced plant height, reduced internode length, increased number of internodes and number of leaves, reduce petiole length, dark-green wrinkled leaves, increased rooting ability, inhibition of flowering	(Giovannini et al. 1997)
Datura sanguinea (Red angel's trumpet)	NCPPB1855	Spontaneous	Compact plant habit, dark-green leaves with reduced length-to-width ratio, reduced flower size, unchanged flower shape	(Giovannini et al. 1997)
Eustoma grandiflorum (Prairie gentian, lisianthus)	MAFF02-10266 (A13), MAFF0301724	Spontaneous	Reduced plant height and internode length, wrinkled leaves, cup-shaped flowers instead of flat-formed, reduced pollen fertility, some lines with viable seeds, some lines with abundant root system	(Handa 1992b; Handa et al. 1995)
	NCPPB1855	Spontaneous	Slightly reduced plant height, reduced internode length, increased number of internodes, bell-shaped flowers instead of cup-like flowers, increased rooting ability, more branched roots with reduced geotropism	(Giovannini et al. 1996)
Gentiana cruciata (Gentian)	ATCC15834	Spontaneous	Short internodes, rolled leaves	(Momcilovic et al. 1997)
Gentiana scabra (Gentian)	MAFF0301724	BAP and NAA	Dwarfness, reduced apical dominance, wrinkled leaves, earlier flowering, increased root growth and plagiotropic roots	(Suginuma and Akihama 1995)
Gentiana (Gentian)	A4 M70GUS	Spontaneous	Reduced growth rate and internode length, wrinkled leaves, formation of flower buds without vernalisation, plagiotropic roots	(Vinterhalter et al. 1999)

Table 1. Continued

Plant species	Strains	Regeneration	Phenotype	Reference
Gentiana purpurea (Gentian)	ATCC15834	Regeneration through callus phase	Short internodes, rolled leaves	(Momcilovic et al. 1997)
Gentiana triflora x G. scabra (Gentian)	ATCC43057 (A4)	TDZ	Reduced plant height and apical dominance, increased number of shoots, with or without wrinkled leaves, increased number of flowers	(Hosokawa et al. 1997)
Ipomoea tricolor (Blue morning glory)	ATCC15834	Spontaneous	Reduced plant height, leaf size and petiole length, wrinkled leaves, delayed flowering, some flower buds did not open, decreased number of flowers, reduced flower size, no change in pollen fertility, abundant roots with extensive branching and plagiotropic growth	(Otani et al. 1996)
	A13 MAFF0301724	Spontaneous	No delay in flowering, slightly reduced flower size, no change in seed set, abundant roots with extensive branching and plagiotropic growth	(Otani et al. 1996)
Kalanchoe blossfeldiana	ATCC15834	CPPU	Reduced plant height, internode length, lateral shoot length, leaf size, leaf wrinkling. Number of lateral shoots either the same, increased or decreased, increased number of internodes, delayed or no change in onset of flowering, compact inflorescence, reduced number of flowers and flower size	(Christensen et al. 2008)
Linum usitatissimum (Flax)	NCPPB1855, A4, TR7	BAP and NAA	Decreased internode length, curly leaves, some lines with a more developed root system with plagiotropic growth	(Zhan et al. 1988)
Nicotiana tabacum 'Xanthi' (Tobacco)	A4	Spontaneous	Reduced apical dominance and internode length, increased leaf width-to-length ratio, wrinkled leaves, delayed flowering, changed flower morphology, reduced pollen fertility and seed set, plagiotropic roots with increased adventitious root formation	(Tepfer 1984)
Nierembergia scoparia (Cupflower)	A13	Spontaneous, but BAP enhanced regeneration	Increased number of nodes, decreased internode length, small and curling leaves, vigorous root growth, normal pollen fertility, viable seeds	(Godo et al. 1997)
Pelargonium graveoles (Rose geranium)	LBA9402 A4	Spontaneous	Reduced plant height, increased branching and number of leaves, highly dentated leaves without leaf wrinkling, improved essential oil quality	(Saxena et al. 2007)
Pelargonium fragans, *P. odoratissimus*, *P. quercifolia* (Scented, apple scented, oak leaved geranium)	A4, HRI, 8196, A4RSI, A4TSNT	Spontaneous	Reduced plant height and internode length, increased number of internodes, shoots and leaves, dark green leaves, fertile, increased rooting ability and abundant root development of highly branched roots	(Pellegrineschi and Davolio-Mariani 1996)
Pelargonium sp. (Lemon scented geranium)	Mixture of ARA4RSII, 15834, LBA9402, 1855	Spontaneous	Reduced plant height and internode length, increased number of node and shoots, reduced root length, increased root branching, resistant to leaf yellowing, changed leaf shape, dark-green and more dentated leaves without leaf wrinkling, faster rooting, increased fragrance, change in essential oil composition, inhibition of flowering	(Pellegrineschi et al. 1994)

Two studies have produced Ri-plants of *E. grandiflorum* using two different *A. rhizogenes* strains, and both studies produced Ri-plants having short internodes and changed flower shape. Interestingly, the flower shape was changed from cup-shaped to bell-formed corollas (Handa 1992b; Giovannini et al. 1996). Furthermore, in the study by Handa (1992b), Ri-plants had small and wrinkled leaves, reduced fertility and some Ri-lines produced an abundant root system. In the study by Giovannini et al. (1996), the Ri-plants exhibited varying degrees of altered morphology with only slight decrease in plant height, but with increased number of internodes and rooting ability with more branched roots. This example demonstrates that different strains of *A. rhizogenes* and different plant genotypes can cause different phenotypic effects within a plant species.

