TRANSFORMATION OF QUINCE (*Cydonia oblonga*) WITH THE *rolB* GENE-BASED CONSTRUCTS UNDER DIFFERENT PROMOTERS

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ABSTRACT

RolB gene (~ 800 bp) was isolated from *Agrobacterium rhizogenes* in PCR with rolB5kod-rolB3kod primers. The identity of gene was confirmed by sequence analysis. Binary constructs basing on pART27 and pNOV2819 plasmids and containing *rolB* gene under (a) own promoter sequence, (b) 35S CaMV, and (c) stress-induced PR promoter were prepared and used to transform quince (*Cydonia oblonga*). 1992 explants derived from clones K.1, K.16 and K.19 were transformed using *A. tumefaciens*. The efficiency of plant regeneration was strongly dependent on concentration of TDZ, and was best with 10 and 32 µM TDZ. The highest number of regenerating untransformed plants was obtained from explants of K.11 (43.8%), meanwhile after transformation the best results of regeneration was observed for explants derived from K.16 (from 22.2% to 2.78% dependent on type of construct). Rooting was observed in plants regenerated from explants transformed with plasmid pART27rolBdir3.

Key words: pear rootstock, rooting, *rolB* gene, genetic modification, efficiency

INTRODUCTION

Good, dwarfing rootstocks are one of the most important problems in effective pear (*Pyrus communis*) production. They not only reduce tree size, but also permit high-density planting, ease management, and increase yield per hectare.

There are only a few dwarfing pear rootstocks on the market. As an alternative, quince (*Cydonia oblonga* Mill.) can be used as a rootstock for grafting pears. However, quince rootstock is sensitive to alkaline soil and is not very winter-hardy (Jacob, 1998; Wertheim et al., 1994).

At the Lithuanian Institute of Horticulture, some new quince rootstocks have been selected. They are sufficiently winter hardy and compatible with many of the pear varieties which were tested. Unfortunately, they do not root very well (Kviklys, 1997; 2000; Kviklys and Kviklienė , 2004).

The RolA, rolB, and rolC genes are located on plasmids pRiA4, pRi2659 and pRi8196 of *Agrobacterium rhizogenes*. These genes induce root formation in most plant species. They have been used in plant transformation to increase rooting capacity (White et al., 1985; Cardarelli et al., 1987; Spena et al., 1987; Capone et al., 1989; Welander et al., 1998; Welander and Zhu, 2000; Zhu et al., 2003; Spena et al., 1987; Ouartsi et al., 2004).

The mechanism of morphological changes in transgenic plants is not clear. However, both endogenous and exogenous auxin activity is higher in plants transformed with *rolB* and *rolC* than in untransformed controls (Sedira et al., 2005; Casanova et al., 2004).

At the Lithuanian Institute of Horticulture, *rolB* was used with different promoters (constitutive 35S CaMV, *rol*B-own and stress-activated PR1) in an attempt to induce rooting in of selected quince rootstock. In this manuscript, we present the results of the first step of this study: the generation of binary constructs containing *rolB* and genes connected with different selection systems. We also discuss their use in successful plant transformation.

MATERIAL AND METHODS

Three quince clones were used in this study: K.11, K.16 and K.19. All had been selected at the Lithuanian Institute of Horticulture.

Young leaves from micro-shoots derived from *in vitro* cultures served as the explants. Isolated leaves were co-cultivated for two hours with transformed *Agrobacterium tumefaciens*, transferred to a dark room for two days, and then maintained in a growing chamber at 21 to 25˚C, with a 16 hour photoperiod.

Explants were regenerated on Murashige and Skoog medium with 30 g/l sucrose, 0.3 μM NAA and TDZ at either 1 μM, 10 μM, 32 μM or 64 μM. Untransformed explants growing under the same conditions served as the control. Explant development was observed over two months.

