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Cell-wall polysaccharide composition and glycanase activity of *Silene vulgaris* callus transformed with *rolB* and *rolC* genes

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ABSTRACT

The aim of this research is to investigate the effects of the *Agrobacterium rhizogenes rol* genes on the composition of cell-wall polysaccharides and glycanase activity in the campion callus. The expression of the *rolC* gene reduces the yield of campion pectin, while the expression of the *rolB* or *rolC* gene inhibits the volumetric production of both pectin and intracellular arabinogalactan. The *rol* genes are involved in regulating the activity of glycanases and esterases, thereby contributing to the modification of polysaccharide structures, their molecular weight (*Mw*) and the degree of pectin methyl esterification (DE). The increase in pectin arabinose residue appears to be connected to a decrease in intracellular and extracellular α-L-arabinofuranosidase activity in transgenic campion calluses. In transgenic calluses expressing the *rolB* and *rolC* genes, the increase in pectin galactose residue is likely due to a decrease in β-galactosidase activity. The decrease in the *Mw* of pectin and its D-galacturonic acid content appears to be connected to an increase in extracellular polygalacturonase activity. Finally, the increase in pectinesterase activity causes a decrease in the DE of pectin. Thus, the expression of *rolB* and *rolC* genes in campion callus has a considerable effect on pectin's sugar composition, DE and *Mw*, while it appears to have an insignificant influence on intracellular and extracellular arabinogalactans.

1. Introduction

The pectic polysaccharides comprise a class of GalA-containing polysaccharides that are abundant in the plant cell wall (Caffall & Mohnen, 2009). The structural classes of the pectic polysaccharides include homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan (AGA), rhamnogalacturonan II (RG-II) and rhamnogalacturonan I (RG-I). Until recently, it was accepted that the rhamnogalacturonan and homogalacturonan domains constituted the "backbone" of pectic polymers. However, an alternative structure has recently been proposed in which HG is a long side chain of RG-I (Vincken et al., 2003). More recently, a widely-accepted cell wall model suggests that the load-bearing component is a cellulose–xyloglucan network interpenetrated by an independent pectin network (McCann, Wells, & Roberts, 1990; Morris, Gromer, Kirby, Bongaerts, & Gunning, 2011).

Pectic polysaccharides are a component of the cell walls of almost all higher aquatic and terrestrial plants and have important biological functions (Albersheim et al., 1994; Mohnen, 2008). They are also of interest due to their high physiological activity and valuable technical properties. Pectin and its derivatives are widely used in the pharmaceutical and food industries as non-toxic, biodegradable, biocompatible polymers for a large number of applications, including binding, thickening, emulsifying and gelling (Sharma & Ahuja, 2011). Pharmaceutical researchers have utilized pectin as an additive in drug delivery systems (DDS) for controlled drug release (Liu, Fishman, & Hicks, 2007).

The biological functions, physiological activity and valuable technical properties of plant polysaccharides are determined by their structural features (Wagner, Stuppner, Schäfer, & Zenk, 1988; Roesler et al., 1991; Ovodov, 2009). Therefore, it is beneficial to search for efficient sources of polysaccharides and methods to modify them in order to obtain polymers with the desired structures and properties. The development of methods for directional changes in the activity of cell wall enzymes provides an opportunity to obtain polysaccharides with particularly valuable properties and a definite structure (Ovodov, 2009). Modifying the structure of pectic

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substances in plant cell cultures using *Agrobacterium* genes is one of these approaches.

The modification of polysaccharides using the regulatory *rol* genes may represent a promising method for engineering plant cells that can produce valuable pectic substances. The *rolA*, *rolB*, and *rolC* genes, isolated from the T-DNA of Ri plasmids of the *Agrobacterium rhizogenes* strain A4 soil bacteria, induce hairy root formation in transformed plants, influencing plant growth and development, modulating ROS levels, and activating the secondary metabolism of plants (Bulgakov, 2008; Shkryl et al., 2008; Bettini et al., 2010; Bulgakov et al., 2012). The transformation of different plant species with the *rolB* and *rolC* genes causes the following morphological abnormalities: a reduction in the plant's height, weight and internode length; a decrease in the size of flowers and leaves; and increased branching. In addition, previous research has shown that gene transformation induces rhizogenesis and improves rooting ability, indicating that the *rol* genes exert an auxin-like activity (Maurel, Brevet, Barbier-Brygoo, Guern, & Tempé, 1990; Bettini et al., 2010). These morphological alterations induced by the *rol* genes indicate that its mechanism of action interferes with the hormones that modulate plant growth and differentiation (Bettini et al., 2010). However, the precise cellular effects of the *rol* gene products still need to be explored.