Catharanthus roseus (Madagascar periwinkle) is appreciated as a flowering plant for garden use and as potted plant. All Ri-plants of *C. roseus* displayed prolific rooting, short internodes, but only half of the obtained

Ri-plants exhibited wrinkled leaves, whereas the leaves of the other half of the Ri-plants appeared morphologically normal. Interestingly, the colour in the proximal region of the petals was white instead of red in flowers of Ri-plants (Choi et al. 2004).

Ri-plants have been produced in *Datura* species. The Ri-plants in *D. sanguinea* (red angel's trumpet) exhibited a compact plant habit due to decreased plant height and reduction in internode length. The Ri-plants had an increased number of leaves, which were dark-green and smaller than control with reduced length-to-width ratio. Moreover, the leaves were dentate or curly, depending on the lines. The Ri-plants also displayed increased rooting ability (Giovannini et al. 1997).

Kalanchoe blossfeldiana is a popular flowering potted indoor and garden plant around the globe and it represents one of the economically most important potted plants in Europe (Flora-Dania Marketing 2003). In *K. blossfeldiana*, one main problem in commercial production is the elongated growth of the inflorescence, which has to be controlled by the application of chemical growth retardants. Interestingly, Ri-lines of *K. blossfeldiana* had a compact growth habit with dense inflorescences due to reduced plant height, internode length and lateral shoot length (Fig. 1). Most of the Ri-lines had delayed time to the onset of flowering, but two lines were identified to have commercial value since these lines had no or only minor delay to the onset of flowering (Christensen et al. 2008). Very exciting was the discovery that the same two Ri-lines were shown to have a better flower longevity and ethylene tolerance than wildtype plants (Christensen and Müller 2009). This suggests that *rol*-genes not only could present an alternative to the use of chemical growth retardants, but concurrently could improve postharvest quality. These characteristics are very valuable to the floricultural industry.

In *Antirrhinum majus* (snapdragon), Ri-plants displayed dwarfism due to short internodes, decreased apical dominance with highly branched stems. The leaves were small and elliptic, fertility was reduced and even sterility was observed. However, it is interesting that the number of flowers was increased due to extensive branching (Handa 1992a; Hoshino and Mii 1998).

Reduced fertility has been reported in Ri-plants from a number of species, but that might not be a problem in ornamentals since many ornamental plants, especially potted plants, are vegetatively propagated. Some fertility, however, is necessary for further breeding. As illustrated above the specific phenotypic and physiological effects of *A. rhizogenes* transformation differ from plant genotype to plant genotype and it is difficult to foresee the exact Ri-phenotype.

Other applications

Transformation with *A. rhizogenes* has been reported to circumvent the need for vernalisation to produce flowering in biennial species (Tepfer 1984; Sun et al. 1991b). *A. rhizogenes* transformation could be applied to biennial ornamental plants in order to stimulate their annual flowering without vernalisation. Increased fragrance has been reported in lemon-scented geranium (*Pelargonium* sp.) (Pellegrineschi et al. 1994) and improvement of essential oil quality in rose geranium (*Pelargonium graveoles*) (Saxena et al. 2007).

