



Optimization of *Rhizobium rhizogenes*-mediated transformation, regeneration and characterization of *Malus domestica* BORKH. Ri lines

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Abstract

This study explored the establishment and optimization of Ri (root inducing) technology for apple breeding, using the bacterium *Rhizobium rhizogenes* to obtain Ri lines with compact shoots and stronger root systems. The transformation and shoot regeneration for *Malus domestica* cultivars was studied in detail. Various *R. rhizogenes* strains, scion and rootstock genotypes, explant types, wounding methods and explant orientations were tested for hairy root induction. Most of the 16 tested strains, especially those with plasmid type III, induced hairy roots in the rootstock genotype ‘M26’. Although apple genotypes differed in response, in most of them roots were successfully induced using strains ATCC 15834, LMG 63 and LMG 150, with leaf blades outperforming petioles as explants. Wounding by scratching or sonication further improved transformation efficiency, as did placing leaf blades with their abaxial side upward on root induction medium. The majority (94%) of roots formed in one transformation experiment were tested PCR-positive for at least one T-DNA gene. Shoot regeneration experiments investigated salt concentrations, gelling agents, cytokinin types, concentrations, and a resting period on hormone-free medium. Shoot regeneration was highly genotype-dependent varying between 0 and 83%, whereas only minor, non-significant effects were observed for the treatments tested. Copy numbers of T-DNA genes were estimated using digital PCR for the first time in apple Ri lines. In the greenhouse, two Ri lines showed compact shoots and shorter leaves, but no enhanced root system. The improved protocol provides a valuable tool for breeders and scientists to obtain and further use Ri lines.

Key Message

Optimized transformation protocol led to high hairy root formation and T-DNA-gene integration rates. Ri plants were successfully regenerated for ‘M26’ and ‘Gala’, differing in integrated T-DNA genes and copy numbers.

Keywords Adventitious shoot regeneration · Apple · *Agrobacterium rhizogenes* · Digital PCR · Hairy roots · Ri phenotype

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Abbreviations

BAP	6-Benzylaminopurine
dPCR	Digital polymerase chain reaction
F	Forward primer
GA ₃	Gibberellic acid
GFP	Green fluorescent protein
GMO	Genetically modified organism
HR	Hairy root
HR1 – HR5	Hairy root formation experiment 1 – 5
IBA	Indole-3-butyric acid
ILVO	Flanders research institute for agriculture, Fisheries and food
LUH	Leibniz University Hannover
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
PCR	Polymerase chain reaction

PGR	Plant growth regulator
PPFD-PAR	Photosynthetic photon flux density [$\mu\text{mol m}^{-2} \text{s}^{-1}$]
R	Reverse primer
Reg1 – 4.2	Regeneration experiment 1 – 4.2,
Ri	Root inducing
RIM	Root induction medium
<i>rol</i>	Root oncogenic locus
<i>R. rhizogenes</i>	<i>Rhizobium rhizogenes</i>
T _A	Annealing temperature
TCM	Transformation/co-culture medium
T-DNA	Transfer DNA
T _L	Left T-DNA
T _R	Right T-DNA
TDZ	Thidiazuron

Introduction

Ri breeding

Apple growers face various challenges, including biotic stresses such as replant disease and abiotic stresses, notably drought, or competition with weeds for water and nutrients. For common cultivation practices, dwarfism is a necessary trait of rootstocks. Modern apple and rootstock varieties are expected to meet these requirements; thus, they should be addressed in breeding programs (Wang et al. 2019).

Recently, a decade-old technique has gained recognition as an additional tool for meeting the current requirements of apple breeding. This technique, known as Ri (root inducing) technology, involves the use of the soil-borne bacterium *Rhizobium rhizogenes* (ex *Agrobacterium rhizogenes*), which can transform plant cells and integrate the T-DNA (transfer DNA) from its Ri plasmid into the plant's genome. The expression of *rol* (root oncogenic locus) genes on the T-DNA alters the hormonal status of plant tissue, leading to the formation of “hairy roots” (HRs) in many species. These hairy roots have already been used for several applications, for example as hairy root cultures for an increased production of the antimalarial artemisinin. The creation of so called “composite plants” with a hairy root system and a non-transformed shoot has also provided a valuable tool for root biology studies, especially in legumes, where investigation of root-microbe-interactions are of interest. The bacterium has also been found useful in several fruit trees to improve propagation of difficult-to-root varieties and rootstocks (De Paolis et al. 2019). For Ri breeding purposes, depending on the plant species and genotype, hairy roots can be cultured on hormone-free medium to spontaneously regenerate shoots, or they can be transferred to shoot regeneration media supplemented with plant growth regulators, especially cytokinins. The new regenerants, called Ri lines,

contain the bacterial T-DNA genes. Ri lines may display typical Ri phenotypes, such as stunted growth due to shortened internodes, shorter and wrinkled leaves, increased and plagiotropic rooting, increased branching, altered flower number, morphology, and timepoint of flowering (Desmet et al. 2020a; Ying et al. 2023).

The introduction of *rol* genes has the potential to enhance apple genotypes in several ways. Rootstocks may have an increased dwarfing effect on the scion (Zhu and Welander 1999). Rugini et al. (2015) showed, that using cherry Ri lines as rootstocks resulted in scion size reduction while maintaining fruit quality at the same time. But the technology could also enable the possibility of own-rooted scion genotypes (Desmet et al. 2020a; Ying et al. 2023). Despite the dwarfing effect, a strong root system is maintained (Desmet et al. 2020a), which may be at the basis of an increased tolerance towards drought and salt stress (Mauro et al. 2017). Regarding the Ri phenotype specifically of apples, *rolB* overexpression notably enhanced the rooting capability of rootstocks ‘M26’ and ‘Jork 9’ (Wang et al. 2019). In other plant species, *rol* gene containing plants may be more resistant to biotic stresses (Arshad et al. 2014; Bettini et al. 2003; Rubnawaz et al. 2020), possibly due to an increased antioxidant potential (Rehman et al. 2023; Shkryl et al. 2022) or due to the promotion of beneficial bacteria through opine secretion (Tanaka et al. 2022; Zhong et al. 2022). For most European countries it has not been decided yet, if Ri lines obtained after cocultivation of plant material with wild *R. rhizogenes* strains are considered genetically modified organisms (GMOs). From a scientific point of view, commonly cultivated crops with *rol* gene integrations are already widely in use, including hop, sweet potato, or tobacco. During evolution, these *rol* genes have been integrated through natural horizontal gene transfer into plant genomes and Ri plants from spontaneous regeneration events emerged and were selected – naturally as well as unintentionally by humans (Matveeva 2021; Matveeva and Otten 2019). In addition, Denmark, an European Union member, explicitly recognized Ri technology as a non-GMO technology (Lütken et al. 2012a).

The creation of Ri lines involves two crucial steps: transformation and regeneration. The current literature regarding apple transformation with *R. rhizogenes* and subsequent regeneration of Ri lines is summarized in Table 1. This was the starting point of our experiments to optimize this process.

Transformation

Different strains have varying efficiencies in inducing hairy roots (Porter and Flores 1991). A limited number of *R. rhizogenes* strains have been tested with apple (Table 1) but their efficiency was not evaluated based on their plasmid

type. Additionally, genotypes show different *Agrobacterium tumefaciens* transformation capacities (Schröpfer et al. 2022) and up to now, only shoots of a few genotypes have been transformed with *R. rhizogenes*. Other types of explants like leaves could prove beneficial, because they are more scalable than shoots and are frequently used in *A. tumefaciens* transformations (Schröpfer et al. 2022). Wounding, a known enhancer of transformation efficiency, provides bacterial entry sites and increases the number of potentially transformed cells. Tissue scratching or ultrasonic treatment can achieve this, with the latter demonstrating significant effectiveness (Trick and Finer 1997). At last, explant orientation has an influence on the regenerative capacity of plant leaves and therefore was another interesting parameter to investigate (Bhatia et al. 2005; Zhang et al. 2020).

T-DNA integration

Determining the ratio of hairy roots to adventitious roots is crucial. Adventitious roots can occur along with hairy roots after infection, as for instance observed in walnut (Falasca et al. 2000). However, *R. rhizogenes* is able to inhibit adventitious root formation due to its endogenous cytokinin synthases (Chagas de Freitas 2021). Distinguishing between these two root types can be challenging, particularly when a

reporter construct like GFP (green fluorescent protein) cannot be used due to its transgenic nature. We aimed to evaluate the formation of adventitious roots in apple by investigating the integration rate of the T-DNA genes in roots formed on leaves using PCR (polymerase chain reaction) amplification of selected T-DNA genes.