RolB was isolated from *Agrobacterium rhizogenes* (strain pRiA4, ATCC-LGC Promochem). Total DNA was isolated from the bacteria using the Genomic DNA purification kit (MBI Fermentas, Lithuania) in accordance with manufacturer recommendations. The total DNA isolated 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. Five primers were used for DNA

amplification: rolB5pro, rolB3ter1, rolB3ter2, rolB5kod and rolB3kod. The *Xba*I sequence was added to all of them (underlined in Tab. 1).

T a b l e 1 . Sequences of the primers used for *rolB* gene amplification

Primer	Sequence
rolB5pro	AATCTAGAGAGCTTGAAAAAGAGAGAACACA
rolB3ter1	AATCTAGAAGCTTGTTAGGCGTGCAAAGG
rolB3ter2	AATCTAGAGGCGGTCTTCGATTCATTTCC
rolB5kod	AATCTAGATGGATCCCAAATTGCTATTCCTT
rolB3kod	AATCTAGATTAGGCTTCTTTCTTCAGGTTTACTG

The amplification was carried out according to the following scheme: 1 cycle of 95ºC for 5 min, 58ºC for 1 min, 72ºC for 2.5 min; then 30 cycles of 95ºC for 1 min, 58ºC for 1 min, 72ºC x 2.5 min.

Isolated genes or genes complex were cloned into cloning vectors using the *Xho* I, *Xba* I, *Pvu* II, *Bcu* I restriction enzymes.

Gene or gene complex	Cloning vector
rolB	pUC57/T
35S CaMV-*rolB*	PCGT
PR-*rolB*	pUGPR1
PT1-PT2-*rolB*	pHanPR1

Plants were transformed with the binary plasmids pART27 and pNOV2819 (Syngenta).pART27 carried the gene for spectinomycin resistance, and pNOV2819 carried the gene for phosphomannose isomerase.

To investigate the effect of genetic constructs on the rate of explant regeneration, the following combinations were used:

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 and 4: constructs of *rolB* gene with CaMV promoter and nos3' terminator (Fig. 2).

RESULTS AND DISCUSSION

A DNA fragment of the expected size of about 800 bp was amplified with rolB5pro, rolB3ter1, rolB3ter2, rolB5kod and rolB3kod primers. The

sequence was 100% identical to the sequence of the *rolB* gene isolated from the plasmid pRiA4 of *A. rhizogenes* and described in Genbank data base (Acc. No. X03433).

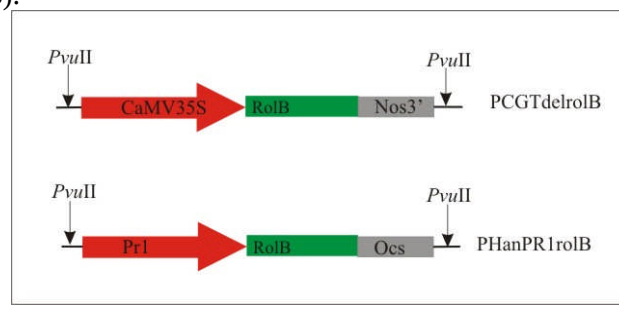

Figure 1. Scheme of constructed *rolB* expression cassettes. CaMV35S – promoter of cauliflower mosaic virus 35S, Nos3‘ – polyadenilation sequences of nopalin syntethase gene, PR1 – salicilate inducing promoter, ocs – terminator of *A. tumefaciens* octopine synthase, rolB – coding sequence of *A. rhizogenes* pRiA4 *rolB* gene

The isolated *rolB* gene was introduced into two cassettes. One contained the constitutive 35S CaMV promoter, and the other contained the stress-induced PR1 promoter (Fig.1). A third combination, consisting of the *rolB* gene with its own promoter, was amplified with rolB5pro, rolB3ter1, rolB3ter2, rolB5kod and rolB3kod. The PCR product was 2.7 kbp long. Homology with the sequence in Genbank (Acc. No. X03433) confirmed that both components were present in the DNA fragment. Quince rootstocks were transformed using binary plasmids carrying the cassettes described above (Fig. 2).