Transformation vector

A. rhizogenes strains are not only used as genetic sources to create new genotypes and phenotypes. They can also

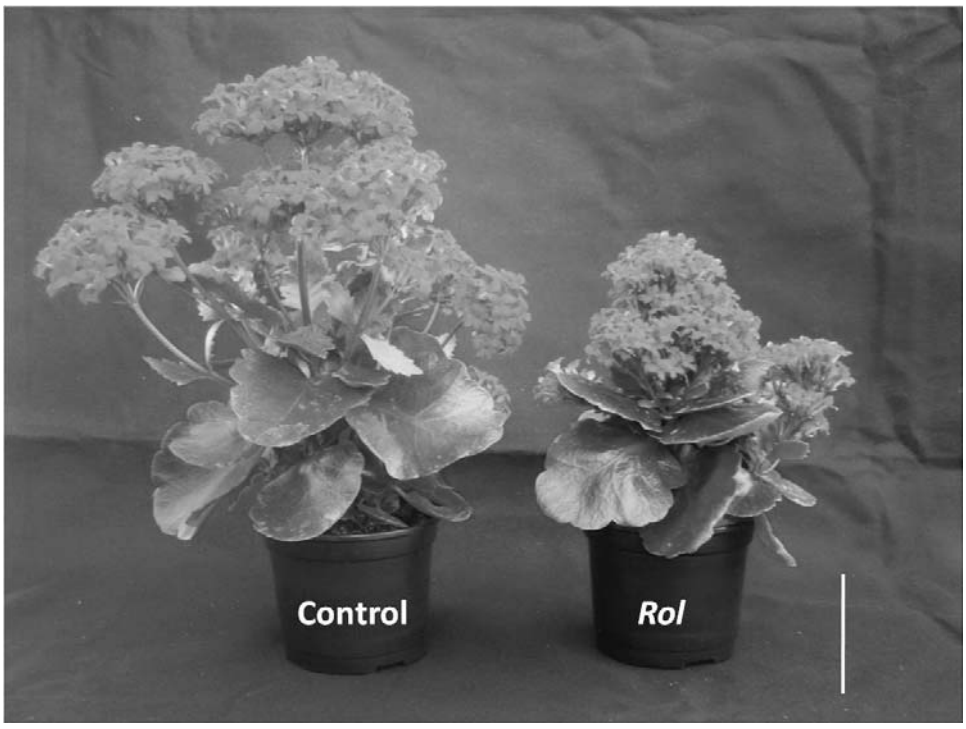


Fig. 1. *Kalanchoe blossfeldiana* 'Molly' control plants and plants transformed with *rol*-genes of *A. rhizogenes* strain ATCC15834. Bar: 10 cm.

serve as transformation vector. Since *A. rhizogenes* is able to co-transfer the T-DNA of a binary vector, it can be used to produce transgenic plants with desired foreign gene(s) by using the *rol*-genes as markers through regeneration from the hairy roots, avoiding the controversial use of antibiotic resistance maker genes (PUDDEPHAT et al. 2001). Antibiotic resistance markers are commonly used in the development of genetically modified (GM) plants, but releasing GM plants containing antibiotic resistance markers is banned from 2008 in the European Union (EUROPEAN UNION 2001). The advantage of *A. rhizogenes* mediated transformation is that it enables the development of transgenic plants by the use of hairy root morphology as the primary indicator of transformation. However, the regenerated transgenic plants often display the Ri-phenotype associated with the presence of Ri T-DNA, and this can be undesirable in some plant species. Since the insertion of Ri and vector T-DNA usually occurs on different chromosomes during co-transformation, their segregation in the progeny allows the recovery of transgenic plants with a normal phenotype, but containing the desired transgene(s) (OTANI et al. 1996; PUDDEPHAT et al. 2001).Transformation of ornamentals using *A. rhizogenes* has been shown in *Ipomoea trichocarpa* (OTANI et al. 1996) and *Rosa hybida* using strain ATCC15834 (FIROOZABADY et al. 1994)and in *Citrus aurantifolia* using ATCC43057 (A4) (PEREZ-MOLPHE-BALCH and OCHOA-ALEJO 1998). FIROOZABADY et al. (1994) showed that transformation using *A. rhizogenes* with a binary vector was as efficient as *A. tumefaciens*.

Transformation and regeneration

Different techniques can be used to obtain infection with *A. rhizogenes* (PORTER 1991), but a commonly used transformation procedure is to co-cultivate explants and *A. rhizogenes* suspension on hormone-free medium for 1–3 days for the infection to take place, and then transfer the explants to hormone-free medium containing antibiotics to eliminate the bacterium. Alternatively, wounds can be inoculated with bacterium suspension (MUGNIER 1988; CHRISTEY 1997).

Most plant tissue and organs, including embryos, hypocotyls, cotyledons, stems, leaves, petioles, shoots and roots have shown capacity to be infected by *A. rhizogenes* resulting in production of hairy roots (OTANI et al. 1996; AZLAN et al. 2002; KANG et al. 2006). However, within a plant species, the different organs differ in susceptibility to *A. rhizogenes* infection, since the response varies depending upon the *A. rhizogenes* strain and its interaction with the plants genes (PELLEGRINESCHI and DAVOLIO-MARIANI 1996; CHRISTENSEN and MÜLLER 2009). Hairy roots developed at the site of infection (Fig. 2) and the morphology of these roots varies considerably among species with differences in thickness, degree of branching and amount of root hair production (MUGNIER 1988).

Production of hairy roots is the normal response after *A. rhizogenes* infection, but some plant species show other responses. For instance in Japanese persimmon (*Diospyros kaki*), calli developed, which regenerated into shoots (TAO et al. 1994). A common characteristic of hairy roots is their hormone-autonomous growth and the hairy roots grow vigorously on hormone free medium (Fig. 3) (MUGNIER 1988). In axenic culture, roots induced by *A. rhizogenes* differ morphologically and physiologically from normal roots as they grow faster, and have reduced apical dominance, which produces highly branched roots and the roots are plagiotropic as they tend to grow horizontally instead of downwards (TEPFER 1984; CHRISTENSEN et al. 2008). The hairy roots are more sensitive to auxin than control roots (SPANÓ et al. 1988). In some species non-inoculated explants produce roots, but these non-transformed roots show poor growth and rare branching compared to the much more vigorous growth of the hairy roots (GIOVANNINI et al. 1997; KOIKE et al. 2003; CHRISTENSEN et al. 2008). In this way, hairy roots can be distinguished from non-transformed roots and selected based on their hairy root phenotype as the primary indicator of transformation without using antibiotic or herbicide resistance genes for selection (CHRISTENSEN et al. 2008).