The effect of the *rol* genes on changes in the production of primary plant metabolites, particularly polysaccharides and enzymes, is not well studied. However, previous research has shown that transforming *Panax ginseng* cell cultures with the *Agrobacterium rolC* gene led to an increase in carbohydrase activity, including that of α- and β-D-galactosidase (Bulgakov, Kusaykin, Tchernoded, Zvyagintseva, & Zhuravlev, 2002) and 1,3-β-D-glucanase (Kiselev et al., 2006). These enzymes influence the polysaccharide composition of the plant cell wall, specifically with regard to the structure of the side chains of pectins and arabinogalactans.

Previously, we demonstrated that expressing the *rolB* gene increased the pectin yield in *Rubia cordifolia* cells, while the *rolC* gene inhibited pectin production, which correlated with its expression level (Günter et al., 2013). Increased expression of the *rolB* and *rolC* genes resulted in significantly reduced arabinose residues in pectin, which was accompanied by increased α-L-arabinofuranosidase activity in cells. Moreover, enhancing *rolB* expression increased galactose residues in pectin due to decreased β-galactosidase activity in cells. Additionally, transgenic cultures exhibited a reduction in pectin D-galacturonic acid residues (Günter et al., 2013).

Preliminary studies of the *Silene vulgaris* (M.) G. (*Oberna behen* (L.) I.) callus examined the synthesis of polysaccharides possessing immunomodulatory activity (Popov, Popova, Ovodova, Bushneva, & Ovodov, 1999). *S. vulgaris* cultures are an alternative source of raw materials for obtaining new and valuable pectins and calcium pectinate hydrogels (Günter et al., 2013). The macromolecule silenan consists of linear and ramified regions (Ovodova, Bushneva, Shashkov, & Ovodov, 2000; Bushneva et al., 2006). The linear region consists of α-1,4-D-galacturonan and α-1,2-rhamno-α-1,4-D-galacturonan, which is also the backbone of the silenan ramified region consisting of rhamnogalacturonan I. The side chains of the ramified region consist of terminal- and α-1,5-linked arabinofuranose and β-1,3-, β-1,4-, and β-1,6-linked galactopyranose residues (Bushneva, Ovodova, Shashkov, Chizhov, & Ovodov, 2003). The core of arabinogalactan consists of different segments of β-1,3-D-galactopyranan, and its side chains contain residues of terminal and 3-*O*-substituted β-galactopyranose, terminal α-arabinofuranose and α-rhamnopyranose, and 2-*O*-substituted α-rhamnopyranose (Bushneva et al., 2006).

The aim of this research is to investigate the effects of *Agrobacterium rol* genes on cell-wall polysaccharide composition and glycanase activity in the campion callus.

2. Materials and methods

2.1. Callus cultures

Callus cultures of the *S. vulgaris* (Moench) Garcke (*O. behen* (L.) I.) (designated S) were maintained on the agarized Murashige and Skoog medium (Murashige & Skoog, 1962) that contained 15 g/l sucrose, 15 g/l galactose, 8 g/l agar, 0.5 mg/l 6-benzylaminopurine, and 1.0 mg/l 2,4-dichlorophenoxyacetic acid. The callus cultures were subcultured for 21 days at 24 °C in the darkness.

The transgenic *rolC* and *rolB* callus cultures of *S. vulgaris* (designated SC and SB, respectively) were established by transformation of the *S. vulgaris* callus cultures with *A. tumefaciens* strains GV3101/pMP90RK containing plasmid vectors pPCV002-35S-*rolC* and pPCV002-35S-*rolB* (Spena, Schmülling, Koncz, & Schell, 1987) as described (Bulgakov, Shkryl, & Veremeichik, 2010). After transformation, the calluses were cultivated for a 3-month period in the presence of 250 mg/l of cefotaxim to suppress the bacteria. Selection of transgenic aggregates was carried out for 5 months using 50 mg/l of kanamycin sulphate.

