Theo P.M. van der Salm

Introduction of *ROL* genes in *Rosa hybrida* L. for improved rootstock performance

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr. C.M. Karssen,
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The cover shows a detail of a flower of *Rosa hybrida* L. 'Extase' (photo: VKC, Aalsmeer)

Stellingen

1. Bij de teelt van snijrozen op eigen wortel wordt niet optimaal gebruik gemaakt van de mogelijkheden om te komen tot een efficiënte produktie.

   Dit proefschrift

2. De aanwezigheid en expressie van *ROL* genen in rozenonderstammen stimuleert de vorming van grondscheuten aan de ongetransformeerde ent.

   Dit proefschrift

3. Bij de beoordeling van rozenonderstammen dient men noch de grootte van het wortelstelsel als onderdeel van een onderstamplant, noch de grootte van het wortelstelsel als onderdeel van een combinatieplant te gebruiken als exclusief kwaliteitscriterium.

   Dit proefschrift

4. Hoewel het onjuist is om de aanwezigheid van een indoxyl-ß-glucosidase activiteit in extracten van *ROLB* getransformeerde planten direct in verband te brengen met de hydrolyse van IAA-ß-glucosiden, moet de betekenis van deze enzymatische activiteit m.b.t. het auxinemetabolisme en de auxinegevoeligheid niet onderschat worden.

   Estruch JJ *et al.*, 1991, EMBO J 10: 3125-3128
   Xia Z-Q and Zenk MH, 1992, Biochem 31: 2695-2697
   Nilsson O *et al.*, 1993, Plant J: 681-689
   Filippini *et al.*, 1995, In: Terzi M *et al.* (Ed) Curr Iss in Plant Mol and Cell Biol. pp 473-479

5. Met het inconsequent beoordelen van het effect van het *ROLB* gen op de beworteling van bladexplantaten van tabak, illustreert de groep van Costantino onbedoeld de controversiële resultaten m.b.t. het *ROLB* gen.

   Spanò L *et al.*, 1988, Plant Physiol 87: 479-483
   Costantino P *et al.*, 1994, In: Smith CJ *et al.* (Ed) Biochemical mechanisms involved in plant regulation. pp 171-177

6. Een energiebesparing voortkomend uit een lagere teelttemperatuur draagt meer bij aan het milieu-imago van de glastuinbouw dan een vergelijkbare besparing gebaseerd op het verhogen van de produktie per m^2.

7. Als de luchthaven Schiphol met het verhogen van de parkeertarieven voor auto's tot doel heeft het milieu te sparen, dan valt meer effect te verwachten als zij de reizigers niet alleen aanmoedigt met de trein te kómen, maar ook met de trein te gáán.

8. Het correct opplakken van rookverbod-stickers lijkt soms moeilijker te zijn dan het opvolgen van de boodschap ervan.

9. Het is onterecht dat de aalscholver de zwartepiet krijgt toegespeeld voor de dalende palingvangsten.

10. Het schrijven van dit proefschrift ging niet altijd over rozen.

Stellingen behorende bij het proefschrift: "Introduction of *ROL* genes in *Rosa hybrida* L. for improved rootstock performance", door T.P.M. van der Salm in het openbaar te verdedigen op dinsdag 4 juni 1996 te Wageningen.

Contents

Chapter 1

Problems and perspectives of rose production

1 Roses

Rose culture

In contrast to the majority of cut flowers, which are harvested entirely at the time of flowering, rose plants (*Rosa hybrida* L.) consecutively produce flowering shoots, which are harvested regularly over a period of five years. Traditionally, flowering shoots are harvested by cutting just above their first or second full-grown leaf from below, leaving the basal stem part of the shoot. This remnant stem part bears several axillary buds, from which new flowering shoots can be formed. Recently, growers developed an alternative cultivation technique of rose, which is referred as 'pollard roses', since all axillary shoots are formed at the base of the plant (Fig. 1). In this system, flowering shoots are completely harvested, resulting in roses with desirable large stems. Non-marketable stems are bent down. Their assimilation capacity serves as a source for the development of new flowering shoots.

The initiation and growth of the flowering shoots requires high energy inputs for heating and supplementary lighting, especially in the winter. However, for environmental reasons the use of fossil reserves for this energy generation should be limited. The energy efficiency of rose production has been already remarkably improved by technical development and modification of cultivation methods. An alternative approach is breeding of roses, which produce more efficiently per unit energy input.

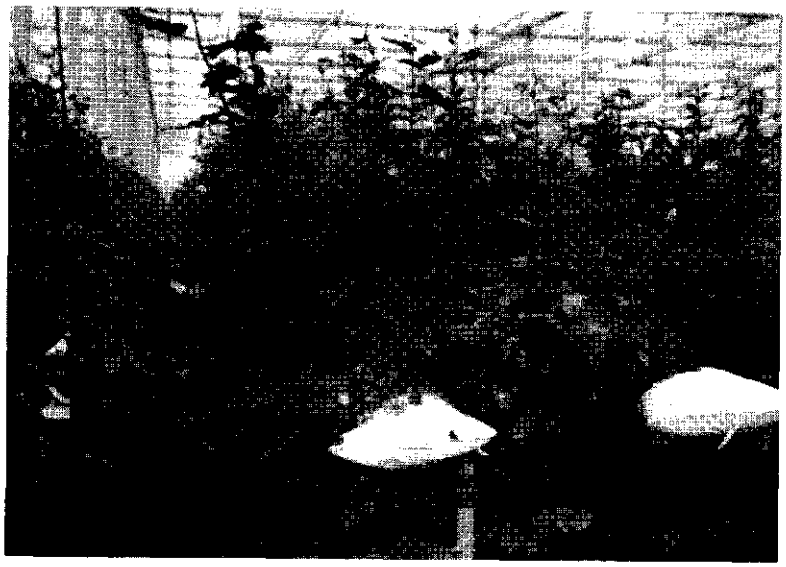

Figure 1. Modern rose culture in the greenhouse. Combination plants are grown on coconut fibres and cultivated as 'pollard roses'.

Economical importance

The cut rose is economically the most important cut flower in the world. Market sales at the leading Dutch auctions valued in 1995 Dfl. 817 million. In the Netherlands the production of roses is a highly developed agricultural industry, comprising an area of 900 ha divided over 950 nurseries. Approximately 50% of the culture area is on substrate, while 60% of the area is provided with supplementary lighting. Rose culture in the Netherlands requires high investments (Dfl. 300 per m^2) and yearly costs amount Dfl. 100-120 per m^2. Especially the categories labour (30%), energy (17%) and depreciation (21%) determine final cost-price. During the past five years the Dutch rose market encountered increasing imports of roses from African countries, such as Kenya, Zimbabwe and Zambia. Costs for labour and energy are relatively low in these countries, whereas costs for transport are substantially high (± 50%). This competition forces Dutch growers to reduce product cost-price, for example by saving energy during the winter, and to focus on high product quality and product diversification. Breeding of superior starting material is a powerful strategy to achieve this. Next to conventional breeding methods, genetic modification offers a new tool to extend the breeding possibilities of roses.

Conventional breeding of cut roses

Roses have been bred since hundreds of years; initially by selection after 'open pollination' or occurrence of sports and more recently by cross-breeding and mutation breeding. Breeding was primarily focused on flower colour, flower shape and stem quality (Thomas, 1994). Nowadays, final yield, vase life, disease resistance and fragrance also become more and more important traits for roses. In principal, it should be possible to breed a superb cut rose, demonstrating all the desired characteristics that are mentioned above. However, in practice it takes an enormous effort to combine all desired characteristics in one cultivar, conceivably resulting in suboptimal compromises between ornamental quality and yield, or between flower and rooting characteristics.

Optimal plant characteristics are more easily obtained by using combination plants, consisting of a rose cultivar grafted on a rootstock (Fig. 2). This approach gives opportunity to a breeding strategy of cut rose cultivars, aimed at optimization of desired properties of the aerial parts, disregarding the rooting characteristics. By analogy, breeding of rootstocks can be fully concentrated on desired root characteristics, such as high adventitious rooting and the ability to support vigorous growth of the scion.

Although the genus *Rosa* includes over 100 species, only eight species have contributed significantly to the genetic basis of modern garden and cut roses (Wylie, 1954). Exploration of the other species for breeding of cut roses was discouraged by crossing barriers, hybrid sterility and a desire to preserve classic combinations of floral characters (Roberts *et al.*,

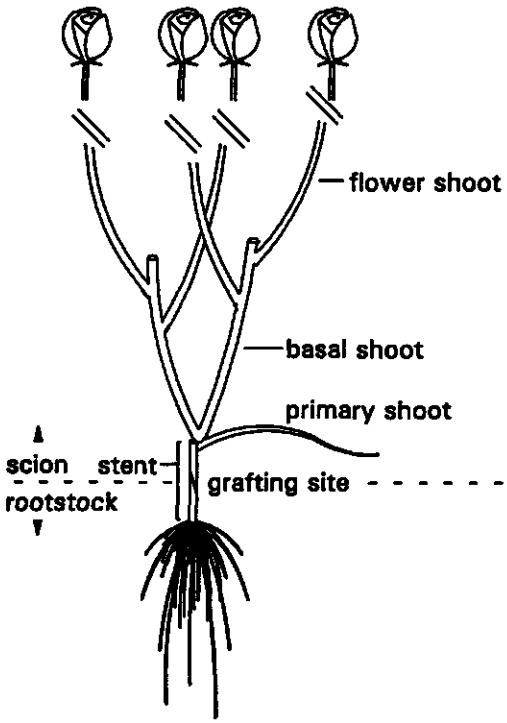

Figure 2. Schematic representation of a combination plant of rose, consisting of a cultivar grafted on a rootstock.

1990; 1995). Therefore, the genetic potential for the improvement of cut roses by cross-breeding appears to be limited. Although breeders succeed in the introduction of a variety of new cultivars each year, the inflow of completely new traits is doubtful and awaits further development of novel breeding technologies.

Conventional breeding of rose rootstocks

In the Netherlands, greenhouse culture of roses in soil is accomplished on 'Edelcanina' seedling rootstocks, while on natural and artificial substrates predominantly clonal rootstocks are used. Table 1 comprises a list of traditionally and presently used rose rootstocks. Wild species and specific selections of these already served as seedling rootstocks for centuries. The major disadvantage of such seedling rootstocks is their lack of uniformity. Breeding of seedling rootstocks was therefore primarily focused on improvement of uniformity and the development of the Edelcaninas ($2n=5x=35$) certainly contributed to this. This was due to an unconventional male gamete formation ($n=x=7$), which results in a limited share of the male genitor in the progeny (Kroon and Zeilinga, 1974). In addition, their thornless stems gave an advantage in grafting.

Complete uniformity was only accomplished after the introduction of clonal rootstocks, which are vegetatively propagated and therefore genetically uniform. Next to the objective to grow an uniform crop, the gradual establishment of natural and artificial substrates in the 1980s finally favoured these clonal rootstocks over seedling rootstocks. Some of the clonal rootstocks, such as *R. chinensis* 'Major' (also known as 'Indica Major') and 'Manettii', are more than 150 years old. The fact that they are still in use in the mediterranean area and the U.S.A. indicates that in contrast to the breeding of cut roses, breeding efforts for rose rootstocks have been relatively poor. This opens perspectives for the development of new clonal rootstocks with improved vigour. Breeders initiative in this field was encouraged by the breeders' rights. Breeding of rootstocks in the Netherlands is localized at the Centre for Plant Breeding and Reproduction Research (CPRO-DLO) and the Department of Horticulture of the Agricultural University Wageningen. This resulted in the development of *R. multiflora cathayensis* 'Multic' and the hybrids 'Marleen' and 'Moneyway' (De Vries and Dubois, 1994). In addition, rose companies selected promising rootstocks such as 'Kuiper', 'Spekine', 'Sturdu' and 'Sturcinq' from seeded populations of *R. canina* 'Inermis'. Furthermore, the South African rootstock 'Natal Briar', which genetic origin is undefined, obtained increasing

Table 1. Seedling and clonal rootstocks for roses and their origin (partly after Leemans and Van der Laar, 1977).

type	name	origin
seedling	*R. rubiginosa* L.	Europe (as a rootstock since 1675)
	R. canina L.	Europe and Western Asia (as a rootstock since 1824)
	R. multiflora Thunb.	Eastern Asia (as a rootstock in England since 1875)
	R. corymbifera 'Laxa'	Froebel, Zürich, Switzerland (1890)
	R. canina 'Inermis' (Edelcanina)	Gamon, Lyon, France (1905)
	R. canina 'Brögs Stachellose' (Edelcanina)	Brög, Reckenbach bei Lindau, Germany (1902)
	R. canina 'Pollmer' (Edelcanina)	Pollmer, Groszenhain, Germany (± 1928)
clonal	*R. chinensis* 'Major' (= 'Indica Major')	Eastern Asia (± 1740)
	R. chinensis 'Manettii'	Crivelli, Italy (± 1835)
	R. multiflora cathayensis 'Multic'	Agricultural University Wageningen (1989)
	R. hybrida 'Moneyway'	CPRO-DLO, Wageningen (1990)
	R. hybrida 'Marleen'	CPRO-DLO, Wageningen (1991)
	R. canina 'Kuiper'	Kuiper B.V., Veendam (1989)
	R. canina 'Inermis' sel. 'Spekine'	Spek Rozen B.V., Boskoop (1992)
	R. canina 'Inermis' sel. 'Sturdu'	STUR[1] (1992)
	R. canina 'Inermis' sel. 'Sturcinq'	STUR[1] (1992)
	'Natal Briar'	South Africa (1993)

[1]Stichting Ter verbetering van Uitgangsmateriaal kasRozen

popularity since 1993. At present, by estimation 50% of the cut roses in the Netherlands is grown on a rootstock, predominantly consisting of 'Natal Briar'.

Before starting a rose culture with combination plants, growers should evaluate an array of rootstock criteria, which can be entitled as 'the rootstock quality' e.g. vigour of the rootstock, its ability to yield flowers of high quality, its rooting ability, disease resistance, possible occurrence of dormancy in wintertime, possible negative scion-rootstock interactions or incompatibility with certain cultivars. The complexity of such an evaluation is obvious, also regarding the variety of rootstocks and rose cultivars. Therefore, rose breeders usually recommend certain rootstock-cultivar combinations on basis of greenhouse trials. Nevertheless, more fundamental research focused on the determination of parameters indicating high rootstock quality is required.

Genetic modification of roses

An alternative tool for the improvement of both cut roses and rose rootstocks, is genetic modification i.e. the application of molecular and cellular breeding techniques. The most obvious beneficial characteristic of this biotechnological approach over conventional breeding and selection is that the recipient plant only gains one or a limited number of genetic characteristics, while its original genetic framework remains unaffected. In addition, gene transfer is no longer determined by crossing potential, but principally unlimited. This makes it possible to utilize suitable genes from either related or unrelated plants, but also from animals, fungi, bacteria or viruses.

A further prerequisite for efficient use of genetic modification in rose breeding is the availability of good transformation procedures. For the genetic modification of plants first the transfer of genetic information to a single plant cell is required. Since many species of the *Rosaceae* are naturally infested by *Agrobacterium* (Porter, 1991), this bacterium is in principle an appropriate vector for the transfer of genetic information to rose. The next step consists of regeneration of whole plants from this cell either by somatic embryogenesis or by organogenesis. Especially for the regeneration process, rose is a recalcitrant species, like most of the other woody species. At the start of the research, described in this thesis, regeneration via organogenesis (Lloyd *et al.*, 1988) or embryogenesis (De Wit *et al.*, 1990; Roberts *et al.*, 1990) has been reported only a few times. At that time genetic modification of roses was not yet described. In course of this research the first positive results on gene transfer to roses were described by Firoozabady *et al.* (1994).

Modification of rose rootstock performance by genetic modification

For the improvement of the rose rootstock performance by genetic modification, genes are required, which affect plant growth and development. In nature, neoplastic growth on various

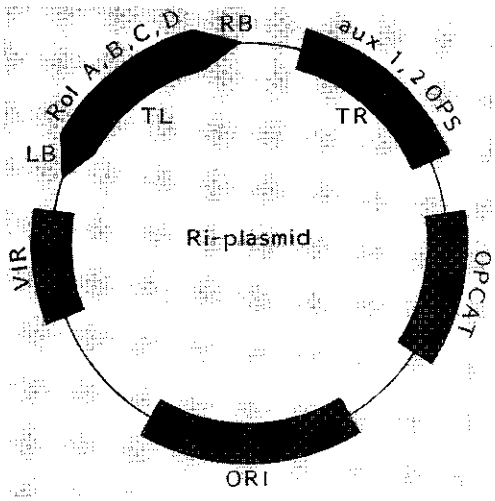

Figure 3. Ri-plasmid of *A. rhizogenes*. TL=left transfer DNA with 18 ORFs, encompassing the *ROLA, B, C, D* genes; LB=left border; RB=right border; TR=right transfer DNA encompassing the genes for auxin synthesis (*AUX1* and *2*) and the opine synthase gene (*OPS*); VIR=virulence region; ORI=origin of replication; *OPCAT*=opine catabolic genes.

plant species is incited upon infection with virulent strains of the soil bacterium *Agrobacterium tumefaciens* and subsequent transfer and integration of genetic information (T-DNA) into the plant genome (Gelvin *et al.*, 1990). Tumour formation is the result of the activity of various hormone genes, such as *IPT*, *TMS1* and *TMS2*, present on the T-DNA, leading to increased levels of cytokinin and auxin in the plant. Analogously, hairy roots are induced upon inoculation with *A. rhizogenes* comprising the *AUX* genes, which are homologous to *TMS1* and *TMS2*, and 18 other open reading frames (Fig. 3). Among these, the *ROLA, B, C* and *D* genes play an important role in the formation of the hairy roots. Especially the *ROLB* and *ROLC* genes are suitable genes for the modification of rooting characteristics, but also for the modification of plant growth and plant shape (Spena *et al.*, 1987; Cardarelli *et al.*, 1987; Schmülling *et al.*, 1988; Capone *et al.*, 1989). Although the biochemical role of their gene products is not well understood, both genes affect hormone metabolism and/or hormone sensitivity. The *ROLB* gene has an auxin like effect in plants (Maurel *et al.*, 1991; 1994; Filippini *et al.*, 1994), resulting in the formation of adventitious roots, whereas the *ROLC* gene has a cytokinin like effect in plants, resulting in decreased apical dominance and increased branching of roots. Moreover, the promotive effect of the *ROLB* gene on adventitious root formation was synergistically enhanced by the *ROLA* and *ROLC* genes. The limited transmissibility of pleiotropic effects from transformed parts to untransformed parts further contributes to the suitability of these genes for the application in rose rootstocks. In conclusion, the *ROLB* gene and especially a combination of *ROLA, B* and *C* genes might be suitable genes for changing the rose rootstock performance.

2 Aim and outline of this thesis

The aim of the research described in this thesis is to improve the performance of combination plants of rose by genetic modification of its rootstock, with special reference to their performance at low energy conditions. The strategy envisaged was based on the idea that the efficiency of a rootstock is determined by the lateral branching of the roots and more in general by the size of the root system. For instance, nutrient uptake and the production of cytokinins seem to occur at a higher degree in large than in small root systems. Alternatively, an altered hormonal status of *ROL* gene transformed roots may also result in an increased production of cytokinin. These elementary processes support the growth of the aerial parts and especially the release of basal shoots (Fig. 2). Since the number of basal shoots is correlated with the successive flower production (De Vries and Dubois, 1989), we expect that the production of a more efficient rootstock provides an opportunity for growers to produce more efficiently.

The first objective of this research was the introduction of the *ROL* genes from *A. rhizogenes* in the recalcitrant species rose. For this purpose the rose rootstock 'Moneyway' was used (Fig. 4). The second objective was to determine the effects of these genes on the rooting characteristics of rootstock plants and their influence on the growth performance of combination plants.

Since rose is a recalcitrant species with respect to genetic modification, special attention had to be paid to methodological aspects such as micropropagation, transformation and regeneration. For the propagation of sterile plant material, which is required for transformation, micropropagation of roses on conventional media was inadequate. Leaves of micropropagated shoots turned chlorotic from the third week on. Therefore first the medium composition was optimized for the in vitro culture of rootstock 'Moneyway' (Chapter 2). In Chapter 3 the effect of *ROL* genes on the formation of adventitious roots was described. A system was developed for the formation of kanamycin resistant roots on stem slices of micropropagated shoots of the rootstock 'Moneyway'. Control constructs harbouring the reporter gene *GUS* enabled us to confirm transformation at this stage. It was shown that the formation of kanamycin resistant roots was stimulated by *ROL* genes, especially by a combination of *ROLA*, *B* and *C*. Since a practical regeneration protocol for rose was not available, a regeneration method was developed, in which shoots were regenerated from excised roots via somatic embryogenesis (Chapter 4). This method showed to be a straightforward and reliable way for the regeneration of whole plants. In Chapter 5 this regeneration method was applied for the regeneration of transformed shoots from kanamycin resistant roots, especially from those with the *ROLB* gene or a combination of *ROLA*, *B* and *C* genes. As a result several transformed plants were obtained. This enabled us to study the

Figure 4. Cultivation of the rose rootstock 'Moneyway' in the field.

effects of the *ROL* genes on the rooting characteristics of shoots and organs of micropropagated material, but also that of cuttings of greenhouse grown plants. In Chapter 6, expression patterns of the *ROL* genes were correlated with the phenotypical characteristics of the transformed rose plants. Two transformants were selected for further study: one with the *ROLB* gene and one with a combination of *ROLA*, *B* and *C* genes. In order to determine the effect of *ROL* genes on the growth and development of a scion, combination plants were made, consisting of the root system of a transgenic rootstock and the aerial parts of the cultivar 'Madelon'. For evaluation of the effects of *ROL* genes on rootstock performance and energy efficiency, the combination plants were studied under climate room conditions at 15 and 20°C. Finally in Chapter 7, an overview is given of the possible applications of *ROL* genes for crop improvement, while the relevance of the results of the research described in this thesis for the progress in this field is discussed.

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Chapter 2

Importance of the iron chelate formula for micropropagation of *Rosa hybrida* L. 'Moneyway'

Theo P.M. van der Salm, Caroline J.G. van der Toorn, Charlotte H. Hänisch ten Cate, Lidwien A.M. Dubois, Dik P. de Vries and Hans J.M. Dons

Abstract

In vitro propagation of the rose rootstock 'Moneyway' was investigated on the following media: Murashige and Skoog (MS), Quoirin and Lepoivre (QL) and Woody Plant medium (WP). Growth, which was measured as length of shoots after a 6-week period, was faster on MS-medium and QL-medium than on WP-medium. In spite of the better growth, chlorosis of newly formed leaves occurred from the third week on and was correlated with a lower chlorophyll content of shoots. Replacement of FeEDTA by FeEDDHA in QL- and MS-medium resulted in the development of green shoots for more than three months. The occurrence of chlorosis was not pH related since the pH of QL-medium with FeEDTA or FeEDDHA had not changed after six weeks of growth. Addition of the light absorbing dye fast yellow 9 to QL-medium with FeEDTA also resulted in green shoots with a higher chlorophyll content. It is suggested that FeEDDHA is a more photostable chelate than FeEDTA, resulting in a higher availability of iron for the rose shoots. The impact of the iron chelate formula on the micropropagation of plant species that are susceptible to iron deficiency, is discussed.

Introduction

To establish vigorous growth and high flower yield, cut rose cultivars (*Rosa hybrida* L.) are generally grown on a rootstock. In vitro plant cultures of cut rose cultivars and rootstocks are used for micropropagation, for obtaining pathogen-free material and for the development of protocols to introduce new characteristics by genetic modification.

In general, growth and development of micropropagated plants depends on factors such as macro-element composition, the total salt strength of the medium, vitamin mixture used, sugar concentration, hormonal composition and climatic conditions (light, day length, temperature). Techniques for the micropropagation of roses have been reviewed by Skirvin *et al.* (1990), Short and Roberts (1991) and Horn (1992). Rose shoots have been grown on WP-medium (Lloyd and McCown, 1981), SH-medium (Schenk and Hildebrandt, 1972), QL-medium (Quoirin *et al.*, 1977; Valles and Boxus, 1987) or MS-medium (Murashige and Skoog, 1962). A high concentration of the cytokinin 6-benzylaminopurine (2-13 μM BAP) and a relatively low concentration of auxin (0.02-0.5 μM α-naphthaleneacetic acid, indole-3-acetic acid or indole-3-butyric acid) were used to promote the outgrowth of axillary buds. For adventitious root formation in vitro media with higher concentrations of auxin (0.5-5 μM NAA, IAA or IBA) and without cytokinin were used. Rooting was enhanced if the macroelements were reduced to a quarter or half of their original concentration (Skirvin

and Chu, 1979; Khosh-Khui and Sink, 1982). This beneficial effect was attributed to reduced nitrogen levels (Hyndman *et al.*, 1982).

Three standard media for the micropropagation of the rose rootstock 'Moneyway' were evaluated. None of these media supported adequate growth and yielded healthy leaves. Here, we describe the effects of two different iron chelates on the shoot length and chlorophyll content of leaves of the rose rootstock 'Moneyway'.

Material and methods

Plant material and plant cultivation

Tissue culture material of the rose rootstock 'Moneyway' was obtained from axillary shoots emerging from surface disinfected nodes of greenhouse-grown plants. Shoots were subcultured every six weeks. For subculture several media were used: MS-medium, QL-medium and WP-medium with 86 μM ferric ethylenediamine tetraacetate (FeEDTA) or ferric ethylenediamine di(o-hydroxyphenylacetate) (FeEDDHA), 58.4 mM sucrose, 4.4 μM BAP, 0.49 μM IBA and 0.8% (w/v) agar (Oxoid, bacteriological agar) at pH 5.6. Three 5-10 mm shoot tips were grown on 50 mL medium in 400 mL glass jars closed with a polycarbonate screw lid and sealed with household foil. Per treatment three or five jars were maintained in a growth chamber at 22°C with 16 h light (Osram L58W/77 fluorescent tubes, 20 μmol m^{-2} s^{-1} at plant level). The experiments were performed with a randomized blocks design.

In three experiments, treatments with FeEDTA were compared to treatments with an equimolar concentration of FeEDDHA. First, a qualitative comparison was performed between the micropropagation on MS-, QL- and WP-medium with FeEDTA and QL-medium with FeEDDHA. Next the influence of the iron chelate formula in QL-medium was quantified. At last the influence of the iron chelate formula was quantified for QL- and MS-medium. Protection of FeEDTA against photodegradation was accomplished by addition of 53 μM 4-amino-1,1'-azobenzene-3,4'-disulfonic acid, which is known as fast yellow 9 (Sigma, St. Louis, U.S.A.) (M. De Block, pers. comm.).

Measurements

The pH of the medium of each jar was measured with a flat surface-polymer body electrode (Sensorex). Shoots in each jar were measured from medium surface to apex after six weeks and mean shoot length per jar was calculated. A sample of 50-70 mg leaf material was taken from three shoots and frozen in liquid nitrogen, ground in an Eppendorf tube and subsequently incubated overnight at room temperature with 1 mL acetone for chlorophyll

extraction. Chlorophyll a and b content was determined spectrophotometrically (Bruinsma, 1963).

The experimental unit was a jar. Data were submitted to analysis of variance. To compare treatment effects, the least significant difference (LSD) was calculated at $p=0.05$.

Results

Different media for rose micropropagation were compared. Shoots on WP-medium were small with poorly developed leaves after six weeks. In contrast, shoots developed on MS-medium had increased in length and had chlorotic leaves which turned necrotic and senescent within six weeks. Shoots on QL-medium also grew fast, but from the third week on chlorosis occurred, which was most pronounced in the youngest leaves. Since chlorosis is indicative of iron deficiency, FeEDTA in the QL-medium was replaced by the more stable iron chelate FeEDDHA. This change indeed resulted in shoots having green leaves after six weeks (Fig. 1). Even after three months newly formed leaves were green.