Regeneration

In general, little is known about regeneration from apple roots (Dinani 2018). The efficiency of Ri plant regeneration from hairy roots can be influenced by numerous factors, with the genotype playing an important role (Wang et al. 2019). In addition, differences in the *rol* gene sequence (Desmet et al. 2019) of different strains could affect regeneration efficiency as well. The influence of apple genotype and bacterial strains with regard to regenerative capacity has not been studied in detail (Table 1). Plant growth regulators significantly affect organogenesis: high BAP (benzylaminopurine) or TDZ (thidiazuron) concentrations increased shoot regeneration from apple roots (Dinani 2018, Table 1) and apple leaves (Pawlicki and Welander 1994; Teixeira da Silva et al. 2019; Wang et al. 2019). According to Dinani (2018), long exposure times to TDZ significantly increased the frequency of regenerating shoot buds as well as using excised root segments in

Table 1 Literature summary of *R. rhizogenes*-mediated transformation of apple and regeneration of Ri lines through organogenesis

Transformation				Regeneration		
Plant species	Genotypes	Explant for transformation	<i>R. rhizogenes</i> strains	Explant for regeneration	Plant growth regulators	References
<i>Malus domestica</i>	'Golden Delicious'	Whole shoots	A4, 232	-	-	Patena et al. (1988)
<i>Malus domestica</i>	'M26'	Whole shoots	A4	Root still attached to shoot	BAP 22.2 µM	Lambert and Tepfer (1992)
<i>Malus domestica</i>	-	Whole shoots	NCPPB 1855	-	-	Rugini (1992)
<i>Malus domestica</i>	'M26'	Whole shoots	A4	-	-	Sutter and Luza (1993)
<i>Malus domestica</i>	'Jork 9'	Whole shoots	NCIB 8196, A4, ATCC 15834	Root still attached to shoot	NAA 1 µM, TDZ 0.1 µM	Pawlicki-Jullian et al. (2002)
<i>Malus prunifolia</i>	'Ringo'	Whole shoots	MAFF-02-10265, MAFF-02-10266, MAFF-03-01724, MAFF-03-01725	Root still attached to shoot	BAP 22.2 µM, IBA 0.5 µM, GA ₃ 0.3 µM,	Yamashita et al. (2004)
<i>Malus baccata</i>	-	Whole shoots	NCIB 8196	Root still attached to shoot	BAP 8.9 µM, IBA 2.5 µM, GA ₃ 0.9 µM, TDZ 2.3 µM	Wu et al. (2012)
<i>Malus domestica</i>	'Melrose', 'Golden Delicious', 'Cadel', 'Gloster'	Whole shoots	ATCC 15834	-	-	Stanišić et al. (2019)

contrast to roots attached to seedlings. Nevertheless, in the context of regeneration from hairy roots, other research groups have exclusively used roots attached to shoots (Table 1) or even reported, that using detached roots did not lead to regeneration (Pawlicki-Jullian et al. 2002; Wu et al. 2012). In addition, a "resting phase" without hormones and antibiotics following hairy root formation may enhance regeneration by reducing stress and may therefore allow for better reaction to the regeneration medium. Similarly, higher cytokinin concentrations during an initial callus induction phase prior to shoot induction may also prove beneficial (Zhang et al. 2020). Several studies of adventitious shoot regeneration from apple leaves have tested different salt concentrations, typically opting for full- or half-strength MS salts (Murashige and Skoog 1962). Some of these studies suggested that lower salt concentrations may be beneficial (Fasolo et al. 1989), but experiments with root explants have not been performed, yet. Gelrite and TDZ induced hyperhydricity in apple (Bethge et al. 2023; Dewir et al. 2018), so salt concentration and gelling agent type were considered in our optimization experiments.

Objectives

The primary objective of this study was to establish an efficient protocol for Ri plant generation as a first step towards the development of an innovative breeding tool in apple. More in detail, our objectives were (i) to induce and optimize hairy root formation on in vitro apple material by testing the effect of plant genotype, bacterial strain, explant type, wounding and explant orientation. (ii) We further investigated treatments for shoot regeneration on the obtained detached hairy roots from different apple genotypes and *Rhizobium rhizogenes* strains, by testing nutrient concentration, gelling agent, resting phase, as well as cytokinin types and concentrations in callus induction and shoot regeneration medium. (iii) The Ri-gene introgression and their copy numbers in the regenerants were determined for the first time in apple using digital PCR. (iv) We performed a

morphological screening of a subset of Ri lines in the greenhouse to investigate their potential for apple breeding.

Materials & methods

The transformation and regeneration experiments were performed by two different institutes, at LUH (Leibniz University Hannover) and ILVO (Flanders Research Institute for Agriculture, Fisheries and Food). Differences in protocols and culture conditions were indicated accordingly.

Plant material and tissue culture conditions

The genotypes listed in Table 2 were sub-cultured every 4–6 weeks on apple propagation medium: Murashige and Skoog (MS) inorganic salts including full MS vitamins, sucrose 3%, benzylaminopurine (BAP) 2.2 μM , indole-3-butyric acid (IBA) 0.5 μM , pH 5.7, Plant Agar (Duchefa, The Netherlands) 0.8%, which was autoclaved at 121°C and 125 kPa for 20 min. Cultures at LUH were maintained at $24 \pm 2^\circ\text{C}$ under a 16 h / 8 h light/dark photoperiod with a photosynthetic photon flux density (PPFD-PAR) of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool daylight fluorescent tubes (Philips Master TL-D 58 W/865; Signify, Hamburg, Germany, or Osram Lumilux 58 W/865, München, Germany). Four weeks prior to the transformation process, the plant material was sub-cultured. Young and fully unfolded leaf blades or petioles were carefully cut and stored in 100 mL of transformation/co-culture medium (TCM: $\frac{1}{2}$ MS medium including vitamins, D-glucose monohydrate 1.5%, pH 5.7, Plant Agar 0.78%) to be collected for transformation. At ILVO, the sub-culturing was identical except for the climate room conditions ($22 \pm 2^\circ\text{C}$). Plant material was not stored but processed immediately.

Bacterial strains and growth conditions

The *R. rhizogenes* strains used in this study are listed in the Online Resource Table S1. The approximate classification

Table 2 Apple genotypes and sources

Genotype	Type of use	Breeder	Plant material kindly provided by
'M26'	rootstock	East Malling Research, UK	Dr. Li-Hua Zhu, Department of Plant Breeding and Biotechnology, Swedish University of Agricultural Sciences, Alnarp, Sweden
'M27'	rootstock	East Malling Research, UK	Nicolas Stevens, Better3fruit, Aarschot, Belgium
'Bittenfelder'	rootstock	No breeding product	Dr. Andreas Wrede, LKSH, Gartenbauzentrum Ellerhoop, Ellerhoop, Germany
'Gala', 'Holsteiner Cox'	scion	'Gala': H.J. Kidd, NZ 'Holsteiner Cox': historic cultivar	Dr. Iris Szankowski, formerly Leibniz University Hannover, Germany
'BTF7', 'BTF17', 'BTF23'	scion	Better3fruit, BE	Nicolas Stevens, Better3fruit, Aarschot, Belgium

of Ri plasmids from the strains ST15.13/057, ST15.13/091 and ST15.13/097 was done with the online platform Phy5 (Nakano et al. 2023) and the plasmid type of the most closely related strain was chosen. For this, the genome sequence data of all 16 strains were used, employing the distance matrix “manhattan” and the agglomeration method “ward.D2”. In some experiments, a transgenic ATCC 15834 strain, carrying the reporter gene *gfp* was applied. The plasmid C757 with the *gfp* construct was obtained from DNA Cloning Service, Hamburg, Germany. At LUH, the bacterial cultures for transformations were prepared as reported in Rüter et al. (2023), by growing the bacteria to an optical density (OD_{600}) of 0.5 ± 0.1 and resuspending the bacteria in liquid TCM with 39 mg L^{-1} acetosyringone again to an optical density of 0.5 ± 0.01 . At ILVO, the bacterial cultures were prepared based on Desmet et al. (2019), where LMG 150 and LMG 63 bacteria were grown to OD_{600} of 0.5 ± 0.1 unless mentioned otherwise in Table 3.

Transformation experiments

The transformation protocol for experiments performed by LUH according to Rüter et al. (2023) was as follows: 30 explants were transferred into 100 mL bacterial suspension. If the treatment included wounding, vessels with explants and bacteria were sonicated for 10 min in an ultrasonic bath (Bandelin Sonorex Super 10 P, type DK 102 P, Germany) with 35 kHz and 60 W L^{-1} . After incubation of the explants with the bacteria for 30 min at room temperature, leaves were placed

with the abaxial side upwards onto solid TCM (Plant Agar 7.5 g L^{-1} , acetosyringone 39 mg L^{-1}) and incubated for 3 days at 25°C in the dark. Afterwards, they were transferred with the same orientation onto root induction medium (RIM: $\frac{1}{2}$ MS medium including vitamins, sucrose 2%, IBA $0.5 \mu\text{M}$, pH 5.8, Plant Agar 0.7%, cefotaxime 0.02%, Timentin 0.01%) and kept at 25°C in the dark for 4 weeks. All antibiotics were added after autoclaving. Four weeks after transformation, the number of emerged roots were counted per explant, as well as fluorescent roots for treatments with the strain ATCC 15834:GFP, using a blue/green LED flashlight and an orange blocking filter (NIPPON Genetics EUROPE GmbH, Germany) for visualization. In contrast to the LUH protocol, the ILVO transformation process according to Desmet et al. (2019) involved direct immersion of explants in bacterial suspension, followed by placement on an orbital shaker at 125 rpm for 30 min. Subsequently, the leaf explants were placed with the abaxial side facing upwards on TCM ($\frac{1}{2}$ MS medium including vitamins, D-glucose monohydrate 1.5%, pH 5.8, micro agar (Duchefa, The Netherlands) 0.73%) with 20 mg L^{-1} acetosyringone and maintained for 2 days in the dark at $22 \pm 2^\circ \text{C}$. The explants were then washed in liquid TCM complemented with 0.05% cefotaxime, again using an orbital shaker set at 125 rpm for 30 min. A bi-weekly transfer of explants to fresh RIM ($\frac{1}{2}$ MS medium including vitamins, sucrose 2%, pH 5.8, micro agar (Duchefa, Netherlands) 0.73%, cefotaxime 0.05%, Timen-tin 0.01%) was carried out. All antibiotics were added after autoclaving. The root count was conducted after 8 weeks. The respective experiment designs can be seen in Table 3.