2.2. RNA isolation, cDNA synthesis and real-time PCR

Genomic DNA was extracted from 100 mg of *R. cordifolia* and *S. vulgaris* callus cultures using a CTAB protocol as described by Echt, Erdahl, and McCoy (1992). DNA samples from each callus line were used as a template for PCR analysis that was carried out in a Bio-Rad C1000 (Bio-Rad Laboratories, Hercules, CA, USA) using ColoredTaq DNA Polymerase (Sileks, Moscow, Russia) as described (Bulgakov et al., 2010). The gene-specific primer pairs used in the PCR analysis are listed in Table 1. To verify the presence of amplifiable DNA, PCR for actin gene was performed in all samples. The PCR amplified products were analyzed by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

Total RNA isolation, analysis and first-strand cDNA synthesis was carried out as described previously (Shkryl et al., 2008; Shkryl, Veremeichik, Bulgakov, & Zhuravlev, 2011). A quantitative real-time PCR (qPCR) analysis was performed using a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories) with 2.5× SYBR green PCR master mix, containing ROX as a passive reference dye (Syntol, Moscow, Russia). All PCR reactions were performed using conditions described previously (Shkryl et al., 2011). Two biological replicates from two different RNA extractions were used for the analysis, and three technical replicates were analyzed for each biological replicate. Non-template controls and RNA-RT controls were included in the analysis to verify the absence of contamination. The absence of non-specific products and primer-dimer artifacts in the samples were confirmed by melting curve analyses performed at the end of each run and by product visualization using electrophoresis on a 1% agarose gel stained with ethidium bromide.

The actin genes of *R. cordifolia* and *S. vulgaris* were used for normalization of the qPCR data, using the delta-Ct method. The primer pairs for the actin genes span an intron sequence that allowed for testing for the absence of DNA contamination in the samples. Stability of the *RcActin* and *SvActin* genes expression was confirmed using the *18S* rRNA gene expression. The results are reported as relative fold expression.

2.3. General methods

Total amounts (% of total amount) of glycuronic acids in polysaccharide fractions were estimated using a reaction with 3,5-dimethylphenol in the presence of concentrated sulfuric acid (Usov, Bilan, & Klochkova, 1995). Total protein content was determined according to the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The degree of methylesterification was calculated

Table 1
List of primer pairs used for PCR analysis.

Gene (GenBank accession no.)	Forward primer	Reverse primer
rolC (X03433)	5′-GCTTGATGACACGCCCAG-3′	5′-CTCCTCACCAACCTTCCCCC-3′
rolB (X03433)	5′-GTGCTGGCGACAACGATTCA-3′	5′-GAACTTGCGAAAATGGCGATG-3′
RcAct (DQ531565)	5′-GATTGAGCACGGTATTGTTAG-3′	5′-ACACCATCACCAGAATCCAAC-3′
SvAct (JX431890)	5′-GCCCCTGCTATGTATGTTGCC-3′	5′-GGCATAACCCTCGTAGAT-3′
Rc18S (HQ730915)	5′-GGAGAGGGAGCCTGAGAAAC-3′	5′-GATTTAGATTGTACTCATTCC-3′
Sv18S (HM562728)	5′-ATTCACTTGGGTGAGACAGAG-3′	5′-TCATCGTTTACGGCATGGACTA-3′

using the method described earlier (Wood & Siddiqui, 1971). The absolute configuration of D-galacturonic acid was evaluated using specific optical rotation determined using a Polartronic MHZ polarimeter (Germany). Spectrophotometric measurements were made with an Ultrospec 3000 instrument (UK). GLC was performed with a Hewlett-Packard 4890A chromatograph (USA) fitted with an RTX-1 (0.25 mm × 30 m, Restek, USA) capillary column with argon as a carrier gas, using a flame-ionization detector and HP 3395A integrator. Molecular masses of polysaccharides were estimated using high performance liquid chromatography. The polysaccharide sample (3 mg) was dissolved in 0.15 M NaCl (1 ml) prepared using bidistilled water, and the solution was filtered and subjected to analysis using the following chromatographic system: a LS-20AD pump (Shimadzu, Japan), a Shodex OHpak SB-804 HQ column (8.0 mm × 30 cm; Shimadzu) with a Shodex GS-26 7B pre-column (7.6 mm × 5 cm; Shimadzu), CTO-10AS thermostat (Shimadzu), and RID-10A refractometer (Shimadzu). Elution was carried out with 0.15 M NaCl at 40 °C with an effluent rate of 0.4 ml/min. The pullulan samples with molecular masses in ranges 1.3, 6, 12, 22, 50, 110, 200, 400 and 800 kDa (Sigma, USA) were employed for column calibration. Molecular masses (*Mw* and *Mn*) of polysaccharides were analyzed using the LC solution program (Version 1.22 SP1).

