Transformation of *Kalanchoë* and oilseed rape with ORFs 11-15 from *Agrobacterium rhizogenes*

B.T. Favero[a], H.B. Hansen, R.K. Rangslang, Z. Liu, J.N. Hegelund, R. Müller, N. Kodahl and H. Lütken

Department of Plant and Environmental Sciences, Faculty of Sciences, University of Copenhagen, Hoejbakkegaard Allé 9-13, 2630 Taastrup, Denmark.

Abstract

Infection by naturally occurring *Agrobacterium rhizogenes* gives rise to hairy root disease in many host plants. Moreover, plants transformed with this bacterium exhibit compact growth and frequently wrinkled leaves that often are reduced in size. The phenotypes are caused by the bacterial T-DNA integration in the plant host genome. The T-DNA includes several genes of which the best characterized are the *root oncogenic loci* (*rol*) genes: *rol*A, *rol*B, *rol*C and *rol*D, corresponding to open reading frames (ORFs) 10, 11, 12 and 15, respectively. Understanding how the *rol*-genes confer compact growth traits in plants is relevant given the potential to reduce the use of chemical growth retardants, thus providing environmentally friendly produced compact ornamental plants. Few studies have investigated the association of genotypes with phenotypic traits caused by the individual *rol*-genes and selected ORFs from *A. rhizogenes*. The current study aims to investigate this association in *Kalanchoë* spp. and oilseed rape by producing plant lines with a selection of single gene constructs comprising *rol*-genes and ORFs. Hence, transformation and tissue culture methods are currently being developed. The aim is implementing a transformation platform to facilitate the regeneration of transgenic lines to study the individual genes. Callus formation of 100% and 80-100% was observed in *K. blossfeldiana* 'Molly' and *K. pinnata* leaf explants, respectively. Whereas in *Brassica napus* 'Elan' cotyledons, the callus formation ranged from 50 to 75% a month after inoculation. Shoot formation in the studied *Kalanchoë* species was between 0 to 10% of the initial number of inoculated explants; whereas in *B. napus* 'Elan' it ranged from 2 to 9%.

Keywords: biotechnology, compact plants, natural transformation, *root oncogenic loci* genes, tissue culture

INTRODUCTION

Transformation methods to create transgenic plants using *Agrobacterium* spp. has been extensively used in modern agricultural biotechnology and plant research has benefited a lot from this technology (Gelvin, 2003). *A. rhizogenes,* which is a soil bacterium, can cause the "hairy root" disease in a diverse range of dicotyledonous plants. This disease is caused once the bacterial root inducing (Ri) plasmid transfer-DNA (T-DNA) is integrated into the genome of the host plant (Chilton et al., 1982; Dubrovsky and Laskowski, 2017). The T-DNA of agropine type Ri plasmid consists of a T_L and T_R DNA (De Paolis et al., 1985) which are independently transferred into the host cell's genome (Nilsson and Olsson, 1997; Nemoto et al., 2009). The T_L strand of the agropine type Ri plasmid consists of 18 open reading frames (ORF) (Slightom et al., 1986), of which the ORFs 10, 11, 12 and 15 correspond to the *root oncogenic loci* (*rol*) genes, *rol*A, *rol*B, *rol*C and *rol*D, respectively (White et al., 1985).

Upon transformation of the T-DNA from the wild type Ri plasmid, the resulting phenotype commonly includes short internodes and altered flower morphology (Tepfer, 1990). In transgenic lines of *Nicotiana tabacum*, for instance, it has been observed that the overexpression of *ORF13* leads to plants with shorter internodes and smaller leaves (Hansen et al., 1993) and in *Arabidopsis thaliana*, ectopic expression of *ORF13* resulted in extremely

[a]E-mail: btf@plen.ku.dk

dwarfed plants having reduced leaf and flower size (Kodahl et al., 2016). The effect of single gene transformation with the *rol*B construct in *Solanum lycopersicum* resulted in wider and shorter leaves that are devoid of wrinkling and plants with decreased apical dominance (van Altvorst et al., 1992); while in *Nicotiana tabacum*, it has been observed that adventitious roots were formed and leaf as well as flower morphology were altered in response to *rol*B transformation (Schmülling et al., 1988). In both transgenic ornamentals and crop plants, *rol*B leads to an increased root to shoot ratio due to rapid proliferation of roots that can produce plants that are more robust than the wild type (Christey, 2001).