Table 3 Listing of all hairy root (HR) formation experiments. Factors tested in the specific experiments are highlighted by grey cells

Experiment	Genotypes	<i>R. rhizogenes</i> strains	Explant type	Wounding	Leaf orientation facing upwards on RIM
HR1 (LUH)	‘M26’	All strains, see Online Resource Table S1	Whole leaves	Sonication 10 min	Abaxial
HR2 (ILVO)	‘M26’, ‘Gala’, ‘Holsteiner Cox’, ‘Bittenfelder’	LMG 63, LMG 150 ^a	Leaf blades (devoid of petiole tissue)	No additional wounding	Abaxial
HR3 (LUH)	‘M26’, ‘Gala’, ‘Holsteiner Cox’, ‘Bittenfelder’	ATCC 15834:GFP	Whole leaves, petioles	Leaves scratched across the midrib	Abaxial
HR4 (LUH)	‘M26’	ATCC 15834:GFP	Whole leaves	No wounding, three midrib scratches, sonication 5 min, sonication 10 min	Abaxial
HR5 (ILVO)	‘Gala’, ‘M27’, ‘BTF7’, ‘BTF17’, ‘BTF23’	LMG 150	Whole leaves	Leaves scratched across the midrib	Abaxial, adaxial

^a OD_{600} were 0.297 (LMG 63) and 0.357 (LMG 150)

Molecular analysis of hairy roots

'M26' leaf explants were transformed with an ATCC 15834 wild type strain, following the same protocol of HR1. The roots were frozen in liquid nitrogen and stored at -80°C . The roots were then crushed in a mixer mill (Retsch MM 400) using two 3 mm steel beads at a frequency of 27 Hz for 90 s. The resulting homogenized material was then utilized for DNA isolation with the "Nucleospin® Plant II" kit from Macherey–Nagel, following the instruction of the manufacturer (buffer: lysis buffer PL1 und 20 μL elution buffer). For the positive bacteria control sample, DNA of ATCC 15834 was extracted by resuspension of one colony in 30 μL sterile deionized water under sterile conditions. The suspension was incubated for 30 min at 95°C and centrifuged for 10 min at 12.000 rcf. The supernatant was stored at -20°C . The PCR reaction was prepared and the PCR conditions chosen according to the polymerase (DCSPol) manufacturer DCS (DNA cloning service, Hamburg, Germany). We first confirmed the DNA integrity with the presence of the *actin* gene and the absence of residual bacteria by testing for the bacterial *virD2* gene. Afterwards, we checked the remaining samples for T_L - and T_R -genes (Table 4). To enable the detection of truncated integration events, we checked genes at the borders of the region for *rol* genes on the T_L -DNA with *rolA* and *rolD* and of the T_R -DNA with *aux2* and *mas1* (Desmet et al. 2020a). As controls, we used DNA of untransformed 'M26' roots and DNA extracts from bacterial colonies.

The PCR samples were separated on a 3% agarose gel (universal agarose "SeaKem® LE" Axon Labortechnik, Germany) with 1 μL of a 1% ethidium bromide solution (10 mg mL^{-1} ; Roth, Germany) per 100 mL TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.5). The TAE buffer also served as a running buffer. To each PCR sample, a gel loading buffer (final concentration: 1.7 mM Tris–HCl (pH 7.6), 0.025% orange G (Bayer, Germany), 10% glycerol, 10 mM EDTA) was added. As a length standard, 5 μL of the

GeneRuler 100 bp Plus DNA Ladder (ThermoFisher Scientific, Germany) was used. Separation was performed at 120 V and gel documentation under UV light of 312 nm (Intas Gel iX 20 Intas Science Imaging, Intas-Capture-Software, program: GelDoc, Version 0.2.14.0).

The molecular analysis of Ri lines at LUH followed the same procedure as for the roots. At ILVO, plants were screened as described in Desmet et al. (2023) using the primers for pRi8196.

Regeneration experiments

ILVO collected roots 8 weeks after transformation and placed them on callus induction medium in the dark at $22 \pm 1^{\circ}\text{C}$ for 4–5 weeks, whereas at LUH, hairy roots were placed directly on shoot induction medium 4 weeks after transformation and incubated at $24 \pm 2^{\circ}\text{C}$ under a 16 light: 8 dark photoperiod. Explants were transferred to fresh shoot regeneration medium every 4–5 weeks, at LUH and ILVO, respectively. The design of the regeneration experiments is summarized in Table 5.

Morphological characterization

Shoots of two independent Ri lines (Ri1 and Ri2) and 'M26' controls were rooted on rooting medium ($\frac{1}{2}$ MS medium including vitamins, sucrose 2%, Phyto Agar 0.9%) for 3 weeks. Afterwards, the root systems were washed, the plants potted in a peat substrate (type VMV800, Einheitserde, Germany) and placed in a foil tent for acclimatization for 4 weeks at 16 h of additional lighting, an average daytime temperature of $22.8^{\circ}\text{C} \pm 1.6^{\circ}\text{C}$ standard deviation and nighttime temperature of $20.3^{\circ}\text{C} \pm 1.0^{\circ}\text{C}$, while gradually lowering the humidity levels. At the end of the acclimation period, 12 plants per genotype were uprooted, and their roots carefully freed from substrate. Subsequently, each plant was planted in a 12 cm diameter pot, layed out with MyPex foil, using disinfected

Table 4 Primer pairs used for amplification of plant and bacterial genes

Gene	Primer sequence 5' → 3'	T_A [$^{\circ}\text{C}$]	PCR product size [bp]	Primer source
<i>actin</i>	F: CGAGGAAGATCTGGCATCA R: AGGAGCTGCTCTTGCCAGT	59	541	Terefe-Ayana et al. (2011)
<i>virD2</i>	F: CCTGACCCAAACATCTCGGCTGCCCA R: ATGCCCCGATCGAGCTCAAGT	59	338	Haas et al. (1995)
<i>rolA</i>	F: CCAATCTGAGCACCACCTCT R: AATCCCGTAGGTTTGTTCG	59	153	Lütken et al. (2012b)
<i>rolD</i>	F: GGTTGAAGTACCCTCTGTCC R: AAGTCCTTTACCGCAACTTC	59	293	Desmet et al. (2020b)
<i>aux2</i>	F: TGCGCCTTGAAGTAACTGTG R: AGCTTTCCGACTGCCATCTA	55	233	Desmet et al. (2020b)
<i>mas1</i>	F: GCTTCGTCATGTTGTAACCGA R: TGGCTCCGCTGCGAATGACA	55	132	This study

Table 5 Overview of all regeneration experiments. All media contained 1 MS vitamins, 3% sucrose and were adjusted to a pH of 5.8. Factors tested in the specific experiments are highlighted by grey cells

Experiment	Genotypes	<i>R. rhizogenes</i> strains	MS strength	Gelling agent	Rest phase	Plant growth regulators	Antibiotics	Experiment evaluation
Reg1 (LUH)	'M26'	ATCC 15834, LMG 63, NCPPB 2655	1, 1/2	Plant Agar 0.8%, Gelrite 0.4%	No	IBA 1 µM + TDZ 9.1 µM	Timentin 0.01%, cefotaxime 0.02%	7 months, 2 repetitions
Reg2 (LUH)	'M26', 'Gala', 'Bittenfelder', 'Holsteiner Cox'	ATCC 15834:GFP	1	Gelrite 0.4%	No	BAP 22.2 µM + NAA 5.4 µM + TDZ 0.1 µM	Timentin 0.01%, cefotaxime 0.02%	12 months
Reg3 (LUH)	'M26', 'Gala', 'Bittenfelder', 'Holsteiner Cox'	ATCC 15834, ATCC 15834:GFP, LMG 150	1	Plant Agar 0.8%	No	BAP 22.2 µM, TDZ 9.1 µM, BAP 8.9 µM + TDZ 9.1 µM (all media: IBA 1 µM)	Timentin 0.01%, cefotaxime 0.02%	12 months
Reg4.1 (ILVO) ^a	'M26', 'Gala', 'Bittenfelder', 'Holsteiner Cox'	LMG 63, LMG 150	1/2	Micro Agar 0.73%	Yes, No	Callus induction: BAP 4.4 µM + TDZ 0.045 µM, BAP 24.2 µM + TDZ 0.09 µM (all media: NAA 2.7 µM) Shoot induction: BAP 4.4 µM + TDZ 0.045 µM	No antibiotics	12 months
Reg4.2 (LUH) ^b	'M26'	ATCC 15834	1	Plant Agar 0.8%	Yes, No	IBA 1 µM + TDZ 9 µM	Timentin 0.01%, cefotaxime 0.02%	7 months, 2 repetitions

^aThe hormone-free rest phase duration was 6 weeks in the dark at 22 ± 1 °C. All media contained only ½ MS vitamins.

^bReg4.1 and Reg4.2 were evaluated together to determine the effect of a hormone-free rest phase. The rest phase duration in Reg 4.2 was 4 weeks in the dark at 24 ± 2 °C. Instead of 1 MS medium including vitamins, the resting medium contained only ½ MS medium including vitamins.

sandy soil from Heidgraben Germany (Mahnkopp et al. 2018). To provide appropriate nutrition, 2 g of a slow-release fertilizer (Osmocote Exact: 16N-9P-12K + 2MgO + TE, Everris International B.V., Netherlands) were added per liter of soil. Finally, the plants were randomly placed in the greenhouse at an average daytime temperature of 26.1 °C ± 4.0 °C standard deviation and nighttime temperature of 21.9 °C ± 4.0 °C, with weekly measurements of shoot length and number of nodes. After eight weeks, plants were removed from pots and soil was cleaned off. The root system was washed and cut from the shoot to collect fresh masses. Shoots and roots were dried for four days (until constant weight) at 70 °C to record dry masses. Leaf parameters including the length and width of the first fully developed leaf and length of the petiole were also measured. The segmentation of the plants in the pictures to remove the background was done separately for roots and shoots. Segmentation of the roots was done in Fiji (version 2024-02-01; ImageJ 1.54f) with the same thresholding for all lines. Segmentation of the shoots was done in Affinity Photo (version 1.10.6.1665) with the Flood Select Tool.