2.4. Isolation of polysaccharides

Isolation of polysaccharides was performed as described earlier (Günter et al., 2007). Yields were calculated in relation to the dry weight of the callus. Volumetric production per liter of medium of intracellular (AG) and extraracellular (AG1) arabinogalactans and pectin silenan (SVC) was estimated. The data obtained are expressed as the means from three separate experiments.

2.5. Complete acidic hydrolysis

Polysaccharide samples (2 mg) were hydrolyzed with 2 M TFA (0.5 m1) at 100 °C for 3–4 h in sealed tubes. The acid was removed by repeated co-evaporation with methanol. The neutral sugars (% of total amount) were quantified by GLC as the corresponding alditol acetates using *myo*-inositol as the internal standard (York, Darvill, McNeil, Stevenson, & Albersheim, 1985). The molar ratios were calculated from the peak areas.

2.6. Molecular weight distributions of pectins

Each fraction of SVC (30 mg) was dissolved in distilled water (50 ml) and separated successively using various ultrafiltration membranes as follows: polysulfone 300, 100 and 50 kDa (Millipore, USA). The fractions were concentrated and lyophilized to furnish the purified SVC-I (*Mw* more than 300 kDa), SVC-II (*Mw* 100–300 kDa), and SVC-III (*Mw* 50–100 kDa).

2.7. Assay of enzymes

Biomass was homogenized in 0.05 M sodium acetate buffer, pH 5.0, at a biomass–buffer ratio of 1:10 and centrifuged at 10,000 × *g* for 20 min. The supernatant was dialyzed for 3 days against 0.05 M sodium acetate buffer, pH 5.0, at 4 °C and centrifuged. The activity of the intracellular enzymes was then evaluated in the supernatant. The activity of the extracellular enzymes secreted into the growing medium was evaluated. The agar culture medium was centrifuged at 10,000 × *g* for 20 min; the supernatant was dialyzed for 3 days against 0.05 M sodium acetate buffer, pH 5.0, at 4 °C and centrifuged. The activity of the extracellular enzymes was then evaluated in the supernatant.

The carbohydrase activities in the callus were detected during the stationary phase (21st day). Activities of polygalacturonase (total), pectinesterase (3.1.1.11), β-galactosidase (3.2.1.23), α-L-arabinofuranosidase (3.2.1.55), β-1,3-glucanase (3.2.1.39) were determined. The data are expressed as the means from four experiments. The enzyme activity is expressed in terms of kat (mol/s) per mg of protein (kat/mg protein × 10^8).

The activity of polygalacturonase was evaluated by the accumulation of reducing sugars after the incubation (10 min) of the enzyme solution with 1% polygalacturonic acid (ICN, United States) in 0.05 M sodium acetate buffer, pH 4.6, at 50 °C. The resulting reducing sugars were measured by the Nelson–Somogyi method (Nelson, 1944). A calibrating plot was constructed using D-galacturonic acid. The amount of pectinase, which releases 1 mol of D-galacturonic acid from polygalacturonic acid per second under the given conditions, was taken as an activity unit of the enzyme.

The pectinesterase activity was analyzed by the titrimetric determination of the carboxyl groups formed upon saponification of the methyl ester of galacturonan. The reaction mixture containing the crude enzyme extract and apple pectin (1%) (Sigma, United States) was incubated for 60 min at 30 °C, then titrated by NaOH (0.1 N) up to pH 7.5 (Gracheva et al., 1982). One unit of pectinesterase activity was defined as the amount of enzyme that catalyzed saponification of one equivalent of the ester bonds in pectin per s at 30 °C.

The β-galactosidase and α-L-arabinofuranosidase activities were determined spectrophotometrically at 400 nm using 2-nitrophenyl β-D-galactopyranoside (Sigma, United States) and 4-nitrophenyl α-L-arabinofuranoside (Sigma, United States) as substrates, respectively. One unit of β-galactosidase or α-L-arabinofuranosidase activity was defined as the amount of enzyme that cleaves 1 mol of substrate per s at pH 4.2 and 30 °C (Polygalina, Cherednichenko, & Rimareva, 2003).