As reviewed by Lütken et al. (2012), the effect of *rol*A, B, C and D has been studied extensively, both in terms of plant compactness and stimulation of secondary metabolism, however little is known about the effect of other ORFs.

To investigate the individual gene functions of the various genes and ORFs present on the T_L DNA, it is necessary to establish a platform of plant transformation, where transgenic lines can be efficiently regenerated. In the present study, transformation and tissue culture techniques were developed in *Kalanchoë* spp. and *Brassica napus*, as model systems for ornamental and agricultural plants, respectively, by transforming them with individual ORFs from the *A. rhizogenes* plasmid pRiA4.

MATERIALS AND METHODS

Plant material

Two species of *Kalanchoë*; *K. blossfeldiana* 'Molly' and *K. pinnata* as well as *B. napus* winter cultivar 'Elan', all grown in greenhouse, were selected for transformation with different ORFs (*rol*B, *rol*C, *ORF13*, *ORF14*) derived from *A. rhizogenes* Ri plasmid of A4 strain. At first, fully developed leaves of *K. blossfeldiana* 'Molly' and *K. pinnata* and seeds of *B. napus* 'Elan' were surface sterilized in 70% ethanol for 1 min, followed by 45-90 g L^{-1} $Ca(ClO)_2$ (ACROS Organics, Geel, Belgium) and 0.03% Tween® 20 (SIGMA, Darmstadt, Germany) for 20 min and rinsed 3 times in sterile water.

To generate explant material, leaves of *K. blossfeldiana* 'Molly' and *K. pinnata* were hereafter cut into approximate 1 cm^2, removing the edge and stem of the leaves (Figure 1A). The sterilized seeds of *B. napus* 'Elan' were germinated for 2 weeks in darkness at RT (22±3°C) on standard MS medium with Gamborg vitamins (Duchefa, Haarlem, The Netherlands) (Gamborg et al., 1968; Murashige and Skoog, 1962), containing 30 g L^{-1} sucrose and 2.5 g L^{-1} gelrite (Duchefa) and with pH adjusted to 6.3 before autoclaving. The explant material consisted of excised cotyledons including approx. Two mm hypocotyls (Figure 1F).

Transformation of *A. tumefaciens*

The ORFs of interest, i.e. *rol*B, *rol*C, *ORF13*, *ORF14*, were cloned into pK2GW7 according to Kodahl et al. (2016) using the Gateway® technology. The plasmid backbone includes the 35S promoter upstream the insertion sites and antibiotic resistance to spectinomycin and kanamycin in bacteria and plants, respectively. These vectors were transformed into rifampicin resistant and competent *A. tumefaciens* C58C1 (pGV3850) by electroporation. Growth of transformed bacteria was maintained at 28°C in LB medium supplemented with 75 mg L^{-1} spectinomycin (Spec75) and 100 mg L^{-1} rifampicin (Rif100), i.e. the selection markers for the plasmid and the bacterial strain. Successful transformations were verified by plasmid purification (GenElute™ Plasmid Miniprep Kit, Sigma) followed by PCR detection of the inserted target gene using gene specific primers (Kodahl et al., 2016).

All antibiotics and plant growth regulators were from Duchefa. The OD of the cultured bacteria was adjusted to 0.4-0.6 with the same medium composition prior to explant transformation.