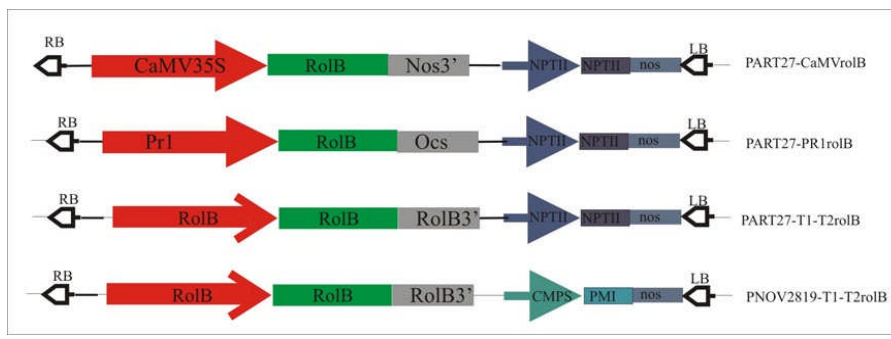

Figure 2. Schemes of binary vectors T-DNR, designed for reception of transgenic *rolB* plants. CaMV35S – promoter of cauliflower mosaic virus, ocs – terminator of *A. tumefaciens* octopine synthase, NPTII – promoter and gene of nopalin synthase, CMPS – promoter of cestrium yellow leaf curling virus, PMI – coding part of phosphomanose isomerase gene, nos – terminator of nopalin synthase gene

The regeneration of transformed plants is an important step of the transformation process. Morphogenesis in the quince was associated with callus formation, unlike in the plum (Cossio and Bassi, 1991). The concentration of TDZ concentration in nutrient medium affects the efficiency of the morphogenesis process (Tab. 2). More regenerated plants were obtained on media containing 10 or 32 µM TDZ than on media containing either 1 or 64 µM TDZ. The proportion of explants which produced regenerated plants was highest with K.11 (43.8%), and ranged from 20.8% to 35.9% with K16 and K19 (Tab. 2).

T a b l e 2. *Cydonia oblonga* shoot regeneration from leaves in *in vitro* culture

Medium	Forms of pear rootstock					
	K.11		K.16		K.19	
	callusogenesis	* regenerated explants [%]	callusogenesis	* regenerated explants [%]	callusogenesis	* regenerated explants [%]
BD 1µM TDZ	+	**2.85 b**	+	**12.5 a**	+	**4.2 c**
BD 10 µM TDZ	+++	**43.75 a**	+++	**35.0 a**	+++	**20.8 b**
BD 32 µM TDZ	+++	**34.35 b**	+++	**27.8 a**	+++	**33.3 a**
BD 64 µM TDZ	++	**29.2 b**	++	**21.9 a**	++	**4.2 c**

*means marked with the same letter do not differ significantly (at P=5%)

Explant co-cultivation with *A. tumefaciens* significantly reduced regenerative capacity (Tab. 3). The regeneration rate of K.11 explants transformed with *A. tumefaciens* was 22.2%. In the control, the rate was as high as 43.8% under optimal medium conditions (Tab. 2 and 3).

T a b l e 3. Plant regeneration after transformation with *A. tumefaciens*, carrying plasmids with different genetic constructs

Clone	pART27rolBrev1		pART27rolB CaMV3		pART27rolBdir3		pART27rolBCaMV4	
	expl. no.	regener. explants [%]	expl. no.	regener. explants [%]	expl. no.	regener explants [%]	expl. no.	regener. explants [%]
K-11	120	2.50 a	192	0.52 b	120	17.50 a	156	0.64 b
K-16	204	2.94 a	192	5.20 a	144	22.22 a	144	2.78 a
K-19	168	0.00 b	204	1.47 b	156	12.18 b	192	1.04 b