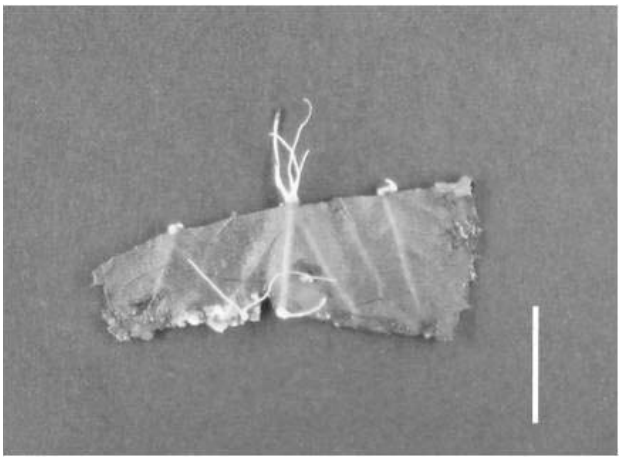


Fig. 2. Development of hairy roots on leaf explants of *Hibiscus rosa-sinensis* 'Cassiopeia Wind Yellow' six weeks after inoculation with *A. rhizogenes* ATCC43057. Bar: 1 cm.

Spontaneous regeneration of shoots from hairy roots grown on hormone-free media has been reported in a number of species whereas in other species cytokinins were needed to promote shoot regeneration. In some species, direct shoot generation from hairy roots has not been possible and plants had to be generated through somatic embryogenesis via a callus phase (Table 1). Species such as *Gentiana lutea*, *G.* acaulis and *Hibiscus rosa-sinensis* have been recalcitrant since no regeneration was achieved from the hairy roots (MOMCILOVIC et al. 1997; CHRISTENSEN et al. 2009). The method for plant regeneration not only depends on plant species, but also on the *A. rhizogenes* strains used (Table 1).

Strains

A. rhizogenes strains can be classified into sub-groups based on the type of opines, the strains are causing the host plant to produce after transformation. The most common *A. rhizogenes* strains identified to date are the agropine-type strains, and hairy roots transformed with these strains produce agropine, mannopine, mannopinic acid and agropinic acid. Mannopine-type strains cause hairy roots to produce only mannopine, mannopinic acid and agropinic acid (PETIT et al. 1983). Cucumopine-type strains cause hairy roots to produce cucumopine (DAV-

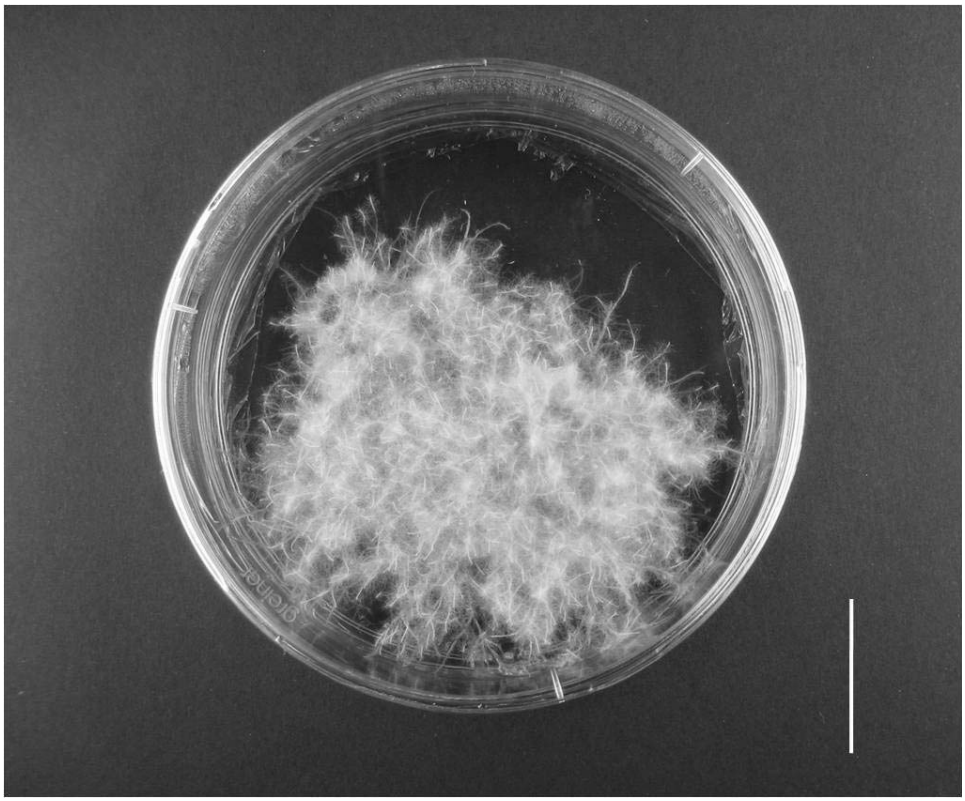


Fig. 3. A six week old root culture showing hormone autonomous growth of hairy roots of *Kalanchoe blossfeldiana* 'Molly' transformed with *rol*-genes of *A. rhizogenes* strain ATCC15834. Bar: 2.5 cm.