Subsequently, the effect of the iron chelate formula in the medium on pH, shoot length, chlorosis and chlorophyll content of shoots was further examined (Table 1). After six weeks, pH of QL-media with FeEDTA or with FeEDDHA were similar and also similar to its initial

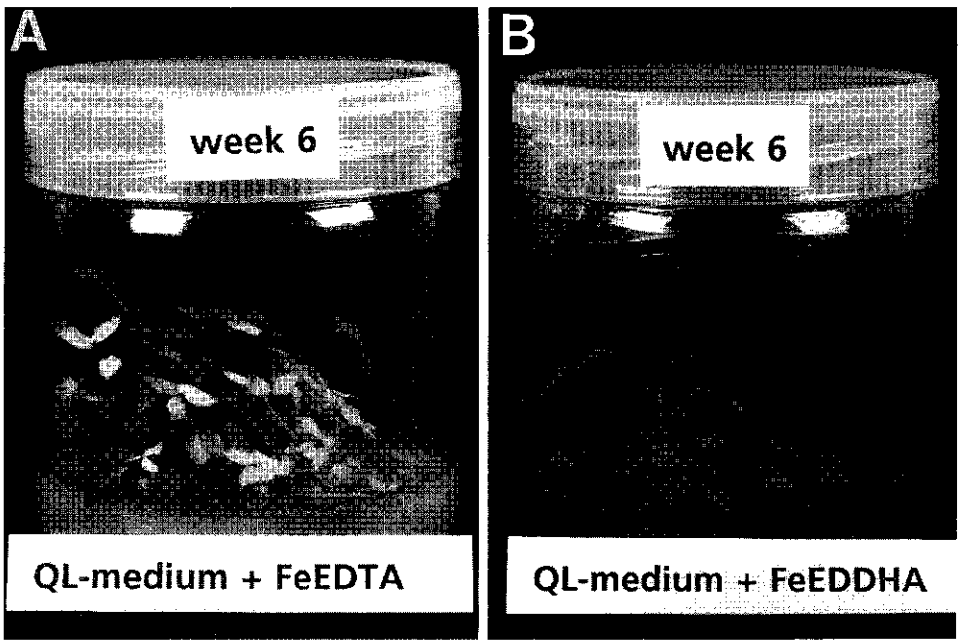

Figure 1. Chlorotic shoots of *Rosa hybrida* L. 'Moneyway' on QL-medium with 86 μM FeEDTA (**A**) and green shoots on QL-medium with 86 μM FeEDDHA (**B**) after six weeks.

value. The length of shoots grown on QL-medium with FeEDDHA was significantly higher ($p=0.013$) whereas the chlorophyll content of shoots was six fold higher than on QL-medium with FeEDTA.

Significant differences in length of shoots grown on QL- or MS-medium were not observed (Table 2A). Addition of FeEDDHA instead of FeEDTA improved growth ($p=0.049$) and resulted in a significantly higher chlorophyll content in shoots grown on QL- or MS-medium (3.3 and 1.7 fold, respectively). A medium chelate interaction was responsible for the higher effect of the substitution on QL-medium than on MS-medium ($p=0.008$).

In the same experiment the light absorbing dye fast yellow 9 was supplemented to QL-medium to analyze the differential influence of FeEDTA and FeEDDHA on shoot length and chlorophyll content. The addition of fast yellow 9 to QL-medium did not affect shoot length (Table 2B). However, it resulted in a two fold higher chlorophyll content of the shoots grown on QL-medium with FeEDTA. In contrast to this positive effect in QL-medium with FeEDTA, fast yellow 9 had a negative effect on the chlorophyll content of shoots grown on QL-medium with FeEDDHA.

Table 1. Effect of iron chelate on the pH of the medium, shoot length and chlorophyll a+b content of leaves of the rose rootstock 'Moneyway' after six weeks (n=3 jars).

	QL-medium with		
	FeEDTA	FeEDDHA	$LSD_{0.05}$
pH	5.7	5.8	1.1
shoot length (mm)	15	29	9
chlorophyll a+b (mg/g FW)	0.27	1.83	0.37

Discussion

For the propagation of the rose rootstock 'Moneyway' three standard media, which differed mainly in their nitrogen content, were tested: MS-, QL- and WP-medium. QL-medium has a lower content of ammonium (5 m*M*) and a similar nitrate content (33 m*M*) as MS-medium (20.6 and 39.4 m*M*, respectively), whereas WP-medium has a lower content of both ammonium (5 m*M*) and nitrate (9.8 m*M*). Faster growth of shoots on MS- and QL-medium was attributed to the higher total nitrogen content of these media. However, on both standard media, leaves became chlorotic suggesting an iron deficiency. Chlorosis did not occur if FeEDTA was substituted by the more stable iron chelate FeEDDHA (Wallace *et al.*, 1955;

Table 2A. Effect of medium and iron chelate formula on shoot length and chlorophyll a+b content of leaves of the rose rootstock 'Moneyway'. Means after six weeks of growth are given (n=5 jars).

	iron chelate		
medium	FeEDTA	FeEDDHA	$\bar{x}^z$
	shoot length[y] (mm)		
QL-medium	24	28	26 a[w]
MS-medium	17	24	21 a
$\bar{x}$	20 b	26 a	
	chlorophyll a+b[x] (mg/g FW)		
QL-medium	0.66 c	2.19 a	
MS-medium	1.43 b	2.40 a	

[z]Mean value of treatments
[y]$LSD_{0.05}$ value for shoot length was 6 mm
[x]$LSD_{0.05}$ value for chlorophyll content was 0.28 mg/g FW
[w]Means followed by the same letter do not differ significantly at $p<0.05$

Table 2B. Effect of iron chelate formula and the presence (+) and absence (-) of fast yellow 9 in QL-medium on shoot length and chlorophyll a+b content of leaves of the rose rootstock 'Moneyway'. Means after six weeks of growth are given (n=5 jars).

	fast yellow 9		
medium	-	+	$\bar{x}^z$
	shoot length[y] (mm)		
QL-medium + FeEDTA	24	21	22 a[w]
QL-medium + FeEDDHA	28	23	25 a
$\bar{x}$	26 a	22 a	
	chlorophyll a+b[x] (mg/g FW)		
QL-medium + FeEDTA	0.66 c	1.54 b	
QL-medium + FeEDDHA	2.19 a	1.60 b	

[z]Mean value of treatments
[y]$LSD_{0.05}$ value for shoot length was 6 mm
[x]$LSD_{0.05}$ value for chlorophyll content was 0.28 mg/g FW
[w]Means followed by the same letter do not differ significantly at $p<0.05$

Halvorson and Lindsay, 1972). The pH of the media had not changed after six weeks of growth, indicating that chlorosis of shoots was not caused by a low iron availability, which might be expected at higher pH values. The greening of the leaves was not only improved by replacement of FeEDTA by FeEDDHA, but also by addition of the light absorbing dye fast yellow 9 to medium with FeEDTA. This suggests that FeEDDHA is more photostable than FeEDTA resulting in a higher availability of iron to the rose shoots. Addition of fast yellow 9 to QL-medium with FeEDDHA resulted in shoots with a significantly lower chlorophyll content. This strongly suggests that next to its protective function, fast yellow 9 also has some negative effect on the chlorophyll content.

Chlorosis has also been reported for in vitro cultured shoots of *Pyrus* (Dolcet-Sanjuan, 1990), *Rhododendron* (Anderson, 1984) and *Rosa* (Horn, 1992) grown on FeEDTA-containing medium. In addition, iron deficiency is often observed in calcifugous plant species such as *Azalea*, *Citrus*, *Rhododendron*, *Vaccinium corymbosum* L., *Vitis* and deciduous fruit trees grown in the field or in the greenhouse (Mengel and Kirkby, 1978).

In summary, iron deficiency in 'Moneyway' cultures can be overcome either by addition of fast yellow 9 or by the replacement of FeEDTA by FeEDDHA. The use of FeEDDHA is more effective. It is tempting to assume that the replacement of FeEDTA by FeEDHHA in media will also improve the in vitro culture of other plant species that are susceptible for iron deficiency, such as the above mentioned plants.

Acknowledgements

We would like to thank dr. J. Roggemans (ISI, Gembloux, Belgium) for providing in vitro material of 'Moneyway', L.C.P Keizer for performing statistical analysis and dr. F. Bienfait for his fruitful suggestions. This research is supported by the Netherlands Agency of Energy and the Environment (Novem).

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Chapter 3

The effects of exogenous auxin and *ROL* genes on root formation in *Rosa hybrida* L. 'Moneyway'

Theo P.M. van der Salm, Caroline J.G. van der Toorn, Charlotte H. Hänisch ten Cate, Wim M. van der Krieken and Hans J.M. Dons

Abstract

The effect of indole-3-butyric acid (IBA) on the formation of non-transformed and *ROL* gene transformed roots on stem slices of in vitro cultured shoots of *Rosa hybrida* L. 'Moneyway' was examined. Formation of adventitious roots on this rootstock was dependent on the IBA dose; it was not affected by the presence of other root primordia on the same explant. Application of 0.32 to 1 μM IBA during five days, followed by transfer to medium without hormones resulted in maximum root formation (90%) after three weeks. The formation of such untransformed roots was completely inhibited by transfer to medium with 5 mg/L kanamycin two days after excision. Ri roots were formed upon inoculation with *A. rhizogenes* LBA9402 harbouring two plasmids: pRi1855, comprising the *ROL* genes and the binary plasmid p*35SGUSINT* with the neomycin phosphotransferase (*NPTII*) gene for kanamycin resistance and the CaMV *35SGUSINT* gene. The formation of these Ri roots on kanamycin-containing medium was independent on the presence of IBA. Stem slices inoculated with a disarmed *A. tumefaciens* GV3101, harbouring only the *NPTII* gene, formed callus and subsequently roots in the presence of kanamycin exclusively on medium with high IBA concentrations (10 or 100 μM). Root formation at 100 μM IBA was considerably improved by transformation with the *ROLB* gene under the influence of the strong CaMV 35S promoter. In addition, low IBA (0.1 and 1 μM) stimulated the formation of roots only on stem slices transformed with *A. tumefaciens* harbouring the *ROLA+ROLB+ROLC* genes; the rooting response at 10 μM IBA was much improved. It was concluded that the *35SROLB* gene and especially a combination of *ROLA*, *B* and *C* genes promote the rooting response.

Introduction

Genetically modified roots of *Nicotiana tabacum* with altered morphology and growth rate have been obtained after introduction of *ROL* genes derived from *Agrobacterium rhizogenes* (Vilaine and Casse-Delbart, 1987a; Spena *et al.*, 1987; Schmülling *et al.*, 1988; 1989), which interact with hormonal metabolism. Rose rootstocks are used in practice to support the growth of cut rose cultivars. Root formation and development of the rooting system of the rootstock are essential for their quality. Hormones play an important role, both in rooting of the rootstock, as well as in its interaction with the cultivar. Introduction of *ROL* genes in such rootstocks can give insight into the hormonal control of root formation and might open possibilities to improve rootstocks.

Both adventitious root formation and shoot formation are preceded by a dedifferentiation process in which plant cells become competent for plant growth regulators (dedifferentiation

phase) (Went, 1939; Meins and Binns, 1979; Meins *et al.*, 1982; Cristianson and Warnick, 1983). Subsequently, plant cells can be induced to differentiate with the appropriate plant growth regulators (induction phase) and finally, the differentiated tissue can develop into roots or shoots (development phase).

In woody species indole-3-butyric acid (IBA) is the most potent auxin used for the induction of adventitious roots. This was clearly shown for the induction of adventitious roots on stem slices of apple (*Malus* 'York') (Van der Krieken *et al.*, 1992). Root induction was dependent on the presence of IBA in the medium. High concentrations of IBA, however, inhibited the further development of the roots. This could be overcome by transfer of tissues to auxin free medium.

Upon infection with *A. rhizogenes*, Ri roots are induced on many plant species (Tepfer, 1980; Porter, 1991). The T-DNA of agropine strains consists of two parts; TL and TR (Huffman *et al.*, 1984). On the TL-DNA four root inducing loci *rolA*, *rolB*, *rolC* and *rolD* have been identified (White *et al.*, 1985), while the TR-DNA harbours two *AUX* genes for auxin synthesis and the genes for agropine/ mannopine synthesis (Vilaine and Casse-Delbart, 1987). Some plant species require either the presence of auxin or the *AUX* genes together with the TL-DNA for Ri root formation, whereas others only need the TL-DNA (Vilaine and Casse-Delbart, 1987). The *ROLB* gene introduced in *N. tabacum* exhibits an auxin like activity due to an increased auxin susceptibility (Maurel *et al.*, 1991; 1994; Filippini *et al.*, 1994). Furthermore it was shown, that ROLB protein hydrolyses indoxyl-ß-glucosides (Estruch *et al.*, 1991b), although intracellular auxin concentrations in *ROLB* transformed plants were not increased (Nilsson *et al.*, 1993; Delbarre *et al.*, 1994). Likewise the *ROLC* gene introduces a cytokinin like activity in plants, which is correlated with a cytokinin-ß-glucosidase activity of the *ROLC* gene product (Estruch *et al.*, 1991a).

The aim of this report is to assess the effect of single or combinations of *ROL* genes on auxin induced root formation in *Rosa*. First, the optimum IBA dose for the formation of adventitious roots on stem slices of the rose rootstock 'Moneyway' was determined. Subsequently Ri roots were induced upon inoculation of stem slices with *A. rhizogenes* in combination with IBA treatment. Finally we studied the effect of single or combinations of *ROL* genes on root formation.

Material and methods

Plant material and culture

Micropropagation of shoots of the rootstock 'Moneyway' was initiated from axillary shoots, emerging from surface disinfected nodes of greenhouse-grown plants. Every six weeks shoots

were subcultured on a QL-medium (Quoirin *et al.*, 1977), that had been improved (Van der Salm *et al.*, 1994) by the following modifications: 86 μM FeEDDHA, a more stable iron chelate, 4.4 μM benzyladenine (BA), 0.49 μM IBA, 58 mM sucrose, 0.8% (w/v) agar (Oxoid, bacteriological agar) at pH 5.6. Shoots were cultured in a growth chamber at 22°C with 16 h light (Osram L58W/77 fluorescent tubes, 20 μmol m^{-2} s^{-1} at plant level).

Root formation

Rooting experiments were performed according to Van der Krieken *et al.* (1993) with some modifications. Stems of 1.5 mm in diameter from six weeks old shoot cultures were cut into slices of 1 mm thick. Stem slices were incubated in petri dishes on a root induction medium (RIM), which consisted of a modified QL-medium of half strength macro elements, 88 mM sucrose and supplemented with various IBA concentrations. To improve root formation riboflavin in concentrations equimolar to IBA was supplemented when indicated (Van der Krieken *et al.*, 1992). Petri dishes were placed upside down and incubated in the dark to prevent breakdown of IBA by light. After various time intervals (1 to 7 days) the stem slices were transferred to the same medium without IBA and riboflavin and incubated upside down at 22°C with 16 h light. Per treatment three groups of 25 stem slices were incubated on 90 mm petri dishes. The number of visible roots per stem slice was counted three weeks after the start of the auxin treatment. Next, the percentage of stem slices with roots and the mean

Table 1. *Agrobacterium* strains with plasmids, T-DNAs and genes.

strain	*vir* plasmid	binary plasmid	T-DNAs (genes)
A. rhizogenes			
LBA9402(pRi1855)	pRi1855[1]	-	TL- (*ROLABCD*) and TR-DNA
LBA9402(pRi1855, p*35SGUSINT*)	pRi1855[1]	pBIN19	TL- (*ROLABCD*), TR- and T-DNA (*NOSNPTII*, *35SGUSINT*)
A. tumefaciens			
GV3101(-)	pMP90	-	-
GV3101(pPCV002-*BGUS1177*)	pMP90RK	pPCV002	T-DNA (*NOSNPTII*, *BGUS*)
GV3101(pPCV002-*CGUS1181*)	pMP90RK	pPCV002	T-DNA (*NOSNPTII*, *CGUS*)
GV3101(pPCV002-*B1100*)	pMP90RK	pPCV002	T-DNA (*NOSNPTII*, *BROLB*)
GV3101(pPCV002-*CaMVBT*)	pMP90RK	pPCV002	T-DNA (*NOSNPTII*, *35SROLB*)
GV3101(pMRK62)	pMP90	pMRK62	T-DNA (*19SNPTII*)
GV3101(pMRK12)	pMP90	pMRK62	T-DNA (*19SNPTII*, *CROLC*)
GV3101(pMRKE15)	pMP90	pMRK62	T-DNA (*19SNPTII*, *AROLA+BROLB+CROLC*)

[1] pRi1855 is the wildtype agropine Ri plasmid of *A. rhizogenes*.

number of roots per stem slice were calculated. To define the distribution of adventitious root formation on stem slices, data on the observed number of roots per stem slice for each treatment (combination of IBA concentration and incubation time) were used. By statistical analysis a Poisson distribution was fitted and a Poisson index was calculated to assess the fit. Further proof for agreement with a Poisson distribution was obtained by using the equation: $m=-\ln P(0)$ (Meins *et al.*, 1982), in which a relationship is given between the percentage of stem slices without roots (P(0)) and the average number of roots per stem slice (m).

Kanamycin susceptibility

The susceptibility of adventitious root formation for kanamycin and the time of application were investigated. Stem slices were incubated on RIM supplemented with 1 μM IBA and riboflavin in the dark during five days, transferred to hormone free medium and incubated in the light. At 2, 3, 5, and 7 days after excision the stem slices were transferred to the same medium supplemented with various concentrations of kanamycin and the antibiotics cefotaxim (200 mg/L) and vancomycin (400 mg/L).

Bacteria and plasmids

Several *Agrobacterium* strains were used as listed in Table 1. *A. rhizogenes* LBA9402(pRi1855) is an agropine strain with TL- and TR-DNA (De Paolis *et al.*, 1985). *A. rhizogenes* LBA9402(pRi1855, p*35SGUSINT*) was obtained by introduction of the binary vector p*35SGUSINT* (Vancanneyt *et al.*, 1990) derived from pBIN19. Furthermore *A. tumefaciens* GV3101 was used in combination with pPCV002 derived binary vectors harbouring the *GUS* gene driven by the promoter of the *ROLB* gene (pPCV002-*BGUS1177*) or by the promoter of the *ROLC* gene (pPCV002-*CGUS1181*), both located on the 1181-bp-long fragment of the *ROLB/ROLC* intergenic region (Schmülling *et al.*, 1989). The same vector was used for the introduction of *ROLB* driven by its own promoter with an approximate size of 1100 bp (pPCV002-*B1100*) or by the CaMV 35S promoter (pPCV002-*CaMVBT*) (Spena *et al.*, 1987). Furthermore GV3101 was used containing pMRK62 derived binary vectors harbouring the *ROLC* gene (ORF 12) driven by its own promoter (pMRK12) and the three *ROL* genes under control of their own promoter located on EcoRI fragment 15 (pMRKE15) (Vilaine *et al.*, 1987). All binary plasmids contained the neomycin phosphotransferase gene (*NPTII*) as a selectable marker. The pPCV and the pMRK constructs were kindly provided by A. Spena and T. Schmülling (Max Planck Institut für Züchtungsforschung, Cologne, Germany) and F. Vilaine (Institut National de la Recherche Agronomique, Versailles, France), respectively.

Transformation

Freshly cut stem slices were incubated for five minutes in a diluted (OD_{550}=0.5) overnight suspension culture of *Agrobacterium* in LB-medium, dried on filter paper and placed on RIM supplemented with 200 μM acetosyringon and various IBA/ riboflavin concentrations. Per petri dish 25 stem slices were incubated. After two days of incubation in the dark, stem slices were transferred to the same medium supplemented with 200 mg/L cefotaxim and 400 mg/L vancomycin to suppress bacterial growth and when indicated with 5 mg/L kanamycin. Three days later the stem slices were transferred to medium without growth hormones and riboflavin, but with the antibiotics and subsequently incubated in the light. After three weeks the number of rooted stem slices was counted. Statistical analysis was carried out using the computer program Genstat (1988).

ß-Glucuronidase assay

Stem slices and roots were histochemically assayed for ß-glucuronidase (GUS) activity with 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as described by Jefferson *et al.* (1987) with modifications (Van Altvorst *et al.*, 1995).

Results

The formation of adventitious roots

Optimum conditions for the formation of adventitious roots on stem slices of the rose rootstock 'Moneyway' were established by varying the IBA concentration and the length of

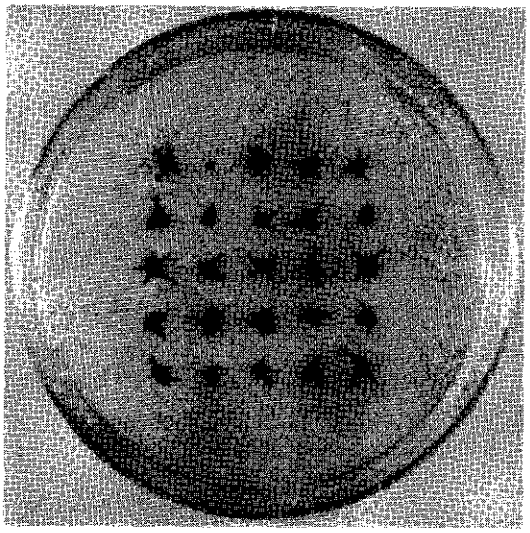

Figure 1. Adventitious roots on stem slices of the rootstock 'Moneyway' after treatment on RIM with 1 μM IBA during 5 days (photo taken after 21 days).

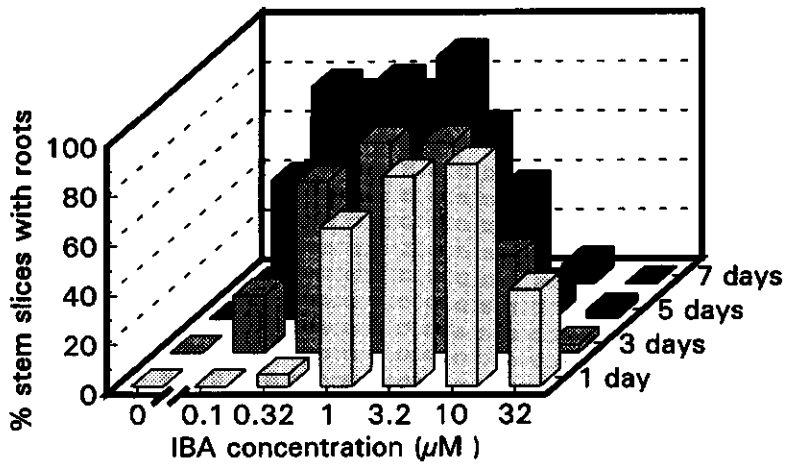

Figure 2. The effect of the IBA concentration and duration of incubation (1, 3, 5 and 7 days) on the percentage of rooted stem slices of the rootstock 'Moneyway', determined 21 days after excision ($LSD_{0.05}$=20.7).

Table 2. Determination of the distribution of the number of adventitious roots formed on stem slices in four treatments. A Poisson index=0 indicates a perfect Poisson distribution. Following the equation m=-lnP(0) (Meins *et al.*, 1982) with P(0) as the fraction of stem slices without roots, the average number of roots per stem slice (m) was calculated. The average number of roots of the treatments is given for comparison.

Treatment	Poisson index	P(0)	$m_{calculated}$ (-lnP(0))	$m_{determined}$
10 *μM* IBA/ 1 day	0.02	0.10	2.3	2.7
3.2 *μM* IBA/ 3 days	-0.03	0.15	1.9	2.2
1 *μM* IBA/ 5 days	0.24	0.10	2.3	2.4
0.32 *μM* IBA/ 7 days	0.13	0.15	1.9	2.1

the auxin treatment period (1 to 7 days). The first roots became visible after seven days. Up to 90% of the stem slices had formed roots after three weeks (Fig. 1). Figure 2 shows the strong dependence of root initiation on the IBA concentration. After a short treatment (one day) with high IBA concentrations (3.2 and 10 *μM*) about 90% of the stem slices produced roots, whereas upon extension of the auxin treatment period, only low auxin concentrations (0.1-1 *μM* during seven days) were effective. The number of roots per stem slice was also dependent on the IBA concentration and the time of incubation (data not shown) and resembled the pattern of the percentage of rooted stem slices. Under optimum conditions

about 2.5 roots per stem slice were formed. These results clearly demonstrated that root formation on stem slices occurred according to an auxin dose response. Statistical analysis on the distribution of the number of roots per explant revealed that the Poisson indices of four highly responding treatments were all proximate to zero, indicating a Poisson distribution (see Table 2). This was confirmed by the correspondence between calculated numbers of roots per stem slice ($m_{calculated}$) and the observed average numbers of roots per stem slice. These results show that there is no interaction between root initials on the same explant.

In the following experiments auxin treatments of five days were used. Under these conditions an IBA concentration of 0.32 to 1 μM was optimal.

Production of transformed roots

a) Kanamycin susceptibility

As part of the programme to obtain transformed roots resistant to kanamycin, we investigated inhibition of adventitious root formation by kanamycin (see Table 3). The system appeared to be very sensitive to kanamycin. Transfer to medium containing only 5 mg/L kanamycin two or three days after start of the incubation resulted in complete inhibition of root formation. Transfer at later times was less effective and resulted in an increasing number of roots that escaped the selection pressure. Then, even high concentrations of kanamycin (50 mg/L) were no longer effective.

To inhibit the formation of adventitious roots in the following experiments, stem slices were transferred to medium with 5 mg/L kanamycin two days after start of the incubation.

Table 3. Inhibition of root formation by kanamycin: effect of concentration and day of transfer on the percentage of rooted stem slices of 'Moneyway'. At the day of transfer stem slices were transferred to the same medium, supplemented with kanamycin. In all treatments stem slices were treated with 1 μM IBA during five days.

	kanamycin (mg/L)				
day of transfer	0	5	10	25	50
2	88	0	0	0	0
3	54	0	0	0	0
5	64	36	24	24	8
7	79	75	64	63	37

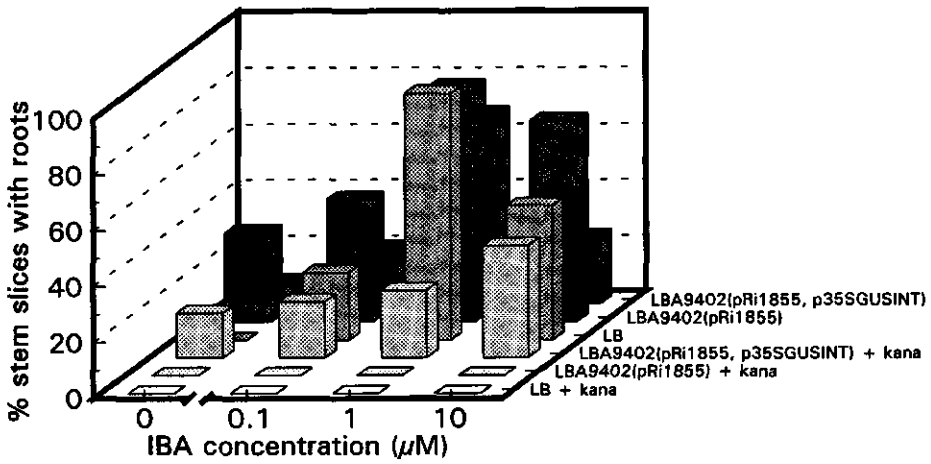

Figure 3. The effect of the IBA concentration on the percentage of rooted stem slices in the presence (+ kana) and absence of kanamycin (5 mg/L) 21 days after inoculation with LB, LBA9402(pRi1855) or LBA9402(pRi1855, p*35SGUSINT*) (n=25 stem slices/ treatment).

b) Production of Ri roots

For the induction of Ri roots stem slices were inoculated with a wildtype *A. rhizogenes* strain LBA9402(pRi1855) and with the same strain harbouring the binary plasmid p*35SGUSINT* and incubated on medium with various concentrations of IBA for five days (Fig. 3). In agreement with the results presented before (Fig. 2), 1 μM IBA resulted in optimum root formation on control slices (LB). After inoculation with *A. rhizogenes*, root formation was extended to lower IBA concentrations and even in the absence of auxin roots were formed. Irrespective of the IBA concentration used, approximately three roots per stem slice were formed (data not shown). Upon transfer to medium with kanamycin (5 mg/L) after two days roots were formed only on stem slices inoculated with *A. rhizogenes* LBA9402(pRi1855, p*35SGUSINT*), containing the kanamycin resistance gene. Since no escapes were formed on control slices on kanamycin containing medium, it is reasonable to conclude that all roots formed in the presence of kanamycin were kanamycin resistant by expression of the *NPTII* gene. This is further supported by the growth characteristics of excised roots. Roots that were induced by LBA9402(pRi1855, p*35SGUSINT*) and selected for kanamycin resistance, grew autonomously on hormone-free medium, showing decreased geotropism and increased lateral branching (data not shown). Upon treatment with X-gluc, blue staining was observed in approximately 50% of the kanamycin resistant roots.

c) Production of transformed roots

Using this system for induction and selection of transformed roots, stem slices were

incubated with *A. tumefaciens* GV3101 harbouring various constructs (Table 1). Compared to the LB control, incubation with GV3101(-), without binary plasmid and in the absence of kanamycin, inhibited the frequency of root formation at 1 μM IBA from 86% to 38% (see Table 4). In the absence of kanamycin, callus formation preceded root formation at 1 to 100 μM IBA (data not shown). In contrast, on medium with kanamycin little callus formation was observed only at high auxin concentrations (10 and 100 μM IBA). Moreover, no roots were formed, showing that the inhibition by kanamycin was complete.

