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Effect of *rolB* transgene on *Prunus cerasus* × *P. canescens* and *Cydonia oblonga* microshoot rhizogenesis

G. Stanienė,

R. Rugienius*,

D. Gelvonauskienė,

V. Stanys

Departament of Orchard Plants Genetics and Biotechnology, Lithuanian Institute of Horticulture, Kauno 30, LT-54333 Babtai, Kaunas distr., Lithuania

With the aim to improve rooting ability, the dwarfing sweet cherry hybrid rootstock (*Prunus cerasus* × *P. canescens)* and pear rootstock (*Cydonia oblonga* P. Mill) were transformed with the *rolB* gene using *Agrobacterium tumefaciens* mediated gene transfer. The binary vectors with *rolB* gene (cloned from *A. rhizogenes* plasmid pRiA4), driven by their own and constitutive CaMV promoter, were used for transformation. More than 400 regenerants of both rootstocks were obtained *in vitro* within seven months. The plant regeneration and the rooting rate of regenerants were found to the depend on the vector used for transformation. The rooting rate was highest when a construct with the *rolB* gene under its own promoter was used for transformation. As compared with the control (untreated plants), after co-cultivation with *A. tumefaciens* the rooting rate of *Cydonia* regenerants *in vitro* increased by 6–44% and of *Prunus* hybrid by 8–30%. DNA (PCR) analysis of regenerants was performed to prove transformation. It was shown that among 40 PCR-tested regenerants 17.5% had a stabile *rolB* insert in their genome. All transformants had well-formed roots characteristic of *rolB* transgenic plants.

Key words: genetic transformation, DNA, rootstocks, pear, cherry

INTRODUCTION

Pear (*Pyrus communis*) and sweet cherry (*Prunus cerasus*) belong to the most widely cultivated fruit tree species in the world. In modern orchard plant production, dwarfing rootstocks are commonly used for reducing tree size to enable high-density planting and easy management and thus a high production efficiency. However, many of rootstocks are difficult to root and propagate, thus limiting their commercial use. There are only few dwarfing rootstocks of *Pyrus* type, available in commercial pear production. Quince (*Cydonia oblonga* P. Mill.) has been used as pear dwarfing rootstock as an alternative, but it is incompatible with some pear cultivars sensitive to alkaline soil conditions and not winter-hardy enough [1, 2]. Therefore, it is of great importance to select new or improve the existing rootstocks. New pear rootstocks – quince clones, which are quite winter-hardy and compatible with most of pear varieties, were selected at the Lithuanian Institute of Horticulture. Unfortunately, insufficient rooting ability, like in many of woody plants, remains its main drawback [3–5].

The *Agrobacterium rhizogenes* genes *rol*A, *rol*B, and *rol*C located on the plasmid pRiA4, as well as plasmids pRi2659 and pRi8196 T-DNA induce root formation in most plant species [6–9]. Those genes are used widely to transform plants in order to increase their rooting ability [8, 10–13]. The *rol*B transgene was reported to often cause plant size reduction [11, 13, 14]. The mechanism of morphological changes in transgenic plants is still not clear. However, plants transformed with the *rol*B or *rol*C genes are more sensitive than untransformed controls to endogenous or exogenous auxins [15, 16].

Vectors carrying the *rolB* gene and driven by their own and constitutive Cauliflower Mosaic Virus (CaMV35S) promoters were constructed and the rootstock transformation technology was optimized during our previous experiments [17]. The aim of our present study was to transform *Prunus cerasus* × *P. canescens* and *Cydonia oblonga* plants with vectors carrying *rolB* gene constructs.

MATERIALS AND METHODS

Young leaves of the pear rootstock *Cydonia oblonga* P. Mill clones K.11, K.16, K.19 and a dwarfing sweet cherry hybrid rootstock (*P. cerasus* × *Prunus canescens)* obtained from micro-shoots *in vitro* served as the explants. The *rolB* gene was cloned from the *Agrobacterium rhizogenes* stem pRiA4. Binary vectors carrying

* Corresponding author. E-mail: r.rugienius@lsdi.lt

this gene were constructed previously and kindly submitted by A. Ražanskienė [17]:

pART27rolBdir3: construct of *rolB* gene with its own 5' (putative promoter) and 3' (putative terminator) sequences in direct orientation;

pART27rolBrev1 – construct of *rolB* gene with its own 5' (putative promoter) and 3' (putative terminator) sequences in opposite orientation;

pART27rolBCaMV3 construct of *rolB* gene with CaMV promoter and nos3' terminator (clone No 3);

pART27rolBCaMV4 construct of *rolB* gene with CaMV promoter and nos3' terminator (clone No 4);

pART27 core plasmid (received from SCIRO, Australia) carried also the gene of spectinomycin resistance for transformant selection.