Analysis of T-DNA copy numbers in regenerants

On a selection of shoots, the copy-number of selected T-DNA genes was determined using digital PCR (dPCR). *EcoRI* and *BfaI* restriction enzymes were selected for fractioning the

DNA, as described in Desmet et al. (2020b). Single copy reference genes *MD03* and *MD05* were obtained as described in the Online Resource. dPCR primers for target and reference genes were developed using Primer3Plus (Table 6). Per sample, 3 × QIAcuity EvaGreen Mastermix (Qiagen) and 200 nM of both primers were added to 10 ng of digested DNA in a total volume of 40 µL. Since EvaGreen was used, target and reference genes were run separately. A “no template control” (NTC) was added for each gene tested. PCR mix was loaded on 26k 24-well QIAcuity Nanoplates (Qiagen). dPCR was performed in a QIAcuity Digital PCR System (Qiagen). PCR conditions consisted of 95 °C for 2 min followed by 40 cycles of 95 °C for 30 s, T_A °C for 30 s followed by 5 min at 35 °C, 5 min at 90 °C and cooling for at least 5 min at 35 °C. Ramp rate was 3 °C/s. QIAcuity Software Suite 2.2 (Qiagen) was used for data analysis. The average of the concentration (copies µL⁻¹) of both reference genes was used as normalization factor (NF) for the target gene copy number. Since apple is diploid, copy number was calculated as 2 × concentration (copies µL⁻¹) of target gene/NF.

Statistical analyses

For statistical analyses, the software R (version 4.3.0) and R Studio (version 2023.09.1) were used. Plots were

Table 6 Primers used for dPCR. Plasmid pRiA4 is present in strain ATCC 15834, plasmid pRi8196 in strains LMG 63 and LMG 150

Plasmid	Gene	Primer sequence 5' → 3'	T _A [°C]	Primer source
pRiA4	<i>rolA</i>	F: TCGGAGTATTATCGCTCGTC R: AAAGGAGTGGTGGCTCAGATT	57	Desmet et al. (2020b)
	<i>rolB</i>	F: GGTAGCTTGCACCTTCTTTC R: TGATATCCCCGAGGGCATTTC	57	Desmet et al. (2020b)
	<i>rolC</i>	F: CCTCAAATGAGCGTAAACCC R: CCTCCATAGAAGCAGAGCAT	57	Desmet et al. (2020b)
	<i>rolD</i>	F: GCAAGGAGAACAAGCATCTC R: CCTCCCGAAATGGAATTGTG	57	Desmet et al. (2020b)
	<i>aux2</i>	F: CAGCCGAGACGATTATCAGA R: CTGACCCCTTCATTGTGATG	57	Desmet et al. (submitted)
	<i>aux1</i>	F: GCCATGCAATAGGGAACAGT R: GTGGTGAGTATGCACGATGG	57	Desmet et al. (submitted)
	<i>rolB_{TR}</i>	F: ATGTCGTGGCTTATGGGTTC R: CCAATTTCAAATCGAGGAA	57	Desmet et al. (submitted)
pRi8196	<i>rolA</i>	F: AAGGCCAAGAAGAGGAAGGC R: TCGTTGACCATCACGGAAGG	61	This study
	<i>rolB</i>	F: GGAGATCCGCTTCGCCTTTA R: TTCAAGAACGTCTCGAGCCC	61	This study
	<i>rolC</i>	F: TTAGTTTCCCTGGCCACGTC R: TATCACGGTCGAGCTCATGC	61	This study
	<i>rolD</i>	F: GCCCACAAGTCTGCACAAAG R: AGCCTGATGCAGCTCCATTT	61	This study
-	<i>MD03</i> (<i>MD04G1230700</i>) ^a	F: CTGTGGATGCACTCTCACTGA R: CCAGCACCTAAGACCGGAAT	57	This study
-	<i>MD05</i> (<i>MD16G1059100</i>) ^a	F: GGAAAAGGGTACTGTAGCCC R: AGGGCCGAGTGTAATACTAA	57	This study

^aSequences of these single copy reference genes were obtained from GDR genome repository (https://www.rosaceae.org/species/malus/malus_x_domestica/genome_GDDH13_v1.1)

generated with the R packages “ggplot2” (Wickham 2016) and “patchwork” (Pedersen 2023). Depending on the data type, a linear model (metric data) or a generalized linear model (count or binomial data) was adjusted and chosen based on comparisons of the dispersion parameter, quantile–quantile (QQ) plots, and the Akaike Information Criterion (AIC). Following the model fitting, an Analysis of Variance (ANOVA) was conducted. If the ANOVA indicated a statistically significant effect, Tukey’s post hoc test was subsequently applied, for comparisons of the respective treatments.

Results

Transformation optimization

In experiment HR1, we tested 16 different wild type strains in a transformation experiment with ‘M26’ leaves, where we measured root formation rates (Fig. 1a) and root numbers per rooted explants (Fig. 1b). The strains had been isolated from different plant genera (Online Resource Table S1) and belonged to one of the three known Ri plasmid types, introduced by Weisberg et al. (2020). They classified Ri plasmids

by their structure and genetic composition. Previous plasmid classification was based on the plasmids opine synthases, but since opine synthases are promiscuously exchanged between strains and type III plasmids contain more than one opine synthase, the type classification proposed by Weisberg et al. (2020) seemed appropriate. The only strains with noteworthy HR formation were of the plasmid type III and one of type I (ST15.13/057 with 53% explants with roots, 6.2 roots/root forming explant). Strain ATCC 15834 resulted in 80% explants with roots and 10.6 roots/root forming explant, LBA 9402 in 73% explants with roots and 10.5 roots/root forming explant and T5/73 in 47% explants with roots and 4.8 roots/root forming explant. Except for strain T155/95, all Type III strains caused high HR formation rates (Fig. 1a), with significant differences to most other strains.

Most strains induced hairy roots, but for the following experiments, the best performing strain ATCC 15834 was chosen. Since the strains LMG 63 and LMG 150 had been isolated from apple plants, they were also included in some of the subsequent experiments, because they can be considered natural pathogens of apple tissue. Thereby, we further investigated strains with the two Ri plasmids types II and III.

In experiment HR2 two scion (‘Gala’, ‘Holsteiner Cox’) and two rootstock genotypes (‘M26’, ‘Bittenfelder’) were

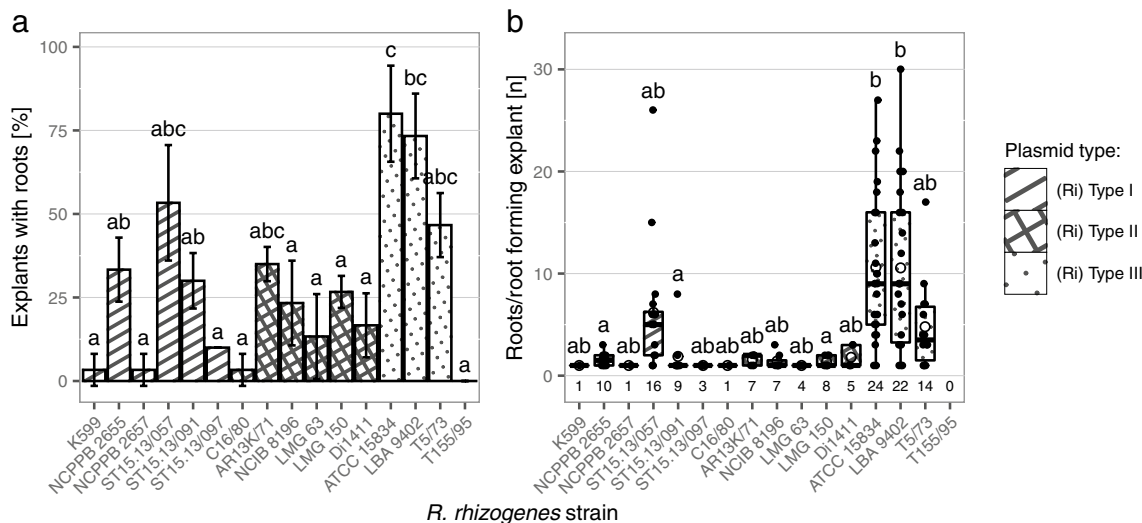


Fig. 1 Root formation of ‘M26’ leaves transformed with different *R. rhizogenes* wild type strains. The strains are grouped according to their plasmid type. Shown are percentages of explants forming roots (a) and numbers of roots per root forming explant (b). Means are indicated by circles in (b), error bars show the SD in (a), letters indicate

significant differences according to Tukey's test with $p < 0.05$ using generalized linear models. **a** A binomial distribution was used for the model, $n = 3$ vessels with 10 explants each. **b** A quasipoisson distribution was used for the model, n (number of root forming explants) is given below each boxplot

checked for their transformability with the two strains LMG 63 and LMG 150, using leaf blades without petiole tissue and no additional wounding. A two factorial ANOVA revealed no significant difference between the two strains or genotypes regarding percentage of explants forming roots or numbers of roots formed. Overall, we observed that all genotypes could be transformed with both strains, except for ‘Bittenfelder’ with LMG 63. In the other combinations, between one third and 93% of all leaf explants formed 1.5 to 3.4 roots on average (Table 7).

To identify the most responsive explant type, in experiment HR3, we employed the most efficient strain ATCC 15834 from experiment HR1, carrying the reporter gene *gfp*, to compare two explant types, i.e. “scratched leaf blades” and “petioles” for the same set of apple genotypes (Fig. 2). A two factorial ANOVA (utilizing a generalized linear model with a quasibinomial distribution) revealed no significant differences between the genotypes ($p = 0.182$), but a significant effect of the explant type ($p = 0.00035$). In all genotypes, we observed a higher percentage of root formation on leaf blades (71 – 81%) than on petioles (14 – 67%) (Fig. 2a). Three of the four genotypes had a significant higher root number per root forming explant on leaf blades (4.3 – 8.5) than on petioles (1 – 2.9) as well (Fig. 2b), while the lack of significance for ‘Bittenfelder’ might be explained by the low number of explants forming roots. Also, if the fluorescent roots are considered, a superiority of leaf blades was recorded for the percentage of explants forming fluorescent roots (Online Resource Fig. S1a). Leaf blades of ‘Gala’ and ‘M26’ formed with 6.2 and 5.0 (in case

of ‘M26’ significantly) more fluorescent roots per fluorescent root forming explant than petioles with 1.5 and 2.3 (Online Resource Fig. S1b).