Stem slices were inoculated with GV3101 containing pPCV002 derived binary vectors harbouring the *NPTII* gene and the *GUS* gene under control of either the *ROLB* promoter or the *ROLC* promoter. In the presence of kanamycin, callus formation was observed again at high auxin concentrations (10 and 100 μM IBA). At these high auxin concentrations kanamycin resistant roots were formed occasionally. The formation of these *BGUS* and *CGUS* transformed roots increased with the extension of the root formation period (2% and 4%, respectively, at 100 μM IBA after five weeks).

The transformation efficiency of *A. tumefaciens* strains harbouring the reporter gene *GUS* was also studied by histochemical staining of stem slices. Upon treatment with X-gluc, 2% and 12% of the stem slices inoculated with *BGUS* or *CGUS*, respectively, stained blue at 100 μM IBA.

On stem slices inoculated with *BGUS* one out of two roots stained blue, especially in the meristematic zone of the root tip (Fig. 4). On stem slices inoculated with *CGUS*, four out of 10 roots stained blue in the meristematic zone of the root tip and the vascular system.

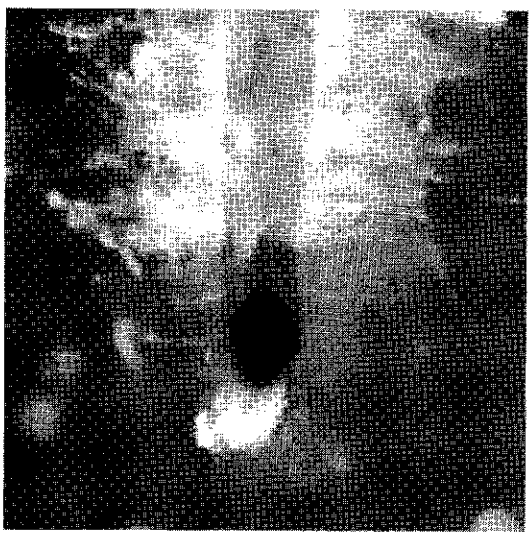

Figure 4. X-gluc stained root transformed with GV3101(pPCV002-*BGUS1177*). Promoter activity is localized in the meristematic zone of the root tip.

Table 4. The percentage of rooted stem slices (± SE) of the rootstock 'Moneyway' in the absence (-) or presence (+) of kanamycin (5 mg/L) 21 or 35 days after IBA treatment and inoculation with LB, GV3101(-) or with GV3101 containing *BGUS*, *CGUS*, *BROLB*, *35SROLB*, *CROLC* or *AROLA+BROLB+CROLC* (n=number of inoculated stem slices per treatment, ND=not determined).

					IBA concentration (μM)			
inoculum	*ROL* genes present on T-DNA	n	kana	days	0.1	1	10	100
LB-control	-	50	-	21	38 ± 6	86 ± 8	26 ± 5	0
GV3101(-)	-	50	-	21	4 ± 2	38 ± 6	36 ± 6	ND
GV3101(-)	-	200	+	21	0	0	0	0
GV3101(pPCV002-*BGUS1177*)	-	100	+	21	0	0	0	0
				35	0	0	0	2.0 ± 0.8
GV3101(pPCV002-*CGUS1181*)	-	125	+	21	0	0	0.8 ± 0.4	0
				35	0	0	3.2 ± 0.8	4.0 ± 1.0
GV3101(pPCV002-*B1100*)	*BROLB*	100	+	21	0	1.0 ± 0.5	3.0 ± 0.9	2.0 ± 0.8
GV3101(pPCV002-*CaMVBT*)	*35SROLB*	75	+	21	0	0	2.7 ± 1.0	14.7 ± 2.4
GV3101(pMRK62)	-	75	+	21	0	0	4.0 ± 1.2	1.3 ± 0.7
GV3101(pMRK12)	*CROLC*	75	+	21	0	0	2.7 ± 1.0	1.3 ± 0.7
GV3101(pMRKE15)	*AROLA+BROLB+CROLC*	75	+	21	1.3 ± 0.7	6.7 ± 1.6	13.3 ± 2.3	0

d) Production of roots containing rol genes

Essentially the same results were obtained after introduction of the *ROLB* gene or the *ROLC* gene under control of their own promoter (*BROLB* and *CROLC*, Table 4). Again roots were formed at low frequency (1 to 4% after three weeks) only at high IBA concentrations, implying that *ROLB* and *ROLC* had no influence in the formation of kanamycin resistant roots. In contrast, the *ROLB* gene driven by the CaMV 35S promoter instead of its own promoter, enhanced root formation considerably. Fifteen percent of the stem slices formed roots at 100 μM IBA. Finally, the effect of a combination of *ROLA*, *B* and *C* genes on root formation was studied using the binary plasmid pMRKE15. Root formation on stem slices was increased at 10 μM IBA and clearly shifted towards lower IBA concentrations (0.1 and 1 μM) compared to the control (pMRK62). This resembled root formation on stem slices treated with the wild type *A. rhizogenes* strain (Fig. 3).

Discussion

The formation of adventitious roots on stem slices of the rose rootstock 'Moneyway' clearly showed a dose response in relation to the concentration of the auxin IBA and the duration of application. Seven days were required before the roots were visible and approximately 2.5 roots were formed per stem slice. Although the average number of roots formed was much lower than on apple shoot slices, the IBA-dependent root induction in rose resembled that in apple, another species of the *Rosaceae* (Van der Krieken *et al.*, 1993).

The formation of adventitious roots on stem slices occurred according a Poisson distribution. This suggests that the formation of adventitious roots was not affected by the presence of other root primordia on the same explant and that single cells or groups of cells precede the formation of adventitious roots. In this respect, adventitious root formation resembled the process of bud initiation in cultured tobacco tissues (Meins *et al.*, 1982).

Root formation on stem slices was inhibited completely when stem slices were transferred to a low concentration (5 mg/L) of kanamycin after two days. Later application of much higher concentrations (50 mg/L) of kanamycin only partly inhibited root formation. This demonstrates that the process of root formation on stem slices is highly susceptible for kanamycin especially during the first two days. The timing coincides well with the root induction phase and the initial differentiation events, such as the first cell divisions, as described for apple (De Klerk and Ter Brugge, 1992).

The formation of Ri transformed roots differed considerably in auxin dependence from normal adventitious root formation. Ri root formation induced by *A. rhizogenes* did not require exogenous auxin at all. This can be attributed to the expression of the *AUX* and/or

ROL genes of the T-DNA in transformed plant cells (Vilaine and Casse-Delbart, 1987). Only half of the kanamycin resistant Ri roots stained blue upon X-gluc treatment. This might be due to inactivation (silencing) of the *GUS* gene or to partial integration.

In contrast to *A. rhizogenes*, *A. tumefaciens* GV3101(-) inhibited root formation on medium without kanamycin. This inhibition might be induced by the secretion of cytokinin due to expression of the *TZS* gene, which is present on Ti plasmid of nopaline strains (Regier and Morris, 1982).

The root formation system was also used for the production of kanamycin resistant roots with selected genes. Upon *A. tumefaciens* transformation the formation of kanamycin resistant callus or roots differed considerably from that on stem slices under non-selective conditions. In the presence of kanamycin callus formation was generally slower and shifted to higher IBA concentrations. Root formation in the presence of kanamycin occurred after callus formation and also shifted to high IBA concentrations. This suggests an accessory role of callus for the root formation such as kanamycin detoxification and local adjustment of the auxin concentration.

The characteristics of introduced genes affected the frequency of transgenic root formation. Much more kanamycin resistant roots were formed at 100 μM IBA upon transformation with *ROLB* under control of a relatively strong CaMV 35S promoter. This is in agreement with the assumed auxin-like activity of the *ROLB* gene product (Maurel *et al.*, 1991; 1994; Filippini *et al.*, 1994) and the promotion of root induction by auxin. However, although the rooting response was increased, no increase in auxin susceptibility was observed after transformation with *ROLB* as observed in protoplasts of *N. tabacum* (Maurel *et al.*, 1991; 1994). This suggests that somatic cells transformed with the *ROLB* gene and competent for root formation behave in a different manner than *ROLB* transformed protoplasts.

Stem slices inoculated with GV3101(pMRKE15), harbouring the *ROLA*, *B*, *C* genes, formed roots also at 0.1 and 1 μM IBA. Root formation at 10 μM IBA was more pronounced than in the control and no roots were formed at 100 μM IBA. This pattern resembled that of stem slices inoculated with *A. rhizogenes*. The results also correspond with the increased root formation on explants of kiwi transformed with the *ROLA*, *B*, *C* genes (Rugini *et al.*, 1991). It suggests that *ROLA* and *ROLC* enhanced the promotive effect of *ROLB* on rooting.

In conclusion, the results presented show that adventitious root formation on stem slices of rose rootstocks is regulated by an exogenous auxin dose. The presence of the CaMV *35SROLB* gene or especially a combination of *ROLA*, *ROLB* and *ROLC* genes promoted the final rooting response three to four fold. In the latter case the auxin range for root induction was extended to low IBA concentrations.

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Chapter 4

Somatic embryogenesis and shoot regeneration from excised adventitious roots of the rootstock *Rosa hybrida* L. 'Moneyway'

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Abstract

Plants were regenerated from excised adventitious roots of the rose rootstock 'Moneyway' via a three phase procedure: callus induction, induction of somatic embryos and shoot development. Callus was induced on excised roots after incubation for four weeks in the dark on SH-medium (Schenk and Hildebrandt) containing 50 μM 2,4-dichlorophenoxyacetic acid. For embryo induction, calluses were transferred to hormone-free SH-medium and incubated for eight weeks. The use of Gelrite instead of agar during callus induction stimulated somatic embryogenesis (up to 16% of the explants formed organized structures), whereas the presence of 6-benzylaminopurine in this phase inhibited subsequent regeneration. Yellow solid calluses with embryo-like cotyledons or primordia and friable calluses with embryos were selected, and upon incubation in the light shoots developed. Shoot development was faster and more frequent on solid callus than on friable callus (64% and 21% of the calluses finally formed one or more shoots, respectively). Eleven out of thirteen regenerants developed similarly to control shoots. Finally, this regeneration method is compared with other systems for somatic embryogenesis and opportunities for the production of transgenic rose rootstocks and rose cultivars are discussed.

Introduction

Methods for the regeneration of whole plants from explants of rose may be used for several purposes such as *Agrobacterium* mediated gene transfer, mutation breeding and fast propagation. Regeneration of rose via adventitious shoot formation or somatic embryogenesis has been reported only a few times. Adventitious shoots and plants have been obtained from leaves, roots and callus of the interspecific hybrid *Rosa persica* x *xanthina* (Lloyd *et al.*, 1988) and from callus induced on internodal segments or leaf discs of *R. hybrida* L. 'Landora' (Rout *et al.*, 1992). Plants were also regenerated after induction of somatic embryos on callus derived from leaf or stem segments (De Wit *et al.*, 1990; Rout *et al.*, 1991), root explants (Roberts *et al.*, 1990) or stamen filaments (Noriega and Söndahl, 1991). Application of such regeneration procedures is generally restricted to one or a few genotypes and is also hampered by low efficiency of regeneration and shoot development.

Since somatic embryos generally are of unicellular origin, such a system is favourable for the production of genetically modified plants. This has been shown recently by Firoozabady *et al.* (1994), who used embryogenic callus cultures of 'Royalty' in an *Agrobacterium* mediated transformation system for the production of transgenic roses.

As part of a research program on the improvement of rose rootstocks by genetic

modification, this study was undertaken to establish a regeneration procedure. For this purpose roots seem to be excellent explants, since shoots (suckers) are formed spontaneously on roots of greenhouse and field grown plants. Moreover Roberts *et al.* (1990) showed that somatic embryogenesis could be induced on roots of a specific hybrid rose *R. persica* x *xanthina*. We have used this observation to further develop a procedure on somatic embryogenesis and whole plant formation using adventitious roots as starting material.

Material and methods

Plant material and production of adventitious roots

Shoots of the hybrid *Rosa persica* x *xanthina* and the rootstock *R. hybrida* L. 'Moneyway' were cultured on a modified QL-medium (Quoirin *et al.*, 1977) in which FeEDTA is replaced by the more stable iron chelate FeEDDHA (Van der Salm *et al.*, 1994). Stem slices of 1 mm thick were incubated for five days on modified QL-medium with half strength macro elements and supplemented with 1 μM indole-3-butyric acid (IBA) (Van der Krieken *et al.*, 1993; Van der Salm *et al.*, 1996). Root outgrowth occurred upon transfer of stem slices to the same medium without auxin. After three weeks adventitious roots of 3-10 mm (Fig. 1A) were excised and used for shoot regeneration experiments.

Shoot regeneration from adventitious roots

Three phases were distinguished during shoot regeneration from excised adventitious roots: callus induction on medium with hormones followed by embryo induction on medium without

Table 1. Experimental overview for somatic embryogenesis and shoot regeneration from excised roots of the rootstock 'Moneyway'.

	experiment 1	experiment 2 and 3
callus induction	- BM/ agar - 2,4-D/ BAP - 8 weeks	- BM or BM+/ agar or Gelrite - 50 μM 2,4-D - 4 or 8 weeks
embryo induction	- BM/ agar or BM+/ Gelrite - hormone-free - 4 weeks	- BM/agar or BM+/ Gelrite - hormone-free - 6 or 8 weeks
shoot development	- BM/ agar or BM+/ Gelrite - hormone-free - 8 weeks	- BM+/ Gelrite - BAP/ IBA - 8 weeks

hormones and shoot development on medium with or without hormones. A scheme of the various treatments during the three phases is presented in Table 1.

Callus induction

The method of Roberts *et al.* (1990) was used with some modifications. Excised roots were incubated on a basal medium (BM), consisting of SH-medium (Schenk and Hildebrandt, 1972) supplemented with 88 m*M* sucrose, 1000 mg/L i-inositol, 600 mg/L L-proline, 5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl and 5 mg/L thiamine HCl at pH 5.8. BM+ was also used: BM enriched with 500 mg/L casein hydrolysate and a combination of additional vitamins, organic acids and sugars, but without coconut milk (Kao and Michayluk, 1975). Media were solidified with 0.8% (w/v) agar (Oxoid, bacteriological agar) or 0.3% (w/v) Gelrite (Phytagel, Sigma).

In the first experiment roots were incubated on 28 combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP), as indicated in Table 2. Per treatment 4 and 30 roots of *R. persica* x *xanthina* and 'Moneyway' were used, respectively, with a maximum of 10 roots per Petri dish. After eight weeks at 25°C in the dark calluses were transferred to hormone-free medium for embryo induction.

In the second and third experiment callus was induced on excised roots of 'Moneyway' by incubation on medium with 50 μM 2,4-D for four or eight weeks. Per treatment 50 and 200 roots were used, respectively.

Embryo induction

Calluses with their original root explant were transferred to hormone-free BM with 0.8% (w/v) agar or hormone-free BM+ with 0.3% (w/v) Gelrite and incubated at 25°C in the dark. The regeneration frequency was calculated as the percentage of calluses with organized structures i.e. embryo- or meristem-like structures, which appeared on friable or yellow solid callus. For statistical analysis of treatment effects on embryo formation, a linear model was fitted with binomial distribution and logit link function.

Shoot development

For shoot development, calluses with organized structures were selected and subcultured in Petri dishes with BM+ with 0.3% (w/v) Gelrite and various concentrations of BAP (0, 1, 3, 10 and 30 μM) and IBA (0 and 0.5 μM). Cultures were incubated in a growth chamber at 25°C with 16 h light (Osram L58W/20 cool white tubes, 20 μmol m^{-2} s^{-1} at plant level) for two months. Shoot propagation was accomplished on the modified QL-medium with FeEDDHA.

Analytical methods

After six weeks of callus induction, the area of formed callus was measured by image analysis, using the following components: CCD camera SONY XC77CE1 with a 16 mm lens (distance 876 mm, f/8 lens aperture), SUN 4/110 SPARC computer and a Data Translation frame-grabber DT1451. The image recording and processing was controlled by the software package SCIL-IMAGE. Data were submitted to analysis of variance after logarithmic transformation. After recording callus areas, incubation on callus induction medium proceeded for another two weeks.

The ploidy level of regenerant clones of 'Moneyway' was determined by flow cytometric determination of the DNA content of nuclei (Galbraith *et al.*, 1983; Sree Ramulu and Dijkhuis, 1986).

Results

Callus induction

First, the effects of 2,4-D and BAP on callus formation on root explants of *R. persica* x *xanthina* and 'Moneyway' were compared.

The results of the highly regenerative *R. persica* x *xanthina*, which was used as a reference, are summarized in Table 2A. For callus formation, 2,4-D in the medium was required. All root explants responded, although the amount of formed callus, as deduced from image analysis, depended on the treatment. Low concentrations of BAP (0.5 and 5 μM) additionally stimulated callus formation, whereas high BAP concentrations (50 μM) inhibited

Table 2. The effect of 2,4-D and BAP on the area (mm^2) and the quality of callus formed on excised roots of *Rosa persica* x *xanthina* (**A**) and 'Moneyway' (**B**) after incubation on BM during six weeks. Numbers between brackets were not used for ANOVA, * = snowy callus.

A)

R. persica x *xanthina* (n=4)		BAP (μM)			
		0	0.5	5	50
2,4-D (μM)	0	(2)	(5)	(4)	(3)
	0.5	38*	52*	123*	51*
	1.6	23	141*	139*	54*
	5	26	251	131*	84*
	16	90	268	170	69*
	50	38	170	140	58
	160	20	98	113	98

B)

'Moneyway' (n=30)		BAP (μM)			
		0	0.5	5	50
2,4-D (μM)	0	(2)	(2)	(3)	(2)
	0.5	15*	31*	18*	3*
	1.6	17	40*	24*	8*
	5	20	36*	32*	10*
	16	27	33	45*	16*
	50	34	37	50*	22*
	160	35	34	39*	12*

the auxin induced callus formation. Also the colour and structure of the callus was influenced by the hormonal concentrations. The presence of only 2,4-D resulted in yellow jelly-like callus, while low 2,4-D together with high BAP resulted in a white snowy type of callus. Adventitious roots of 'Moneyway' also formed callus on 2,4-D and BAP, without exception, but the amount of callus was much smaller (Table 2B). Statistical analysis revealed that optimum callus formation on roots of *R. persica* x *xanthina* and 'Moneyway' was established at similar hormonal concentrations, ranging from 1.6-50 μM 2,4-D and 0.5-5 μM BAP. 'Moneyway' callus induced on 2,4-D alone was yellow and turned brown in time (Fig. 1B), while snowy white callus (Fig. 1C) was formed on a wider range of 2,4-D and BAP than in the case of *R. persica* x *xanthina*.

Induction of organized structures

For embryo induction, calluses formed on 5 to 160 μM 2,4-D and various BAP concentrations were transferred to hormone-free BM with agar or hormone-free BM+ with Gelrite.

Calluses of *R. persica* x *xanthina* all developed into friable callus with embryos within four weeks, with the exception of calluses induced on medium with 50 μM BAP. Upon further cultivation in the light the embryos developed into green plants within two months.

In contrast, embryogenesis on callus of 'Moneyway' roots was much more restricted. Only two out of 15 calluses, induced on medium with 50 μM 2,4-D and without BAP, initiated a yellow solid callus with aberrant embryo-like cotyledons when subcultured on hormone-free BM+ with Gelrite for four weeks. Upon further subculture, one of these calluses formed a few primordia with linearly organized cells and finally shoots.

For succeeding experiments, 50 μM 2,4-D was chosen for callus induction and the effect of the callus induction period, the addition of organic components and the type of gelling agent was investigated. Table 3 shows that the frequency of calluses with organized structures was increased by shortening the callus induction period from eight to four weeks. Moreover, enrichment of the medium with organic components and the presence of Gelrite stimulated callus growth and increased the frequency of callus with organized structures. Two types of callus with organized structures were distinguished, sometimes appearing side by side: a white, friable callus and the already mentioned yellow, solid callus. The friable callus grew very fast and formed embryogenic structures from eight weeks on (Fig. 1D, E). In contrast, the solid callus grew much slower, but formed already within two to eight weeks aberrant embryo-like cotyledons (Fig. 1F, G) and/or linearly organized cells, possibly as a result of organogenesis (Fig. 1H).

Finally, the effect of gelling agent and additional organic components during callus induction was studied to elucidate their individual effect on the formation of organized

Table 3. The effect of length of the callus induction period, additional organic components and gelling agent on the percentage of calluses with organized structures. Excised roots of 'Moneyway' were incubated on callus induction medium with 50 μM 2,4-D during 4 or 8 weeks and subsequently on embryo induction medium without growth regulators during 6 weeks.

		% of calluses with organized structures	
		embryo induction	
callus induction		BM/ agar	BM+/ Gelrite
4 weeks	BM/ agar	4	8
	BM+/ Gelrite	16	10
8 weeks	BM/ agar	0	2
	BM+/ Gelrite	4	2

n=50 excised roots, effect of callus induction period and use of additional organic components/ Gelrite during callus induction and/or embryo induction was significant at p=0.005 and at p=0.05, respectively.

Table 4. The effect of additional organic components and gelling agent present during callus induction on the percentage of calluses with organized structures. Excised roots of 'Moneyway' were incubated on callus induction medium with 50 μM 2,4-D during 4 weeks and subsequently on BM with agar during 8 weeks for embryo induction.

	% of calluses with organized structures	
	agar	Gelrite
BM	0.5	3.5
BM+	0.5	6.0

n=200 excised roots, effect of gelling agent was significant at p=0.005.

structures (Table 4). The variation in this experiment was minimized by using 200 root explants per treatment. It was striking that in this experiment a much lower frequency of calluses with organized structures was found. Nevertheless the results clearly show that the presence of Gelrite during callus induction increased the frequency of organized structures, whereas the addition of organic components had no significant effect.

Shoot development

To investigate shoot development, 14 friable and 11 solid calluses with organized structures were selected from the last experiments. Calluses were partitioned in portions of about 100 mg, transferred to BM+ with Gelrite supplemented with various concentrations of BAP (0,

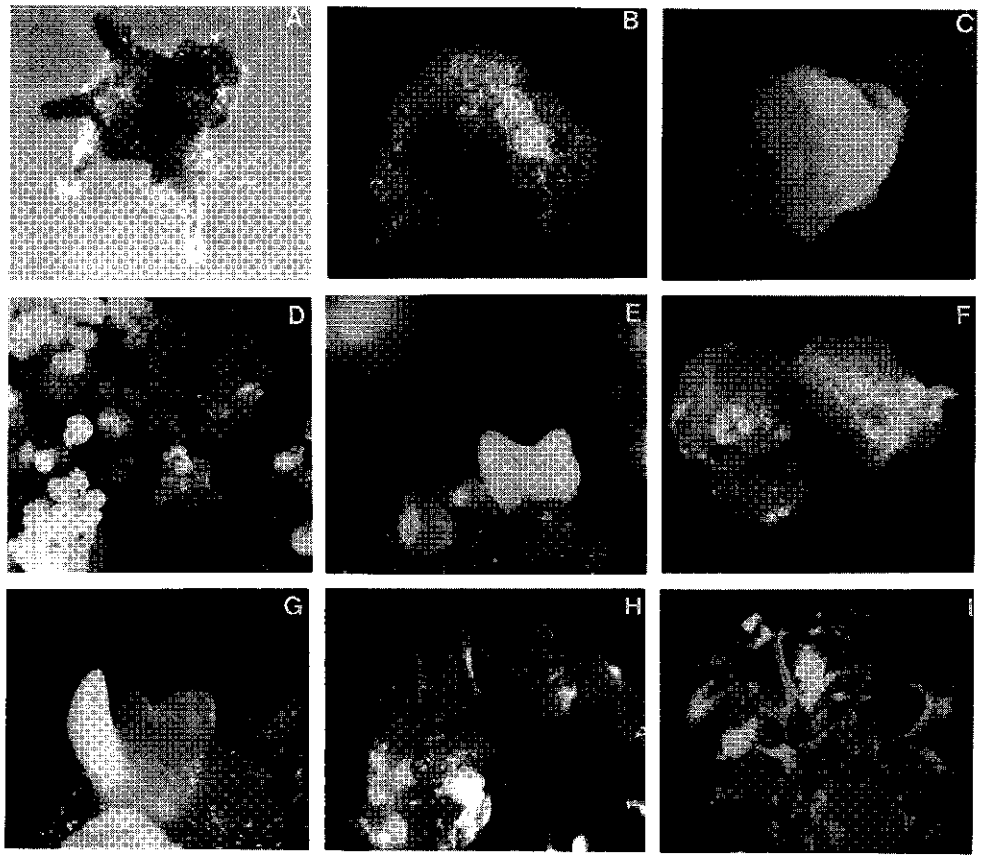

Figure 1. Shoot formation by somatic embryogenesis on root explants of *R. hybrida* L. 'Moneyway'. **(A)** Adventitious root formation on a stem slice after three weeks. **(B)** Yellow callus formed on excised roots after incubation on BM with 50 μM 2,4-D for six weeks. **(C)** White, snowy callus formed on excised roots after incubation on BM with 50 μM 2,4-D and 50 μM BAP for 6 weeks. **(D)** Friable callus with embryogenic structures. **(E)** Detail of friable callus with an embryo. **(F)** Yellow solid callus. **(G)** Detail of yellow solid callus with embryo-like cotyledons. **(H)** Primordia with linearly organized cells. **(I)** Regenerated shoots.

1, 3, 10 and 30 μM) and IBA (0 and 0.5 μM) and incubated in the light for two periods of four weeks. Independently of the hormone treatment, 3 out of 14 friable calluses and 7 out of 11 solid calluses formed one or more shoots. The development of shoots from friable callus with embryogenic structures was slow. It took more than two months before the first swollen embryogenic structures developed into shoots. In contrast, the primordia on the solid callus turned green within a few days and developed into fast growing shoots after two weeks (Fig. 1I). Thus shoot development on solid callus was more frequent and much faster than on friable callus. Finally, shoots were excised, transferred to propagation medium and subcultured every six weeks.

Genetic stability of regenerated shoots

To examine the effect of the regeneration procedure on the ploidy level, DNA content of nuclei of 13 independent regenerants of 'Moneyway' was analyzed by flow cytometry; 12 regenerants were triploid, similar to the control micropropagated shoots of 'Moneyway', while one regenerant was hexaploid (2n=6x). Upon further cultivation 11 out of 12 regenerants developed similarly to control shoots, whereas one shoot had variegated leaves.