β-1,3-Glucanase activity was measured by release of reducing sugars from the β-1,3-glucan, namely, laminaran (Sigma, United States). Reducing sugars were assayed according to the procedure of Nelson (1944). Laminaran (0.1%) in 0.05 M sodium acetate buffer (pH 5.0) was used as a substrate. The reaction mixture containing the crude enzyme extract and laminaran was incubated for 40 min at 37 °C (Lozovaya, Waranyuwat, & Widholm, 1998). Glucose was used as a standard. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that produced 1 mol of reducing sugar per s under the above conditions.

2.8. Statistical analysis

The results are given as the mean ± standard deviation (S.D.). The statistical significance of the differences between two means was evaluated by Student's *t*-test and a value of $p < 0.05$ was

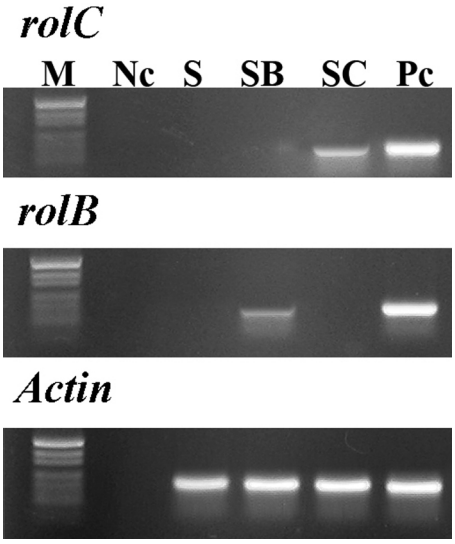

Fig. 1. Electrophoretic separation of the PCR products. M—pUC19/MspI marker; PC—positive controls; S, SB and SC—nontransformed, *rolB*-, and *rolC*-transformed callus cultures of *S. vulgaris*, respectively.

considered significant. Statistical calculation was performed with the Excel Microsoft software (version Windows 2002).

3. Results and discussion

3.1. The generation and expression analysis of transgenic S. vulgaris cultures over-expressing rolC and rolB

Suspension cultures of *S. vulgaris* (S) were inoculated with *Agrobacterium* strains harbouring *rolC* or *rolB* genes, controlled by the 35S promoter of the cauliflower mosaic virus. After co-cultivation with bacteria, the suspension cells were transferred onto solid media, and transgenic callus lines were selected through five passages on media supplemented with kanamycin. After kanamycin selection, callus lines were grown for three months on media without antibiotics to allow cells to reach homeostasis and to ensure that the obtained lines were stable during multiple subcultures.

Gene-specific PCR analysis revealed that the DNA samples from the *rolB* and *rolC* gene-treated calluses contained the sequences of the *rol* genes (Fig. 1), thus confirming that the cells were successfully transformed.

cDNA samples were prepared from the control and transgenic cultures, and qPCR analysis of *rol* gene expression was performed. cDNA samples isolated from the RBH and RCH cell lines of *R. cordifolia* were also analyzed, as these lines have the highest expression level of *rolB* and *rolC* genes among the different transgenic callus cultures previously tested (Shkryl et al., 2008). The expression levels of *rolB* and *rolC* in SB and SC cells were 2.1 and 1.6-fold lower, respectively, than the levels found in the RBH and RCH callus lines (Fig. 2). Thus, the level of *rol* gene expression in *S. vulgaris* transgenic calluses is moderate compared with its expression in RBM and RCM *R. cordifolia* callus lines (Shkryl et al., 2008).

3.2. The growth characteristics of calluses transformed with rol genes

In this study, the effects of transforming campion callus cultures with *rolB* and *rolC* genes on the growth parameters were examined. Cell growth was reduced overall, specifically, the dry biomass concentration per litre of medium, growth index, the specific cell growth rate, the dry biomass produced per day, and the time of dry

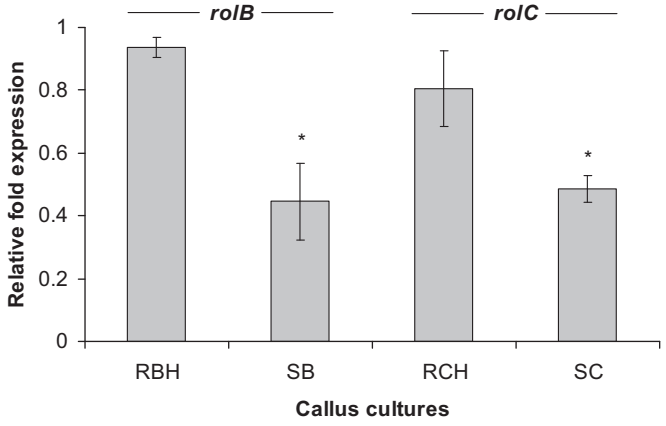

Fig. 2. The expression levels of the *rolB* and the *rolC* genes in transgenic callus cultures of *R. cordifolia* (RBH and RCH) and *S. vulgaris* (SB and SC) measured by qPCR. The results are reported as relative fold expression. * Statistically significant differences of means ($p < 0.05$) for each of the *rol* gene.