These plasmids were transferred to *A. tumefaciens* and used for plant transformation.

Plant transformation. Isolated leaves were co-cultivated for two hours with *Agrobacterium tumefaciens* carrying the binary vector with *rolB* gene, then kept in a dark room for two days, and maintained in a growing chamber at 21 to 25 °C, on 50 μM $m^{-2}s^{-1}$ light with a 16-h photoperiod. Explants were regenerated on Murashige–Skoog [18] medium with 30 g/l sucrose, 0.3 μM NAA and 32 μM TDZ. Untransformed explants were grown under the same conditions and served as the control. In order to suppress the remaining *Agrobacteriam,* the explants were kept on a growth medium with 250 μg/ml cefatoxyme for three days after the transformation. The explants were subcultivated on the same medium every 3–4 weeks. Regenerated plants were transferred onto selective medium with 100 μg/ml spectinomycine upon regeneration (after 4–6 weeks). The condition and regeneration of explants was estimated every 10 days. Explant development was followed for over two months. Transformation with *rolB* gene was confirmed by the PCR method.

PCR reaction. The total DNA was isolated using the CTAB method [19]. Isolated DNA was used as a template for PCR. The PCR mixture contained: 1 unit of *Taq* DNA polymerase (MBI Fermentas, Lithuania), 1.5 mM $MgSO_4$, 0.2 mM dNTP and 1μM of each oligonucleotide primer. The primer pairs used for confirmation of *rolB* transformation are given below:

ROLBPF1 5′-ATGGATCCCAAATTGCTATTCCTTC CACGA-3′,

ROLBPR1 5′-TTAGGCTTCTTTCTTCAGGTTTAC-TGCAGC-3′.

The amplification was carried out according to the following scheme: 1 cycle of 95 °C for 4 min, then 30 cycles of: 95 °C for 1 min, 49 °C for 1 min, 72 °C × 1.30 min.

PCR products were analyzed using DNA electrophoresis.

RESULTS AND DISCUSSION

Cydonia transformation results showed that plant regeneration rate (ReR) was reduced significantly after cocultivation of explants with *A. tumefaciens*. The maximum ReR without treatment with bacteria reached 43.8% and after cocultivation decreased to 22.22% (Table 1). The highest ReR in the control variant had K.11 clone and after cocultivation clone K.16 plants. The ReR of clone K.19 was the lowest, like in the control; the ReR of explants after cocultivation depended on the vector construction. The highest value of this trait (ReR 12.2–22.22%) was obtained when the construct pART27rolBdir3 with its own *rolB* gene promoter in direct orientation was used.

After cocultivation with *A. tumefaciens* carrying pART27rolBrev1 construct, one *Cydonia* regenerant was obtained and successfully rooted. Only 36.1% of control plants had roots (Table 2). 67 regenerants were obtained after cocultivation with *A. tumefaciens* carrying the pART27rolBrdir3 construct. The rooting rate of those regenerants was close to the control variant. The screening of regenerants for presence of the *rolB* transgene using PCR showed that a transgenic *Cydonia* plant was obtained only in one case when the pART27rolBrev1 construct was used for the transformation. A specific, altered morphology of the plants (dense "hairy" roots) was characteristic of some of regenerants. No significant difference in the number of roots between control regenerants and transformants were found.

The localization of *rol* gene expression in tissues and the character of expression (decreased size, deformed leaves, short internodes, etc.), according to the li-

Table 1. ***Cydonia*** **regeneration depending on plant genotype and vector construction**

Construct	Clone					
	K.11		K.16		K.19	
	Treated explants	Regenerated, %*	Treated explants	Regenerated, %*	Treated explants	Regenerated, %*
pART27rolBdir3	120	17.50b	144	22.22b	156	12.18b
pART27rolBrev1	120	2.50c	204	2.94c	168	0.00c
pART27rolB CaMV3	192	0.52c	192	5.20c	204	1.47c
pART27rolBCaMV4	156	0.64c	144	2.78c	192	1.04c
Control	96	43.75a	96	35.00 a	96	20.80a

* Data marked with same letter did not differ significantly (LSD ≤ 0.01).