Discussion

In this paper we have described a three phase procedure for somatic embryogenesis and shoot regeneration from adventitious roots formed on stem slices of the rose rootstock 'Moneyway'. First, excised roots were incubated on callus induction medium containing 2,4-D. For embryo induction calluses were transferred to medium without growth regulators. Calluses with organized structures were selected and shoots were formed after further incubation in the light.

A wide range of 2,4-D concentrations followed by incubation on hormone-free medium resulted in fast and abundant embryo formation on excised adventitious roots of *R. persica* x *xanthina*. Previously, Roberts *et al.* (1990) observed somatic embryogenesis in the presence of 14 μM 2,4-D only after four months. This suggests that embryo induction was accelerated by the transfer of callus from medium with 2,4-D to medium without growth regulators. Such an effect of the auxin 2,4-D has been observed in other systems as well, e.g. *Daucus carota* (De Vries *et al.*, 1988) and *Medicago sativa* (Dudits *et al.*, 1991). Application of such a 2,4-D treatment on adventitious roots of 'Moneyway' also successfully led to embryo induction.

Application of other methods for induction of somatic embryogenesis (De Wit *et al.*, 1990; Rout *et al.*, 1991) did not result in shoot regeneration from adventitious roots of 'Moneyway' (data not shown). Although not tested, this might be due to the cultivar and/or explant specificity of used methods.

Embryo formation of *R. persica* x *xanthina* and *R. hybrida* L. 'Moneyway' was inhibited by high concentrations of BAP during callus induction. For another rose, *R. hybrida* L. 'Landora', Rout *et al.* (1991) used a BAP containing medium for the induction of somatic embryogenesis, but callus induction in the absence of BAP was not tested. Organized structures of 'Moneyway' only developed in the absence of BAP. This agrees well with the inhibition of somatic embryogenesis in *Dactylis glomerata* (Wenck *et al.*, 1988) and *Daucus* (Paek *et al.*, 1985) by endogenous and exogenous cytokinins, respectively. Although essential for organogenesis, cytokinins inhibit embryogenesis.

The formation of organized structures of 'Moneyway' was improved by using Gelrite instead of agar as gelling agent. The superiority of Gelrite compared to agar in promoting regeneration was also observed in other systems e.g. *Mangifera indica* L. (DeWald *et al.*, 1989) and *Poa pratensis* L. (Van Ark *et al.*, 1991).

The presence of embryos and embryo-like cotyledons on the friable and the yellow solid callus, respectively, indicates the induction of somatic embryogenesis. However, shoot development on the solid callus was mediated via organogenic-like shoot meristems. Therefore, it cannot be excluded that next to somatic embryogenesis, adventitious shoot formation was also involved. Both somatic embryogenesis and adventitious shoot formation were also observed in regeneration experiments with wounded leaves of a *Cichorium* hybrid and proved to be temperature dependent (Decout *et al.*, 1994).

The formation of adventitious roots on stem slices of 'Moneyway' was very efficient. From an in vitro shoot of 30 mm about 30 stem slices were cut, yielding approximately 100 roots after three weeks. In the presented regeneration method 6 or 16% of the root explants formed callus with organized structures. This appeared to be more than for a method described for the regeneration of the cut rose 'Royalty', in which only two embryogenic calluses were obtained from several experiments in a three year period (Firoozabady *et al.*, 1994).

Application of this procedure for other roses requires adventitious roots from stem slices or other explants. Regeneration of plants from excised adventitious roots has already successfully been accomplished for various cut rose cultivars (F.H.M. Derks, pers. comm.). For improvement of rootstock quality, genetically modified roots of the rootstock 'Moneyway' with the *ROL* genes derived from *Agrobacterium rhizogenes* were produced (Van der Salm *et al.*, 1996). In conclusion, the presented regeneration method gives new opportunities for the production of transgenic rose rootstocks and rose cultivars.

Acknowledgements

This research is supported by the Netherlands Agency for Energy and the Environment (Novem). The authors are grateful to A. Roberts for providing the hybrid *R. persica* x *xanthina*, J.G. van der Vooren for performing image analysis and L.C.P. Keizer for performing the statistical analysis.

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Chapter 5

Production of *ROL* gene transformed plants of *Rosa hybrida* L. and characterization of their rooting ability

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Abstract

Transgenic plants of the rootstock *Rosa hybrida* L. 'Moneyway' were produced via a two step procedure. First, kanamycin resistant roots were generated on stem slices from micropropagated shoots, which were cocultivated with *Agrobacterium tumefaciens* containing the neomycin phosphotransferase (*NPTII*) gene for conferring kanamycin resistance, together with individual *ROL* genes from *A. rhizogenes*. Root formation was quite efficient and up to two kanamycin resistant roots per stem slice were produced. In the second step these roots were used to regenerate transgenic plants via somatic embryogenesis. Although regeneration lasted up to 12 months, production of several transformants was successfully accomplished. Non-transformed escapes were not found, indicating that the initial selection on kanamycin resistance was reliable.

The presence of the *ROL* genes stimulated lateral root formation. In addition, adventitious root formation on micropropagated shoots and leaf explants was also enhanced, even on medium without auxin. It appears that the auxin sensitivity was increased to such a degree that cells were able to respond to endogenous auxins present in shoots and leaves. In line with this, adventitious root formation on stem explants taken from *ROLA, B, C* transformed plants was also enhanced. However, root formation on stem explants from the *ROLB* transformed plant was decreased. This was probably due to overexpression of the *ROLB* gene. Rooting experiments in the greenhouse demonstrated that adventitious root formation on cuttings was improved threefold upon introduction of *ROLB* or a combination of *ROLA, B* and *C* genes. It is concluded that a method was developed for the production of *ROL* gene transformed roses with improved rooting characteristics.

Introduction

Rose is one of the most important ornamental crops worldwide. To increase vigour and disease resistance, roses are often grafted on a rootstock. As part of a research program to improve growth characteristics of rose, we aim at the production of genetically modified rose rootstocks with an improved root system. For this purpose the introduction of the root loci, the *ROL* genes from *Agrobacterium rhizogenes* was chosen. These genes are well-known for their phenotypical effects in the hairy-root disease (Costantino *et al.*, 1984; Tepfer *et al.*, 1984). Four genes (*ROLA, B, C* and *D*) are involved in the observed changes in root formation and root morphology (White *et al.*, 1985). The individual *ROL* genes have been isolated and upon introduction in plants it was found that they interact with several plant hormonal processes. *ROLB* introduced in *Nicotiana tabacum* was responsible for an auxin

like activity, such as enhanced adventitious root formation, which is correlated with an increased auxin sensitivity (Maurel *et al.*, 1991; 1994; Filippini *et al.*, 1994). Likewise, *ROLC* has a cytokinin like action, such as a diminished apical dominance. For the gene products of *ROLB* and *ROLC* an indoxyl- and a cytokinin-ß-glucosidase activity, respectively, were found in vitro (Estruch *et al.*, 1991a and b). It is questionable whether such enzymatic activity is operative in vivo, since auxin and cytokinin levels were not enhanced in transgenic plants (Nilsson *et al.*, 1993a and b).

Since the release and development of axillary shoots of plants such as rose are hormonally regulated, these processes might be affected by application of *ROL* gene transformed rootstocks. As more axillary shoots lead to more flower-bearing shoots, the *ROL* genes may offer a tool for improvement of rose by genetic modification. However, rose is considered, like many other woody species, as a recalcitrant species for transformation.

For introduction of the *ROL* genes, a two step transformation procedure was developed. First, transgenic roots were formed on stem slices, followed by regeneration of whole plants from excised roots. For the production of transformed roots, stem slices from micropropagated 'Moneyway' shoots were cocultivated with *A. tumefaciens*, comprising the *ROL* genes and the neomycin phosphotransferase (*NPTII*) gene for kanamycin resistance and treated with auxin for root induction (Van der Salm *et al.*, 1996b). As a result, kanamycin resistant roots with altered root characteristics were formed. Subsequently, a method was developed for the regeneration of whole plants from excised adventitious roots by somatic embryogenesis (Van der Salm *et al.*, 1996a). In the present paper, both procedures were combined for the production of *ROL* gene transformed plants of the rootstock 'Moneyway'. For evaluation of the rooting characteristics in vitro, transformed roots and explants of transformed shoots were treated with auxin in order to elucidate the effects of *ROL* genes on lateral and adventitious root formation, respectively. Finally, the effect of *ROL* genes on the rooting characteristics of cuttings was tested in the greenhouse.

Material and methods

Plant material and production of transformed roots

Micropropagated shoots of the rootstock *Rosa hybrida* L. 'Moneyway' were cultured on a modified QL-medium (Quoirin *et al.*, 1977), in which FeEDTA was replaced by the more stable iron chelate FeEDDHA, containing 4.4 μM 6-benzylaminopurine (BAP) and 0.49 μM indole-3-butyric acid (IBA) (Van der Salm *et al.*, 1994). For the production of transformed roots, stem slices were inoculated in a diluted overnight suspension (OD_{550} = 0.5) of *Agrobacterium tumefaciens* GV3101 and incubated on root induction medium with high IBA

concentration (1, 10 or 100 μM) during five days (Van der Salm *et al.*, 1996b). The following binary vectors were used: p*35SGUSINT* (Vancanneyt *et al.*, 1990) with the *GUSINT* gene driven by the CaMV 35S promoter, pPCV002-*B1100* with the *ROLB* gene driven by its own promoter (Spena *et al.*, 1987) and pMRKE15 with the *ROLA, B, C* genes under control of their own promoters (Vilaine *et al.*, 1987). Next to the *ROL* genes, all binary vectors contained the *NPTII* gene for conferring kanamycin resistance. Two days after infection stem slices were transferred to the same medium with 5 mg/L kanamycin, 200 mg/L cefotaxim and 400 mg/L vancomycin for selection on kanamycin resistance and inhibition of bacterial growth. Three days later stem slices were transferred to the same medium without hormones. Upon sustained incubation on this medium kanamycin resistant roots were excised from the stem slices after 3, 8 and 13 weeks.

Characterization of lateral root formation

First, *ROLB* transformed roots were propagated by lateral root formation. Ten independent kanamycin resistant roots containing the *ROLB* gene were excised from the stem slices three weeks after root induction and incubated in Erlenmeyer flasks (100 mL) with 10 mL liquid MS-medium (Murashige and Skoog, 1962) supplemented with 0.5 μM IBA, 88 mM sucrose, 50 mg/L cefotaxim and 50 mg/L vancomycin at pH 5.8. Five roots per flask were incubated on a rotary shaker (60 rpm) at 25°C in the dark. After four weeks lateral roots were excised and subcultured on fresh medium. After three subcultures lateral roots were used to quantify lateral root formation. For this, 25 mm root segments were incubated on the same MS-medium with 0.8% agar and various IBA concentrations. Per treatment four replications with five kanamycin resistant roots were incubated in Petri dishes at 22°C in the dark. The number of lateral roots was counted after three weeks.

For the effect of *ROLA, B, C* genes on lateral root formation similar experiments were carried out with primary kanamycin resistant roots, excised from stem slices after 13 weeks.

Regeneration of plants from transformed roots

For regeneration of shoots with the *ROLB* gene, first callus was induced on excised propagated roots of approximately 25 mm on basal medium (BM) (Van der Salm *et al.*, 1996a), which consisted of a modified SH-medium (Schenk and Hildebrandt, 1972) with 5, 16 or 50 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 600 mg/L L-proline and 0.8% agar (Oxoid, purified agar) at pH 5.8. Calluses were subcultured on the same medium in the dark for three months. For embryo induction calluses were transferred to the same medium with a reduced 2,4-D concentration (0, 5 or 16 μM, respectively) and incubated during one month. For each 2,4-D treatment, three lateral roots of 10 independent root clones were used. Subsequently, calluses were transferred to the same medium without 2,4-D and subcultured

during five months. For the outgrowth of shoots embryogenic structures were selected and subcultured on the same medium during three months in the light.

Based on the experience with the regeneration of *ROLB* transformed shoots, the regeneration procedure was improved and applied for roots with the *35SGUSINT* gene or a combination of *ROLA, B, C* genes. Callus was induced on excised primary roots on BM+, which consisted of an enriched SH-medium, supplemented with 50 μM 2,4-D, 200 mg/L cefotaxim, 400 mg/L vancomycin and 0.3% Gelrite (Phytagel, Sigma) at pH 5.8 (Van der Salm *et al.*, 1996a). Petri dishes with 10 roots each were incubated in the dark during one month. For embryo induction calluses were transferred to hormone-free BM with the same antibiotics and 0.8% agar instead of Gelrite and incubated during two months. Subsequently, calluses with organized structures were selected for shoot outgrowth and initially subcultured on BM+ with 8.9 μM BAP, 0.49 μM IBA and 0.3% Gelrite during three months in the light. After this period shoot outgrowth was accomplished on BM without hormones and 0.8% agar during six months.

Finally, transformed shoots were excised and propagated on the modified QL-medium with 4.4 μM BAP and 0.49 μM IBA (Van der Salm *et al.*, 1994). For transfer to the greenhouse shoots were rooted on the modified QL-medium with 1 μM IBA and transplanted into the soil after 10 days. After maintaining high relative humidity (RH) for two weeks, plants were acclimatized to greenhouse conditions.

Characterization of adventitious root formation

The effect of *ROL* genes on adventitious root formation was studied with in vitro grown plant material and with cuttings of greenhouse grown plants.

Rooting experiments were carried out with leaflets and stem explants of micropropagated plants, according to Van der Krieken *et al.* (1993) with some modifications (Van der Salm *et al.*, 1996b). Explants were treated with various concentrations of IBA for five days and subsequently transferred to hormone-free medium. After three weeks the percentage of rooted explants was counted.

Rooting experiments were also carried out with cuttings of *ROL* gene transformed plants taken from the greenhouse and cuttings taken from untransformed regenerants and plants derived from micropropagation, as a control. Per treatment 10 replicates with 10 cuttings each were used. Cuttings were treated with or without rooting powder (0.4% IBA), placed in a mixture of perlite, vermiculite and sand (1:3:1) and incubated in the greenhouse at 25°C, 100% RH and under daylight, supplemented with 6h illumination with Philips 400W HPI-T lamps. After two weeks cuttings were acclimatized by lowering RH. The number of roots per cutting was counted after one or three weeks.

Data analysis

Lateral and adventitious root formation of in vitro material were determined as the percentage of explants with lateral or adventitious roots, respectively. Adventitious root formation of greenhouse grown cuttings was characterized by the percentage rooted cuttings and the mean number of roots per rooted cutting. Data were analyzed by ANOVA, using the computer program Genstat (Genstat 5 Committee, 1988).

ß-Glucuronidase assay

Roots, embryogenic calluses and leaf explants with the *35SGUSINT* gene were histochemically assayed with 5-bromo-4-chloro-3-indolyl ß-glucuronide (X-gluc) for ß-glucuronidase (GUS) activity according to Jefferson *et al.* (1987) with the modifications of Van Altvorst *et al.* (1995).

DNA analysis

DNA was extracted from 0.5 g tissue of micropropagated shoots (Van der Beek *et al.*, 1992). DNA of a *ROLB* transformed *N. tabacum* cv. Petit Havana SR1 plant was used as a positive control. DNA samples were digested by restriction endonucleases, separated on a 0.7% agarose gel and blotted onto a 'Hybond-N+' membrane. A *ROLB* probe of 730 basepairs was obtained by PCR amplification of the *ROLB* gene of pMRKE15 using the oligonucleotide primers 5'-GAA CAC GGA GCA TCT CCT GGA-3' and 5'-ACT CCT CCC AAT TAT GGG CAT C-3'. For the *NPTII* probe a fragment of 465 basepairs was used according to Van Wordragen *et al.* (1993). After hybridization with random primer [^{32}P]-labelled fragments (Feinberg and Vogelstein, 1983), banding patterns were visualized by autoradiography and/or scanning with a bio imaging analyzer (Fuji BAS2000).

RNA analysis

Total RNA was extracted from micropropagated shoots and callus developed at the base of these shoots (De Vries *et al.*, 1991). Samples of 20 μg of total RNA were denatured with glyoxal, electrophorized on a 1.4% agarose gel, transferred to 'Hybond-N+' membranes for Northern analysis (Sambrook *et al.*, 1989) and hybridized with random primer [^{32}P]-labelled fragments, using the same *ROLB* probe as used in the Southern analysis.

Results

Formation of transformed roots

For the production of transformed rose plants, first kanamycin resistant roots were produced

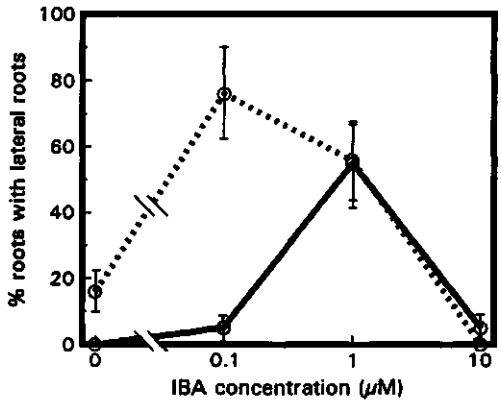

Figure 1. Lateral root formation on *35SGUSINT* (——) and *ROLA, B, C* transformed (·····) roots of the rose rootstock 'Moneyway', three weeks after excision. Lateral roots were induced with various concentrations of IBA. Each value is the mean of four replications, with five roots per replication ± SE.

on stem slices, which were cocultivated with *A. tumefaciens* harbouring the *35SGUSINT* gene, the *ROLB* gene or a combination of *ROLA, B, C* genes. Kanamycin resistant roots harbouring the *ROLB* gene were produced as described by Van der Salm *et al.* (1996b) and used for propagation on liquid MS-medium. Kanamycin resistant roots harbouring the *35SGUSINT* gene or the *ROLA, B, C* genes were produced on 1125 and 1000 cocultivated stem slices, respectively. In total 945 roots with the *35SGUSINT* gene and 1976 roots with the *ROLA, B, C* genes were excised after 3, 8 and 13 weeks. Since on average one to two roots per stem slice were harvested, this proved to be an efficient way for the production of transgenic roots.

At the second harvest after eight weeks, 131 kanamycin resistant roots formed on stem slices cocultivated with *35SGUSINT*, were treated with X-gluc. Blue staining was detected in 66% of the roots. This confirmed the presence and expression of the *35SGUSINT* gene in the roots. Apparently, the method allows the production of hundreds of transformed roots in subsequent harvests.

Lateral root formation on transformed roots

To study the effect of *ROL* genes on lateral root formation, kanamycin resistant roots were excised from the stem slices and incubated on various concentrations of IBA. Control roots harbouring the *35SGUSINT* gene, but no *ROL* genes, showed optimum lateral root formation at 1 μM IBA (Fig. 1), whereas roots transformed with the *ROLA, B, C* genes showed an optimum response at a lower IBA concentration (0.1 μM IBA). Even on medium without IBA lateral roots were formed on *ROLA, B, C* transformed roots. This suggests that the *ROL* gene

transformed roots were more sensitive to auxin. In contrast, *ROLB* transformed roots, which were propagated in liquid MS-medium, showed equal lateral root formation as control roots (data not shown), indicating that the *ROLB* gene had no effect on the auxin sensitivity.

Regeneration of plants from transformed roots

The first regeneration experiments were carried out with *ROLB* transformed roots, which were propagated in liquid MS-medium. To obtain embryogenic callus, a large number of root explants was cultured on various concentrations of 2,4-D and subcultured as described in materials and methods. Of the 90 combinations examined, only one was successful. After three months of incubation on 16 μM 2,4-D, one month on 5 μM 2,4-D and five months on hormone free medium one embryogenic callus was obtained. Three months later eight transformed shoots were raised from this embryogenic callus.

After the successful development of these *ROLB* transformed shoots from a single root, the procedure was further optimized and applied for the regeneration of plants from roots transformed with *ROLA, B, C* genes or *35SGUSINT* (Table 1). After one month callus induction on 50 μM 2,4-D and two months embryo induction on hormone-free medium the regeneration frequencies of *35SGUSINT* and *ROLA, B, C* transformed explants were comparable; 2 and 3% of the calluses formed organized structures, respectively. Compared to the method used for *ROLB* transformed roots, the period to induce embryogenesis was reduced from 9 to 3 months. Moreover, regeneration efficiency was higher. All twelve *35SGUSINT* transformed calluses with organized structures showed expression of the marker gene. Finally, four independent shoots with *35SGUSINT* and four independent shoots with *ROLA, B, C* genes were generated from the 6th till the 12th month after callus induction on roots. All four plants with the *35SGUSINT* gene stained blue upon treatment with X-gluc, indicating that the regenerant shoots were really transformed.

Table 1. Regeneration of transgenic 'Moneyway' plants from kanamycin resistant roots. *35SGUSINT* or *ROLA, B, C* transformed roots were induced to form embryogenic callus (after 12 weeks) and shoots (after 48 weeks).

	time (weeks)	*35SGUSINT*	*ROLA, B, C*
number of transformed roots	0	650	210
number of calluses with embryogenic structures	12	12 (2%)	6 (3%)
number of shoots	48	4 (1%)	4 (2%)

DNA analysis

To confirm the presence of *ROL* genes in the genome of rose, the eight shoots obtained from

one single *ROLB* transformed embryogenic callus were analyzed by Southern blotting. Hybridization of EcoRI or EcoRI/HindIII digested DNA with the *ROLB* probe generated the expected bands of 2.6 and 1.7 kb, respectively (Fig. 2A and 2C), similar as in a *ROLB* transformed plant of *N. tabacum* cv. Petit Havana SR1. These corresponding patterns demonstrated that the *ROLB* gene was fully integrated. Due to the smaller genome size (rose=100-800 Mb/1C, tobacco=4221-4646 Mb/1C), signal strength of rose was much higher than that of tobacco. Hybridization of HindIII digested DNA revealed two bands, that corresponded with two individual integrations. Furthermore all *ROLB* transformed shoots revealed the same banding pattern (data not shown), indicating that the eight regenerated subclones were indeed genetically uniform.

Southern analysis of DNA from the four putative transformants with the *ROLA, B, C* genes also affirmed their transgenic character (Fig. 2B and 2C). Hybridization with the *ROLB* probe revealed at least one internal XbaI fragment of 6.5 kb in each transformant, and proves the presence of an intact fragment harbouring the *ROLA, B, C* genes in these transgenic plants. Besides, transformants ABC2 and ABC3 showed a second fragment, indicating a recombined or partially integrated copy carrying at least the *ROLB* gene. To determine the copy number, XbaI digested DNA was hybridized with the *NPTII* probe. In the DNA of transformant ABC1 two fragments hybridized: one of 2.9 kb and one of approximately 5 kb. Since the 2.9 kb band was also present in DNA of the transformants ABC2 and ABC3, we suggest that this band represents a tandem repeat. We deduced that the transformants ABC1 and ABC2 contained one tandem repeat. Transformant ABC3 contained one tandem and at least three other copies, whereas ABC4 contained only one copy.

Phenotype of transformants

To reveal the effect of *ROL* genes on whole plant development, the phenotype of the transformants was studied during micropropagation. Transgenic plants with the *35SGUSINT* gene developed normally without any phenotypic change. Shoots of the *ROLB* transformant (B1) initially developed as untransformed shoots, but after six weeks they started to form adventitious roots at their base, whereas no roots were formed on the control shoots (Fig. 3). The four transformants with *ROLA, B, C* genes showed a more distinct phenotype than the *ROLB* transformant. Next to the formation of adventitious roots at their base after six weeks, three transformants (ABC1, ABC2 and ABC3) showed decreased shoot length and reduced apical dominance, while leaves of the fourth transformant (ABC4) were also wrinkled and epinastic (Fig. 3). The *ROLA, B, C* transformant ABC1 with a moderate phenotype, showing only reduced apical dominance and no wrinkled leaves, together with the *ROLB* transformant B1 were chosen for transfer to the greenhouse and characterization of rooting.

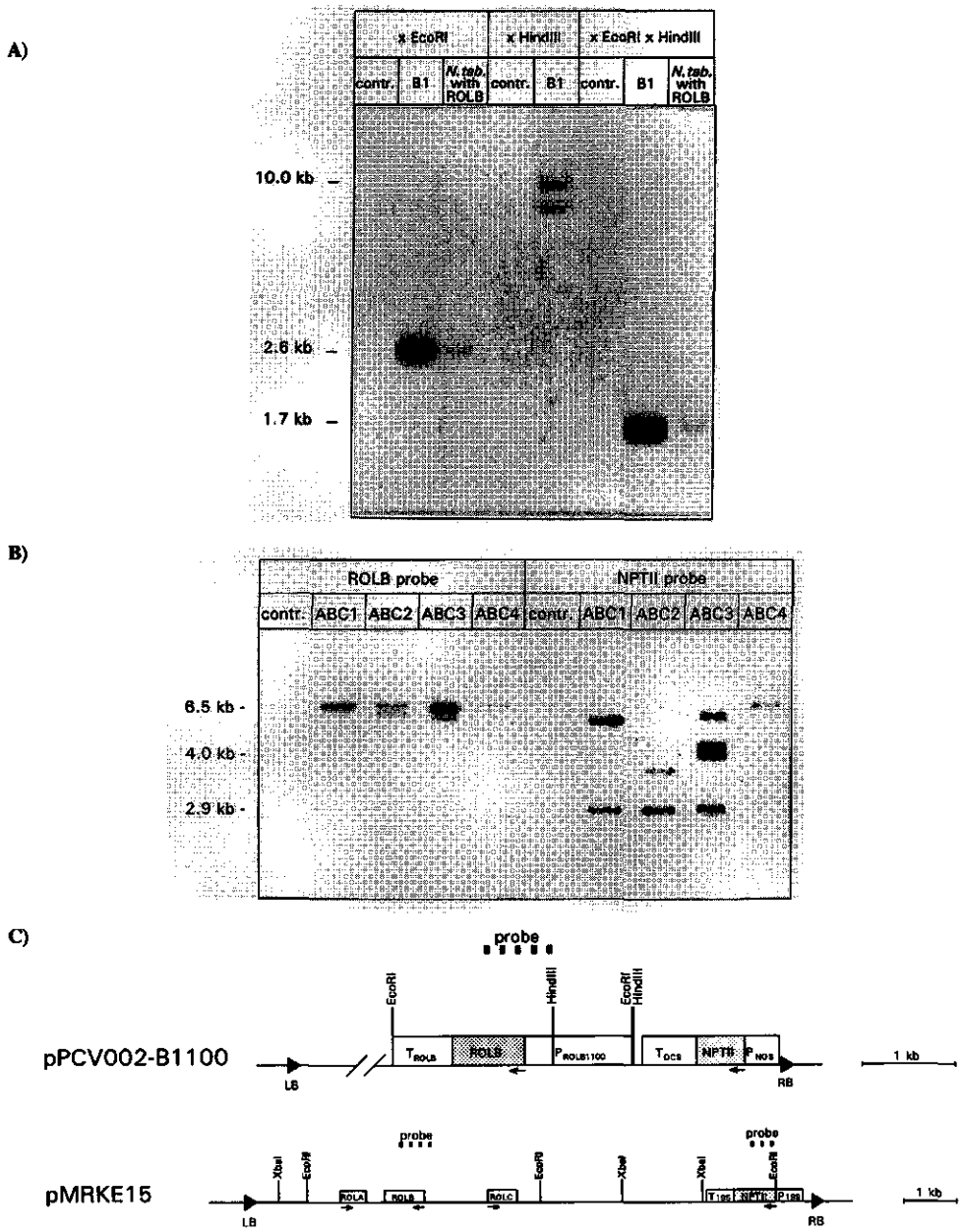

Figure 2. Southern blot analysis of *ROLB* (**A**) and *ROLA, B, C* (**B**) transformed shoots of the rose rootstock 'Moneyway'. Untransformed rose shoots and shoots of a *ROLB* transformed *Nicotiana tabacum* cv. Petit Havana SR1 served as a negative and positive control, respectively. DNA was digested with EcoRI and/or HindIII (**A**) and XbaI (**B**) and hybridized with a probe specific for *ROLB* (**A** and **B**) or *NPTII* (**B**). The T-DNA regions of pPCV002-*B1100* and pMRKE15 are shown in the lower panel (**C**). Fragments used as probe are indicated by dotted lines.