IOUD et al. 1988) and mikimopine-type strains mikimopine (ISOGAI et al. 1988). Table 2 lists the commonly used *A. rhizogenes* strains and the plasmid they carry.

Strain LBA9402 is a rifampicin resistant derivate of NCPPB1855 (OOMS et al. 1985). Strain R1000 and A4T have a chromosomal background of *A. tumefaciens* strain C58 into which pRiA4 has been conjugated (MOORE et al. 1979; WHITE et al. 1985; HAN et al. 1997) and R1600 and R1601 are similar, but carry additional cosmids. The strains A4RS and A4RSII are derivates of A4, which are rifampicin and spectinomycin resistant (JOUANIN et al. 1986; SUN et al. 1991b). Agropine-type strains of *A. rhizogenes* are the most widely used strains for transformations, although mikimopine-type strains are popular among Japanese researchers (Table 1).

T-DNA structure

The Ri plasmid of cucumopine and mannopine-type strains consist of only one T-DNA region (HANSEN et al. 1991; MORIGUCHI et al. 2000) whereas agropine-type strains carry a second T-DNA (JOUANIN 1984), which contains genes involved in auxin biosynthesis (*aux1* and *aux2*) (Camilleri et al. 1991) along with genes of unknown function like *rol*B_{TR} (Bouchez et al. 1990). Ri-plasmids containing two T-DNAs are termed split T-DNA and the two DNAs are designated left (T_L) and right (T_R) T-DNA. T_L- and T_R-DNA are ranging in size from ~15–20 kb each separated by approximately 16 kb of non-integrated DNA (WHITE et al. 1985). T_L- and T_R-DNA are known to be transferred and integrated independently

Table 2. *A. rhizogenes* strains classified according to the type of opines they are causing the host plant to produce after transformation.

Strain type	Plasmid	Strain	Source	Biotype	Reference
Agropine	pRiA4	A4, ATCC43057	Unknown	2	(PETIT et al. 1983)
	pRi15834	ATCC15834	Unknown	2	(COSTANTINO et al. 1981; PETIT et al. 1983)
	pRi1855	NCPPB1855	Rosa sp.	2	(COSTANTINO et al. 1981; FILETICI et al. 1987)
	pRiHRI	HRI	Unknown	Unknown	(PETIT et al. 1983)
Mannopine	pRi8196	NCIB8196	Unknown	2	(COSTANTINO et al. 1981; FILETICI et al. 1987)
Cucumopine	pRi2659	NCPPB2659	Cucumber	1	(COMBARD et al. 1987; FILETICI et al. 1987)
Mikimopine	pRi1724	MAFF301724	Melon	1	(ISOGAI et al. 1988, 1990)

into the host plant genome (JOUANIN et al. 1987), but only the transfer of T_L-DNA is essential for induction of hairy roots. However, in some cases the information carried on the T_L-DNA is not sufficient and the presence of the T_R-DNA extends the host range (WHITE et al. 1985; CARDARELLI et al. 1987; JOUANIN et al. 1987).

rol-genes

Unless otherwise specified, the description of individual genes in this review refers to the agropine-type plasmids. Studies of the T_L-DNA of the agropine-type have identified at least 18 open reading frames (ORF) (SLIGHTOM et al. 1986; LEMCKE and SCHMULLING 1998). ORF 10, 11, 12 and 15 coincided with *rol*A, *rol*B, *rol*C and *rol*D, respectively (WHITE et al. 1985; SLIGHTOM et al. 1986). The combination of *rol*A, *rol*B and *rol*C loci is sufficient for producing the hairy root phenotype (SCHMULLING et al. 1988; MARIOTTI et al. 1989), and the capacity of *rol*A, *rol*B and *rol*C genes to induce roots with faster growth rates than normal roots is equivalent to that of the whole T_L-DNA (SPANÓ et al. 1988). Since *rol*A, *rol*B and *rol*C are essential for the initiation of hairy roots, most research has focused on characterizing these genes, whereas *rol*D is the least studied among the *rol*-genes (SCHMULLING et al. 1988; SPANÓ et al. 1988).

rolA

The *rol*A gene seems to play an essential role in the establishment of Ri-phenotype since a number of morphological alterations like leaf wrinkling, short internodes and stunted growth (Table 1) also are seen in plants transformed with *rol*A only; these plants are termed *rol*A-plants (Table 3). Furthermore, revertant shoots, which means normal looking, lateral shoots, have been observed in tobacco plants with the Ri-phenotype transformed with wildtype *A. rhizogenes*. The transcription of *rol*A has been shown to be inactivated in the revertant shoots whereas transcripts of other *rol*-genes were detected in some cases (SINKAR et al. 1988b). Furthermore, *Anthirrhinum* plants transformed with an *A. rhizogenes* strain, in which the *rol*A gene has been knocked out, displayed a less severe Ri-phenotype than plants transformed with wildtype strain (NEWBURY and SENIOR 2001).