To investigate the influence of different wounding treatments on whole leaves, we performed experiment HR4 with ‘M26’ leaves transformed with ATCC 15834:GFP (Online Resource Fig. S2). We compared no additional wounding besides the cut on the petiole section, three midrib scratches, 5 min and 10 min sonication. The ANOVA for total root numbers per root forming explants (Online Resource Fig. S2b) displayed a significant effect of the treatments with $p = 0.0378$. However, no treatment effects could be verified in pairwise comparisons in the Tukey test, only for the comparison of the treatments “no wounding” (4.4 roots per

Table 7 Root formation on leaf explants of two scion and two root-stock genotypes, transformed with the wild type strains LMG 150 or LMG 63. No significant effects of strain or genotype were detected according to ANOVA

<i>R. rhizogenes</i> strain	Apple genotype	Explants with roots [%] ($n = 15$)	Roots/root forming explant [n]
LMG 150	‘Gala’	93	2.4
	‘Holsteiner Cox’	40	1.5
	‘M26’	60	3.4
	‘Bittenfelder’	33	2.2
LMG 63	‘Gala’	27	2
	‘Holsteiner Cox’	47	3.1
	‘M26’	47	2.6
	‘Bittenfelder’	0	-

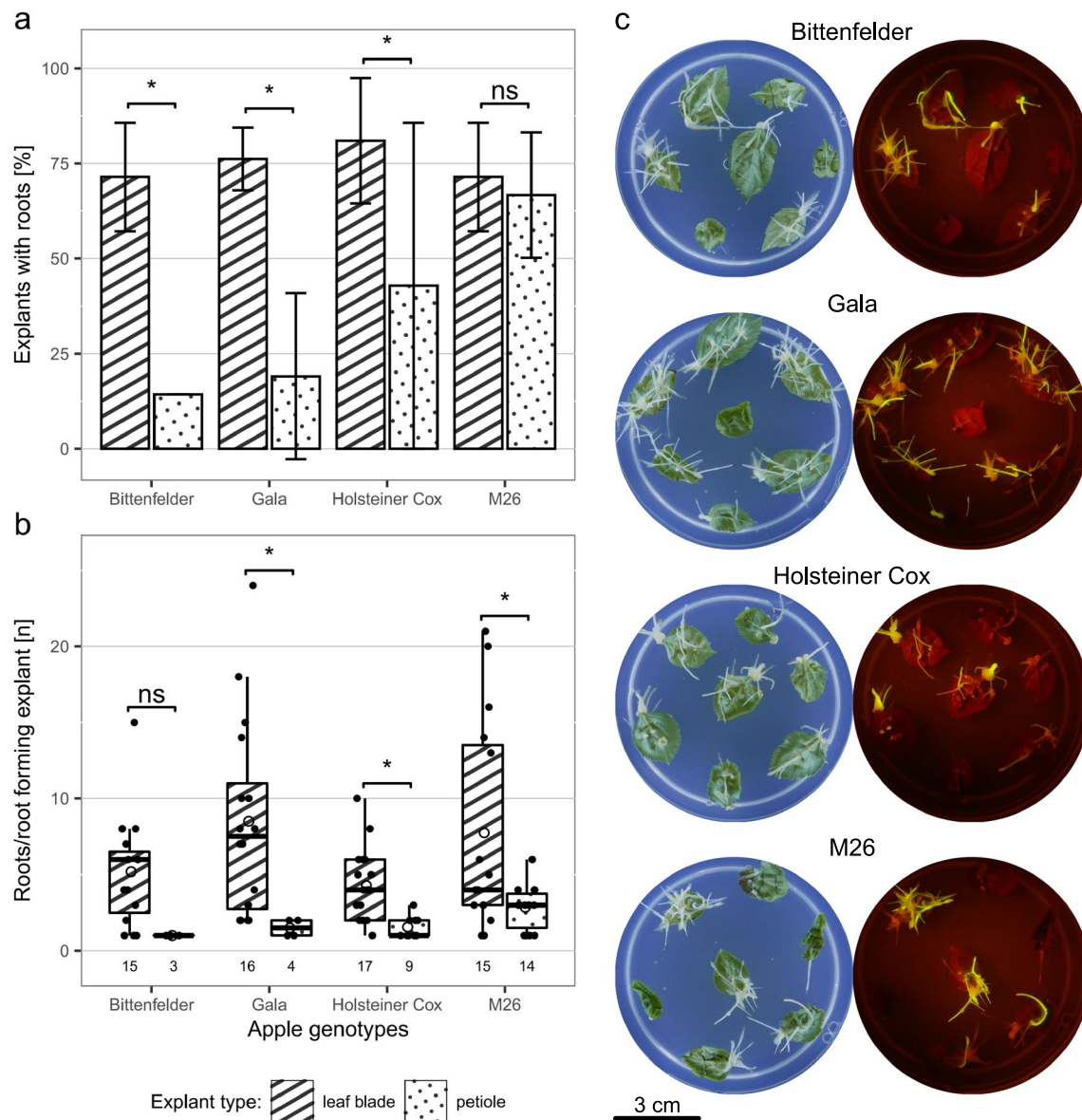


Fig. 2 Root formation of four apple genotypes depending on the explant types “leaf blade” and “petiole” after transformation with the strain ATCC 15834:GFP. **a** Percentage of explants forming roots. Asterisks indicate significant differences according to Tukey's test with $p < 0.05$ using a generalized linear model with a quasibinomial distribution, $n = 3$ vessels with 7 explants each, error bars show the SD. **b** Root number per root forming explant (n). Means shown as circles, asterisks indicate significant differences according to Tukey's

test with $p < 0.05$ using a generalized linear model with a quasipoisson distribution, n is labeled below each boxplot. **c** Pictures of the leaf blade variant are shown and were taken 5 weeks after transformation. Next to each bright light photo there is a photo of blue-green-LED excited fluorescence of hairy roots expressing GFP with an orange filter. Data on fluorescent roots is provided in Online Resource Fig. S1

rooted explant) and “10 min sonication” (9.3 roots per rooted explant) a low p -value was observed ($p = 0.0755$). Even though being not significant, the same beneficial effects of midrib scratches and 10 min sonication were noticed for the percentage of explants forming roots. The same trend, although not significant as well, could be observed for fluorescent roots.

In experiment HR5, we integrated the results of all preceding experiments by testing new cultivars with the strain LMG 150. We used scratched, whole leaves with ‘Gala’ as a control genotype. At the same time, we further investigated whether there is an effect of the orientation of the explant and the plant genotype (Fig. 3). A two factorial ANOVA (generalized linear model with a binomial distribution)

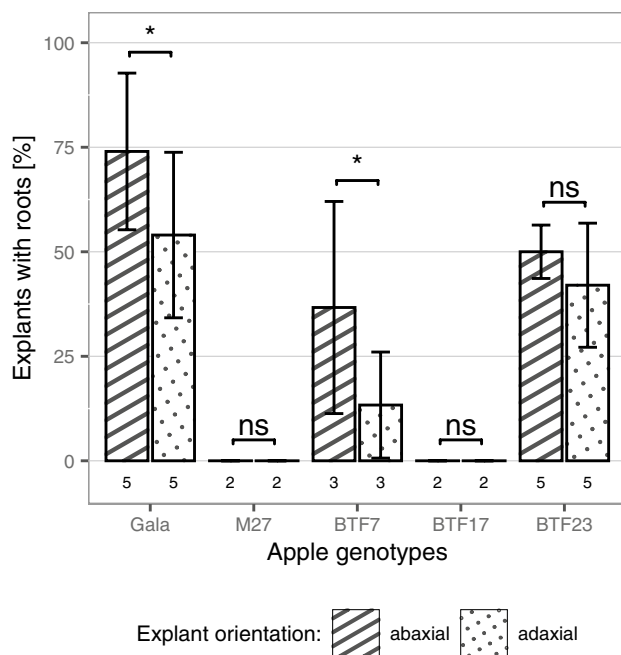


Fig. 3 Effect of explant orientation on root induction in different genotypes. Whole leaf explants were wounded by scratching and placed on root induction medium either with the abaxial or adaxial side facing upwards. The leaves were transformed with the wild type strain LMG 150. Asterisks indicate significant differences among the orientations within one genotype according to Tukey's test with $p < 0.05$ using a generalized linear model with a binomial distribution, error bars show the SD. N is given below each boxplot and represent repetitions, each with 10 explants, combined from two experiments. In the first experiment, the genotypes 'Gala', 'M27', 'BTF17' and 'BTF23' with two repetitions were transformed, in the second experiment the genotypes 'Gala', 'BTF7' and 'BTF23' with three repetitions

revealed a significant difference between the genotypes ($p = < 2.2 \times 10^{-16}$) and a significant difference between the orientations ($p = 0.0061$). For all genotypes that formed roots ('Gala', 'BTF23', 'BTF7') we could observe either a tendency or a significant effect towards a higher percentage of explants forming roots, if the explant was placed on root induction medium with the abaxial side facing upwards. This orientation resulted in 74%, 37% and 50% of the explants forming roots of 'Gala', 'BTF7' and 'BTF23', respectively.

A general comparison of the results of the transformation experiments performed at ILVO and LUH involving the same genotypes and strains is presented in the Online Resource Table S2. Despite the differences in methods and culture conditions, the results indicate similar HR formation with a tendency for higher root formation at LUH. In contrast to ILVO, non-transformed control explants formed some roots in the experiments carried out at LUH.

To evaluate the T-DNA gene integration rates, we analyzed 95 roots formed on 'M26' leaf explants transformed

with the wild type strain ATCC 15834. The isolated DNA was first evaluated for the presence of the *actin* gene and absence of the *virD2* gene. *Actin* could be detected in all samples, except for one. In 44 root samples (46%) we could still detect *virD2* from residual bacteria, which left us with 50 roots (53%) for further analysis. We tested each sample for the presence of the T_L genes *rolA* and *rolD* and of the T_R genes *aux2* and *mas1* (Table 8).