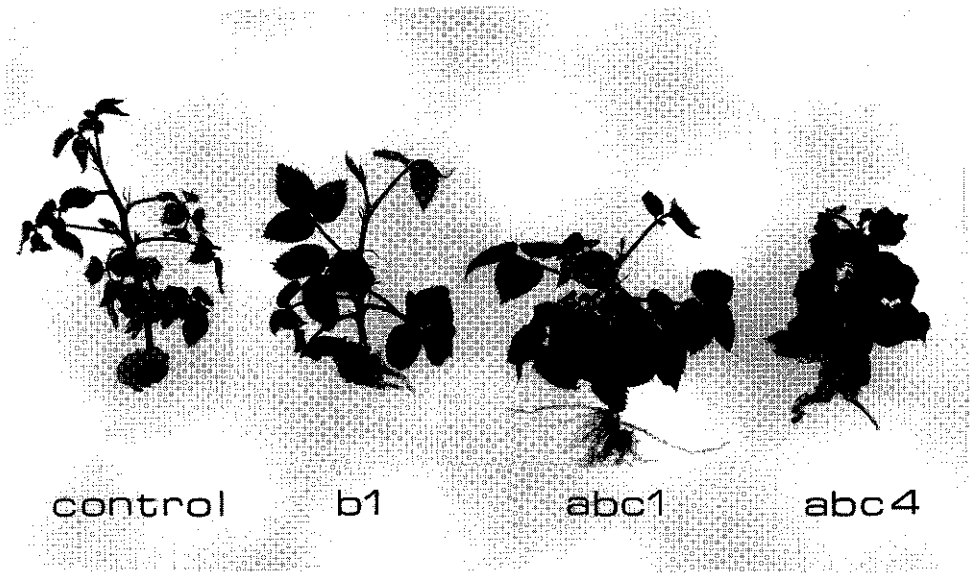

Figure 3. Transformed shoots from the rose rootstock 'Moneyway' after six weeks in micropropagation. From left to right: an untransformed shoot; a transformant with the *ROLB* gene (B1) showing adventitious roots; and two transformants with the *ROLA, B, C* genes, both with adventitious roots, one shoot showing decreased apical dominance (ABC1), while leaves of the other are wrinkled (ABC4).

Rooting in vitro

Leaflets and stem explants from micropropagated shoots were treated with various concentrations of IBA. Adventitious root formation on excised leaflets from untransformed shoots was initiated at a low concentration of exogenous auxin (0.1 μM IBA) and further stimulated by higher concentrations (1 and 10 μM IBA) (Fig. 4A). Root formation on leaflets from the *ROLB* transformant B1 already started (20% rooted explants) at 0 μM IBA and was further enhanced (60% rooted explants) at 0.01 μM IBA. On auxin-free medium, adventitious root formation on leaflets from transformant ABC1 was even higher (60% rooted explants). The results clearly show that adventitious root formation on excised leaflets is enhanced by the presence of the *ROLB* gene and even more pronounced by the *ROLA, B, C* genes.

On stem explants, the effects of auxin and *ROL* genes on adventitious root formation were different (Fig. 4B). First, adventitious root formation only occurred in the presence of exogenous auxin. Furthermore, stem explants from untransformed shoots showed a nice optimum curve for rooting with a maximum of nearly 100% rooting at 1 μM IBA. Remarkably, adventitious root formation on stem explants from transformant B1 was significantly lower; only 33% rooting at 1 μM IBA. Lower doses of auxin (down to 0.01 μM IBA during one day) were ineffective, indicating that a super sensitive response of this transformant could be excluded. In contrast to the stem explants from transformant B1, stem

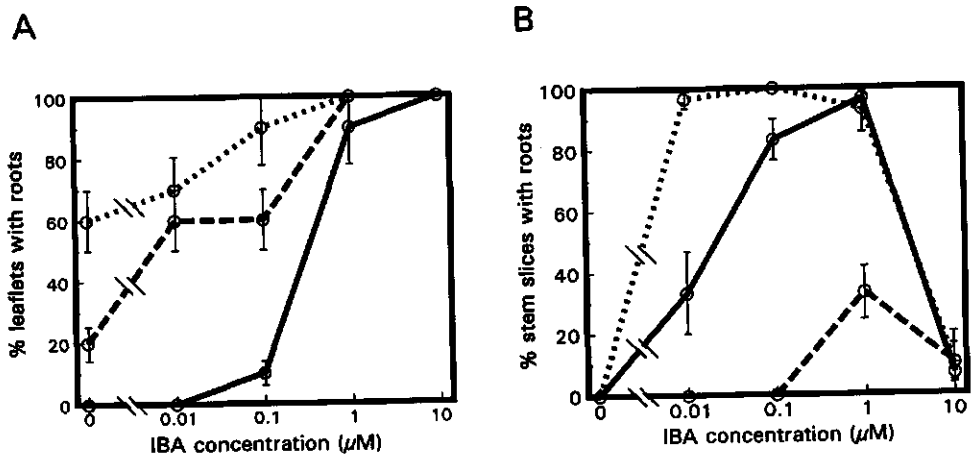

Figure 4. Adventitious root formation on leaflets (**A**) and stem slices (**B**) of control (——), *rolB* transformed (----) and *ROLA*, *B*, *C* transformed (·····) shoots of the rose rootstock 'Moneyway'. Adventitious root formation was determined three weeks after the start of root induction with various concentrations of IBA. Each value is the mean of three (**A**) or two (**B**) replications, with 10 explants per replication ± SE.

explants from transformant ABC1 showed a higher rooting response at low auxin concentrations (0.01 and 0.1 μM IBA). These results indicate that the sensitivity of stem explants for exogenous auxin was decreased by the *ROLB* gene and increased by a combination of *ROLA*, *B* and *C* genes.

To explain the unexpected rooting characteristics of the *ROLB* transformant B1, expression

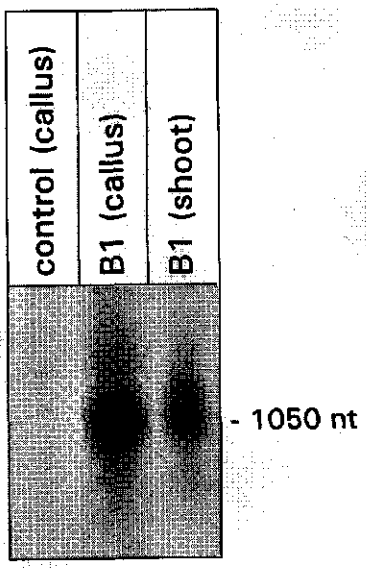

Figure 5. Northern blot analysis of *ROLB* transformed shoots and callus of the rose rootstock 'Moneyway'. The blot was hybridized with a *ROLB* specific probe.

of the *ROLB* gene was studied by Northern blot analysis of total RNA, which was isolated from micropropagated shoots and callus from the base of these shoots. After hybridization with the *ROLB* probe a strong signal of the expected size (± 1050 nt) was present in both lanes, while no signal was present in callus RNA of an untransformed control (Fig. 5). It demonstrated that the *ROLB* gene was active in this transformant. In RNA from micropropagated shoots a faint additional band of 1200 nt also hybridized (see also Chapter 6).

Rooting of cuttings

The effect of *ROL* genes on the rooting ability of cuttings was also studied with the transformants B1 and ABC1. Upon treatment with rooting powder, no differences in adventitious root formation were found between cuttings of B1 and control cuttings after one and three weeks (data not shown). However, in the absence of rooting powder, cuttings of B1 did not only show a higher rooting percentage than control cuttings after three weeks, but also yielded more roots per rooted cutting (Table 2A). This demonstrates that the *ROLB* gene promoted adventitious root formation on cuttings, but only in the absence of exogenous auxin.

Table 2. Rooting performance of cuttings of transgenic rootstocks of *R. hybrida* L. 'Moneyway'. Cuttings from *ROLB* transformed plants (B1) were treated without rooting powder and measured after three weeks (**A**), while cuttings from *ROLA*, *B*, *C* transformed plants (ABC1) were treated with rooting powder and measured after one week (**B**). n=10 replications with 10 cuttings per replication, ***=significant at $p=0.001$.

A)

	rooting%	number of roots/rooted cutting
control	29	1.7
B1	82	4.2
genotype-effect	***	***
$LSD_{0.05}$	25	1.2

B)

	rooting%	number of roots/rooted cutting
control	20	2.6
ABC1	60	8.4
genotype-effect	***	***
$LSD_{0.05}$	18	3.1

Adventitious root formation was even more intense on cuttings of the transformant ABC1. After treatment with rooting powder, the majority of cuttings were already densely rooted after one week, whereas control cuttings were only poorly rooted (Table 2B). This indicates that the presence of the *ROLA* and *ROLC* genes, together with the *ROLB* gene, even further stimulated the rooting ability.

Discussion

This research has successfully led to a transformation procedure for the recalcitrant species *R. hybrida* L.. The rose rootstock 'Moneyway' was transformed via a two step procedure. First, kanamycin resistant roots were formed on stem slices, followed by regeneration of transformed plants from these roots via somatic embryogenesis. The formation of kanamycin resistant roots was efficient; up to two roots per stem slice were formed and hundreds of roots could be easily produced within a period of 13 weeks. However, regeneration of organized callus from transformed roots was less efficient (2-3%), also compared to that from untransformed roots in the same system (6 or 16%) (Van der Salm *et al.*, 1996a). This suggests that the presence of kanamycin during the selective formation of roots had a negative effect on the subsequent formation of embryogenic callus. Finally, 19 independent embryogenic callus lines were obtained. From these calluses nine transgenic plants were obtained; four with the reporter gene *35SGUSINT*, one with the *ROLB* gene and four with a combination of *ROLA*, *B* and *C* genes. Among these regenerants no escapes were found, showing that the initial selection on kanamycin resistance was reliable.

This transformation procedure gives opportunities for transformation of other rose cultivars (Derks *et al.*, 1995) and recalcitrant species, which are easy to root and which regenerate from roots, such as: *Citrus* (Burger and Hackett, 1986), *Faidherbia* (Ahée and Duhoux, 1994), *Prunus* (Kolova, 1993) and *Malus* (Lambert and Tepfer, 1992). Recently, another transformation method for rose was reported (Firoozabady *et al.*, 1994). Although transformation of embryogenic callus and subsequent development of transformed plants occurred very efficient for the cut rose 'Royalty', use of this method was limited due to the poor induction of embryogenic callus. Furthermore, regeneration was only accomplished on callus induced on stamen filaments, which are usually not available for rose rootstocks.

The production of hundreds of kanamycin resistant roots enabled us to study the effect of *ROL* genes on lateral root formation. Lateral root formation on roots transformed with a combination of *ROLA*, *B* and *C* genes was at 0 and 0.1 μM IBA considerably higher than that on control roots with *35SGUSINT*. In contrast, the *ROLB* gene had no effect on lateral root formation. These findings correspond with an increased lateral root formation, which was

found in *ROLC* transformed roots of tobacco (Schmülling *et al.*, 1988), suggesting a role of the *ROLC* gene in lateral root formation.

Plant morphology of the four *35SGUSINT* transformants was normal. Despite the long callus phase necessary to obtain plant regeneration, somaclonal variation was not observed. In contrast to these *35SGUSINT* transformed shoots, micropropagated shoots of the *ROLB* and the *ROLA, B, C* transformants formed adventitious roots at their base after six weeks, while shoots with the *ROLA, B, C* genes also showed reduced apical dominance, decreased shoot length and occasionally wrinkled leaves. These observations agree well with the phenotypes of *ROL* gene transformed plants of *N. tabacum* (Schmülling *et al.*, 1988).

Adventitious root formation on micropropagated rose shoots and excised leaflets was increased by the presence of the *ROLB* gene and especially by the *ROLA, B, C* genes. The latter finding agrees well with the increased rooting ability of microcuttings and leaf discs of *ROLA, B, C* transformed kiwi (Rugini *et al.*, 1991). The observation that adventitious root formation on leaflets of the *ROLB* transformed roses even occurred in the absence of exogenous auxin, is in line with the idea that the *ROLB* gene increases the auxin sensitivity. Endogenous auxin levels in leaflets of the *ROLB* transformed plants seem to be sufficiently high for the induction of adventitious roots. This increase in sensitivity corresponds with the increased auxin sensitivity in tobacco mesophyll protoplasts upon transformation with *ROL* genes (Maurel *et al.*, 1991; 1994). The higher rooting response on *ROLA, B, C* transformed leaflets compared to the *ROLB* transformed leaflets might be due to the *ROLA* gene, which facilitated adventitious rooting on leaf disks of tobacco on hormone-free medium (Carneiro and Vilaine, 1993).

In contrast, adventitious root formation on stem explants was only stimulated by a combination of *ROLA, B* and *C* genes, whereas the *ROLB* transformant remarkably showed decreased rooting. The latter might be due to overexpression of the *ROLB* gene, as suggested for a non-root-forming callus of *N. tabacum* transformed with the *ROLB* gene (Spena *et al.*, 1987). Therefore *ROLB* expression in micropropagated shoots and callus was studied by Northern analysis. Only 20 μg of total RNA was required to obtain a strong signal and this was far less than the 8 μg of poly(A)$^+$ RNA, as described for the only reported Northern analysis of *ROLB* plants so far (Schmülling *et al.*, 1988). The elevated *ROLB* expression in callus developed at the base of shoots might be incited by the basipetal flow of auxin from the apex to the callus, since the promoter of the *ROLB* gene is regulated by auxin (Maurel *et al.*, 1990). In stem slices, treated with the auxin IBA, we expect a similar rise in *ROLB* expression. This elevated expression might be too high to stimulate adventitious root formation.

To summarize, in this research we developed a method for the production of transformed roses. With the production of *ROL* gene transformed rootstocks with improved rooting

characteristics, an useful trait was introduced in rose. As an important practical implication it was also demonstrated for the first time that the rooting characteristics of a woody species grown in the greenhouse, were remarkably improved upon introduction of *ROL* genes. The transformed rootstocks will be further assessed in the greenhouse and will be used for the production of rootstock-scion combination plants.

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Chapter 6

Stimulation of scion bud release by *ROL* gene transformed rootstocks of *Rosa hybrida* L.

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Abstract

For improvement of the root characteristics of roses, *ROL* genes from *Agrobacterium rhizogenes* were introduced in the cultivar Moneyway, which is used as a rootstock. One transformant with the *ROLB* gene (B1) and four independent transformants with a combination of *ROLA, B, C* genes (ABC1 to ABC4) were examined. Transformation with *ROL* genes drastically affected the phenotype of the rootstock plant: increased (*ROLB*) or decreased (*ROLA, B, C*) apical dominance, altered leaf shape and a reduced plant weight. The presence of wrinkled, round-edged or small lanceolate leaves in the transformants was correlated with the transcription of a *ROLA* messenger of 650 nt, a *ROLB* messenger of 1050 nt and a *ROLC* messenger of 850 nt, respectively. These observations suggest that only one of the *ROL* genes was abundantly transcribed at a time. The *ROLB* transformant and one *ROLA, B, C* transformant were used as rootstocks with the cut rose cultivar Madelon as scion. The growth and development of the non-transformed aerial parts were not affected by the *ROLB* gene in the rootstock, although *ROLB* transformed roots in combination plants were smaller than those of the non-transformed control. In contrast, grafting on the *ROLA, B, C* transformed rootstock resulted in a stimulation of both root development of the rootstock and axillary bud release of the untransformed scion. Axillary bud release increased from 0.1 to 0.6 and from 0.3 to 1.3 basal shoot per plant at 15 and 20°C, respectively. Since axillary shoots (the basal shoots) form flowers, this altered plant architecture will presumably lead to a more efficient flower production.

Introduction

Most cut roses are grown on a rootstock to increase flower production, but also to overcome deficiencies in adventitious root formation and to obtain disease resistance in the root part. The use of rootstocks can be rationalized by the fact that breeding activities of cut roses were usually focused on flower quality and colour, and not on the characteristics of the hidden roots. To provide rose plants with a root system that supports vigorous growth, rootstocks with specific root traits are used.

Plant developmental processes, such as adventitious root formation and axillary shoot formation, are controlled by plant hormones. For the induction of adventitious roots auxin is considered as the key hormone (Epstein and Ludwig-Müller, 1993). Auxin is produced in apices and leaves of growing plants and is transported basipetally. Adventitious root formation is antagonistically and synergistically affected by cytokinin and ethylene, respectively. Control of axillary bud release is also regulated by auxin and cytokinin (Cline,

1991). Upon obstruction of the basipetal auxin flow by decapitation, the level of cytokinin in the xylem increased, leading to the release of axillary buds (Bangerth, 1994). It clearly demonstrates the importance of a balance between auxin and cytokinin. Since cytokinin is predominantly synthesized in the roots (Chen *et al.*, 1985) and transported to the aerial parts of the plant, a prominent role in axillary bud release might be attributed to the hormonal status of rootstocks. Therefore, genetic modification of rootstocks with genes that influence hormone metabolism or hormone sensitivity, presumably results in rootstocks with changed hormonal status, which may lead to improved properties of the scion such as stimulation of axillary bud release.

Suitable genes are genes from *Agrobacterium*, and especially the *ROL* genes from *A. rhizogenes*, which interfere with hormone metabolism and plant development. Transformation with the *ROLB* gene resulted in increased adventitious root formation, indicating increased auxin activity (Cardarelli *et al.*, 1987). It has also been shown that the presence of a *ROLB* gene affected auxin sensitivity in tobacco mesophyll protoplasts (Maurel *et al.*, 1991; 1994). Likewise, transformation with the *ROLC* gene resulted in plants with reduced apical dominance and internodal length, indicating a cytokinin-like effect (Schmülling *et al.*, 1988). Furthermore, an indoxyl-ß-glucosidase and a cytokinin-ß-glucosidase activity were found for the ROLB and ROLC proteins, respectively (Estruch *et al.*, 1991a and b). However, such enzymatic activities do not seem to be expressed in transgenic plants since auxin and cytokinin levels were not increased in *ROLB* and *ROLC* transformed plants (Nilsson *et al.*, 1993a and b).

The *ROL* genes were already successfully utilized for improvement of the rooting ability of several woody species. For instance, the rooting ability of microcuttings of kiwi was increased considerably upon introduction of a combination of *ROLA*, *B*, *C* genes (Rugini *et al.*, 1991). Unfortunately the introduction of *ROL* genes gives rise to pleiotropic effects in the aerial parts of the plant due to the role that hormones play in various aspects of growth and development. Many of these problems can be overcome by using combination plants as in rose culture. This gives the opportunity for genetic modification of the rootstock without direct interfering with the habitus of the scion, which yields the desired horticultural products i.e. the cut flowers of rose.

Upon the introduction of *ROL* genes in the rose rootstock 'Moneyway', we investigated the influence of these transgenic rootstocks on the growth of combination plants. Since growth parameters very much depend on temperature, growth and development of the combination plants were followed at two temperatures. It was shown that not only the growth characteristics of the *ROL* gene transformed roots were profoundly changed, but also those of the untransformed scion.

Material and methods

Production of transformed plants

Transformed plants of the rootstock *Rosa hybrida* L. 'Moneyway' were produced via a two step procedure. First, transformed roots were produced on stem slices (Van der Salm *et al.*, 1996b), which were cocultivated with *Agrobacterium tumefaciens* GV3101(pPCV002-*B1100*) harbouring the *ROLB* gene driven by its own promoter (Spena *et al.*, 1987) or with GV3101(pMRKE15) harbouring a combination of *ROLA*, *B*, *C* genes under control of their own promoters (Vilaine *et al.*, 1987). Both strains contained the neomycin phosphotransferase (*NPTII*) gene for conferring kanamycin resistance. In the second step, whole plants were regenerated from these roots via somatic embryogenesis (Van der Salm *et al.*, 1996a).

Finally, one *ROLB* transformant (B1) and four independent transformants with a combination of *ROLA*, *B*, *C* genes were obtained (ABC1, ABC2, ABC3 and ABC4). Their transgenic character was confirmed by Southern analysis (Chapter 5). Transgenic shoots were propagated on a modified QL-medium (Quoirin *et al.*, 1977; Van der Salm *et al.*, 1994), in which FeEDTA was replaced by the more stable iron chelate FeEDDHA and that contained 4.4 μM 6-benzylaminopurine (BAP) and 0.49 μM indole-3-butyric acid (IBA). For transfer to the greenhouse, shoots were rooted on the modified QL-medium with 1 μM IBA and transplanted to the soil after 10 days. After maintaining high relative humidity (RH) for two weeks, plants were acclimatized to greenhouse conditions.

Propagation of rootstock plants

Cuttings were taken from greenhouse grown plants, treated with rooting powder (0.4% IBA), placed in a mixture of perlite, vermiculite and sand (1:3:1) and incubated in the greenhouse at 25°C, 100% RH and under daylight supplemented with 6h illumination with Philips 400W HPI-T lamps. After two weeks cuttings were acclimatized by lowering RH. Rooted cuttings were transferred to hydroculture after three weeks.

Production of combination plants

Combination plants were made by cutting-grafting according to Van de Pol and Breukelaar (1982) with some modifications. First, rootstock cuttings were taken and treated with rooting powder. After one week, when root primordia and small roots were already formed, the apical end of the stem was excised. On top of the rooted internode, a nodal stem segment of the cultivar Madelon was grafted. Further culture and treatment of these combination plants continued, as described for the propagation of rootstock plants. After three weeks combination plants were used for growth analysis on hydroculture.

Growth analysis of rootstock and combination plants

Performance of rootstock and combination plants was studied using hydroculture in climate rooms at 15 or 20°C, with 16 h light (Philips 400W HPI-T lamps, 150 μmol.m^{-2}.s^{-1}) and 70% RH. Plants were grown on tanks with 500 L circulating nutrient solution, which was developed by De Kreij and Kreuzer of the Research Station for Floriculture and Glasshouse Vegetables in Aalsmeer, the Netherlands. Plants were positioned according to a randomized block design.

For morphological analysis, 20 rooted cuttings of the *ROLB* transformant and four rooted cuttings of the four *ROLA, B, C* transformants were grown in hydroculture at 20°C. After six weeks the number of axillary shoots was counted and plants were harvested for determination of leaf area (measured with a Li-Cor area meter Model Li-3100) and dry weight of various plant organs.

For evaluation of the effects of *ROL* genes on growth and development of the scion, per treatment 20 rooted combination plants were placed on hydroculture at 15 or 20°C. The length of the primary shoot was measured weekly. After 11 (15°C) or 9 (20°C) weeks the number of axillary shoots was counted, while leaf area and dry weight of various organs were also determined. The various organs of a combination plant are schematically represented in Figure 1.

Data were submitted to statistical examination by analysis of variance (ANOVA) or by

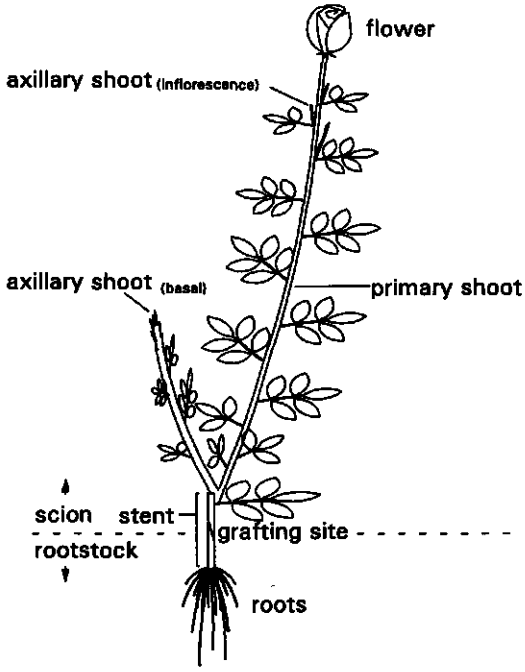

Figure 1. Schematic representation of a combination plant with measured elements.

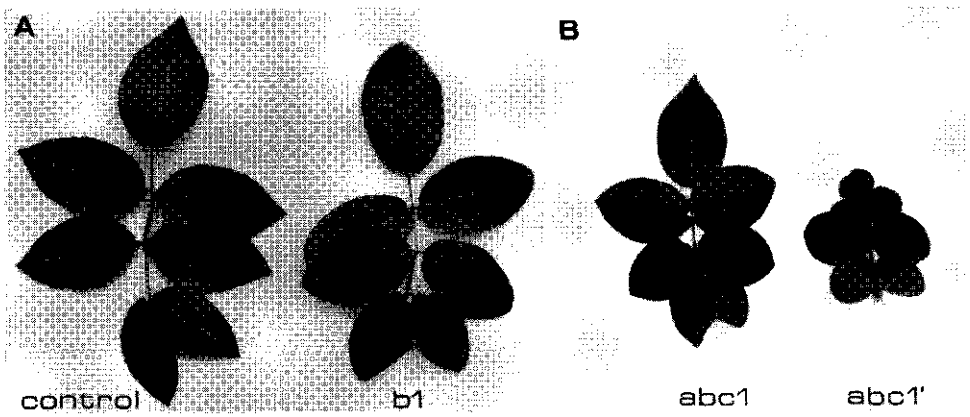

Figure 2. Morphological characteristics of leaves of control and transformed plants of 'Moneyway'. From left to right: (**A**) control (lanceolate), B1 (round-edged). (**B**) ABC1 (lanceolate), ABC1' (round-edged).

using a generalized linear model (GLM) with a log link function and Poisson distribution, according to Jansen and Hoekstra (1993), using the computer program Genstat (Genstat 5 Committee, 1988). When relevant, a LSD (ANOVA) or an approximate LSD (GLM) was given ($p=0.05$).

RNA-analysis

For Northern analysis RNA was extracted from various organs (De Vries *et al.*, 1991). Samples of 20 μg of total RNA were denatured with 1.5 *M* glyoxal and electrophorized on a 1.4% agarose gel, transferred to a 'Hybond-N+' membrane and hybridized with a random primer [^{32}P]-labelled probe (Sambrook *et al.*, 1989), specific for the *ROLA*, *ROLB* or *ROLC* gene. These DNA fragments (420, 820 and 640 bp in length) were obtained by PCR amplification of pMRKE15 (Vilaine *et al.*, 1987), using the following sets of primers, which were deduced from the sequence of the TL-DNA of *A. rhizogenes* (Slightom *et al.*, 1986): for *ROLA*: 5'-CTAAGATCATGCTGTAACGCTTC-3' and 5'-GCGTATTAATCCCGTAGGTTTGT-3', for *ROLB*: 5'-GAACACGGAGCATCTCCTGGA-3' and 5'-ACTCCTCCCAATTATGGGCATC-3' and for *ROLC*: 5'-TTGTGGACCTATACCTAACTCAA-3' and 5'-TCCATCTGCTCATTCAGCTTGA-3'. After hybridization filters were washed, exposed to X-ray film at -80°C using intensifying screens and/or scanned with a bio imaging analyzer (Fuji BAS2000) for quantitative measurement.

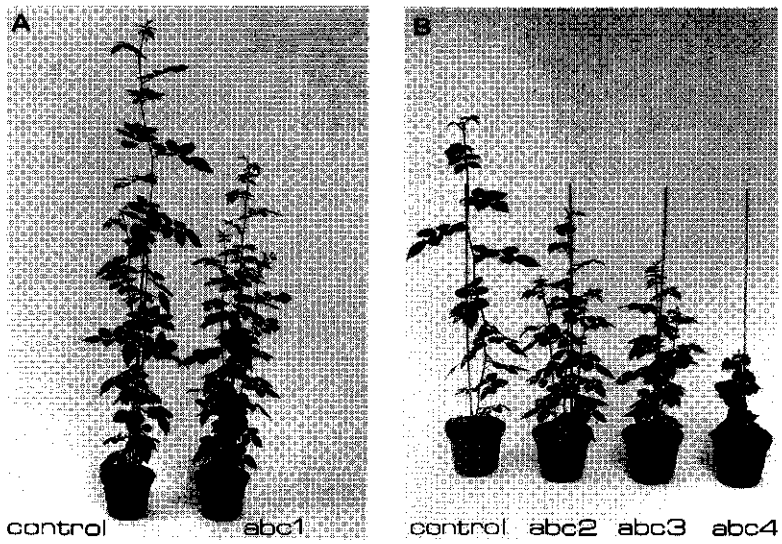

Figure 3. Phenotypical characteristics of control and *ROLA, B, C* transformed plants of 'Moneyway' in the soil. (A) Left: control, right: ABC1 after 12 weeks. (B) From left to right: control, ABC2, ABC3 and ABC4 after 9 weeks.