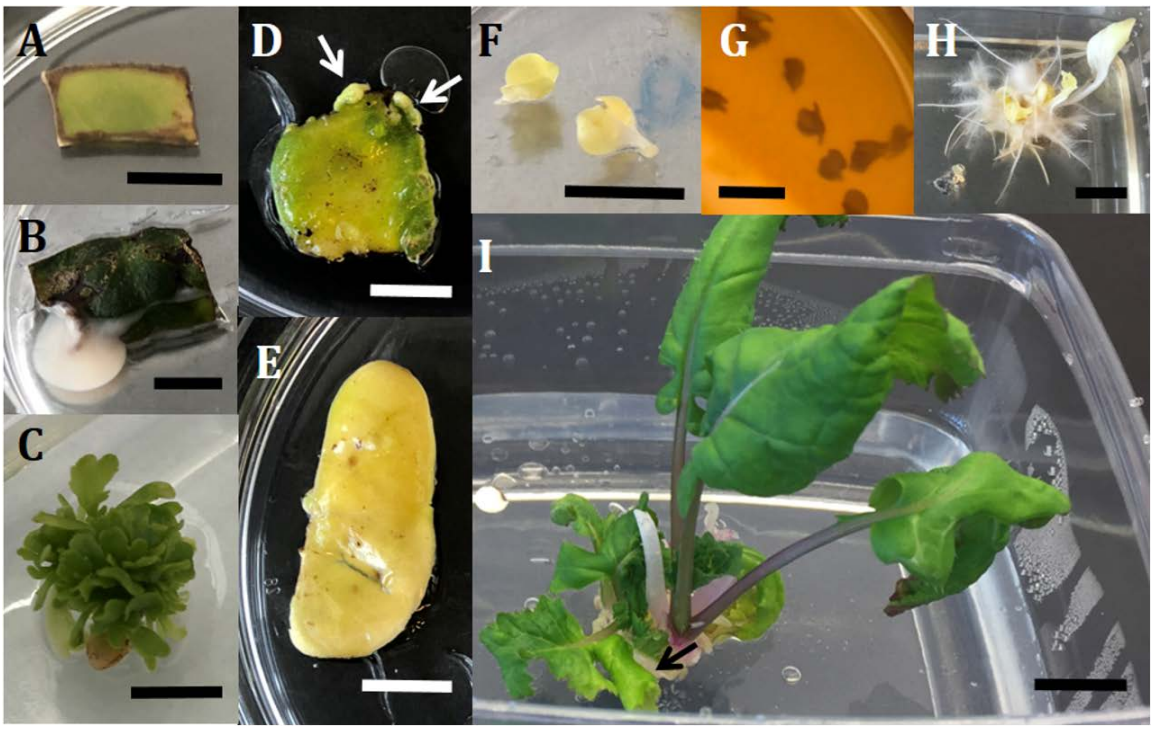

Figure 1. Plant transformation and regeneration. *K. pinnata* healthy explant (A) and with *A. tumefaciens* overgrowth (B); shoot regenerated from *K. blossfeldiana* 'Molly' (C), explant with callus (D) and senescing explant (E); excised cotyledon from *B. napus* 'Elan' (F), *A. tumefaciens* inoculation (G), explant during regeneration (H) and regenerated shoot (I). The bar represents 1 cm.

Transformation and regeneration of *Kalanchoë*

Cut leaves of *K. blossfeldiana* 'Molly' and *K. pinnata* were inoculated for 30 min with *A. tumefaciens* harboring the destination vectors supplemented with acetosyringone 15 mg L^{-1} (Aceto15) (Lütken et al., 2010). For controls, leaves were likewise placed in LB media with Spec75, Rif100 and Aceto15 without the bacteria.

After the inoculation, explants of *K. blossfeldiana* 'Molly' and *K. pinnata* were transferred to co-cultivation plates with standard MS media containing Aceto15 and kept in darkness for 2 days at 20±2°C. The plant materials were hereafter washed with 1000 mg L^{-1} ticarcillin disodium/clavulanate potassium (15:1 mixture, timentin) (Tim1000) and transferred to regeneration media, i.e., standard MS plus 200 mg L^{-1} timentin (Tim200), 0.009% (w/v) sodium benzoate (SB) (Atamon, Haugen-group, Hvidovre, Denmark), 100 mg L^{-1} kanamycin (Kan100, selection marker for plants, Figure 1E). The regeneration media contained either 1 mg L^{-1} thidiazuron (TDZ) or 2 mg L^{-1} TDZ. The healthy explants were moved to new regeneration media approximately every third week.