Table 2. **Rooting of *Cydonia* N.11 shoots *in vitro* after co-cultivation with *A. tumefaciens***

Construct	Regenerants			Number of obtained transformants
	Planted, number	Rooted		
		Number	%*	
pART27rolBdir3	67	31	46.2b	0
pART27rolBrev1	1	1	100.0a	1
Control	36	13	36.1b	0

* Data marked with same letter did not differ significantly (LSD ≤ 0.01).

Table 3. **Rooting of sweet cherry rootstock *Prunus cerasus* × *P. canescens in vitro* after co-cultivation with *A. tumefaciens***

Construct	Regenerants			Transformants	
	Planted, number	Rooted		Number	%*
		Number	%*		
pART27rolBdir3	26	26	100.0a	3	11.5a
pART27rolBrev1	113	89	78.8b	3	3.4b
pART27rolB CaMV3	243	185	76.1b	1	0.5c
pART27rolBCaMV4	72	56	77.8b	1	1.8bc
Control	121	85	70.2c	0	0.0c

* Data marked with the same letter did not differ significantly (LSD £ 0.01).

terature, depend on the promoter and even on the occurrence of a special segment of promoter [20].

In our study, *Cydonia* regenerants also showed a decreased size and misshapen upper leaves. Regenerants obtained after co-cultivation with *A. tumefaciens* carrying the *rolB* gene with the constitutive Couliflower Mosaic Virus promoter (CaMV) were smaller and stronger misshapen than plants treated with this gene driven by its own promoter. Taking into account that the *rolB* transgene could affect plant reaction to endogenous and egzogenous phytohormons [15, 16], it could be supposed that the construct with the constitutive CaMV promoter is hyperactive, distorts morphological signals and negatively impacts the morphogenesis. Those regularities were characteristic of all treated clones irrespective of the genotype.

Comparable results were obtained on sweet cherry rootstock *P. cerasus* × *Prunus canescens* transformation. It was shown that all *Prunus* hybrid regenerants rooted after leave explant co-cultivation with *A. tumefaciens* carrying the pART27rolBdir3 construct (Table 3). The rooting rate in control (untreated) plants was significantly lower and reached 70.2% only.

Microplants having 1–8 roots and shoots 7–19 mm in length were transferred into peat moss substrate *ex vitro*. 80.3% of the plants survived in such conditions for more than two months (control plants – 77.8%). Transformation events were proven using the PCR and *rolB* gene specific primers (Figure). The number of transformants was different and depended on the vector construct. Six *Prunus* hybrid transformants after treatment with pART27rolBdir3 and pART27rolBrev1 constructs

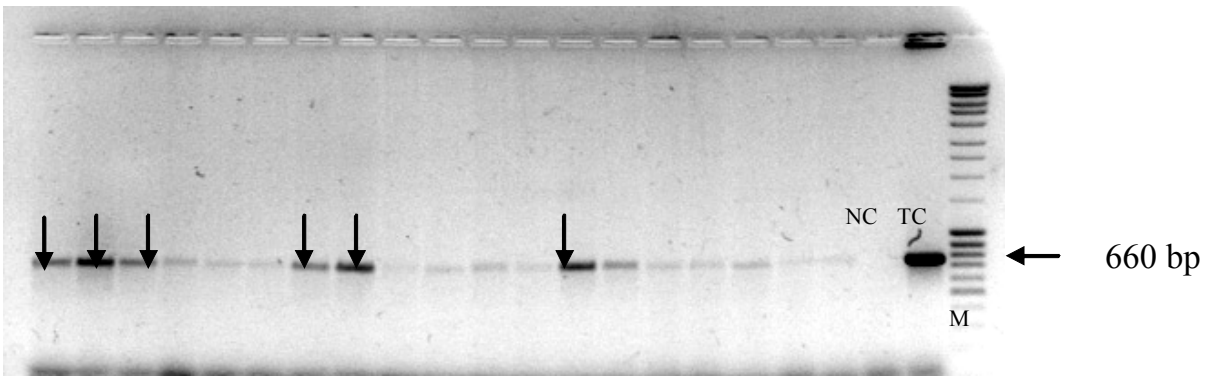

Figure. Electrophoregram of *Prunus cerasus* L. × *P. canescens* regenerant DNA using *rolB* gene specific primers ROLBPF1 and ROLBPR1

Transformants are shown by arrows, NC – negative control, TC – positive control (plasmid DNA), M – DNA fragment size marker.

were obtained (this was proven by PCR). Constructs with Cauliflower Mosaic Virus promoter CaMV35S yielded only two transformants. As in the case of *Cydonia* transformation, it seems that this construct is less favorable for rootstock transformation. We continue our work on checking the rootstock regenerants for presence of the *rolB* transgene and hope we'll find even more transformants.