Results

Morphological characterization of transformed plants

Transgenic plants of the rose rootstock 'Moneyway' harbouring either the *ROLB* gene or the *ROLA, B, C* genes were grown in the greenhouse. The *ROLB* transformant B1 resembled control plants in size. However, leaves of the *ROLB* transformant were round-edged (Fig. 2A). *ROLA, B, C* transformed plants were smaller, had shorter internodes and exhibited increased lateral branching as shown for ABC1 in Fig. 3A. Leaves of this transformant were lanceolate, as in the control, but smaller (Fig. 2B). Occasionally, the morphology of these plants changed, giving rise to even smaller leaves with a round-edged leaf shape, which are denoted as ABC1' for transformant ABC1. There was also considerable variation between the four independently transformed *ROLA, B, C* plants (Fig. 3B). The transformants ABC1, ABC2 and ABC3 slightly differed in phenotype, whereas transformant ABC4 showed a more severe phenotype with a poorly developed primary shoot and wrinkled leaves.

The performance of the *ROLB* transformant and the four *ROLA, B, C* transformants was analyzed by growing them in hydroculture. The results of this growth analysis are shown in Table 1A and B. Compared to the untransformed 'Moneyway' plants, the *ROLB* transformant showed a nearly complete inhibition of axillary shoot formation. This increase in apical dominance was also reflected by a decreased total dry weight (W_{dry} total) and a decreased total leaf area. Since the root system of the *ROLB* transformant was proportionally reduced in weight, the shoot/root ratio was the same in control and *ROLB* transformant. This

Table 1. Growth parameters of transgenic rootstocks of *R. hybrida* L. 'Moneyway'. Cuttings from the *ROLB* transformant B1 (A) or the *ROLA, B, C* transformant ABC1 (B) were grown on hydroculture at 20°C and harvested after six weeks. as=axillary shoots, ps=primary shoot, n=5 (A) or n=1 (B) replications with four plants per replication.

A)

				significance	
parameter	unit	control	B1	genotype	$LSD_{0.05}$
number of as[x]		1.7	0.1	***	0.3
W_{dry} ps[y]	g	11.3	9.3		
W_{dry} as[y]	g	2.7	0.0	***	1.1
W_{dry} cutting[y]	g	1.1	1.1		
W_{dry} roots[y]	g	1.2	0.9	**	0.2
W_{dry} total[y]	g	16.3	11.3	***	2.6
shoot/root[y]		13.6	13.6		
leaf area[x]	cm^2	1998	1176	***	259

B)

							significance	
parameter	unit	control	ABC1	ABC2	ABC3	ABC4	genotype	$LSD_{0.05}$
number of as[x]		2.0	10.5	6.0	6.8	0.0	***	3.2
W_{dry} ps[y]	g	10.7	4.1	4.1	2.4	1.0	***	1.4
W_{dry} as[y]	g	2.4	5.5	5.3	3.0	0.0	***	1.8
W_{dry} cutting[y]	g	0.6	0.6	0.4	0.3	0.1	***	0.1
W_{dry} roots[y]	g	1.5	1.1	0.9	1.0	0.1	***	0.5
W_{dry} total[y]	g	15.3	11.2	10.6	6.7	1.1	***	3.0
shoot/root[y]		9.2	9.6	11.0	6.5	31.6	***	3.0
leaf area[x]	cm^2	1928	2324	2350	1558	184	***	602

[x]=GLM, *when relevant a maximum LSD is given since SEs are proportional for a non-linear model*
[y]=ANOVA
, *=significant at p=0.01 and 0.001, respectively.

transformant was further used for analysis of its properties as a rootstock.

In contrast with the *ROLB* transformant, more axillary shoots were formed on the *ROLA, B, C* transformed 'Moneyway' plants. These *ROLA, B, C* transformed plants formed 3-5 times more axillary shoots, except for transformant ABC4. This plant was highly aberrant, showing dwarf growth and wrinkled leaves (see also Fig. 3B). Of course the increase in number of axillary shoots was correlated with an increased dry weight of axillary shoots

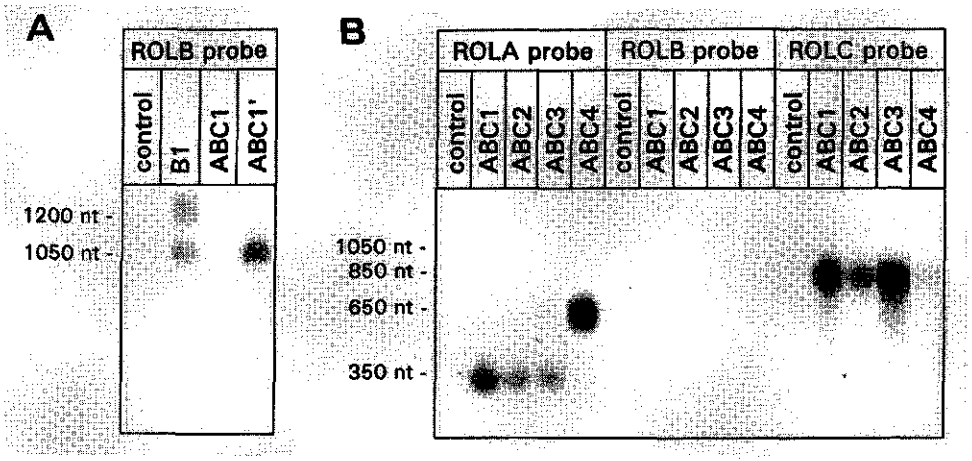

Figure 4. Northern analysis of transformants. **(A)** RNA was extracted from leaves of an untransformed control, round-edged leaves of the *ROLB* transformant (B1) and lanceolate leaves (ABC1) and round-edged leaves (ABC1') of the *ROLA, B, C* transformant ABC1. The blot was hybridized with a *ROLB* specific probe. **(B)** RNA was extracted from leaves of an untransformed control and of the *ROLA, B, C* transformants ABC1 to ABC4. Blots were subsequently hybridized with a *ROLA*, a *ROLB* and a *ROLC* specific probe.

(W_{dry} as), but the total dry weight (W_{dry} total) of these plants was lower than that of the control. This was due to a strongly reduced primary shoot of the transgenic plants. As for the *ROLB* transformant, also the root systems of the *ROLA, B, C* transformants were reduced in weight. Since transformant ABC1 showed a moderate phenotype with good growth and clearly decreased apical dominance, this plant was selected to examine the effect of a *ROLA, B, C* transformed rootstock on growth and development of combination plants.

RNA analysis

Expression of the *ROL* genes was studied in greenhouse grown plants. Using a probe for the *ROLB* gene, the mRNA of 1050 nt was detected in various organs of the *ROLB* transformant. In leaves an unknown additional band of 1200 nt hybridized with this probe (Fig. 4A). Transcription of the 1050 nt mRNA was quantified in various parts of the plant (Table 2) and showed to be organ dependent. It was very high in the shoot apex, while also axillary buds, roots, stem and young leaves showed a clear signal. In contrast to young leaves, the expression level in old leaves was very low.

Expression of individual *ROL* genes was studied in the leaves of the *ROLA, B, C* transformants. After hybridization with a *ROLA* specific probe, only transformant ABC4

showed the expected messenger of 650 nt. In RNA isolated from the three other transformants another signal was found, indicating a much shorter messenger of approximately 350 nt (Fig. 4B). Remarkably, no hybridization was observed with the *ROLB* specific probe, indicating that the *ROLB* gene was not expressed in the leaves of these plants. After hybridization with the *ROLC* probe, a messenger of 850 nt was observed in RNA from all four transformants, although the expression level in the aberrant transformant ABC4 was low. The expression in stems and roots of ABC1 showed a similar pattern as that in lanceolate leaves; only expression of the *ROLC* gene (data not shown).

The expression of the *ROLB* gene was studied in more detail. As already mentioned, the *ROLA, B, C* transformants also possessed 'aberrant' round-edged leaves, which resembled the leaves of the *ROLB* transformant. In these small, round-edged leaves of ABC1, a *ROLB* messenger of 1050 nt was observed (Fig. 4A), similar to the *ROLB* transformant. However, the *ROLA* and *ROLC* genes were not expressed in these leaves (results not shown), while in lanceolate leaves, *ROLC* expression was clearly observed (Fig. 4B). This implies that the round-edged leaf shape is correlated with a *ROLB* messenger of 1050 nt. It also suggests that expression of the *ROLB* gene did not coincide with that of the *ROLA* and *ROLC* genes.

Table 2. Quantitative representation of the relative expression of the *ROLB* gene in RNA extracted from various organs of the transformant B1. Expression in the shoot apex was indexed as 100% (n=2 independent quantifications).

plant organ	*ROLB* mRNA (%)
shoot apex	100
axillary buds	58
stem	41
young leaves	25
old leaves	4
roots	50

Growth and development of combination plants

Combination plants, consisting of the cultivar Madelon grafted on a *ROLB* or *ROLA, B, C* transformed 'Moneyway' rootstock, were grown on hydroculture at 15 and 20°C. The growth of primary shoots of combination plants with *ROLB* transformed rootstocks followed a sigmoid curve and the plants reached a maximum length after six weeks at 20°C (Fig. 5A). Growth of primary shoots at 15°C was prolonged until week 9, resulting in larger flower stems. Scions grafted on the *ROLB* transformed rootstock and those on control rootstocks grew synchronously; the growth curves coincide at both temperatures. After 11 (15°C) or 9 (20°C) weeks all combination plants were harvested for measurement of several parameters (Table 3A). We were primarily interested in genotypical differences at both temperatures. First, a distinction was made between axillary shoots formed right below the apical end of

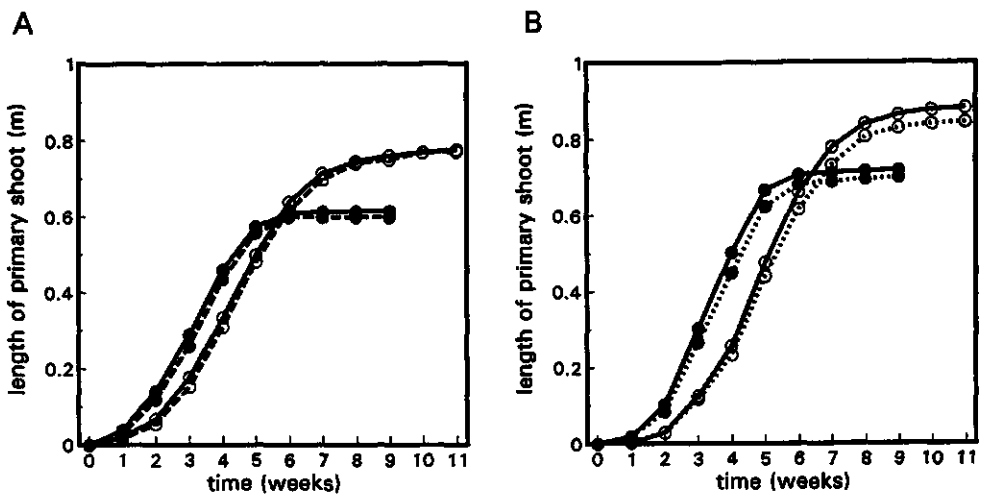

Figure 5. Growth of the length of the primary shoot of combination plants at 15°C (open circles) or 20°C (closed circles). The performance of untransformed rootstocks (——) was compared with that of (A) the *ROLB* transformed rootstock B1 (----) and (B) the *ROLA, B, C* transformed rootstock ABC1 (·····).

the primary shoot ($as_{inflorescence}$) and axillary shoots formed at the basis of the primary shoot (as_{basal}) (see also Fig. 1). Regarding these parameters, no differences were found between plants growing on control or *ROLB* transformed rootstocks. This also holds for the other parameters (e.g. dry weight of primary shoot and flower) except for the dry weight of the roots (W_{dry} roots). The root system of the *ROLB* transformed rootstocks was less developed, resulting in a decreased dry weight, both at 15 and 20°C. Despite this difference in root weight, total dry weights of both types of combination plants were not significantly different. This also resulted in a higher shoot/root ratio of combination plants with a *ROLB* transformed rootstock. This suggests that the *ROLB* transformed rootstock was more efficient in supporting growth of aerial parts.

Effects observed in combination plants, consisting of 'Madelon' grafted on the *ROLA, B, C* transformed rootstock ABC1, were much more pronounced. Already at the start of analysis, root systems of combination plants with ABC1 as a rootstock, were much further developed than control root systems (14 vs. 8 roots per plant, respectively). Despite this difference in root quantity, growth of the primary shoots of combination plants grafted on the rootstock ABC1 did not differ significantly from that of the control (Fig. 5B). As described for combination plants with *ROLB* transformed rootstocks, growth at 15°C was much slower and lasted until week 9. During culture and at the time of harvest after 11 (15°C) or 9 (20°C) weeks, root systems of the transformed rootstock ABC1 were still more

Table 3. Growth parameters of combination plants, consisting of the cultivar 'Madelon', grafted on a transformed or untransformed rootstock of *R. hybrida* L. 'Moneyway'. Combination plants with the *ROLB* transformed rootstock B1 (A) or the *ROLA, B, C* transformed rootstock ABC1 (B) were grown on hydroculture during 11 (15°C) or 9 (20°C) weeks. as_{basal}=axillary shoots at the base of the primary shoot, $as_{infl.}$=axillary shoots in the inflorescence, ps=primary shoot, n=5 replications with 4 plants per replication.

A)

		15°C		20°C		significance		
parameter	unit	control	B1	control	B1	genotype	genotype x temp.	$LSD_{0.05}$
number of as_{basal} [x]		0.2	0.2	0.6	0.5			
number of $as_{infl.}$ [x]		1.7	2.2	3.2	2.8		*	0.7
W_{dry} ps [y]	g	10.5	11.5	7.8	7.6			
W_{dry} as_{basal} [x]	g	1.1	1.4	1.6	2.3			
W_{dry} $as_{infl.}$ [x]	g	5.5	8.2	11.3	9.5		*	1.6
W_{dry} flower [y]	g	3.6	3.7	1.6	1.6			
W_{dry} stent [y]	g	1.7	2.0	1.5	1.5			
W_{dry} roots [y]	g	3.1	2.4	1.8	1.4	***		0.3
W_{dry} total [y]	g	25.5	29.2	25.7	23.8			
shoot/root [y]		7.4	11.2	14.0	16.4	***		1.1
leaf area [y]	cm^2	1579	1904	2977	2857			

B)

		15°C		20°C		significance		
parameter	unit	control	ABC1	control	ABC1	genotype	genotype x temp.	$LSD_{0.05}$
number of as_{basal} [x]		0.1	0.6	0.3	1.3	***		0.3
number of $as_{infl.}$ [x]		2.0	3.5	3.0	3.3		***	0.5
W_{dry} ps [y]	g	13.3	11.0	9.9	7.8	***		0.7
W_{dry} as_{basal} [x]	g	0.4	0.8	0.3	2.6	***		0.8
W_{dry} $as_{infl.}$ [x]	g	5.3	9.3	11.2	11.4		*	2.5
W_{dry} flower [y]	g	3.0	1.9	2.2	1.4	***		0.2
W_{dry} stent [y]	g	1.9	1.5	1.2	1.1	***		0.1
W_{dry} roots [y]	g	3.6	4.4	1.8	2.6	***		0.4
W_{dry} total [y]	g	27.6	28.9	26.6	26.9			
shoot/root [y]		7.0	5.7	14.2	10.0		***	1.2
leaf area [y]	cm^2	1708	1890	3118	3375	*		196

[x]=GLM, when relevant a maximum LSD is given since SEs are proportional for a non-linear model
[y]=ANOVA
*, ***=significant at p=0.05 and 0.001, respectively.

Figure 6. (A) Root development of combination plants after 9 weeks at 20°C, left: control, right: transformed rootstock ABC1. (B) Aerial parts of combination plants, consisting of a 'Madelon' scion grafted on a rootstock, after 9 weeks at 20°C, left: scion grafted on a control rootstock, right: scion grafted on the transformed rootstock ABC1.

extensively developed than control roots (Fig. 6A). The roots were shorter, but much more branched.

These strong effects on the phenotype of the root system were also clearly observed in the growth analysis performed after harvest of all plants (Table 3B). In contrast to the *ROLB* transformed rootstocks, the dry weight of the roots (W_{dry} roots) of combination plants with *ROLA, B, C* transformed rootstocks was significantly higher than in the control. This was also represented by a lower shoot/root ratio. Regarding the aim of the present studies, the most important observation was that the numbers of axillary shoots at the base of the primary shoot (number of as_{basal}) were higher on combination plants with ABC1 as a rootstock than on those with control rootstocks (Table 3B, Fig. 6B). The number of basal shoots increased from 0.1 to 0.6 and from 0.3 to 1.3 basal shoot per plant at 15 and 20°C, respectively. It indicates that scion bud release was stimulated by the presence of the *ROLA, B, C* genes in the rootstock. In contrast to this positive effect some other traits were negatively affected. The dry weights of primary shoot (W_{dry} ps) and flower (W_{dry} flower) were lower in combination plants with *ROLA, B, C* transformed rootstocks. On the other hand leaf area was higher, possibly as a result of the decreased apical dominance in combination plants with ABC1 as a rootstock. Remarkably, at 15°C the number of axillary shoots in the inflorescence

(number of $as_{infl.}$) of combination plants with a *ROLA, B, C* transformed rootstock was significantly higher than in the control, whereas this was not the case at 20°C. In summary, these results demonstrate that use of a *ROLA, B, C* transformed rootstock in combination plants results in a drastic change in plant architecture such as an increased axillary bud release at the base of the untransformed scion.

Discussion

In this research it was shown that the phenotypical effects of *ROL* genes, when applied only in the root part of combination plants of rose, clearly differed from their effects in completely transformed roses. To illustrate this contrast, first the various effects of *ROL* genes on completely transformed roses are discussed and then the effects of *ROL* genes on the development of combination plants.

Completely transformed plants

ROLB transformed roses showed an increased apical dominance compared to control plants. Such an auxin-like effect might be expected in *ROLB* transformed plants. Although the precise mechanism is not yet known, auxin sensitivity has been shown to be affected by the presence and expression of the *ROLB* gene (Maurel *et al.*, 1991; 1994). The increase in apical dominance contradicts with observations in *Lycopersicon esculentum* (Van Altvorst *et al.*, 1992) and *Medicago* (Frugis *et al.*, 1995), in which enhanced lateral shoot formation was observed (lower apical dominance). The high transcription level of the *ROLB* gene, especially in the aerial parts, might explain the increased apical dominance as observed in this study. The decreased apical dominance, which was observed in *ROLA, B, C* transformed roses, corresponds with the phenotype of tobacco transformants (Schmülling *et al.*, 1988). This stimulating effect can be interpreted as a cytokinin-like effect, due to the dominating expression of the *ROLC* gene in these transformed roses.

Introduction of *ROL* genes also led to peculiar changes in leaf shape. In untransformed 'Moneyway' plants the leaves were lanceolate, whereas *ROLB* transformed plants and in some cases *ROLA, B, C* transformed plants exhibited round-edged leaves. This round-edged leaf shape was correlated with the presence of an expected *ROLB* messenger of 1050 nt. Next to this messenger, another unknown messenger of approximately 1200 nt was found in leaves of the *ROLB* transformant. Although two different transcripts were already described for both the *ROLA* and the *ROLC* gene (Spena *et al.*, 1987; Schmülling *et al.*, 1988), this is the first time that a second *ROLB* transcript was found.

One of the *ROLA, B, C* transformed plants showed a strongly aberrant phenotype with

wrinkled leaves. Northern analysis revealed the presence of a *ROLA* mRNA of 650 nt. In *ROLA* and *ROLA, B, C* transformed tobacco plants similar leaf morphology was found, also together with a mRNA of 650 nt (Schmülling *et al.*, 1988). In three other *ROLA, B, C* transformed roses the 650 nt mRNA was not detectable, but we observed a mRNA of only 350 nt. Such a *ROLA* transcript was also found in hairy roots of cucumber (Amselem and Tepfer, 1992). Since the coding region of the *ROLA* gene comprises 300 nt, only 50 nt should remain for the leader and the 3' non-coding region. Therefore it is plausible that this 350 nt mRNA is incomplete. This deficiency might be explained by premature polyadenylation. Alternatively, in *Arabidopsis thaliana*, an intron of 76 nt was found in the untranslated leader of the pre-m RNA of *ROLA*. Splicing showed to be a prerequisite for expressing the phenotype (Magrelli *et al.*, 1994). In our experiments, we have no indications for such a posttranscriptional regulation in the aberrant transformant with wrinkled leaves. However, the presence of a 350 nt mRNA in the three independent rose transformants might be interpreted as the result of incorrect splicing.

The Northern analysis has also shown that abundant expression of the *ROLA*, *ROLB* and *ROLC* genes never occurred simultaneously in leaves of the *ROLA, B, C* transformants. This might be based on the neighbouring positions of these genes on the EcoRI fragment 15 from *A. rhizogenes*. The complementary transcription of the *ROLB* and *ROLC* genes might be based on the dual promoter between both genes. Transcription of the *ROLA* and *ROLB* genes might also be antagonistically related. These genes are transcribed in cis in reverse directions, thus transcription of one gene might be hampered by readthrough transcription of the other gene.

Transformation with the *ROL* genes not only affected apical dominance and leaf shape. The growth analysis also showed that the growth of the transformed plants as measured by dry weight was reduced compared to control plants. This means that growth and development of the transgenic plants changed in several ways upon introduction of *ROL* genes. Due to these pleiotropic effects, practical applications of *ROL* genes in completely transformed roses are questionable.

Combination plants

The growth analysis of combination plants with a *ROLB* transformed rootstock demonstrated that root development was negatively affected by the presence of the *ROLB* gene in the root part. This observation corresponds with the already mentioned auxin-like action upon transformation with the *ROLB* gene (Maurel *et al.*, 1991; 1994) and the auxin induced inhibition of root growth (Van der Krieken *et al.*, 1992). Despite the smaller root system, the performance of the untransformed scion remained unaffected. Since in completely transformed *ROLB* roses both root and shoot growth were negatively affected, we conclude

that shoot growth is directly inhibited by local expression of the *ROLB* gene, and not indirectly via a poorly developed root system.

In contrast to the inhibiting effect of the *ROLB* gene, root development of combination plants was enhanced by the presence the *ROLA, B, C* genes. Axillary bud release at the base of the untransformed scion was also stimulated, possibly due to an increased production of cytokinin in such well developed and highly branched root systems. Alternatively, this stimulation of axillary bud release might be the result of an altered hormonal status of this *ROLA, B, C* transformed rootstock, reflecting a cytokinin-like action as mentioned for *ROLC* transformed plants (Schmülling *et al.*, 1988). The relevance of this gene was confirmed by the dominating expression of the *ROLC* gene in *ROLA, B, C* transformed roots. Comparison with the relatively poor performance of combination plants with a *ROLB* transformed rootstock also suggests a role of the *ROLA* and/or the *ROLC* genes in this stimulative effect.

Next to the already mentioned stimulation of scion bud release in combination plants with *ROLA, B, C* transformed rootstocks, we also observed an inhibitory effect on the growth of the primary shoot and flower. It appears that the stimulative effect on axillary shoot formation occurred at the expense of other plant parts. Nevertheless, the total dry weight of combination plants with a *ROLA, B, C* transformed rootstocks equalled that of the control. Severe pleiotropic effects, as mentioned for the completely transformed roses, were not observed.

In conclusion, this research demonstrates that the elementary plant architecture of an untransformed scion was profoundly altered by the presence of the *ROLA, B, C* genes in transformed rootstocks. The root system of such transformed rootstocks was much more developed than that of untransformed rootstocks. As a result of this *ROL* gene transformed rootstock, more basal shoots were formed at the base of the untransformed scion, while the leaf area was also increased. These two parameters illustrate an improved growth performance of such combination plants. The promotive effects of the *ROL* genes occurred both at 15 and 20°C. Since the number of axillary shoots at the base of a rose plant is correlated with flower production (De Vries and Dubois, 1989), we expect that application of *ROLA, B, C* transformed rootstocks provides an opportunity for growers to produce more efficient both at 20°C and at 15°C. A better production at low energy conditions might be commercially attractive for greenhouse production of cut roses.

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Chapter 7

General discussion: Prospects for applications of *ROL* genes for crop improvement

Theo P.M. van der Salm, Charlotte H. Hänisch ten Cate and Hans J.M. Dons

1 Introduction

Various aspects of the interaction between the soil bacterium *Agrobacterium rhizogenes* and plants have been studied extensively: the biology of the plant pathogenic bacterium, the transfer of genetic information to the genome of plants, identification and characterization of transferred genes and their use as an instrument to interfere with plant development. During the last two decades extensive research has been carried out in this field, which has been summarized in various reviews (Tepfer, 1989; Gelvin *et al.*, 1990, Costantino *et al.*, 1994). First, only the pathogenic rooting response was described, but after production of the first genetically modified tobacco plants using *A. rhizogenes* (Ackermann, 1977), many other phenotypical alterations attracted attention, such as altered plant shape, growth, rooting, flowering and fertility. These combined symptoms were later denominated as the 'hairy root syndrome' or the Ri phenotype (Costantino *et al.*, 1984; Tepfer *et al.*, 1984). The mentioned characteristics might have an agronomical importance and as a consequence can be valuable for crop improvement. The objective of this chapter is to evaluate useful characteristics from the diversity of phenotypical alterations, which occur upon *A. rhizogenes* application. First, a general introduction on the biology of *A. rhizogenes* and its functional genes is given. Next, effects of *A. rhizogenes* on rooting, plant growth, plant shape and flowering and fertility are discussed. For the evaluation of useful characteristics for crop improvement, not only the results obtained with Ri transformed plants are regarded, but also alternative approaches are considered, such as: *A. rhizogenes* as a rooting agent, transformation with a restricted number of genes from *A. rhizogenes*, either driven by their own or heterologous regulatory signals and production of plants which are only partially genetically modified. In contrast to reviews about more fundamental aspects, this review deals with the practical implications of *A. rhizogenes* use. Besides comments on reported studies, in which *A. rhizogenes* or *ROL* genes were used for practical purposes, we also give recommendations for applications of *ROL* genes for crop improvement.

2 *Agrobacterium rhizogenes*

Agrobacterium

Both *A. tumefaciens* and *A. rhizogenes* are pathogenic soil bacteria, which induce tumours (crown gall) or roots (hairy root disease) at the site of bacterial infection on various plant species (Fig. 1). Since their discoveries (Smith and Townsend, 1907; Riker *et al.*, 1930; Elliot *et al.*, 1951) the morphogenetic effects of these pathogens on plant development have been studied extensively (Tepfer, 1989; Gelvin *et al.*, 1990; Costantino *et al.*, 1994).

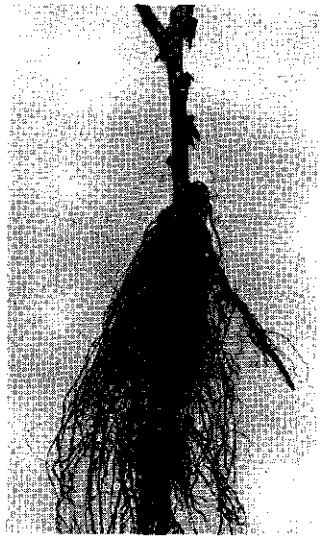

Figure 1. Symptoms of the hairy root disease on apple infested by *A. rhizogenes*.