The molecular analysis revealed that nearly all roots (94%) were true hairy roots, containing at least one T_L -DNA gene. Most of them contained genes from the T_R -DNA as well (82%). The data also suggests that in nearly half of all roots tested (42%), only truncated T-DNA fragments have been inserted into the genome, according to our PCR-based analysis.

Regeneration optimization

The hairy roots were submitted to various treatments in five regeneration experiments Reg1 – 4.2 (Table 5). Significant effects were only observed for the regeneration capacity of the different genotypes, whereas the treatments, i.e. MS salt strength, gelling agent, cytokinin type and concentration or a resting phase on plant growth regulator-free medium only had minor, non-significant effects. Therefore, we summarized the mean shoot formation rates in the Online Resource Table S3. The regeneration of adventitious shoots from callus formed by the hairy roots (Fig. 4) started about 10 weeks after separating the roots from the explants. The apple genotypes 'M26' and 'Gala' had a high regeneration capacity (up to 83% of hairy roots used for regeneration produced shoots), whereas 'Bittenfelder' and 'Holsteiner Cox' did not regenerate at all, except for one shoot, regenerated from a hairy root of 'Holsteiner Cox' in experiment Reg4.1. For the different treatments, the following observations seem worth mentioning: in experiment Reg1, the combination of full-strength MS salts with Plant Agar led to the lowest regeneration rate compared to the other MS-strength and gelling-agent combinations. Thus, half-strength MS medium and Gelrite may be recommended for further use. When comparing cytokinin concentrations and combinations (experiment Reg3), there was generally a higher regeneration rate on medium containing only a high TDZ concentration compared to media with BAP. In experiment Reg4.1, more shoots were obtained by using a callus induction medium with a lower cytokinin concentration, as seen for hairy roots from 'M26' and 'Gala', induced by the strain LMG 150, without a resting phase. In general, a resting phase on hormone-free medium did not improve shoot regeneration (experiments Reg4.1 and Reg4.2).

The regenerated shootlets were placed on standard shoot multiplication medium (Fig. 4a, b). At first, they often

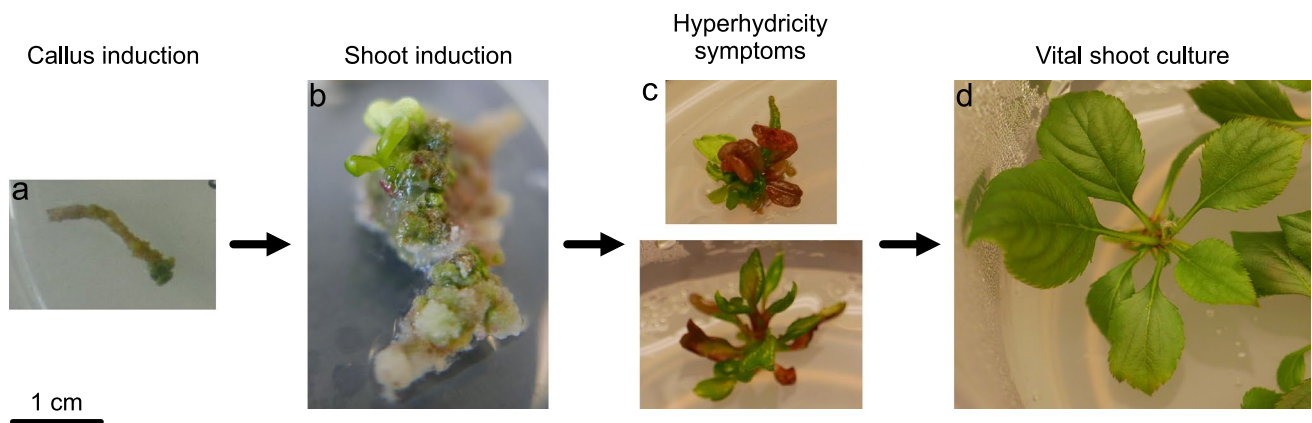


Fig. 4 Representative pictures of the shoot regeneration process from ‘M26’ hairy roots (experiment Reg2 with PGRs BAP 22.2 μ M, NAA 5.4 μ M, TDZ 0.1 μ M). **a** Callus induction after 4 weeks on shoot induction. **b** Shoot regeneration starting after 3 months and last-

ing > 1 year. **c** During the first 2–3 culture passages, stress symptoms were often observed, such as reddish, hyperhydric shoots. **d** Vital, multiplying shoots after 6 culture passages on shoot multiplication medium

displayed abnormal morphologies, like hyperhydricity, stunted growth, red leaves, low multiplication rates and several of them died, independent of the regeneration treatment (Fig. 4c). When observing 182 excised shoots of ‘M26’ over five to six culture passages, 76 (42%) of them developed into healthy and well multiplying shoot cultures (Fig. 4d). For ‘Gala’ this was true for 6 from a total of 9 (67%) excised shoots. The remaining shoots were either multiplying but hyperhydric, stagnating and forming callus, or died.

Shoot cultures that were actively growing and multiplying were analyzed in order to test the T-DNA integration. The lines were independent events, since only one shoot culture was established from one hairy root explant. Nearly all shoots (91%) were proven to be true Ri lines (Table 9).

Copy number detection in regenerants

A subset of Ri lines that multiplied well in vitro or had been acclimatized for morphological analyses was characterized further for the copy numbers of the integrated genes (Table 10). In Ri lines Ri1 and Ri2 transformed with strain ATCC 15834:GFP, only T_L-DNA got integrated, two

copies of all four *rol* genes in Ri1 and a single copy in Ri2. Lines Ri3 and Ri4 transformed with wildtype ATCC 15834 carried 3 and 1 copy of the T_L-DNA, respectively, whereas a single copy of the T_R-DNA was detected in both lines. Lines Ri5–12 were derived from transformation with strains LMG 150 and LMG 63, which contain a single T-DNA only. The T-DNA insertion ranged from 1 to 3 copies, which was consistent over all 4 *rol* genes measured. The only exception was the line Ri11, in which the number of copies of *rolC* and *rolD* doubled that of *rolA* and *rolB*, what might indicate the insertion of a full and a truncated T-DNA fragment in this Ri line. There was no clear correlation between copy number and the used strain or apple genotype (‘M26’ versus ‘Gala’).

Morphology of greenhouse-grown Ri plants

To evaluate apple Ri lines for their above- and belowground morphology, we rooted and acclimatized wild type ‘M26’ and two independently regenerated Ri lines Ri1 and Ri2. Both lines originated from a transformation experiment with ATCC 15834:GFP and contained only the T_L-DNA, with Ri1

Table 8 Integration of T_L and T_R genes of ATCC 15834 in ‘M26’ roots proven by PCR. A total of 50 roots was analyzed for which no residual bacteria and a positive PCR of an actin gene fragment were shown

Total: 50 roots (100%)		T _L -DNA				
		<i>rolA</i> +/ <i>rolD</i> +	<i>rolA</i> -/ <i>rolD</i> +	<i>rolA</i> +/ <i>rolD</i> -	<i>rolA</i> -/ <i>rolD</i> -	
T _R -DNA	<i>aux2</i> +/ <i>mas1</i> +	23 (46%)	3 (6%)	0 (0%)	0 (0%)	T _R -DNA: 41 (82%)
	<i>aux2</i> -/ <i>mas1</i> +	8 (16%)	4 (8%)	0 (0%)	0 (0%)	
	<i>aux2</i> +/ <i>mas1</i> -	3 (6%)	0 (0%)	0 (0%)	0 (0%)	
	<i>aux2</i> -/ <i>mas1</i> -	3 (6%)	1 (2%)	2 (4%)	3 (6%)	
		T _L -DNA: 47 (94%)				

Table 9 Independent regenerants from hairy roots and their T-DNA status. Strain ATCC 15834 contains both T_L and T_R-DNA, strains LMG 63, LMG 150 and NCPPB 2655 only have a single T-DNA

Institute	Apple genotype	<i>R. rhizogenes</i> strain	Number of lines tested by PCR	+ all tested T-DNA genes	+ truncated T-DNA	- T-DNA
LUH	'Gala'	ATCC 15834	3	1 (T _L + T _R), 1 (T _L)	1 (T _L + T _R)	0
	'Gala'	LMG 150	2	0	0	2
	'M26'	ATCC 15834	11	5 (T _L + T _R), 3 (T _L)	3 (T _L + T _R)	0
	'M26'	LMG 63	7	5	0	2
	'M26'	NCPBP 2655	14	10	1	3
ILVO	'Gala'	LMG 150	13	13	0	0
	'Gala'	LMG 63	1	1	0	0
	'M26'	LMG 150	6	6	0	0
	'M26'	LMG 63	4	4	0	0
	Sum		61 (100%)	49 (80%)	5 (8%)	7 (11)

containing two copies and Ri2 one copy (Table 10). After 8 weeks of growth in a greenhouse pot experiment, we scored shoot and root dry mass, shoot length, number of nodes, leaf length and width and petiole length (Fig. 5).

In the majority of parameters, we observed notable and significant differences for the Ri lines when compared to the wild type 'M26'. For instance, the Ri lines exhibited a reduced shoot dry mass (reduction of 24% and 50%, Fig. 5a) and shorter internode length (shoot length in Fig. 5c / node number Fig. 5d; reduction of 26% and 39%). Additionally, both Ri lines were characterized by smaller leaves (Fig. 5e, f) and shorter petioles (Fig. 5g). Interestingly, the Ri lines also exhibited differences amongst each other: Although Ri1 matched the control's height, it had a higher node count (control 21, Ri1 27, Ri2 20, Fig. 5d). Conversely, Ri2, while having a similar number of nodes as the control, was shorter (control 16.5 cm, Ri1 15.9 cm, Ri2 10.1 cm, Fig. 5c). The root

dry mass in Ri1 (0.19 g) was comparable to the control (0.18 g), but it was significantly lower in Ri2 (0.09 g) (Fig. 5b).