The *Kalanchoë* spp. explants were kept in a climate chamber with a photoperiod of 16 h of light and a photosynthetic photon flux density of approximately 50 µmol m^{-2} s^{-1} at 20/18±2°C day/night temperature. Shoots were transferred to MS standard media, Tim200, SB1, Kan100 and 1.7 mg L^{-1} gibberellin (GA_3) and afterwards transferred to pots with soil and placed in greenhouse.

Transformation and regeneration of oilseed rape

The cotyledons from *B. napus* 'Elan' were inoculated (Figure 1G) for 30 s with *A. tumefaciens* harboring the destination vectors amended with acetosyringone 15 mg L^{-1} (Aceto15) (Bhalla and Singh, 2008). For controls, cotyledons were similarly placed in LB media with Spec75, Rif100 and Aceto15 without the bacteria.

Immediately after inoculation, the *B. napus* 'Elan' cotyledons were moved to co-

cultivation plates with standard MS medium containing 5 mg L^{-1} $AgNO_3$, 0.75 mg L^{-1} benzylaminopurine, 0.2 mg L^{-1} 1-naphthalenoacetic acid and 0.01 mg L^{-1} GA_3 and kept in darkness for 2 days at 20±2°C (Bhalla and Singh, 2008). Subsequently, the cotyledons were transferred to callus induction medium, i.e. co-cultivation medium amended with Tim200 and kept in darkness at 20±2°C for 3-4 weeks. Hereafter, only green explants were moved to the shoot initiation (SI) media, i.e. co-cultivation medium amended with Tim200 and 25 mg L^{-1} kanamycin (Kan25), and placed for 4 weeks under the same light and temperature regimes used for *Kalanchoë*.

The regenerated shoots were moved to shoot outgrowth medium, i.e. SI medium with 50 mg L^{-1} kanamycin (Kan50). The grown shoots were transferred to root inducing media, half strength MS medium with 1 mg L^{-1} indole-3-butyric acid (IBA) for the time necessary to observe multiple (min. Two cm) roots. The rooted explants were transferred to sterile soil and moved to the greenhouse.

Each experiment for the individual loci of interest was repeated 3-6 times in displaced periods. The number of explants per repetition was approximately 80-100 explants per locus of interest and a similar quantity for controls.

RESULTS AND DISCUSSION

Kalanchoë

The number of explants of *K. blossfeldiana* 'Molly' and *K. pinnata* were determined after inoculation and the senescence and infection ratios were monitored over time. After some weeks, calli (Figure 1D) and shoots (Figure 1C) were visible. The experiment proceeded for 20-30 weeks (Table 1).

Table 1. *Kalanchoë* spp. tissue culture parameters from *rol*B, *rol*C and *ORF13* independent transformation events after approx. Twenty-seven weeks from *A. tumefaciens* inoculation. Data are shown as average±standard deviation.

Spp.		Total n. of explants	Contamination (%)	Senescence (%)	Calli (%)	Shoot[a] (%)	Moved to soil (%)
K. blossfeldiana 'Molly'	Control	263	49±21	47±19	83±21	11±4	5±7
	*rol*B[b]	120	97	3	100	1	0
	*rol*C	338	89±4	10±4	100	8±11	7±10
	ORF13[b]	100	88	12	100	1	0
K. pinnata	Control	183	20±14	65±18	100	11±8	1±2
	*rol*B	341	20±5	70±14	100	9±4	1±2
	*rol*C	285	28±18	63±16	100	0	0
	ORF13	205	40±17	50±20	98±2	9±7	0

[a]Number of explants with shoots in *K. blossfeldiana* 'Molly': control 22, *rol*B 1, *rol*C 19, *ORF13* 1; in *K. pinnata* control 19, *rol*B 30, *rol*C 0, *ORF13* 19.