Stable expression of *rol*A in transgenic plants produces a highly aberrant phenotype, characterized by dwarf or semi-dwarf plants due to reduced internode length. The plants have small, wrinkled, dark-green leaves with changes in the length-to-width ratio. The onset of flowering might be delayed and the inflorescence condensed bearing abnormal flowers, which often are male sterile (Table 3). The aberrant phenotype is much stronger in *rol*A-plants, where *rol*A is under a constitutive promoter than in *rol*A-plants, where *rol*A is under the control of its native promoter (DEHIO et al. 1993). Due to the highly aberrant phenotype caused by *rol*A, this gene has not much practical application except for creating dwarf root stocks (ZHU et al. 2001a).

rolB

The *rol*B-gene (ORF11) seems to be the most important of the *rol*-genes in hairy root formation since *rolB* alone is

Table 3. Examples of phenotypical changes induced by *rol*A-gene in different plant species.

Plant species	Promoter	Phenotype	Reference
Daucus carota (Carrot)	Native	Increased number of shoots. Some plants converted from biennial to annual flowering	(LIMAMI et al. 1998)
Lycopersicon esculentum (Tomato)	Native	Small, dark-green, strongly wrinkled leaves, high ratio between dry weight and leaf area, long internodes, reduced flower bud length, hyperstyly, reduced pollen production. Male sterility	(VAN ALTVORST et al. 1992)
Malus x domestica M26 (Apple rootstock)	Native	Reduced stem growth, leaf area and internode length, some transformants showed reduced leaf area, shoot dry weight and plant dry weight	(HOLEFORS et al. 1998)
Nicotiana tabacum (Tobacco)	Native	Wrinkled leaves, condensed flower inflorescence, increased stigma size, larger flowers	(SCHMULLING et al. 1988)
	Native	Severely stunted, extremely reduced internode length, altered leaf morphology, severe leaf wrinkling, low length-to-width ratio	(SINKAR et al. 1988a)
	Native	Wrinkled leaves, reduced internode length, deficient root growth	(CARNEIRO and VILAINE 1993)
	Native	Male sterility, abnormal flower morphology, wrinkled leaves and shortened internodes	(SUN et al. 1991a)
	35S	Wrinkled leaves, reduced leaf size, reduced number of flowers, altered flower morphology, short internodes, inhibited or delayed flowering, compact inflorescence, shorter flowers, female and male sterility	(MARTIN-TANGUY et al. 1993, 1996)
	35S	Stunted growth, dark green, wrinkled leaves, changed leaf length to width ratio, delayed flowering, reduced number of flowers, condenses inflorescence, reduced length of styles	(DEHIO et al. 1993)
Oryza sativa (Rice)	35S	Dark, severely, wrinkled leaves, reduced number of branches	(LEE et al. 2001)

able to produce roots in several plants species although in some cases auxin was required (Cardarelli et al. 1987; Spena et al. 1987). The *rol*B hairy root lines show the typical hairy root traits such as high growth rate, high branching and absence of geotropism (Capone et al. 1989). The importance of *rol*B is further illustrated by mutation studies where *A. rhizogenes* strains containing a mutated *rol*B-gene were avirulent (White et al. 1985).

Plants transformed with only *rol*B, *rol*B plants, have changed morphology such as reduction of apical dominance and altered leaf morphology with or without leaf wrinkling. A general characteristic of *rol*B-plants is the improved rooting ability (Table 4). Due to the positive effect of *rol*B on rooting, *rol*B has been used as a rooting gene in a number of plant species to improve rooting ability (Table 4).

rolC

The *rol*C-gene appears to play an important role in hairy root formation since *rol*C alone is able to produce roots in some species like tobacco (*Niccotiana tabacum*) (Spena et al. 1987). *rol*C has a cytokinin-like action in plants, and when regulated by its native promoter, *rol*C plants show reduced plant height, reduced apical dominance, increased number of lateral shoots, earlier flowering, reduced flower size and reduced pollen production (Table 5). These effects are much more pronounced if *rol*C is driven by a constitutive promoter. In addition, plants, where *rol*C is under control of a constitutive promoter, are often male sterile (Schmulling et al. 1988; Nilsson et al. 1993).