Cellular and molecular studies revealed that the molecular basis of the disease was the transfer of genetic information from the bacterium to the plant cell (Hooykaas *et al.*, 1992). Fragments of transfer DNA (T-DNA), originating from the tumour inducing (Ti) or root inducing (Ri) plasmid, are integrated in the plant genome (Chilton *et al.*, 1977; 1982). In general, a T-DNA bears two categories of genes: hormone genes for the stimulation of cell division on the place of infection, leading to tumorous or root growth and genes for the synthesis of non-protein amino acids (opines), which can be used as a substrate by the bacterium.

The Ri plasmid of A. rhizogenes

The pathogenicity of an infection depends both on the *A. rhizogenes* strain and the plant species that are used (Tepfer *et al.*, 1989; Porter *et al.*, 1991). Strains of *A. rhizogenes* are classified by the type of opine synthesis genes that are transferred: agropine, cucumopine or mannopine (Petit *et al.*, 1983). Agropine strains of *A. rhizogenes* carry two different T-DNAs on their Ri plasmid: the TL- (T left) and the TR-DNA (T right). On the TR-DNA the gene for the synthesis of agropine and genes for the biosynthesis of auxin (*AUX1* and *AUX2*) are localized (Cardarelli *et al.*, 1985). By transposon-mediated mutagenesis it was shown that four root loci (*rolA*, *B*, *C*, and *D*) on the TL-DNA were responsible for the Ri phenotype (White *et al.*, 1985). Sequence analysis revealed a total of 18 open reading frames (ORFs) on the TL-DNA. Among these, ORFs 10, 11, 12 and 15 corresponded with *ROLA*, *ROLB*, *ROLC* and *ROLD* genes, respectively (Slightom *et al.*, 1986).

The ROL genes

The Ri phenotype was also established upon isolation of the *ROLA*, *B*, *C* and *D* genes from *A. rhizogenes* and subsequent transfer to plants by *A. tumefaciens*. Little is known about the *ROLA* and *ROLD* genes. The *ROLA* gene in plants is responsible for the formation of wrinkled leaves, shortened internodes (Schmülling *et al.*, 1988; Sinkar *et al.*, 1988) and changes in polyamine metabolism (Sun *et al.*, 1991), while the *ROLD* gene seems to encode a transportable factor which induces early flowering in tobacco (Costantino *et al.*, 1994). Transcripts of the *ROLD* gene were predominantly found in roots (Leach and Aoyagi, 1991). Since both the *ROLB* and *ROLC* genes contribute to a large extent to the Ri phenotype, much more attention has been paid to these *ROL* genes. *ROLB* was identified as the critical *ROL* gene for the induction of roots (Spena *et al.*, 1987; Cardarelli *et al.*, 1987; Capone *et al.*, 1989a, b). An increased sensitivity to auxin and possibly a role of the ROLB protein in auxin perception or transduction was suggested by Maurel *et al.* (1991, 1994). It was shown that mesophyll protoplasts from *ROLB* transformed tobacco plants were 10,000 times more sensitive to auxin than those from control plants. Moreover, auxin binding activity of membranes of *ROLB* transformed plants was higher than that of control plants (Filippini *et al.*, 1994). This difference in auxin binding was abolished by anti-ROLB antibodies, suggesting an increased number of auxin receptors due to transformation with the *ROLB* gene. On the other hand an indoxyl-ß-glucosidase activity of the ROLB protein was found, suggesting a role in the release of active auxins (Estruch *et al.*, 1991b). This is highly questionable since several tested auxin conjugates did not serve as a substrate, while auxin levels were not increased in *ROLB* transformed plants (Nilsson *et al.*, 1993a). The *ROLB* gene is mainly expressed in root caps, regions of cell division (Schmülling *et al.*, 1989) and in the vascular system of mature organs (Altamura *et al.*, 1991).

The *ROLC* gene has a cytokinin like effect on the phenotype of plants i.e. decreased apical dominance and shorter internodes. In addition, plants show a decreased chlorophyll content. The gene product of *ROLC* is a cytokinin-ß-glucosidase, which hydrolyses cytokinins from N-glucoside conjugates (Estruch *et al.*, 1991a). Despite this enzymatic activity, levels of cytokinin were lower, while gibberellin levels were unexpectedly higher in *ROLC* transformed tobacco plants compared to control plants (Nilsson *et al.*, 1993b). Under control of its own promoter the *ROLC* gene is expressed mainly in the phloem of roots, stems and leaves (Schmülling *et al.*, 1989), while at cellular level its gene product is especially localized in the cytosol (Estruch *et al.*, 1991c).

In conclusion, although much information is available about the *ROLB* and *ROLC* genes, knowledge about the biochemical action of their gene products is still incomplete.

3 Use of *Agrobacterium* suspensions as a rooting agent

Strobel (1985) was the first to mention *Agrobacterium* for agricultural use. Inoculation of rootstock almond trees with *A. rhizogenes* resulted in a larger root number and root mass, while shoot growth was also enhanced. Similar results were obtained with olive seedlings (Strobel *et al.*, 1988). Over a course of three years, trees with inoculated root systems grew more quickly and produced more flowers, fruits and oil than control trees. Also in pea, flowering was enhanced upon inoculation with a suspension of *A. rhizogenes* (Lucretti *et al.*, 1986). Suspensions of *A. rhizogenes* were also used as rooting agents for the induction of adventitious roots on cuttings of woody plant species, which are difficult to root. For example root formation on in vitro cuttings of *Populus deltoides* x *P. nigra* and stem cuttings of hazelnut (*Corylus avellana* L.) was stimulated by treatment with various *A. rhizogenes* strains (Charest *et al.*, 1992; Bassil *et al.*, 1991). Inoculation with *A. rhizogenes* also promoted adventitious root formation on stems of the rootstock *Rosa canina* (Van der Mark *et al.*, 1990). It was shown that such plants were able to grow on a mass of 'hairy roots'. Even rooting of cuttings from three year old trees of the gymnosperm *Pinus monticola* was improved upon inoculation with *A. rhizogenes* (McAfee *et al.*, 1993). The use of *A. rhizogenes* as a rooting agent is therefore not restricted to angiosperms, but also effective for gymnosperms.

Treatment with *A. rhizogenes* did not always result in improved root formation. For example, root induction on stems of chestnut (*Castanea sativa*) initially failed upon inoculation with *A. rhizogenes* in combination with an auxin treatment, etiolation and air layering, whereas similar treatment with *A. tumefaciens* harbouring the *ROLB* gene resulted in viable rhizogenesis (Rinallo *et al.*, 1993). This was the first described method on adult chestnut propagation by cuttings. Also the rooting efficiency of in vivo and vitro cuttings of apple rootstocks (*Malus*) was not affected by inoculation with *A. rhizogenes* (Lambert *et al.*, 1991). Surprisingly, inoculation with *A. tumefaciens* harbouring the *ROLC* gene under control of the CaMV 35S promoter resulted in an increased rooting efficiency of in vitro micro-cuttings. These results suggest that when treatment with a wildtype *A. rhizogenes* was insufficient for improvement of rooting, inoculation with *A. tumefaciens* harbouring individual *ROL* genes might be more effective.

In conclusion, suspensions of *A. rhizogenes* or *A. tumefaciens* with *ROL* genes are suitable tools for the induction of adventitious roots on plants of various crops, although the response is very much dependent on the plant species. *A. rhizogenes* incited root formation sometimes led to accelerated growth of untransformed aerial parts. Since plants or organs were treated with active bacteria, this stimulative effect on plant growth may be also attributed to the direct presence of *A. rhizogenes* or to altered population dynamics of other soil bacteria

(Tepfer, 1989). Such an altered microbiological balance in the rhizosphere may indirectly affect the coherent nutritional status of the plant. Despite the numerous positive effects, *A. rhizogenes* has to be considered as a pathogenic soil bacterium and therefore hazardous for other crops. Hence, to prevent undesired spread of *A. rhizogenes* in the environment, the use of suspensions for rooting should be avoided.

4 Rooting

Adventitious root formation

The most prominent characteristic of *A. rhizogenes* is its ability to induce adventitious roots. Auxin is the key hormone for adventitious root formation that usually occurs after a dedifferentiation process, in which plant cells become competent for the hormone. Subsequent treatment with auxin can induce differentiation of plant cells, finally leading to the formation of root meristems (Christianson and Warnick, 1983). Auxins also play a role in root growth. Auxin is produced in the apex and leaves of plants and transported basipetally. Free auxins are inactivated by conjugation with amino acids or sugars (Cohen and Bandurski, 1982) and upon hydrolysis the active auxin is released. Auxins bind to specific receptors, which are located within the lumen of the endoplasmic reticulum, but also on the outside of the plasma membrane. It is assumed that this binding directs the onset of cellular signal systems, such as hyperpolarisation, the start of an ATPase dependent H^+ excretion and the modulation of gene expression. These processes lead to a change of membrane properties resulting in cell enlargement and for instance root growth by cell elongation (Barbier-Brygoo, 1995; Venis and Napier, 1995). Next to the stimulative effect of auxin, root development is antagonistically or synergistically affected by cytokinin and ethylene, respectively.

Many woody species are recalcitrant in the formation of adventitious roots. The root inducing and root growth stimulating characteristics of *A. rhizogenes* may therefore be useful for improvement of especially woody species. Next to the use of *Agrobacterium* suspensions as a rooting agent, which was mentioned before, root formation was also stimulated by the production of genetically modified plants. Plants were either transformed with the complete TL/TR-DNA, harbouring many genes which are largely unidentified, or with only one or a combination of individual *ROL* genes.

Root formation of Ri transformed plants

Genetically modified shoots with TL-DNA were obtained via Ri transformed callus or 'hairy roots' of various species (Tepfer, 1984). As mentioned before, the presence of TL-DNA

greatly affected a series of physiological and phenotypical characteristics including adventitious root formation. The effect of such modifications was mainly tested in woody species.

Ri transformed plants of *Allocasuarina verticillata* Lam. (Phelep *et al.*, 1991) and *Populus alba* x *P. glandulosa* (Chung *et al.*, 1989) showed improved rooting in vitro. In addition, abundant root development in the soil was observed on Ri transformed plants of the woody legume *Robinia pseudoacacia* L. and *Actinidia deliciosa* (kiwi) (Han *et al.*, 1993; Yazawa *et al.*, 1995). Studies on the formation of adventitious roots on leaf explants of Ri transformed tobacco plants demonstrated that both the rooting response and the sensitivity to auxin were increased (Spanò *et al.*, 1988). The hormone response was changed even more drastically in *Malus* (Lambert *et al.*, 1992). Adventitious roots were induced on Ri transformed shoots after treatment with cytokinins, whereas auxins were toxic, suggesting a completely altered organogenic response.

Ri transformed root systems also showed enhanced growth and development. It was shown that the meristematic zone in apices of Ri transformed roots was larger than in the control, also showing a higher degree of cell division (Mengoli *et al.*, 1992). Next, the formation of lateral roots was increased (Tepfer, 1984). Regardless these improved root characteristics, transformation with *A. rhizogenes* frequently resulted in plants suffering harmful pleiotropic effects in the aerial parts, such as reduced apical dominance, curled leaves and shorter internodes. It appears that these aberrations restrict practical application of Ri transformed plants.

Root formation via introduction of individual ROL genes

Stimulation of root development was also accomplished by the introduction of individual *ROL* genes. Since only one or few *ROL* genes are introduced, less harmful pleiotropic effects are expected, and therefore this might be a more straightforward approach.

Transformation of tobacco with a combination of *ROLA, B, C* genes resulted in plants with the Ri phenotype, exhibiting the formation of 'hairy roots' (Spena *et al.*, 1987; Schmülling *et al.*, 1988; Spanò *et al.*, 1988). Further analysis revealed an elevated rooting response and an increased sensitivity of leaf explants to auxin, resembling that of Ri transformed leaves (Spanò *et al.*, 1988). Introduction of the *ROLA* gene also promoted adventitious root formation on tobacco leaf discs, which were incubated on hormone-free medium, although further root growth was inhibited (Carneiro *et al.*, 1993). Transformation with the *ROLB* gene alone also increased adventitious root formation on stems of tobacco, similarly as the *ROLA, B, C* genes (Schmülling *et al.*, 1988). For the *ROLC* gene an increased lateral root formation was found. It appeared that presence of the *ROLB* gene is essential for stimulation of adventitious root formation, while the *ROLA* and *ROLC* genes

play an accessory role.

To test the effect of *ROL* genes on rooting of woody species, kiwi was transformed with a combination of *ROLA*, *B* and *C* genes (Rugini *et al.*, 1991). As a result the rooting ability of microcuttings was increased from 40 to 100%. Research carried out at our laboratory and which was focused on the improvement of rose rootstocks (*R. hybrida* L.) by genetic modification (Van der Salm *et al.*, 1996a and b; Chapter 3 and 4) further strengthened these results. Adventitious root formation on microcuttings was increased upon introduction of the *ROLB* gene and especially upon introduction of a combination of *ROLA*, *B* and *C* genes, while rooting on explants of these in vitro grown transformants showed a tenfold increase in auxin sensitivity (Chapter 5). After transfer of these transformants to the greenhouse, it was demonstrated that introduction of the *ROLB* gene or a combination of *ROLA*, *B* and *C* genes also resulted in a threefold increase in rooting efficiency and rooting capacity of cuttings. Together these results clearly demonstrate, that the *ROLB* gene or especially a combination of *ROLA*, *B* and *C* genes are suitable for promotion of adventitious root formation in woody species.

5 Growth characteristics

Since the *ROL* genes of *A. rhizogenes* interfere in hormone metabolism and/or hormone sensitivity of the plant, these genes are suitable tools to modify whole plant growth and development. To evaluate their effects on plant growth, first Ri or *ROL* gene transformed plants are described. Next, to elucidate the influence of Ri- or *ROL* gene transformed roots on growth of the aerial parts, studies are described on combination plants, in which untransformed scions were grafted on genetically modified root systems.

Growth of Ri transformed plants

The effect of the TL-/TR-DNA on the growth of Ri transformed plants has been analyzed in various plant species, but especially in potato. The fresh weight of in vitro plants of TL-DNA transformed potato was higher than that of untransformed plants, partly because they had a higher water content (Ooms *et al.*, 1986a). This was confirmed by a decreased potassium concentration and osmotic water pressure in cell sap extracted from transformed shoots. In field studies performed with potato plants, tuber yield per plant was lower for Ri transformed plants and more variable than in the control (Ooms *et al.*, 1986b). These results were confirmed by Van de Geijn *et al.* (1988). After in vitro propagation, Ri transformed potato plants always had a higher fresh weight than the control. However, upon transfer to nutrient solution the relative growth rate of Ri transformed plants was lower than that of the

control, while respiration did not increase as in the control plants. Similar results were also obtained with tobacco (Václavík *et al.*, 1988). Upon transplanting Ri transformed plants from in vitro conditions to the soil, leaf area and dry weights were initially greater than in the control, but 14 days later no differences were observed. Photosynthesis and water uptake efficiency were found to be lower. In conclusion, Ri transformed plants did grow faster under in vitro conditions than control plants, whereas upon transfer to the soil it was the other way around. Analysis of opine synthesis in Ri transformed potato plants showed that normal plant development and tuber weight was negatively correlated with the synthesis of opines (Ottaviani *et al.*, 1991).

With respect to woody species, it was observed that Ri transformed in vitro plants of *Allocasuarina verticillata* Lam. were heavier than control plants (Phelep *et al.*, 1991). In addition, Ri transformed plants of *Rauvolfia serpentina* produced more fresh weight in the soil than control plants (Benjamin *et al.*, 1993). These results are in contrast with those obtained with potato and tobacco. It shows that the effect of *A. rhizogenes* transformation on plant growth in the soil was dependent on the plant species.

Growth of plants transformed with individual ROL genes

Because of the numerous pleiotropic effects observed in Ri transformed plants, it is expected that effects of *ROL* genes on plant growth are easier to interpret in plants transformed with single or a combination of *ROL* genes than in plants containing the complete TL-DNA. Effects of individual *ROL* genes on plant growth were studied especially in potato and tobacco.

Potato plants, transformed with the *ROLC* gene under control of the strong CaMV 35S promoter, had an increased number of tubers per plant compared to the control. Moreover, tubers were longer with a higher numbers of eyes (Fladung *et al.*, 1990). However, the total tuber yield in pots was lower than in the control and showed to be negatively correlated with *ROLC* expression (Fladung *et al.*, 1992). After transformation with the *ROLC* gene behind the leaf specific rbcS promoter, plants were slightly reduced in size with a few more tillers compared to the control. Although chlorophyll content and photosynthetic rate were always lower in these transformed plants, tuber yield remained unaffected. Remarkably, transformation with *ROLC* decreased the amount of reducing sugars in tubers (Fladung *et al.*, 1993).

Transformation of tobacco with the *ROLA* gene driven by its own promoter resulted in normal sized plants with wrinkled leaves (Schmülling *et al.*, 1988), whereas plants with the *ROLA* gene driven by the CaMV 35S promoter showed stunted growth with dark, wrinkled leaves (Dehio *et al.*, 1993). Transformed tobacco plants with the *ROLB* gene were smaller than the control (Mariotti *et al.*, 1989). Leaves of *ROLB* transformants were normal sized,

but round-edged (Schmülling *et al.*, 1988). *ROLC* transformed tobacco plants also showed reduced height, with small (Scorza *et al.*, 1994), pale green and lanceolate leaves, which resembled those of flowering plants (Schmülling *et al.*, 1988). Although photosynthesis rate per unit leaf area was not affected by the *ROLC* gene, plant growth was decreased (Scorza *et al.*, 1994). Similar to tobacco plants transformed with the *ROLC* gene, plants with a combination of *ROLA*, *B* and *C* genes were also smaller than control plants, with reduced apical dominance and smaller internodes and leaves (Schmülling *et al.*, 1988; Mariotti *et al.*, 1989). Therefore, we suggest that the effects of the *ROLA*, *B*, *C* genes on aerial growth of tobacco plants could be ascribed to the *ROLC* gene. However, in *Medicago* apical dominance was not only decreased upon introduction of the *ROLC* gene, but also upon transformation with the *ROLB* gene. Interestingly, nutritional value of these transformants was improved by decreased lignification of the stems (Frugis *et al.*, 1995).

Together, these results lead to the conclusion that leaf shape (wrinkled, round, lanceolate) was correlated with the presence of the individual *ROL* genes (*ROLA*, *ROLB* or *ROLC*, respectively). Although plant development was affected in various ways, plants transformed with individual *ROL* genes were generally lighter in weight than control plants. However, some interesting qualitative features were found in *ROL* gene transformed plants that might have practical implications.

Growth of chimeric plants with a Ri or ROL gene transformed root system

To study the effect of a *ROL* gene transformed root system on growth of the aerial parts, grafts were made between Ri or *ROLC* transformed plants and untransformed plants of potato (Ooms *et al.*, 1986c; Fladung *et al.*, 1990). At first sight these experiments suggested that effects of *ROL* genes were not transmissible from roots to the aerial parts of an untransformed scion.

On the other hand, also experiments were described in which transformed roots affected the growth and development of an untransformed scion. A changed mineral uptake by Ri roots of potato diminished chlorophyll content of the grafted tomato shoot (Van de Geijn *et al.*, 1988). This transmissible chlorosis was correlated with a high phosphorus content in roots and aerial parts and ascribed to an unfavourable Fe/P ratio. Furthermore, roots transformed with ORF13 of the TL-DNA, promoted axillary bud release of non-transformed scions of tobacco (Hansen *et al.*, 1993). Taking all these results into account, this leads to the conclusion that Ri transformed roots certainly are able to affect growth and development of the scion.

Growth of roses grafted on ROL gene transformed rootstocks

In our studies, performed during the already mentioned research which was focused on the

improvement of rose rootstocks (*R. hybrida* L.) by genetic modification, we further analyzed the effect of *ROL* gene transformed roots on the growth of aerial parts (Chapter 6). For this research, combination plants were made, consisting of an untransformed scion grafted on a *ROLA, B, C* transformed rose rootstock. The presence of *ROLA, B , C* genes in the rootstock stimulated the formation and the initial growth of adventitious roots. Also after three months on hydroculture the transformed rootstocks were larger than the control rootstocks, while the release of basal shoots of the scion was significantly higher ($p<0.001$) on transformed rootstocks than on control rootstocks (see Fig. 6A and B, Chapter 6), with on average 1.3 and 0.3 basal shoots per plant, respectively. This demonstrated that these *ROLA, B, C* transformed rootstocks were more efficient in supporting axillary bud release. Since the development of basal shoots is correlated with the final flower production (De Vries and Dubois, 1989), we presume that use of such genetically modified rootstocks will lead to an increased flower production. Since rose culture requires high energy inputs, it might be commercially attractive for growers to use rootstocks, which produce more efficiently per unit energy input.

In orthodox strategies with completely transformed plants, plant growth in the soil was generally negatively affected upon transformation with *ROL* genes. In addition, agronomical use of such *ROL* gene transformed plants appeared to be limited by the presence of various harmful pleiotropic effects in the aerial parts. In *R. hybrida* L., this obstacle was circumvented by a new approach in which combination plants were used with *ROL* gene transformed rootstocks. The presence of *ROLA, B, C* genes in the rootstock not only stimulated the growth of the root system but also the release of axillary buds in the untransformed scion, presumably leading to a more (energy) efficient flower production. We conclude that this result opens new perspectives for the application of *ROL* genes in rootstocks.

6 Plant shape

Axillary shoot formation

Inhibition of bud release (apical dominance) is ascribed to the basipetal flow of auxin from the shoot apex (Cline, 1991; 1994). Termination of the auxin flow by decapitation results in the release of axillary buds. Since auxin does not move into the axillary buds, second messengers, such as ABA or ethylene, might be involved in the transduction of the final inhibitory signal into the buds. A role of endogenous cytokinins has also been suggested. Transport of endogenous cytokinins in the xylem was initiated upon obstruction of the auxin

flow (Bangerth, 1994). Since auxins and cytokinins are both locally involved in axillary bud release, determination of *ROL* gene induced changes of hormone concentrations in entire plants or organs can't be related to a phenotypical change. The multiple effects of *ROL* genes on hormone metabolism even further troubles the comprehension of such phenotypical effects of *ROL* genes.

Plant architecture of Ri or ROL gene transformed plants

For improvement of ornamental characters, *Pelargonium* was transformed with the TL- and TR-DNA from *A. rhizogenes*, resulting in an increased production of branches, leaves and aromatic substances as well (Pellegrineschi *et al.*, 1994). Individual *ROL* genes were also used for modification of plant architecture of ornamentals. *R. hybrida* L. was transformed with the *ROLC* gene or a combination of *ROLA*, *B* and *C* genes (Souq *et al.*, 1995; Chapter 6). As a result apical dominance and plant height were decreased considerably compared to the control. Also *Eustoma grandiflorum* (Handa *et al.*, 1994) and *Salpiglossis sinuata* (Rietveld *et al.*, 1995) were transformed with individual *ROL* genes. In the latter case the *ROLC* gene induced the expected bushy phenotype, though not as drastic as in tobacco.

Introduction of the *ROLB* gene in *N. tabacum* and *Lycopersicon esculentum* Mill. resulted in thinner (Mariotti *et al.*, 1989) or shorter stems (Van Altvorst *et al.*, 1992). As mentioned before, *ROLB* reduced apical dominance in *Medicago* (Frugis *et al.*, 1995), but also in *L. esculentum* Mill. (Van Altvorst *et al.*, 1992). However, we found that axillary shoot formation of a *ROLB* transformed rose rootstock was significantly ($p < 0.001$) lower than that of control rootstocks (0.1 and 1.7 lateral shoots per rootstock plant, respectively). This demonstrates that apical dominance was increased by the *ROLB* gene (Chapter 6). Since the *ROL* genes affect the endogenous hormone balance, which is presumably plant specific, it seems that the effect of the *ROLB* gene on plant shape is not very predictable, as shown by the diverse observations.

Modification of plant architecture is of commercial interest in many crops. Decreased or increased apical dominance may lead to improvement of ornamental quality, while increased apical dominance will prevent the laborious budding during the culture of various crops such as carnation and tomato. Creating fruit trees with an column-like shape will also facilitate mechanical harvesting. In general, transformation with the *ROLC* gene and especially a combination of *ROLA*, *B* and *C* genes decreases apical dominance, whereas the *ROLB* gene both decreases or increases apical dominance, depending on the plant species. In principle *ROL* genes are suitable genes for the modification of plant architecture.

7 Flowering and fertility

Regulation of flower induction and flower development

Research on flower induction and flower development is accomplished with mutants and genes from *Arabidopsis thaliana*, *Antirrhinum majus* and *Petunia hybrida* (Weigel and Meyerowitz, 1993; Ma, 1994; Angenent *et al.*, 1995). These developmental processes are controlled by a hierarchy of genes: late flowering genes, floral meristem identity genes, cadastral genes and homeotic genes. Plant hormones control elementary processes of plant growth and development by regulation of gene expression. The onset of flowering and the regulation of flower development was affected by elevated levels of cytokinin (Bernier *et al.*, 1990). Since the *ROL* genes also interfere in hormone metabolism, it is suggested that flower development and fertility might also be affected by these genes. Access to the process of flowering gives opportunity to valuable applications such as: the modification of flower number and flower shape of ornamental crops, the acceleration of flowering of ornamentals and the reduction of the regenerative cycle of woody species in breeding programs.

Effects of ROL genes on flowering and fertility

Many papers indeed report on effects of *ROL* gene transformation on flower development and fertility. Ri transformed *Brassica* flowered, but did not set seed (Ooms *et al.*, 1985). In Ri transformed plants of *S. tuberosum* and *Hyoscyamus muticus* flowering and fruit set were delayed (Ooms *et al.*, 1986b; Oksman-Caldentey *et al.*, 1991). Flowers of the latter plants were morphologically abnormal with only four petals instead of five, while the stamens were shorter than the pistils (hyperstyly) preventing self-pollination. In general, transformation with *A. rhizogenes* seems to have negative effects on flower development, resulting in retardation of flowering and male or female sterility. However, it is questionable whether these effects are due to the poor plant quality or specifically to Ri transformation.

The effects of individual *ROL* genes on flowering were analyzed in transgenic plants with *ROLA*, *ROLB* or *ROLC*. On tobacco plants transformed with the *ROLA* gene, small flowers with altered petals and anthers were formed, with a delay of 3-4 weeks (Sun *et al.*, 1991). Female and especially male fertility were reduced drastically. These effects were more pronounced in plants transformed with the *ROLA* gene driven by the strong CaMV 35S promoter, resulting in flowers with shortened styles (Dehio *et al.*, 1993). Flowers of *ROLA* transformed tomato plants were also small, showing hyperstyly (Van Altvorst *et al.*, 1992). Pollen production and pollen viability were reduced, whereas female fertility appeared normal. In general, transformation with the *ROLA* gene resulted in retarded and diminished flowering with decreased fertility.

Transgenic tobacco plants with the *ROLB* gene driven by its own or the CaMV 35S

promoter also showed hyperstyly (Schmülling *et al.*, 1988), while plants with the *ROLB* gene under control of the anther specific *TAP1* promoter from *Anthirrhinum* showed more compact flowers with shrivelled anthers (Spena *et al.*, 1992). More severe symptoms were observed in transgenic tobacco plants with *ROLB* under control of a tetracycline-dependent promoter (Röder *et al.*, 1994). Induction of the *ROLB* gene with tetracycline resulted in extremely stunted plants, with necrotic and wrinkled leaves. Vegetative meristems were severely affected, while floral meristems were not developed. Withdrawal of tetracycline allowed the development of normal meristems and shoots, even from severely affected meristems. This demonstrated that the *ROLB* gene reduced flower induction and development.