[b]Results obtained in a single experiment.

At the end of the experiment, less than 5% of explants of *K. blossfeldiana* 'Molly' were viable. Moreover, the explants of *K. blossfeldiana* 'Molly' exhibited *A. tumefaciens* overgrowth (Figure 1B), indicating that the elimination of *A. tumefaciens* by the Tim1000 wash was not sufficient to inhibit bacterial growth. The relatively high levels of bacterial contamination in the controls indicate that further optimization of the sterilization of explants are necessary.

The presence of regenerated shoots in *K. blossfeldiana* 'Molly' were under 11%. Additionally, only two shoots from the control group of *K. blossfeldiana* 'Molly' moved to soil survived and started developing roots.

Sanikhani et al. (2006) observed approximately 83.5% of shoot regeneration in *K. blossfeldiana* 'Molly' leaf explants after 5 weeks when adding 1 mg L^{-1} TDZ to the regeneration media. Furthermore, a 50-70% regeneration ratio was observed in *K. blossfeldiana* 'Hillary' and 'Tenorio' by García-Sogo et al. (2010) in transformations using *A. tumefaciens*. Both studies had similar experimental setup as ours, however explants in their study were kept at

approximately 5°C lower temperature during regeneration, potentially impairing shoot induction in this tropical species.

Viability of the explant material rather than contamination was the main cause of the explant number diminution in *K. pinnata*, with a large percentage of the explants senescing after one week of the transformation. The *K. pinnata* leaves were especially sensitive to the surface sterilization procedure with >50 g L^{-1} of $Ca(ClO)_2$, which led to vitreous explants soon after the transformation. Despite the reduction of the concentration to 45 g L^{-1} during the sterilization procedure, the explants continued exhibiting premature senescence yet showing better appearance after transformation. This indicate that the cultivation procedure can be optimized further.

Regenerated plants from *K. pinnata* explants were obtained, however they did not survive the transition from tissue culture to soil. For future studies, it has been shown that the use of hormone free medium for 2-3 weeks is a good preparation before transferring explants to soil (J.N. Hegelund, pers. commun.).

***B. napus* 'Elan'**

The transformation with four individual constructs, *rol*B, *rol*C, *ORF13* and *ORF14*, and the respective control group were carried out on explants obtained from cotyledons derived from *B. napus* 'Elan' in separate experiments (Table 2).

Table 2. *B. napus* 'Elan' data from *rol*B, *rol*C, *ORF13* and *ORF14* independent transformation events after approx. Nineteen weeks from *A. tumefaciens* inoculation. Data are shown as average ± standard deviation.

	Total n. of explants	Contamination (%)	Senescence (%)	Callus (%)	Shoot (%)	Moved to soil (%)
Control	188	3±3	95±4	70±12	8±4	-
*rol*B	196	6±1	89±2	48±6	7±6	0.4±0.4
*rol*C	196	1±1	95±2	61±11	6±2	0.4±0.4
ORF13	198	5±5	88±7	66±3	10±4	0.4±0.4
ORF14	192	3±3	94±4	69±7	2±0.1	0.4±0.4

Callus was visible at the cut end of cotyledons after 2 weeks of the *A. tumefaciens* inoculation (Figure 1H). The contamination and senescence rate was noted at the commencement of the callus induction stage according to the protocol by Bhalla and Singh (2008) and continued throughout the entire experimental process.

Callus induction at 34 days after transformation was quite high with more than half of the explants producing callus. It was observed that the control produced callus more abundantly as compared to the explants that were inoculated with *A. tumefaciens*, this can be attributed to the fact that the explants in the control were not subjected to stress during co-cultivation which was a result of the presence of *A. tumefaciens*.

The calli transferred to shoot initiation medium resulted in 2-10% of generated shoots, owing to the selection pressure due to the presence of 25 mg L^{-1} kanamycin in the medium (Öz et al., 2009). The selection of putatively transformed calli was based on the explant capability to grow in medium supplemented with kanamycin for several weeks.