Among the *rol*-genes, *rol*C is the most applied gene due to its cytokinin like-effect on plant cells and it has been used as a dwarfing gene in a number of plant species to reduce plant height and apical dominance and thereby increasing the number of lateral shoots (Table 5). Furthermore, the effect of *rol*C on rooting has also been exploited to increase rooting ability. *rol*C is also highly used in the establishment of hairy root cultures to produce secondary metabolites (Palazon et al. 1998).

The *rol*C promoter has been used as a phloem specific promoter in plant biotechnology research (Geigenberger et al. 1996; Saha et al. 2007), and the *rol*C promoter has been used in front of disease resistance genes to create systemic disease resistance in *Nicotiana benthamiana* (Pandolfini et al. 2003) and potato (Graham et al. 1997).

rolABC

As described earlier, each of the *rol*A-, *rol*B- and *rol*C-genes is able to produce roots in transformed plants and the genes have a synergistic effect on root formation, since the combination of *rol*ABC greatly increases the capacity to induce root formation (Spena et al. 1987).

Plants transformed with *rol*ABC show the typical Ri-phenotype, but the Ri-phenotypic expression in *rol*ABC is often weaker compared to plants transformed with *rol*A alone. For instance, tomato (*Lycopersicon esculentum*) *rol*A-plants had small dark-green, strongly wrinkled leaves and long internodes, whereas tomato plants transformed with *rol*ABC showed only a slight deviation in morphology compared to control plants (Van Altvorst et al. 1992).

The combination of *rol*A-, *rol*B- and *rol*C-genes also has a synergistic effect on stimulating the production of secondary metabolites in transformed tobacco hairy roots and regenerated plants (Palazon et al. 1998). The objectives of transformation of plants with the combination of *rol*A-, *rol*B- and *rol*C-genes is the same as for using wildtype *A. rhizogenes* strains. For example to introduce dwarfism, reduced apical dominance and increasing the rooting ability.

rolD

The *rol*D gene (ORF15) is found only in Ri T-DNA of agropine type strains. The *rol*D gene is the only *rol*-gene incapable of triggering root formation (Mauro et al. 1996), and for that reason *rol*D is the least studied gene among

Table 4. Examples of phenotypical changes induced by *rol*B-gene in different plant species.

Plant species	Promoter	Phenotype	Reference
Actinidia deliciosa (Kiwi)	Native	Normal phenotype, increased rooting ability	(Rugini et al. 1997)
Lycopersicon esculentum (Tomato)	Native	Wider and shorter leaves without leaf wrinkling, strong reduction of apical dominance, reduced number of flowers	(Van Altvorst et al. 1992)
Malus x domestica 'Florina' (Apple)	Native	Increased rooting ability and root fresh weight. No other change in phenotype	(Radchuk and Korkhovoy 2005)
Malus x domestica M.9/29 (Apple rootstock)	Native	Increased *in vitro* rooting, reduced number of nodes, reduced stem length, same relative growth rate, root length and morphology as control plants	(Zhu et al. 2001b)
Medicago sativa (Alfalfa, lucerne)	Native	Delayed flowering, increased stem number, increased root and shoot dry weight	(Frugis et al. 1995)
Nicotiana tabacum (Tobacco)	Native	Altered leaf morphology, increased stigma, increased flower size, abnormal flowers, adventitious roots on stems, slightly reduced pollen production	(Schmulling et al. 1988)
	Native	Wrinkled, dark-green leaves, excessive, partially non-geotropic roots	(Cardarelli et al. 1987)
	35S	Necrotic leaves, rounded leaf edge, heterostyly	(Schmulling et al. 1988)

Table 5. Examples of phenotypical changes induced by *rolC*-gene in different plant species.

Plant species	Promoter	Phenotype	Reference
Atropa belladonna (Deadly nightshade)	35S	Reduced apical dominance, pale and lanceolated leaves, smaller flowers, increased flowering	(Kurioka et al. 1992)
	Native	Normal phenotype	(Kurioka et al. 1992)
Cichorium intybus (Belgian Endive)	Native	Conversion from biennial to annual flowering	(Kamada et al. 1992)
Chrysanthemum morifolium (Chrysanthemum)	Native	Reduced plant height and internode length, increased number of shoots	(Kubo et al. 2006)
Dianthus caryophyllus (Carnation)	35S	Reduced apical dominance, increased number of lateral shoots, flowering and rooting ability	(Zuker et al. 2001)
Diospyros kaki (Japanese persimmon)	35S	Reduced plant height and internode length and leaf size, increased number of lateral shoots	(Koshita et al. 2002)
Nicotiana tabacum (Tobacco)	Native	Altered leaf morphology, increased branching, reduced flower size, pollen production and size of seed capsules	(Schmulling et al. 1988)
	Native	Reduced plant height, internode length and leaf size, earlier flowering, reduced flower size, pollen viability, size of seed capsules and number of seeds	(Scorza et al. 1994)
	Native	Reduced plant height, apical dominance, and flower size	(Oono et al. 1987)
	35S	Dwarf and bushy due to decreased internode length, increased number of shoots and leaves, small lanceolated leaves, reduced female fertility, male sterility	(Schmulling et al. 1988)
	35S	Reduced apical dominance and internode length, increased number of nodes, light green, lanceolate leaves, reduced flower size, male sterility	(Nilsson et al. 1993)
	35S	Reduced height and flower size, altered leaf shape, slightly increased petiole length, male sterile	(Martin-Tanguy et al. 1993)
	35S	Reduced plant height, lanceolated leaves, earlier flowering, reduced fertility	(Faiss et al. 1996)
Osteospermum ecklonis (African daisy)	35S	Pale green leaves, erect plant habit, earlier flowering, increased number of flowers	(Giovannini et al. 1999)
Pelargonium x *domestica* (Regal pelargonium)	35S	Reduced plant height, leaf area and flower diameter, earlier flowering	(Boase et al. 2004)