To conclude, one PCR confirmed a transformant of pear (*Cydonia oblonga*), and eight transformants of cherry (*Prunus cerasus* L. × *P. canescens)* rootstocks were obtained after co-cultivation with *A. tumefaciens* carrying a plasmid with the *rolB* gene. All transformants were rooted, whereas the rooting rate of control (unstransformed) plants reached only 36.1% in *Cydonia* and 70.2% in the *Prunus* hybrid. The morphology of roots was characteristic of *rolB* gene transformants.

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References

1. Jacob HB. Acta Hort 1998; 475: 169–77.
2. Wertheim SJ, Wagenmakers PS, Sugar D. Acta Hort 1994; 367: 138–51.
3. Kviklys D. Investigation of dwarf pear rootstocks. In: Modern Orchards: Achievements and Tendencies. Babtai, 1997: 23–7.
4. Kviklys D. Sodininkystė ir daržininkystė. 2000; 19: 23–5.
5. Kviklys D, Kviklienė N. Acta Hort 2004; 658: 359–64.
6. White FF, Tayor BH, Huffman GA et al. J Bacteriol 1985; 164: 33–44.
7. Cardarelli M, Mariotti D, Pomponi M et al. Mol Gen Genet 1987; 209: 475–80.
8. Spena A, Schmüllin T, Koncz C et al. Embo J 1987; 6: 3891–9.
9. Ouartsi A, Clérot D, Meyer AD et al. Plant Sci 2004; 166: 557–67.
10. Capone IL, Spano L, Cardarelli M et al. Plant Mol Biol 1989; 13: 43–52.
11. Welander M, Zhu LH. Acta Hort 2000; 521: 133–8.
12. Welander M, Pawlicki N, Holefors A et al. J Plant Physiol 1998; 53: 371–80.
13. Zhu LH, Li XY, Ahlman A et al. Plant Sci 2003; 165: 829–35.
14. Zhu LH, Welander M. Acta Hort 2000; 521: 139–46.
15. Sedira M, Butler E, Gallagher T et al. Plant Sci 2005; 168: 1193–8.
16. Casanova E, Valdés AE, Zuker A et al. Plant Sci 2004; 167(3): 551–60.
17. Ražanskienė A, Stanienė G. Rugienius R et al. J. of Fruit and Ornament Plant Res 2005; 14 (Suppl 1): 95–102.
18. Murashige T, Skoog F. Physiol Plantarum 1962; 15: 473–9.
19. Doyle JJ, Doyle JL. Focus 1990; 12(1): 13–15.
20. Guivarch A, Carneiro M, Vilaine F et al. Plant Mol Biol 1996; 30: 125–34.

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ROLB TRANSGENO POVEIKIS _PRUNUS CERASUS_ × _P. CANESCENS_ IR _CYDONIA OBLONGA_ MIKROŪGLIŲ RIZOGENEZEI

Santrauka

Siekiant gauti gerai besišaknijančius hibridinio poskiepio *Prunus cerasus x P. canescens* ir kriaušių poskiepio *Cydonia oblonga* augalus, atlikta jų genetinė transformacija *rolB* genu taikant *Agrabacterium tumefaciens* transformacijos sistemą. Transformacijai naudoti binariniai vektoriai su *rolB* genu (klonuotu iš *A. rhizogenes* plazmidės pRiA4) ir savo bei konstitutyviu CaMV35S promotoriumi. Po kokultivavimo su agrobakterija *in vitro* iš viso gauta per 400 regenerantų. Nustatyta, kad abiejų rūšių augalų regeneracija ir šaknijimasis priklauso nuo vektoriaus konstrukcijos. Augalai geriausiai įsišaknijo *in vitro*, kai transformacijai buvo panaudotas vektorius su *rolB* genu ir savo promotoriumi. Lyginant su kontroliniais augalais (nepaveiktais *A. tumefaciens*), *Cydonia* regenerantų šaknijimasis po kokultivavimo su *A. tumefaciens* padidėjo 6–44%, o *Prunus* hibrido – 8–30%. Polimerazinės grandininės reakcijos būdu ištyrus regenerantų DNR, paaiškėjo, kad tarp 40 tirtų regenerantų 17,5% turėjo *rolB* geno intarpą genome. Visi transformantai buvo gerai įsišakniję, o šaknų morfologija būdinga transformantams su *rolB* genu.