The putatively transformed shoots obtained were under 5% after the final selection process, which involved an increase of the selection agent, i.e. kanamycin, to 50 mg L^{-1} (Figure 1I). Of the putatively transformed shoots obtained, the ones that exhibited root formation when placed in root induction media with 1 mg L^{-1} IBA (Bhalla and Singh, 2008) were moved to soil. An explant from each construct was moved to soil whereby the explants putatively transformed with *rol*B and *ORF13* constructs made it into full grown plants while the ones putatively transformed with *rol*C and *ORF14* did not survive this process.

The rate of contamination was quite low as the seeds were germinated in vitro after surface sterilization with 45 g L^{-1} $Ca(ClO)_2$ and the treatments that followed were done under sterile environment. Quite the opposite was observed when petioles from approx. One month

old greenhouse grown plants were used as explant source (data not shown). Senescence was reportedly very high in all of the explants following the treatment with the plant selection agent (Kan25, Kan50) which exhibited a requisite effect on the explants which were not putatively transformed.

Here we report of the initiation a platform for exploring ORFs from the *A. rhizogenes* plasmid pRiA4 *in planta* with the aim to study the individual functions of *rol*B, *rol*C, *ORF13* and *ORF14.*

CONCLUSIONS

Regeneration after transformation of *K. blossfeldiana* 'Molly' and *K. pinnata* resulted in 1 to 10% shoot recovery. Transformation of *B. napus* 'Elan' cotyledons and subsequent regeneration resulted in few shoots and is still ongoing. For both genera, regeneration and transfer of plants to soil are challenging. Successful transformants need to be verified by PCR to confirm that transgenic plants have been produced.

ACKNOWLEDGEMENTS

The Danish Council for Independent Research supported this research (Grant n. DFF-7017-00197).

Literature cited

Bhalla, P.L., and Singh, M.B. (2008). *Agrobacterium*-mediated transformation of *Brassica napus* and *Brassica oleracea*. Nat. Protoc. *3* (*2*), 181–189 https://doi.org/10.1038/nprot.2007.527. PubMed

Chilton, M.-D., Tepfer, D.A., Petit, A., David, C., Casse-Delbart, F., and Tempé, J. (1982). *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. Nature *295* (*5848*), 432–434 https://doi.org/10.1038/295432a0.

Christey, M.C. (2001). Use of Ri-mediated transformation for production of transgenic plants. Vitr. Cell. Dev. Biol. Plant. *37*, 687–700 https://doi.org/10.1007/s11627-001-0120-0.

De Paolis, A., Mauro, M.L., Pomponi, M., Cardarelli, M., Spanò, L., and Costantino, P. (1985). Localization of agropine-synthesizing functions in the TR region of the root-inducing plasmid of *Agrobacterium rhizogenes* 1855. Plasmid *13* (*1*), 1–7 https://doi.org/10.1016/0147-619X(85)90050-2. PubMed

Dubrovsky, J.G., and Laskowski, M. (2017). Lateral root initiation. In Encyclopedia of Applied Plant Sciences (Elsevier), p.256–264 https://doi.org/10.1016/B978-0-12-394807-6.00126-X.

Gamborg, O.L., Miller, R.A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. *50* (*1*), 151–158 https://doi.org/10.1016/0014-4827(68)90403-5. PubMed

García-Sogo, B., Pineda, B., Castelblanque, L., Antón, T., Medina, M., Roque, E., Torresi, C., Beltrán, J.P., Moreno, V., and Cañas, L.A. (2010). Efficient transformation of *Kalanchoe blossfeldiana* and production of male-sterile plants by engineered anther ablation. Plant Cell Rep. *29* (*1*), 61–77 https://doi.org/10.1007/s00299-009-0798-8. PubMed

Gelvin, S.B. (2003). Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. Microbiol. Mol. Biol. Rev. *67* (*1*), 16–37 https://doi.org/10.1128/MMBR.67.1.16-37.2003. PubMed

Hansen, G., Vaubert, D., Heron, J.N., Clerot, D., Tempe, J., and Brevet, J. (1993). Phenotypic effects of overexpression of *Agrobacterium rhizogenes* T-DNA ORF13 in transgenic tobacco plants are mediated by diffusible factor(s). Plant J. *4* (*3*), 581–585 https://doi.org/10.1046/j.1365-313X.1993.04030581.x.