Discussion

Transformation

The transformation protocols used in this study consistently resulted in hairy root formation, but their frequencies varied between replicates and between the LUH and ILVO protocols (Online Resource Table S2). Variation among repetitions is not uncommon and most likely are caused by different physiological states of the plant material. The observed discrepancies between the laboratories may be due to differences in media composition, culture conditions, and protocol specifics as described in the materials and methods

Table 10 Estimated copy numbers of inserted T-DNA genes in Ri lines as revealed by dPCR. NA: genes not present in the bacterial strain; -: genes not transferred to the Ri line

Ri line	Apple genotype	<i>R. rhizogenes</i> strain	<i>rolA</i>	<i>rolB</i>	<i>rolC</i>	<i>rolD</i>	<i>aux1</i>	<i>aux2</i>	<i>rolB_T_R</i>
Ri1	'M26'	ATCC 15834:GFP	1.9	1.9	1.9	1.8	-	-	-
Ri2	'M26'	ATCC 15834:GFP	1.0	1.0	0.9	0.9	-	-	-
Ri3	'M26'	ATCC 15834	2.9	3.0	3.0	2.9	1.0	1.0	1.0
Ri4	'M26'	ATCC 15834	1.1	1.1	1.1	1.1	1.0	1.1	1.1
Ri5	'M26'	LMG 150	3.0	3.0	2.9	3.0	NA	NA	NA
Ri6	'M26'	LMG 63	1.0	1.0	1.0	1.0	NA	NA	NA
Ri7	'M26'	LMG 150	1.0	0.9	1.1	0.9	NA	NA	NA
Ri8	'M26'	LMG 63	2.1	2.1	2.0	2.1	NA	NA	NA
Ri9	'Gala'	LMG 150	1.0	1.0	1.0	1.0	NA	NA	NA
Ri10	'Gala'	LMG 150	1.0	1.0	1.0	1.0	NA	NA	NA
Ri11	'Gala'	LMG 150	1.0	1.0	2.0	1.9	NA	NA	NA
Ri12	'Gala'	LMG 63	2.0	2.0	1.9	2.0	NA	NA	NA

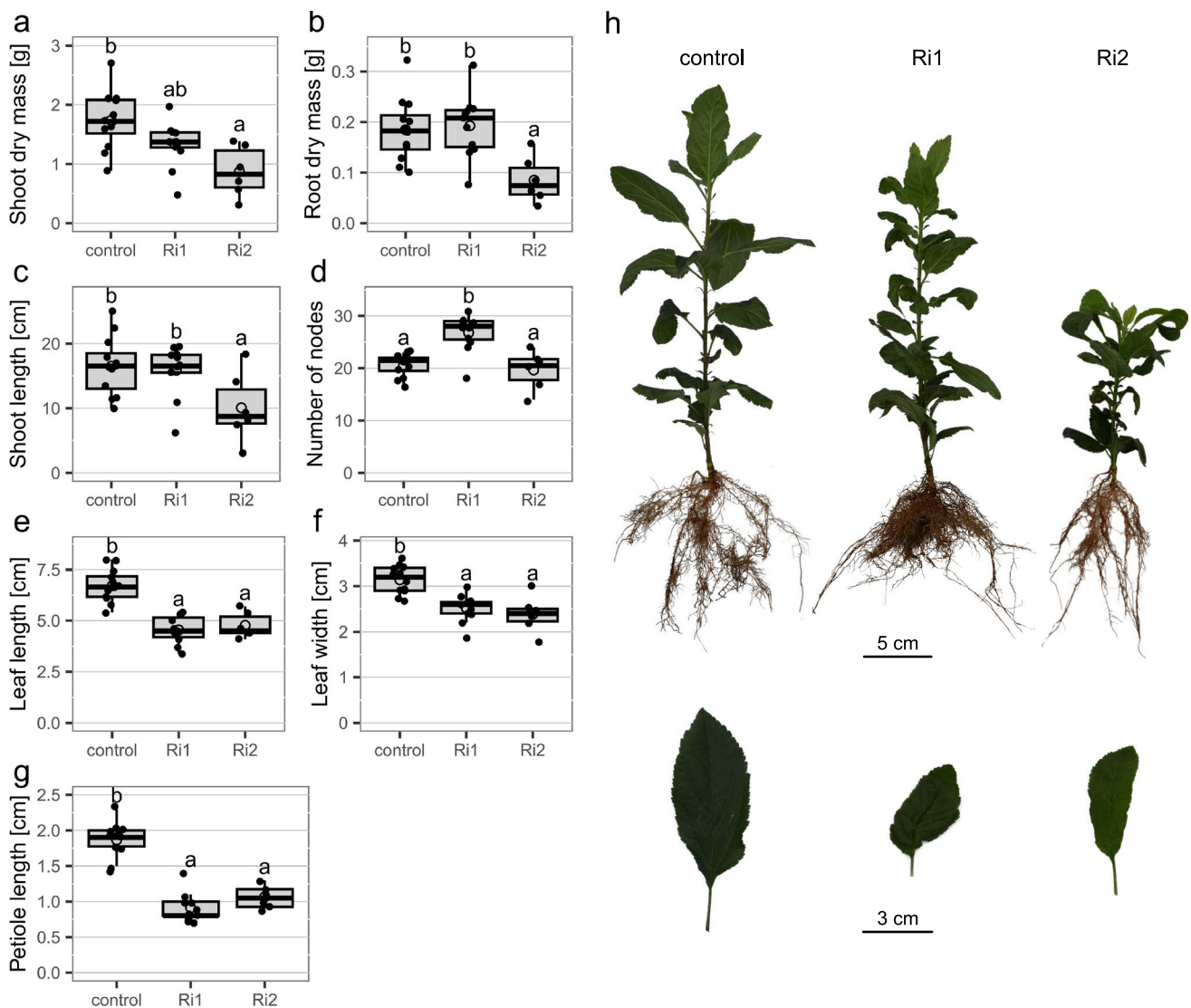


Fig. 5 Morphological parameters of acclimatized 'M26' wild type plants and two Ri lines. **a–g** Means shown as circle, letters indicate significant differences according to Tukey's test at $p < 0.05$ using a linear model, except for figure d, where a generalized linear model

with a quasipoisson distribution was used. $N = \text{control}: 12, \text{Ri1}: 11, \text{Ri2}: 6$. Figure h shows representative pictures of plants and leaves of the wildtype and the two Ri lines

section. Notably, explants in the control treatments without bacteria produced roots in LUH experiments, in contrast to ILVO experiments. Although the adventitious root formation in control treatments could be considered problematic, the molecular analysis (Table 8) proved most roots formed on explants treated with *R. rhizogenes* to be true hairy roots carrying T-DNA genes. This could be due to the cytokinin synthesis genes in *R. rhizogenes*, encoded on the Ri plasmid, potentially suppressing adventitious root formation in favor of hairy roots (Chagas de Freitas 2021; Pujari and Babu 2022).

We evaluated 16 different *R. rhizogenes* strains across the three known Ri plasmid types. All type III strains, except T155/95, exhibited high HR formation rates and

significantly outperformed other strains (Fig. 1). T155/95, isolated from grapes (Vitaceae, Online Resource Table S1), did not induce root formation, possibly due to its different host origin compared to other Type III strains isolated from roses (Rosaceae). Our findings are consistent with those of Desmet et al. (2019) on *Daucus carota*, where ATCC 15834 (Type III) was most efficient in root induction, followed by NCPPB 2659 (Type I), and then LMG 150 (Type II). All strains tested in more than one experiment resulted in comparable numbers of roots across the experiments: For example, LMG 150, on average, led to the formation of 1.4 roots (experiment HR1, 'M26'), 2.4 and 3.4 roots (experiment HR2, 'Gala' and 'M26') and 2.5 roots per root forming explant (experiment HR5, abaxial orientation, 'Gala').

Likewise, ATCC 15834 showed strongly consistent root numbers, but on a much higher level, with 10.6 roots formed per rooted explant (experiment HR1, ‘M26’), 8.5 and 7.7 roots (experiment HR3, leaf blades of ‘Gala’ and ‘M26’) and 9.3 roots (experiment HR4, sonication 10 min, ‘M26’).

Three of our four most intensively analyzed scion and rootstock genotypes ‘M26’, ‘Gala’ and ‘Holsteiner Cox’ have been reported to be transformable by *Agrobacterium tumefaciens* (Puite and Schaart 1996; Szankowski et al. 2001; Welander et al. 1998). For ‘M26’, also transformation by *R. rhizogenes* had been reported previously (Table 1). Indeed, in all transformation experiments of this study, these genotypes, as well as ‘Bittenfelder’, were successfully transformed with *R. rhizogenes*. Nevertheless, we found two of the BTF genotypes to be recalcitrant to transformation, hinting at a genotypic variation of transformability when using *Rhizobium*-mediated transformation (Schröpfer et al. 2022).

Regarding the explant type, leaf blades showed a higher root formation rate than petioles. In contrast to whole shoots, which had been used in all previous studies (Table 1), leaves may allow for more scalable and controllable experiments due to their homogeneity in terms of tissues in contrast to shoots. Wounding, a known enhancer of transformation efficiency, was investigated by sonication-assisted transformation, a method successfully applied to apple and other species of the Rosaceae family in *A. tumefaciens*-mediated transformation (Breitenbücher et al. 2012; Szankowski et al. 2001; Wada et al. 2020). Although our data did not show a significant difference between wounded and unwounded leaf blades, a clear trend in favor of 10 min sonication at 60 W L⁻¹ and midrib scratches was observed. Our test of explant orientation showed that leaf blades with the abaxial side facing upwards significantly formed more roots compared to the opposite orientation in all genotypes that formed roots. Explant orientation is already known to have an effect on shoot regeneration in tomato and apple (Bhatia et al. 2005; Zhang et al. 2020).