Table 6. Examples of phenotypical changes induced by *rol*D-gene in different plant species.

Plant species	Promoter	Phenotype	Reference
Daucus carota (Carrot)	Native	Severely dwarfed, wrinkled leaves, curved petioles	(Limami et al. 1998)
Lycopersicon esculentum (Tomato)	Native	Early flowering, increased number of inflorescences, higher fruit yield, perfect normal fruits and fertile seeds.	(Bettini et al. 2003)
Nicotiana tabacum (Tobacco)	Native	Early flowering, increased number of inflorescences	(Mauro et al. 1996)
	Native/35S	Normal phenotype	(Lemcke and Schmulling 1998)

the *rol*-genes and *rol*D-plants have only been produced in a few plant species (Table 6). Severe dwarfism was observed in *rol*D carrot (*Daucus carota*) plants (Limami et al. 1998), whereas tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*) *rol*D plants showed early flowering with an increased number of flowers without any aberrant morphology (Mauro et al. 1996; Bettini et al. 2003). However, in another study of tobacco *rol*D plants, there was no phenotypic effect of *rol*D expression, and the plants were phenotypically normal (Lemcke and Schmulling 1998).

GMO?

A very interesting aspect is whatever plants transformed with *rol*-genes using wildtype strains of *A. rhizogenes* are considered to be GM plants or not. In the European Union, the release of GMOs into the environment is regulated by directive "2001/18", which is defining a genetic modified organism as an organism in which the genetic material has been altered in a way that does not occur naturally by mating or natural recombination. Further-

more, techniques for genetic modification are defined as any technique involving heritable material prepared outside an organism and insertion of this heritable material via a vector (virus, plasmid or other system) or by direct introduction (injection) into a host organism wherein the heritable material do not naturally occur, but in which it is capable of continued propagation. Natural processes such as conjugation, transduction and transformation are not considered to result in genetic modification (EUROPEAN UNION 2001). Following these definitions, the Ri-plants, resulting from transformation using wildtype strains of *A. rhizogenes*, should not be considered to be GMO. However, this interpretation of the EU directive has to be confirmed by the authorities. In Japan, transformants derived from wildtype *A. rhizogenes* transformation are free from the legal controls of GMOs in Japan (MISHIBA et al. 2006).

Despite the many successful reports about the positive effects of transformation of ornamentals with *rol*-genes, to our knowledge no *rol*-transformants have been released to the market. One reason is the low acceptance of GMO's, also in ornamentals. Another important reason are the costs of registration for GM crops, which are much higher than the capital gained by the beneficial effects of the *rol*-genes in ornamentals. If Ri-plants derivated by transformation using wildtype *A. rhizogenes* strains are considered to be free from legal control in the EU, this situation could change.

Conclusion

Several ornamental species have been successfully transformed with *rol*-genes of *A. rhizogenes* and the *rol*-genes cause changes in many morphological traits with relevance to ornamental value. Most striking is the growth retarding effect documenting that the *rol*-genes are very effective as a mean to regulate growth. Beside compact growth, the *rol*-genes have shown to improve the quality of ornamental plants by increasing the rooting ability, causing early flowering, increasing flowering, improving flower longevity, enhancing ethylene tolerance and fragrance. Furthermore, the *rol*-genes might have the possibility to convert biennial plants to annual flowering without the need for vernalisation. *A. rhizogenes* can be used as a transformation vector to transfer other genes of interest to plants without the need of marker genes e.g. antibiotic resistance. The plants' morphological changes in response to transformation with *rol*-genes are dependent on plant genotype. Additionally, *rol*-genes of different *A. rhizogenes* strains can cause different morphological responses within a plant genotype. The specific phenotypic and physiological effects of *A. rhizogenes* transformation are difficult to foresee. However, this variability also makes transformation with *A. rhizogenes* and its *rol*-genes a promising method in molecular breeding for creating new diversity in ornamental species.

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Addresses of authors: Brian Christensen and Renate Müller (corresponding author), University of Copenhagen, Faculty of Life Sciences, Department of Agricultural Sciences, Crop Science, Højbakkegård Allé 21, DK-Taastrup, Denmark, e-mail: ren@life.ku.dk.