biomass doubling (Table 2). It was also determined that *rolB* gene expression leads to greater inhibition of cell growth than *rolC* gene expression.

A similar phenomenon has been observed by Bettini et al. (2010), in particular, insertion of the *rolC* gene resulted in a decreased indoleacetic acid level in the shoot apical region of the transgenic clone and inhibited tomato plant growth. Additional research has provided supporting evidence that the *rol* genes' mechanism of action interferes with hormones that modulate plant growth, thereby inducing morphological alterations such as reduced height, decreased flower and leaf size, rhizogenesis, etc. (Maurel et al., 1990; Bettini et al., 2010).

Therefore, the growth characteristics of the campion callus cultures transformed with *rol* genes were changed depending on the gene type.

3.3. The content and general chemical characteristics of pectic substances in transgenic cultures

Pectin SVC and intracellular (AG) and extracellular (AG1) arabinogalactans were isolated from callus cultures of the campion *S. vulgaris*, which were either not transformed (control) or transformed with the *rolB* and *rolC* genes. The pectin yield in the *rolB* transgene SB culture was similar to that of the control culture S, whereas the yield of pectin in the *rolC* transgene SC culture was 1.6-fold lower than that of the control culture (Table 3). These results indicate that expression of the *rolC* gene inhibits the synthesis of campion pectin. A similar phenomenon has been observed in madder *R. cordifolia* cells, where the inhibition effect of the *rolC* gene was related to its expression level; i.e., a decrease in pectin biosynthesis was typical only for transgenic cultures with high levels of transgene expression (Günter et al., 2013).

The yield of pectin SVC in the transgenic cultures varied depending on the gene type, whereby the yield was 1.9-fold higher in the *rolB* transgene culture than in the *rolC* transgene culture. The same pattern occurred in madder cell lines with low and medium expression levels (Günter et al., 2013).

The yield of AG in SB and SC cultures was unchanged compared to the control S culture. A similar observation was made for madder; i.e., the yield of AG did not change in the cultures with medium and high expression levels (Günter et al., 2013). The volumetric production of SVC and AG in both SB and SC cultures was 1.5–3.1-fold lower than that of the control. The volumetric production of AG1 was unchanged.

Table 2
The growth characteristics of *S. vulgaris calluses* transformed with *rol* genes.

Line	Biomass (g/l)		Growth index		μ (day^{-1})	P (g/L/day)	T (day)
	Fresh	Dry	Of fresh biomass	Of dry biomass			
S (control)	512.5 ± 1.3	11.9 ± 0.9	20.6 ± 3.4	14.3 ± 2.6	0.16 ± 0.01	0.54 ± 0.02	4.4 ± 0.2
SB	136.6 ± 28.0*	5.9 ± 0.5*	2.9 ± 0.8*	7.4 ± 1.3*	0.10 ± 0.01*	0.25 ± 0.02*	6.9 ± 0.5*
SC	240.9 ± 20.9*#	8.0 ± 0.5*#	6.0 ± 2.2*#	6.9 ± 2.1*	0.10 ± 0.01*	0.34 ± 0.02*#	7.2 ± 0.9*

Note: S—untransformed culture (control); SB—the culture transformed with the *rolB* gene; SC—the culture transformed with the *rolC* gene; μ—the specific cell growth rate on dry biomass (day^{-1}); P—production of dry biomass per day (g/L/day); T—time of dry biomass doubling (day); data are presented as the mean ± S.D.
* $p < 0.05$ vs. the control.
# $p < 0.05$ vs. the SB line.