In contrast to plants transformed with *ROLA* or *ROLB*, flowering was earlier in transgenic tobacco plants with the *ROLC* gene under control of its own promoter (Oono *et al.*, 1990) or the strong CaMV 35S promoter (Scorza *et al.*, 1994). In the latter case, plants flowered earlier by up to 35 days, but flowers were smaller. Similar phenotypic changes were found in *Atropa belladonna* (Kurioka *et al.*, 1992). Flowering was earlier with more flower buds than in the control. Transformed plants were even able to flower in vitro. However, in tobacco transformed with CaMV 35S *ROLC* pollen viability was reduced (Scorza *et al.*, 1994), or even male sterility was observed, due to a severely damaged pollen production (Schmülling *et al.*, 1988; Oono *et al.*, 1993). This is remarkable since the CaMV 35S promoter is supposed not to be expressed in pollen. Moreover, crossings with *ROLC* transformed potato plants failed (Fladung *et al.*, 1990). It is concluded that the *ROLC* gene accelerated flowering in time and number, whereas female and male fertility were decreased.

To summarize, introduction of the *ROLC* gene resulted in improved and accelerated flowering, whereas transformation with the *ROLA* or *ROLB* gene generally inhibited flower development. In addition, fertility was generally decreased by the *ROL* genes. Since the *ROL* genes are expressed throughout the whole plant and not only in the flowers, other elements of plant development will also be affected. Although studies with the *ROLC* gene are encouraging, applications of *ROL* genes for modification of flower development and fertility are still far-away. In order to regulate gene activity more precisely, studies on the use of *ROL* genes driven by flower specific promoters are required.

8 Concluding remarks and future prospects

The *ROL* genes of *A. rhizogenes* and especially the *ROLB* and *ROLC* genes, are known to affect plant hormone metabolism and/or sensitivity. These genes are therefore in principle suitable tools to modify plant development.

For the application of the *ROL* genes for crop improvement, transformed plants with the

complete TL-DNA from the Ri plasmid of *A. rhizogenes* can be used or even more favourably, with individual *ROL* genes. By introduction of the *ROLB* gene and especially by a combination of *ROLA*, *B* and *C* genes, rooting characteristics of various woody species were shown to be improved. In general, introduction of *ROL* genes resulted in a lower plant weight in the field, indicating that these genes were inappropriate to increase crop yield. However, in some cases plant growth was improved qualitatively. The plant shape of various ornamental crops was modified by transformation with the *ROLC* gene or a combination of *ROLA*, *B* and *C* genes, resulting in a smaller and bushy-shaped plant. Finally, introduction of the *ROLC* gene might be a tool to modify flowering of plants.

Despite the variety of elements of plant development that can be affected by introduction of the *ROL* genes, it has to be concluded that the final step to practical application is not yet realized. This might be due to the many pleiotropic effects observed in *ROL* gene transformed plants. Therefore alternative approaches need to be developed to overcome these undesired effects. A novel approach is the application of *ROL* genes in combination plants, consisting of an untransformed scion grafted on a *ROL* gene transformed rootstock. For example, in combination plants of rose, consisting of a cultivar grafted on a *ROLA*, *B*, *C* transformed rootstock, the root system developed faster and more heavily than in the control. The *ROL* genes stimulated the release of axillary buds of the untransformed scion. We expect that this acceleration will result in a changed plant architecture, which is able to yield more flowers. Alternatively, pleiotropic effects may also be overcome by attributing more emphasis to the application of *ROL* genes, driven by organ or tissue specific promoters, leading to a more defined expression of *ROL* genes. This approach will reveal the effect of *ROL* gene induced changes in specific organs on the development of other plant organs. The experiments with combination plants of rose suggest that the transmissibility of pleiotropic effects to other organs is limited.

In conclusion, the *ROL* genes from *A. rhizogenes* are in principle suitable genes for the improvement of crops, especially for woody species. Further studies on the biochemistry of the gene products and more in general on hormone metabolism and perception are required for full comprehension of the fundamental mechanism of *ROL* genes. Together with the development of transformation methods for woody species, this will lead to the development of new and more defined applications of *ROL* genes for agricultural use.

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Summary

In contrast to many other cut flowers, which are harvested completely at the time of flowering, rose plants (*Rosa hybrida* L.) continuously produce flowering shoots, which are harvested on a regular basis. For the release of axillary buds and development of flowering shoots high energy inputs are required in rose culture. However, for environmental reasons the use of fossil fuels should be limited i.e. the energy efficiency of rose production needs to be improved. The energy efficiency in horticulture was already remarkably improved by technical and cultural measures, which has been evolved since the energy crisis in the seventies. Breeding of roses, which produce more efficiently per unit energy input, is another powerful tool to save energy. The aim of the research described in this thesis is to genetically change roses in such a way that they can be cultured at low energy input.

The production of flowering stems of roses is dependent on the number of axillary shoots or basal shoots. Axillary bud release is a plant developmental process, which is controlled by plant hormones, such as auxin and cytokinin. Auxin is produced in the apex and leaves of growing plants and is transported to the roots, while cytokinin is produced in the root tips and transported to the aerial parts. This means that the hormonal status of the roots influences the development of the whole plant. Many cut roses are therefore grafted on a rose rootstock; not only to enhance axillary bud release and flower production, but also to overcome deficiencies in adventitious root formation. The separation of rose plants in a scion and a rootstock part gives opportunity to specific breeding programmes for flower and root characteristics. In contrast to a high breeding activity in cut roses, breeding efforts for rose rootstocks have been relatively poor. This opens perspectives for the development of new rose rootstocks, either via cross breeding or more recently also via genetic modification. The latter approach has the advantage that gene transfer is no longer determined by crossing potential, but principally unlimited from one species to another species. In this thesis we aimed at the improvement of rootstock performance by the introduction of the *ROL* genes from the bacterium *Agrobacterium rhizogenes*. These genes influence plant development by interference in hormone metabolism or hormone sensitivity.

A prerequisite for genetic modification is the availability of reliable transformation procedures. At the start of this research such a transformation protocol was not available for rose. Since rose is a recalcitrant species in this respect, much attention has been paid to the development of methodological principles of genetic modification, such as micropropagation, transformation and regeneration.

First, the micropropagation of the rose rootstock 'Moneyway' was examined. On standard media either shoot growth was inadequate or leaves became chlorotic, suggesting an iron deficiency. Replacement of FeEDTA by the more stable iron chelate FeEDDHA in the

medium resulted in the development of green shoots for more than three months.

Adventitious root formation of rose was studied on stem slices of micropropagated shoots. First it was shown that the formation of adventitious roots was dependent on the auxin dose and was not affected by the presence of other root primordia on the same stem slice. Secondly, to study the effects of *ROL* genes, a method was developed for the production of transformed roots, using 5 mg/L kanamycin for selection. Kanamycin resistant roots were formed on stem slices, which were inoculated with *A. rhizogenes*, harbouring the complete Ri plasmid encompassing the *ROL* genes and a binary plasmid with the neomycin phosphotransferase (*NPTII*) gene for kanamycin resistance. In contrast to adventitious root formation, the formation of these Ri transformed roots was independent of the presence of auxin. This autonomous formation of Ri roots might be due to the transfer of *AUX* genes, which offer an alternative synthesis route for auxin. Stem slices, inoculated with *A. tumefaciens* harbouring only the *NPTII* gene, formed on kanamycin containing medium with high auxin concentrations first callus and subsequently roots. The formation of such kanamycin resistant roots was significantly improved upon transformation with the *ROLB* gene under the strong CaMV 35S promoter and even more by transformation with a combination of *ROLA, B, C* genes. These experiments demonstrated that the *ROLB* gene and especially the *ROLA, B, C* genes are in principle suitable genes for improvement of the rooting ability of rose rootstocks.

For the production of transformed rose plants a study was undertaken to establish a regeneration procedure. Plants were regenerated from excised adventitious roots via somatic embryogenesis. First, the roots were incubated on callus induction medium containing a high concentration of auxin (50 μM 2,4-D). For embryo induction calluses were transferred to hormone-free medium. The use of Gelrite instead of agar during callus induction and/or embryo induction stimulated embryogenesis; up to 16% of the calluses formed organized structures. Approximately 40% of these structures further developed into shoots. Despite the long lasting callus phase during regeneration, the majority of the regenerant plants did not show any signs of somaclonal variation, indicating that we developed a suitable regeneration procedure.

This regeneration procedure was applied for the regeneration of plants from excised kanamycin resistant roots. For this purpose first hundreds of kanamycin resistant roots were produced, with an efficiency of up to two roots per stem slice. Regeneration of plants from these roots lasted up to 12 months, but finally nine independent transformants were produced: four transformants with the reporter gene *35SGUSINT*, one transformant with the *ROLB* gene behind its own promoter (B1) and four transformants with a combination of *ROLA, B, C* genes also driven by their own promoters (ABC1 to ABC4). Rooting experiments in vitro showed that adventitious root formation on shoots and leaves was enhanced by the presence

of these *ROL* genes. Even in the absence of exogenous auxin roots were induced, probably due to an increased sensitivity for endogenous auxin. The presence of the *ROLA, B, C* genes also enhanced adventitious root formation on stem slices of micropropagated plants, whereas the *ROLB* gene surprisingly decreased it. We suggested that this might be due to overexpression of the *ROLB* gene. The rooting experiments in vitro were confirmed by rooting experiments with cuttings from greenhouse grown plants; adventitious root formation was improved threefold upon introduction of the *ROLB* gene or a combination of *ROLA, B, C* genes.

Next to an increased rooting ability of *ROL* gene transformed plants, many pleiotropic effects were observed in the greenhouse: increased (*ROLB*) or decreased (*ROLA, B, C*) apical dominance, reduced plant weight and altered leaf morphology. Northern analysis showed that altered leaf shapes were correlated with the presence of specific *ROL* transcripts; wrinkled leaves with a *ROLA* messenger of 650 nt, round-edged leaves with a *ROLB* messenger of 1050 nt and small and lanceolate leaves with a *ROLC* messenger of 850 nt.

The *ROLB* transformant (B1) and one *ROLA, B, C* transformant (ABC1) were used as rootstocks in combination plants with the cut rose cultivar Madelon as a scion. Growth and development were followed on hydroculture at two temperatures during three months. Although the formation of adventitious roots was initially enhanced by the *ROLB* gene, further root development was inhibited. Despite the smaller root system, the performance of the untransformed scion was as in the control. In contrast, root development was enhanced by the presence of *ROLA, B, C* genes, while the development of the untransformed aerial part was also affected. Regarding the aim of this research, the most important observation was that the release of axillary buds was increased from 0.1 to 0.6 and from 0.3 to 1.3 basal shoot per plants at 15 and 20°C, respectively. This enhanced axillary bud release might reflect either an increased production of cytokinin in such well developed root systems or a cytokinin-like action as mentioned for *ROLC* transformed plants. In this respect it should be mentioned that the *ROLC* gene was indeed active in *ROLA, B, C* transformed roots. Since the formation of these basal shoots is correlated with flower production, we expect that application of *ROLA, B, C* transformed rootstocks will lead to a more energy efficient flower production both at 20°C and 15°C.

In the final part of this thesis, prospects for the applications of *ROL* genes for crop improvement are discussed. As suggested in many reports, the *ROL* genes are principally suitable tools to modify plant developmental processes such as adventitious root formation and axillary bud release. However, practical application of *ROL* gene transformed plants might be hampered by the many pleiotropic side effects, as observed in completely transformed roses. Based on the research described in this thesis, we offer a novel approach for application, which is clearly different from earlier strategies. It was demonstrated for the

first time that expression of *ROL* genes in the rootstock led to a beneficial stimulation of axillary bud release of the untransformed scion, without the transmissibility of many undesired pleiotropic effects.

Samenvatting

In tegenstelling tot planten, die gedurende een beperkte periode één of enkele snijbloemen produceren, vormt de roos (*Rosa hybrida* L.) voortdurend nieuwe bloemtakken, die vlak voor het bloeistadium geoogst worden. In de rozenteelt is voor het uitlopen van axillaire ogen en de ontwikkeling van bloemtakken veel energie vereist. Vanuit milieu oogpunt bezien dient het gebruik van fossiele brandstoffen echter beperkt te worden en ook voor de produktie van snijrozen wordt een efficiënter gebruik van energie nagestreefd. Na de oliecrisis in de jaren zeventig is de energie-efficiëntie in de glastuinbouw al aanzienlijk verbeterd door toepassing van technische maatregelen en aanpassingen in de teelt. Daarnaast kunnen door de veredeling van rozen, die efficiënter produceren per gebruikte eenheid energie, ook forse energiebesparingen behaald worden. Het doel van het in dit proefschrift beschreven onderzoek is dan ook om genetisch veranderde rozen te produceren, die geteeld kunnen worden onder energie-arme omstandigheden.

De produktie van rozentakken is afhankelijk van het aantal axillaire scheuten of grondscheuten dat uitloopt. Axillaire scheutvorming is een ontwikkelingsproces, dat wordt gereguleerd door plantenhormonen, zoals auxine en cytokinine. Auxine wordt geproduceerd in de apex en bladeren van groeiende planten en wordt getransporteerd naar de wortels, terwijl cytokinine geproduceerd wordt in worteltopjes en getransporteerd wordt naar de bovengrondse delen van de plant. Hierdoor is de hormonale status van de wortels van groot belang voor de ontwikkeling van de gehele plant. Veel snijrozen worden daarom geënt op een onderstam; niet alleen om de axillaire scheutvorming en bloemproduktie te stimuleren, maar ook om bewortelingsdefecten op te vangen. Een opdeling in ent en onderstam maakt het tevens mogelijk om afzonderlijk te veredelen op bloem- en worteleigenschappen. In tegenstelling tot de hoge veredelingsinspanning op het gebied van snijrozen, is de veredeling van rozenonderstammen altijd beperkt geweest. Dit biedt perspectief voor de ontwikkeling van nieuwe rozenonderstammen d.m.v. kruisingsveredeling of sinds kort via genetische modificatie. Deze laatst genoemde aanpak heeft het voordeel dat de uitwisseling van genetische eigenschappen niet langer bepaald wordt door kruisbaarheid, maar in principe ongelimiteerd is tussen verschillende soorten. In dit proefschrift hebben we ons gericht op het verbeteren van de onderstammen door introductie van de *ROL* genen uit *Agrobacterium rhizogenes*. Deze genen beïnvloeden de ontwikkeling van de plant door verandering van het hormoonmetabolisme of de hormoongevoeligheid.

Een voorwaarde voor genetische modificatie is de beschikbaarheid van een goed transformatiesysteem. Bij de aanvang van dit onderzoek was een dergelijk transformatieprotocol niet voorhanden. Aangezien roos in dit opzicht als een recalcitrant gewas wordt beschouwd, is veel aandacht besteed aan de ontwikkeling van methodologische

principes nodig voor genetische modificatie, zoals weefselkweekvermeerdering van planten, transformatie en regeneratie.

Allereerst is de weefselkweekvermeerdering van de rozenonderstam 'Moneyway' onderzocht. Standaardmedia resulteerden in onvoldoende scheutgroei of de vorming van chlorotische bladeren, mogelijk veroorzaakt door ijzergebrek. Vervanging van FeEDTA in het medium door het meer stabiele ijzerchelaat FeEDDHA resulteerde in de ontwikkeling van groene scheutjes gedurende meer dan drie maanden.

Adventieve wortelvorming van roos werd onderzocht aan stengelplakjes van in vitro vermeerderde scheutjes. Allereerst werd aangetoond dat de vorming van adventieve wortels afhankelijk was van de auxine dosis en niet beïnvloed werd door de aanwezigheid van andere wortelprimordia op het zelfde stengelplakje. Om daarnaast de effecten van *ROL* genen te bestuderen werd een methode ontwikkeld voor de produktie van transgene wortels, waarbij geselecteerd werd met 5 mg/L kanamycine. Op stengelplakjes werden kanamycine-resistente wortels gevormd na inoculatie met *A. rhizogenes* met de *ROL* genen gelegen op het Ri plasmide en het neomycine fosfotransferase (*NPTII*) gen, voor het verkrijgen van kanamycine-resistentie, gelegen op een binair plasmide. In tegenstelling tot adventieve wortelvorming, was de vorming van deze Ri getransformeerde wortels onafhankelijk van exogeen auxine. Deze autonome vorming van Ri wortels werd waarschijnlijk veroorzaakt door de overdracht van *AUX* genen, die een alternatieve synthese van auxine mogelijk maken. Stengelplakjes die geïnoculeerd waren met *A. tumefaciens* met alleen het *NPTII* gen, vormden op medium met een hoge concentratie auxine en in aanwezigheid van kanamycine eerst callus en vervolgens wortels. Deze vorming van kanamycine-resistente wortels werd gestimuleerd door transformatie met het *ROLB* gen met de CaMV 35 promoter en nog sterker door transformatie met een combinatie van *ROLA, B, C* genen. Deze experimenten toonden aan dat het *ROLB* gen en met name een combinatie van *ROLA, B, C* genen in principe geschikt zijn voor het verbeteren van de beworteling van onderstammen.

Voor de produktie van transgene planten werd onderzoek verricht aan de ontwikkeling van een regeneratieprocedure voor roos. Planten werden geregenereerd vanuit afgesneden adventieve wortels via somatische embryogenese. Hiervoor werden wortels allereerst geïncubeerd op callusinductiemedium met een hoge concentratie auxine (50 μM 2,4-D). Voor de inductie van embryo's werden calli vervolgens overgezet op hormoonloos medium. Het gebruik van Gelrite in plaats van agar gedurende callusinductie en/of embryoinductie stimuleerde embryogenese; tot 16% van de explantaten vormde georganiseerde structuren. Ongeveer 40% van deze structuren ontwikkelde uiteindelijk verder tot een scheut. Ondanks de lange callusfase, vertoonde de meerderheid van de regeneranten geen tekenen die duiden op somaklonale variatie. Hiermee werd gedemonstreerd dat een geschikt regeneratiesysteem was ontwikkeld.

Deze regeneratieprocedure werd vervolgens toegepast voor de regeneratie van planten uit afgesneden kanamycine-resistente wortels. Hiervoor werden allereerst honderden transgene wortels geproduceerd, met een maximale efficiëntie van twee wortels per stengelplakje. Het regenereren van planten uit deze wortels duurde tot 12 maanden, maar uiteindelijk werden negen onafhankelijke transformanten geproduceerd: vier transformanten met het merkergen *35SGUSINT*, één transformant met het *ROLB* gen achter de eigen promoter (B1) en vier transformanten met een combinatie van *ROLA, B, C* genen, gelegen achter de eigen promoter (ABC1 tot en met ABC4). Bewortelingsexperimenten in vitro toonden aan dat adventieve wortelvorming aan scheuten en bladeren werd bevorderd door de aanwezigheid van *ROL* genen. Zelfs in afwezigheid van exogeen auxine werden wortels geïnduceerd, wellicht door een toegenomen gevoeligheid voor endogeen auxine. De aanwezigheid van *ROLA, B, C* genen stimuleerde ook de adventieve wortelvorming aan stengelplakjes van in vitro planten, terwijl het *ROLB* gen dit juist tegenging. Deze remming hield mogelijk verband met overexpressie van het *ROLB* gen. De resultaten van de bewortelingsexperimenten in vitro werden bevestigd door experimenten uitgevoerd met stekken van in de kas geteelde planten; adventieve wortelvorming was verdrievoudigd na introductie van het *ROLB* gen of een combinatie van *ROLA, B, C* genen.

Naast een verbeterde beworteling van met *ROL* genen getransformeerde planten, werden veel pleiotrope effecten waargenomen in de kas: verhoogde (*ROLB*) of verlaagde (*ROLA, B, C*) apicale dominantie, een lager plantgewicht en een veranderde bladmorfologie. Northern analyse liet zien dat de verschillende bladvormen gecorreleerd waren met de aanwezigheid van specifieke *ROL* transcripten; gekrinkelde bladeren met een *ROLA* transcript van 650 nt, ronde bladeren met een *ROLB* transcript van 1050 nt en kleine, spitse bladeren met een *ROLC* transcript van 850 nt.

De *ROLB* transformant (B1) en één *ROLA, B, C* transformant (ABC1) werden gebruikt als onderstam in combinatieplanten, met de cultivar Madelon als entras. Gedurende drie maanden werden bij twee temperaturen de groei en ontwikkeling van combinatieplanten op hydrocultuur gevolgd. Hoewel de vorming van adventieve wortels aanvankelijk versneld werd door het *ROLB* gen, werd de verdere groei ervan geremd. Ondanks een kleiner wortelstelsel, was de ontwikkeling van de ongetransformeerde ent vergelijkbaar met die van controle planten. Daarentegen werd de verdere wortelontwikkeling juist gestimuleerd door de aanwezigheid van *ROLA, B, C* genen, terwijl de ontwikkeling van de ongetransformeerde ent ook beïnvloed werd. Met betrekking tot het uiteindelijk doel van het onderzoek, was een toename van het aantal grondscheuten de meest belangrijke waarneming. Bij 15 en 20°C nam het aantal axillaire scheuten respectievelijk toe van 0,1 tot 0,6 en van 0,3 tot 1,3 grondscheut per plant. Dit versnelde uitlopen van axillaire knoppen weerspiegelt óf een verhoogde cytokinine produktie in deze goed ontwikkelde wortelstelsels óf een cytokinine-achtige

werking zoals bekend van *ROLC* getransformeerde planten. Relevant hierbij was dat het *ROLC* gen inderdaad tot expressie kwam in *ROLA, B, C* getransformeerde wortels. Aangezien de hoeveelheid gevormde grondscheuten bepalend is voor de latere bloemproduktie, is de verwachting dat toepassing van *ROLA, B, C* getransformeerde onderstammen zal leiden tot een energie-efficiëntere produktie bij 20°C en 15°C.

In het laatste deel van dit proefschrift worden de vooruitzichten voor de toepassing van de *ROL* genen voor gewasverbetering bediscussieerd. Zoals in vele artikelen gesuggereerd wordt, zijn de *ROL* genen in principe geschikt voor het aanpassen van ontwikkelingsprocessen in planten, met name voor adventieve wortelvorming en axillaire scheutvorming. Praktische toepassingen van met *ROL* genen getransformeerde planten worden echter verhinderd door het optreden van veel pleiotrope effecten, zoals waargenomen in transgene rozen. Op basis van het in dit proefschrift beschreven onderzoek wordt een nieuwe benadering geboden, die duidelijk afwijkend is van eerdere strategieën. Voor het eerst werd aangetoond dat expressie van *ROL* genen in een onderstam de vorming van grondscheuten in de ent stimuleert, zonder het optreden van veel ongewenste pleiotrope effecten.

Account

Parts of this thesis have been or will be presented elsewhere:

Chapter 2:

Van der Salm TPM, Van der Toorn CJG, Hänisch ten Cate ChH, Dubois LAM, De Vries DP and Dons JJM (1994) Importance of the iron chelate formula for micropropagation of *Rosa hybrida* L. 'Moneyway'. Plant Cell Tissue Org Cult 37: 73-77

Chapter 3:

Van der Salm TPM, Van der Toorn CJG, Hänisch ten Cate ChH, Van der Krieken WM and Dons JJM. The effects of exogenous auxin and *ROL* genes on root formation in *Rosa hybrida* L. 'Moneyway'. Plant Growth Reg (in press)

Chapter 4:

Van der Salm TPM, Van der Toorn CJG, Hänisch ten Cate ChH and Dons JJM (1996) Somatic embryogenesis and shoot regeneration from excised adventitious roots of the rootstock *Rosa hybrida* L. 'Moneyway'. Plant Cell Rep 15: 522-526

Chapter 5:

Van der Salm TPM, Van der Toorn CJG, Bouwer R, Hänisch ten Cate ChH and Dons JJM. Production of *ROL* gene transformed plants of *Rosa hybrida* L. and characterization of their rooting ability. Plant Mol Biol (submitted)

Chapter 6:

Van der Salm TPM, Bouwer R, Van Dijk AJ, Keizer LCP, Hänisch ten Cate ChH, Van der Plas LHW and Dons JJM. Stimulation of scion bud release by *ROL* gene transformed rootstocks of *Rosa hybrida* L. Plant Phys (submitted)

Chapter 7:

Van der Salm TPM, Hänisch ten Cate ChH and Dons JJM. Prospects for applications of *ROL* genes for crop improvement. Nature Biotechnol (in preparation)

Other publications:

Van der Salm TPM, Van der Toorn CJG and Hänisch ten Cate ChH (1994) Energie besparen met transgene roos 'Moneyway'. Vakblad voor de Bloemisterij 30: 25

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Curriculum vitae

Theo van der Salm werd op 23 september 1960 in Leiden geboren. In 1978 behaalde hij het VWO diploma aan het Fioretti College te Lisse. In hetzelfde jaar werd gestart met de studie Tuinbouwplantenteelt aan de Landbouwhogeschool te Wageningen. In 1986 werd deze studie afgesloten met het behalen van het doctoraal examen met de afstudeervakken Plantenfysiologie, Tuinbouwplantenteelt en Onkruidkunde en een stage aan de Colorado State University.

Van november 1986 tot mei 1988 werkte hij bij de afdeling Moleculaire Biologie van de Stichting Ital, later het DLO-Centrum voor Plantenveredelings- en Reproduktieonderzoek (CPRO-DLO), aan de isolatie en karakterisering van *Bacillus thuringiensis* kristaleiwitgenen.

Daarna werd tot augustus 1991 bij dezelfde afdeling gewerkt aan een door het veredelingsbedrijfsleven gefinancierd project, gericht op het verkrijgen van insektenresistentie bij tomaat door genetische modificatie. Dit onderzoek resulteerde in de produktie van insektenresistente planten door *B. thuringiensis* genen met een veranderde coderende sequentie te gebruiken.

In oktober 1991 werd hij aangesteld bij de afdeling Ontwikkelingsbiologie van het CPRO-DLO. Gedurende vier jaar werd het in dit proefschrift beschreven onderzoek uitgevoerd.

Nawoord

Bij de start van het in dit proefschrift beschreven onderzoek waren, voordat de allereerste rozenstengel was gesneden, al twee scalpelmesjes gebroken en had ik met één ervan mezelf in de vingers gesneden. De boodschap was duidelijk: deze gestekelde plant zou zich niet zomaar op een zonnige achternamiddag laten transformeren. Nu, meer dan vier jaar later en dankzij het inzicht en de inspanning van velen is het transformeren van roos gelukt, zijn de effecten van *ROL* genen geanalyseerd en staan de resultaten beschreven in dit proefschrift. Graag wil ik op deze plaats hulde en dank betuigen aan alle mensen die hieraan hebben bijgedragen.

Allereerst wil ik mijn co-promotor Hans Dons bedanken voor de gecreëerde mogelijkheid om dit onderzoek uit te voeren bij de afdeling Ontwikkelingsbiologie van het CPRO-DLO. Zijn deskundigheid en vriendelijkheid hebben mij enorm gestimuleerd. Ik wil hem dan ook hartelijk bedanken voor de begeleiding en het becommentariëren van de hoofdstukken.

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Uiteraard wil ik ook Lot Hänisch ten Cate bedanken. Haar ervaring met de *ROL* genen van *Agrobacterium rhizogenes* vormde de basis voor dit onderzoek. Tijdens het onderzoek en het schrijven van de hoofdstukken was ze altijd een uitnodigend aanspreekpunt en mede dankzij haar originele ideeën is het gelukt om roos te transformeren.

In het bijzonder wil ik Caroline van der Toorn bedanken voor haar praktische bijdrage gedurende de eerste drie jaren van het project. Haar enorme inzet is van groot belang geweest voor het slagen van het project o.a. voor de produktie van de eerste transgene rozen. Daarnaast wil ik natuurlijk ook Reinoud Bouwer bedanken voor het uitvoeren van de moleculaire analyses aan de transgene rozen en Arie van Dijk voor de efficiënte samenwerking tijdens de groeianalyses. Zonder jullie flexibele inzet in het vierde en laatste jaar was het zeker niet gelukt om het onderzoek op een dergelijke wijze af te ronden.

Dit project is tot stand gekomen en uitgevoerd in samenwerking met de afdeling Siergewassen en hiervoor wil ik Hein van Holsteijn, Dik de Vries en Lidwien Dubois bedanken. Na de start van een tweede transformatieproject, gericht op verbetering van het vaasleven van roos, is deze samenwerking verder uitgebreid. In direct verband hiermee wil ik mijn kamergenote Sonja Derks bedanken voor de stimulerende en prettige wijze van samenwerken.

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