*means marked with the same letter do not differ significantly (at P=5%)

K.16 had the highest regeneration capacity for transformed explants, and K.11 had the highest regeneration capacity for untransformed explants. Efficiency of explant regeneration also depended on which plasmid was introduced. The plants transformed with pART27rolBdir3 had the highest regeneration rate. *rolB* with its own promoter and terminator sequences in direct 5'-3' orientation was the most effective and least toxic to plants and bacteria. Rooting took place only in plants regenerated from explants transformed with pART27rolBdir3. No rooting took place in control plants or plants regenerated from explants transformed with the other constructs.

In all, 78 plants were regenerated from explants transformed with pART27, which contains *rolB* with it own promoter. Twenty one plants from explants transformed with *rolB* gene with constitutive CaMV35S promoter were obtained.

The role of the *rol* genes of *A. rhizogenes* for plant root system is well known. Their natural activity is correlated with a change in auxin activity (Sedira et al., 2005; Casanova et al., 2004). Therefore, these genes can be used in plant transformation to improve plant rooting (White et al., 1985; Cardarelli et al., 1987; Spena et al., 1987; Capone et al., 1989; Welander et al., 1998; Welander and Zhu, 2000; Zhu et al., 2003; Spena et al., 1987; Ouartsi et al., 2004). 67 to 100 % of transgenic clones of the pear rootstock BP10030 (*Pyrus communis*) formed roots on medium without auxins, whereas none of the untransformed control plants did so (Zhu et al., 2003). *RolB* caused a reduction in size of some transgenic plants (Welander and Zhu, 2000; Zhu and Welander, 2000).

The expression of *rolB* gene strongly depends on promoters. When *rolB* with its own promoter was used, expression was observed only in certain tissues such as stem vascular bundles. The different domains of the promoter (A, B and C) caused different morphological changes such as wrinkled leaves, short internodes, or dwarf phenotype (Guivarch et al., 1996).

The results of our study confirmed that successful transformation of quince is possible using constructs containing *rolB*. Further study is needed to develop the best strategy for modifying *Cydonia oblonga* and increasing rooting capacity in this species.

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TRANSFORMACJA ROŚLIN *Cydonia oblonga* KONSTRUKCJAMI BINARNYMI Z GENEM *rolB* POD RÓŻNYMI PROMOTORAMI

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S T R E S Z C Z E N I E

Gen *rolB* (~800 pz) wyizolowano z *Agrobacterium rhizogenes* w PCR ze starterami rolB5kod-rolB3kod. Sekwencja uzyskanego produktu PCR wykazała bardzo wysoką homologię (100%) z sekwencjami genów *rol*, publikowanymi w GenBanku. Uzyskany fragment DNA został wprowadzony do konstrukcji binarnych, opartych na plazmidach pART27 i pNOV2819 pod promotorami a) 35S CaMV, b) własnym promotorem *rol* oraz c) promotorem PR (indukowanym przez stres). Wymienione konstrukcje wykorzystano do modyfikacji genowej roślin *Cydonia oblonga* (klony K1, K16 i K19 – łącznie 1992 eksplantaty) przez *Agrobacterium tumefaciens*. Wydajność regeneracji zależała od koncentracji TDZ (10, 32 µM) i genotypu. Największą liczbę zregenerowanych, nietransformowanych eksplantatów uzyskano z roślin K11 (43,8%), podczas gdy wydajność regeneracji post-transformacyjnej była największa dla klonu K16 (2,8-22,2%, zależnie od konstrukcji genowej). Jak dotychczas prawidłowe ukorzenianie zaobserwowano tylko dla eksplantatów transformowanych plazmidem pART27rolBdir3.

Słowa kluczowe: podkładki śliwy, ukorzenianie, *rolB*, modyfikacja genetyczna, wydajność transformacji/regeneracji