Kodahl, N., Müller, R., and Lütken, H. (2016). The *Agrobacterium rhizogenes* oncogenes rolB and ORF13 increase formation of generative shoots and induce dwarfism in Arabidopsis thaliana (L.) Heynh. Plant Sci. *252*, 22–29 https://doi.org/10.1016/j.plantsci.2016.06.020. PubMed

Lütken, H., Jensen, L.S., Topp, S.H., Mibus, H., Müller, R., and Rasmussen, S.K. (2010). Production of compact plants by overexpression of *AtSHI* in the ornamental *Kalanchoë.* Plant Biotechnol. J. *8* (*2*), 211–222 https://doi.org/10.1111/j.1467-7652.2009.00478.x. PubMed

Lütken, H., Clarke, J.L., and Müller, R. (2012). Genetic engineering and sustainable production of ornamentals: current status and future directions. Plant Cell Rep. *31* (*7*), 1141–1157 https://doi.org/10.1007/s00299-012-1265-5. PubMed

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. *15* (*3*), 473–497 https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Nemoto, K., Hara, M., Suzuki, M., Seki, H., Oka, A., Muranaka, T., and Mano, Y. (2009). Function of the aux and *rol*

genes of the Ri plasmid in plant cell division in vitro. Plant Signal. Behav. *4* (*12*), 1145–1147 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2819440/ https://doi.org/10.4161/psb.4.12.9904. PubMed

Nilsson, O., and Olsson, O. (1997). Getting to the root: the role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. Physiol. Plant. *100* (*3*), 463–473 https://doi.org/10.1111/j.1399-3054.1997.tb03050.x.

Öz, M.T., Eyidogan, F., Yücel, M., and Öktem, H.A. (2009). Optimized selection and regeneration conditions for *Agrobacterium*-mediated transformation of chickpea cotyledonary nodes. Pak. J. Bot. *41* (*4*), 2043–2054.

Sanikhani, M., Frello, S., and Serek, M. (2006). TDZ induces shoot regeneration in various *Kalanchoë blossfeldiana* Poelln. cultivars in the absence of auxin. Plant Cell Tissue Organ Cult. *85* (*1*), 75–82 https://doi.org/10.1007/s11240-005-9050-6.

Schmülling, T., Schell, J., and Spena, A. (1988). Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO J. *7* (*9*), 2621–2629 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC457048/ https://doi.org/10.1002/j.1460-2075.1988.tb03114.x. PubMed

Slightom, J.L., Durand-Tardif, M., Jouanin, L., and Tepfer, D. (1986). Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. Identification of open reading frames. J. Biol. Chem. *261* (*1*), 108–121 http://www.jbc.org/content/261/1/108.long. PubMed

Tepfer, D. (1990). Genetic transformation using *Agrobacterium rhizogenes.* Physiol. Plant. *79* (*1*), 140–146 https://doi.org/10.1111/j.1399-3054.1990.tb05876.x.

van Altvorst, A.C., Bino, R.J., van Dijk, A.J., Lamers, A.M.J., Lindhout, W.H., van der Mark, F., and Dons, J.J.M. (1992). Effects of the introduction of *Agrobacterium rhizogenes rol* genes on tomato plant and flower development. Plant Sci. *83* (*1*), 77–85 https://doi.org/10.1016/0168-9452(92)90064-S.

White, F.F., Taylor, B.H., Huffman, G.A., Gordon, M.P., and Nester, E.W. (1985). Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes.* J. Bacteriol. *164* (*1*), 33–44 https://doi.org/10.1128/JB.164.1.33-44.1985. PubMed