Our investigation of the T-DNA genes integration rates in roots formed on ‘M26’ leaf blades aimed to determine the ratio of hairy roots to adventitious roots (Table 8). Of the 50 bacterial residue-free roots analyzed, 94% were confirmed to be true hairy roots, each containing at least one T-DNA gene. Dhooche et al. (2023) also reported a high transformation rate for *rol* genes in roots of different genotypes of *Osteospermum fruticosum*, with rates ranging from 35 to 100%. We observed that while T_L genes were integrated without T_R genes, T_R genes were never inserted in the absence of T_L genes. Most roots contained both, T_L and T_R genes (82%). Interestingly, almost half of all roots tested (42%) had only truncated T-DNA fragments integrated into the genome, as not all T-DNA genes could be detected. This suggests that truncated T-DNA integration should be considered when testing roots or regenerated shoots for *rol* genes. Genes from

both ends of the T-DNA must be tested for a proper identification and molecular characterization of hairy roots or Ri shoots. For both T_L and T_R, we observed more truncations on the left border side (32%) in contrast to the right border side (10%), consistent with observations in the literature (Krizkova and Hrouda 1998; Thomas and Jones 2007). According to Gelvin (2021), this might be caused by the protection of the right border by interaction with the virD2 protein, while the sequence downstream to the left border in 3' direction is uncoated from the virE2 protein, which leaves the strand unprotected for exonuclease trimming. For our analysis, we used an agarose gel-based system for detection of PCR amplicates. Despite its low false positive rate, there may be cases where PCR products were not detected in a gel. Alternative systems such as qPCR or probe detection using a system such as Taqman™ could reduce the false negative rate. However, given that positive results can be trusted with appropriate controls, we are confident in the high integration rate, suggesting that nearly all roots can be considered true hairy roots, which is an important information for scientists and breeders for regeneration or Ri lines from roots formed after *R. rhizogenes*-mediated transformation of apple leaves.

Ri plant regeneration

While the existing literature only reported regeneration of Ri plants from roots attached to shoots, we successfully achieved regeneration from excised hairy roots formed on leaf explants of ‘M26’ and ‘Gala’. However, ‘Bittenfelder’ and ‘Holsteiner Cox’ were recalcitrant to regeneration, but were also tested in much lower numbers. Except for these strong genotypic differences, if a genotype has the ability to regenerate, various treatments such as bacterial strains used for hairy root induction, plant growth regulator combinations, gelling agents and nutrient concentration did only affect regeneration to a minor extent, in this study.

However, some trends were identified and should be considered: Full strength MS salts solidified with agar did not perform as well as half-strength MS salts and Gelrite as gelling agent. Yepes and Aldwinckle (1994) reported no difference between full and half strength MS salts, but they did show an increase in shoot bud formation on leaves on medium N6 with a lower salt content. Fasolo et al. (1989) also found that N6 was beneficial for adventitious shoot formation, suggesting that N6 may be another medium to test to further improve regeneration efficiency from hairy roots in future experiments. Our observation of slightly improved shoot formation using Gelrite versus agar as gelling agent is in agreement with findings for shoot regeneration from leaf explants (Chevreau et al. 1997; Pawlicki and Welander 1994; Saito and Suzuki 1999; Sun et al. 2008; Welander

and Maheswaran 1992). Further, a tendency was noted towards a higher shoot regeneration rate from hairy roots on medium containing high concentrations of TDZ, as the only cytokinin. This is consistent with the results of Pawlicki and Welander (1994), who demonstrated that on medium with 10 μ M TDZ shoot regeneration was better than on media with other TDZ concentrations or 22 μ M BAP. For the apple genotype ‘Gala’, Dinani (2018) found that root segments cultured on 20 μ M or 5 μ M TDZ for 20 days gave optimal results regarding shoot regeneration. Longer exposure to TDZ also significantly increased the frequency of regenerating buds. In our experiments, the use of a resting phase and higher cytokinin concentrations in the callus induction phase did not improve regeneration.

For the regeneration of shoots from the recalcitrant genotypes ‘Bittenfelder’ or ‘Holsteiner Cox’, further experiments should be considered. The single regenerated shoot in experiment Reg4.1 shows that it is in principle possible to induce shoots in ‘Holsteiner Cox’. A major difference in this experiment compared to experiments Reg2 and Reg3 was the use of a callus induction phase, which may have a beneficial effect for this genotype. Testing different callus induction treatments could therefore prove promising.

The regenerated shoots often showed an abnormal morphology compared to non-regenerated shoots. Over a six-month period with 5 to 6 culture passages, at least one third of all regenerated shoots developed into healthy, proliferating shoot cultures. The abnormal morphologies, still prevalent at a lower rate after 6 months, are likely due to TDZ in the regeneration medium, as mentioned in reports of TDZ-induced abnormalities in apple in vitro culture, such as hyperhydric shoots (Teixeira da Silva et al. 2019), but an influence of the integrated bacterial genes, that might cause stress reactions, cannot be excluded. In addition, lower adventitious root formation percentages were observed during in vitro rooting of regenerated Ri plants compared to control shoots (data not shown), which was also presumably due to high TDZ concentrations used for shoot regeneration (Dewir et al. 2018; Nielsen et al. 1993; Teixeira da Silva et al. 2019).

Molecular characterization of regenerants

The vast majority (91%) of all regenerants appeared to be true Ri lines containing *rol* genes (Table 9). This high number can be explained by the similarly high rate (94%) of hairy roots formed on ‘M26’ leaf explants (Table 8). It is noteworthy that in LVO all regenerants contained *rol* genes, while in LUH some regenerants did not, similar to the root analysis. This may be due to protocol differences, such as the use of IBA in the root induction medium at LUH, which possibly enhanced root formation in a way that allowed

adventitious roots to emerge from which shoots without *rol* genes could regenerate. Nevertheless, both protocols were similarly able to regenerate a high proportion of *rol*-containing shoots.

Our analysis of 12 regenerants revealed T-DNA copy numbers ranging from 1–3 copies (Table 10). A truncated integration event was also observed in line Ri11, where *rolA* and *rolB* were present as one copy and *rolC* and *rolD* as two copies. Desmet et al. (2020b) regenerated *Sinningia speciosa* Ri lines with similar observations, where copy numbers ranged from 1–3, truncated T-DNA integrations occurred with genes lacking or being present in less copy numbers on the left border of both T_L and T_R . In *Osteospermum*, copy-numbers were much higher in the majority of Ri lines, but truncated T-DNA fragments were present as well (Desmet et al. submitted). In both cases, lines were available, in which T_L was integrated independently of T_R .

Morphology of Ri plants

In our greenhouse experiment, morphological analysis revealed typical Ri traits such as compact shoots and smaller leaves in our two tested ‘M26’ Ri lines. Although the root system did not appear to be stronger in terms of root dry mass, the representative pictures suggest an altered root architecture in the line Ri1, which may be more branched and worth investigating, since root architecture is a relevant trait for breeding programs as well (Wang et al. 2019). Two possible explanations for the unexpected results in terms of root dry mass are suggested: (i) The Ri lines might still be affected by TDZ used in the regeneration medium, as in vitro rooting was also negatively affected. (ii) The effect of the respective Ri trait in a specific apple genotype is Ri line-dependent, since the copy number and chromosomal location of the integration site can influence the expression of the T-DNA genes. Likewise, in other studies some Ri lines of apple and other species showed stronger root systems, whereas other Ri lines showed no differences compared to the respective control or even lower root dry mass as well (Desmet et al. 2020a; Holfors et al. 1998; Sedira et al. 2001; Welander and Zhu 2000; Zhu et al. 2007; Zhu and Welander 2000). Desmet et al. (2020b) also evaluated two Ri lines of *Sinningia speciosa*, one line (Reg1) with single copy *rol* genes, and one line (Reg2) with two copies of *rolA*, *rolB*, *rolC* and 3 copies of *rolD*, while also containing a single copy of *aux1* and *rolB-T_R*. In their study, Reg1 displayed a strong aboveground Ri phenotype, which was less pronounced in Reg2. This aligns with our findings, where our line Ri2, in contrast to Ri1, had only one copy of T_L and showed a more compact shoot architecture. Both studies investigated only a limited number of plants with different copy numbers, so the results need to be considered carefully. To further

investigate these findings, more Ri lines need to be tested along with *rol* gene free controls that have undergone the same regeneration process. The longevity of the influence of TDZ on root morphology should also be investigated in order to use comparable lines in morphology experiments.

In conclusion, apple Ri plants were reproducibly generated in this study, with factors affecting transformation and shoot regeneration having been identified. Thereby, a set of Ri lines was obtained differing in T-DNA gene composition and copy numbers which should be characterized regarding their morphology and their plant hormone profiles in order to be able to link transgene status to the respective Ri phenotypes. This will be necessary to specify, which breeding aims can be approached by implementing the Ri technology in apple breeding, for example the use of Ri lines with low copy numbers as rootstock material (Rugini et al. 2015).

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Author contributions ED, EDK, and TW did the funding acquisition. All authors contributed to designing and planning the experiments. PR, TE, ED, EDK, MHD and JB performed the experiments. EDK analyzed the copy number data and wrote the respective sections, PR analyzed the other data, calculated the statistics, created the graphics and wrote the first draft. PR, TE, EDK and TW reviewed and edited the manuscript. All authors discussed the results, read and approved the final manuscript.

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Data availability The raw experimental data (hairy root counts, dPCR data, qPCR data and greenhouse morphological parameters of Ri lines) that support the findings of this study are available in Figshare with the identifier <https://doi.org/10.6084/m9.figshare.25012433>.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose. Traud Winkelmann is an Associate Editor of the journal. As such, she was fully excluded during all the evaluation period of this work, had no access to its handling during peer-refereeing, and her status had no bearing on the editorial consideration of the manuscript.

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