The sugar composition of the pectin in campion transgenic cultures SB and SC was evaluated and compared with the control culture S (Table 4). The pectins from the transgenic and control calluses of the campion contained large amounts of D-galacturonic acid residues (61–81% of the total amount). The main neutral components were composed of galactose (2.7–9.1%) and arabinose (1.9–8.8%) residues (Table 4). Small amounts of rhamnose, glucose, xylose, and mannose residues were also found in the polysaccharide samples. An increase in neutral sugar content was observed in the SB and SC transgene cultures in comparison with the control. The amounts of galactose residues in the pectins from the SB and SC cultures were 2.3 and 3.4-fold higher, respectively, than that of the control culture S. The amounts of arabinose residues in the pectins from the SB and SC cultures were 4.3 and 5.2-fold higher, respectively, than that of the control. Moreover, a 1.3-fold decrease in D-galacturonic acid residues in the pectins from SB and SC was observed in comparison with the control. DE of pectin was 12.2% for the untransformed callus, while DE decreased to 4.1–4.4% in the pectins from the SB and SC transgenic cultures.

Ratios of sugar composition may be gathered to obtain information on the polymeric level. Using sugar composition data, three 'sugar ratios' that express the occurrence and properties of certain cell wall polysaccharides were calculated, based on methods from a previous study (Houben, Jolie, Fraeye, Van Loey, & Hendrickx, 2011). The first sugar ratio measures the linearity of pectin, comparing the pectic backbone sugar GalA to the neutral pectic sugars (Ara + Gal + Rha + Xyl) of the side chains. The second ratio compares the proportion of Rha to GalA, indicating the amount of RG in pectin overall. The third ratio compares the number of RG-I side-chain sugars (Ara + Gal) to Rha, measuring the extent of branching of RG-I (Houben et al., 2011).

Pectins from the SB and SC transgene cultures showed lower linearity (sugar ratios 3.5 and 2.9, respectively), a higher RG content (sugar ratio 0.03) and more RG-I branching (sugar ratios 7.9 and 8.6, respectively) than the pectin of the untransformed culture S. The pectin of the S culture was more linear (sugar ratio 11.2), had low RG content (sugar ratio 0.01), and had less RG-I branching (sugar ratio 4.6). Therefore, compared with the untransformed cells, the data showed that the pectin of transgenic cultures was very branched, with low linearity.

The *Mw* of the pectin from the SB and SC transgenic cultures was 1.1 and 1.4-fold lower, respectively, than that of the control. The *Mn* of the pectin from the SB and SC cultures was 2.4 and 1.5-fold higher, respectively, than that of the control (Table 4). The same decrease in *Mw* was observed in the pectin from the madder cell line that had a high *rolB* gene expression level, whereas in the *rolC* transgenic cultures with low, medium, and high expression levels, the *Mw* of pectin increased (Günter et al., 2013).

The polysaccharide fractions of SVC were obtained using ultrafiltration membranes. The results showed that the fractions differed in molecular weight as follows: SVC-I, *Mw* > 300 kDa; SVC-II, *Mw* 100–300 kDa; and SVC-III, *Mw* 50–100 kDa. The SVC-I fraction had the greatest yield compared with the SVC-II and SVC-III fractions. The molecular weight distribution of SVC extracted from the *rolB* transgene callus showed that the SVC-I fraction yield was 1.2-fold higher than that of the control (Table 5). The yields of SVC-II and SVC-III fractions were 5.1 and 1.8-fold higher, respectively, than those of the control. The galactose and arabinose residues in SVC-I were 2.6 and 4.2-fold higher, respectively, than that of the control. The amounts of galactose and arabinose residues in SVC-II were 1.4 and 1.3-fold higher, respectively, than that of the control. A 1.1–1.2-fold decrease in the D-galacturonic acid residues of the SVC-I and SVC-II fractions was observed compared to the control. The *Mw* distribution of SVC extracted from the *rolC* transgene callus showed that the yields of SVC-I, SVC-II and SVC-III were 1.1, 6.4 and 1.5-fold higher than those of the control (Table 5). The galactose and arabinose residues in SVC-I were 4.0 and 3.4-fold higher, respectively, than that of the control. The galactose residues in SVC-II were 1.3-fold higher. A 1.2-fold decrease in the D-galacturonic acid residues of the SVC-I and SVC-II fractions was observed compared to the control. Thus, an increase in galactose and arabinose residues in pectins from the SB and SC transgenic cultures was confirmed using the *Mw* distributions of pectins.

The main components of intracellular arabinogalactan AG included galactose (47.3–49.2%), arabinose (10.4–11.0%) and D-galacturonic acid residues (6.0–8.2%) (Table 4). Small amounts of rhamnose, glucose, xylose, and mannose residues were also found in the polysaccharide samples. The galactose/arabinose ratio in AG from the SB and SC transgenic cultures was unchanged compared to the control (4.3–4.7%). The amounts of arabinose, galactose and D-galacturonic acid residues were unchanged. The *Mw* of AG from

Table 3
The content of polysaccharides in *S. vulgaris* callus cultures transformed with *rol* genes.

Line	Yield (%)		Volumetric production (%)		
	SVC	AG	SVC	AG	AG1
S (control)	2.6 ± 0.7	4.4 ± 1.1	0.22 ± 0.03	0.39 ± 0.02	0.24 ± 0.06
SB	3.1 ± 0.4	5.6 ± 0.2	0.15 ± 0.01*#	0.26 ± 0.01*	0.25 ± 0.01
SC	1.6 ± 0.2*#	5.4 ± 0.2	0.07 ± 0.01*#	0.26 ± 0.01*	0.24 ± 0.05

Note: S—untransformed culture (control); SB—the culture transformed with the *rolB* gene; SC—the culture transformed with the *rolC* gene; SVC—pectin; AG—intracellular arabinogalactan; AG1—extraracellular arabinogalactan; data are presented as the mean ± S.D.
* $p < 0.05$ vs. the control.
# $p < 0.05$ vs. the SB line.

Table 4
Characteristic of polysaccharides isolated from *S. vulgaris* callus cultures transformed with *rol* genes.

Line	Contents (% of total amount)								DE (%)	*Mw* (kDa)	*Mn* (kDa)
	Gal	Ara	Rha	Glc	Xyl	Man	GalA	Protein			
Pectin SVC											
S (control)	2.7	1.9	1.0	2.1	1.6	0.7	80.8	9.2	12.2	416	32
SB	6.2*	8.1*	1.8*	1.6*	1.6	1.1*	61.2*	18.5*	4.4*	384*	78*
SC	9.1*	9.8*	2.2*	2.3*	1.0*	1.3*	64.0*	10.2	4.1*	302*	47*
Intracellular arabinogalactan AG											
S (control)	48.6	10.4	2.4	5.9	5.0	2.2	7.4	18.2		197	20
SB	47.3	11.0	3.0*	3.3*	2.6*	1.9	6.0	24.9*		159*	25
SC	49.2	11.0	3.2*	10.8*	7.1*	3.7*	8.2	6.8*		162*	24
Extraracellular arabinogalactan AG1											
S (control)	55.4	10.1	2.0	4.8	6.3	0.6	11.6	9.1		125	24
SB	61.0	12.1	2.3	1.4*	4.5*	0.6	10.4*	7.8		200*	26
SC	62.1	9.1	2.2	1.6*	4.3*	0.7	12.6	7.2		164*	27

Note: S—untransformed culture (control); SB—the culture transformed with the *rolB* gene; SC—the culture transformed with the *rolC* gene; data are presented as the mean ± S.D.
* $p < 0.05$ vs. the control.

the SB and SC transgenic cultures was 1.2-fold lower than that of the control, while the *Mn* of AG from the SB and SC cultures was 1.2 and 1.3-fold higher, respectively (Table 4). The madder cell line with a high *rolC* gene expression level exhibited a decrease in the *Mw* of AG, whereas the line with a high *rolB* expression level exhibited an increased *Mw* of AG (Günter et al., 2013). A similar pattern of increased *Mn* has been observed in the *rolB* and *rolC* transgenic cultures of madder cells.

The main components of extracellular arabinogalactan AG1 included galactose (55.4–62.1%), arabinose (9.1–12.1%) and D-galacturonic acid residues (10.4–12.6%) (Table 4). Small amounts of rhamnose, glucose, xylose, and mannose residues were also found in the polysaccharide samples. The amounts of galactose, arabinose and D-galacturonic acid residues and the galactose/arabinose ratio in AG1 from the SB and SC transgenic cultures were close to the control. The *Mw* of AG1 from the SB and SC cultures was 1.6 and 1.3-fold higher, respectively, than that of the control, while *Mn* increased 1.1-fold (Table 4).

Thus, the expression of the *rolB* and *rolC* genes in the campion callus has a considerable effect on sugar composition and DE of pectin, but an insignificant influence on intracellular and extracellular arabinogalactans. Neutral sugar residues in pectin, in particular, galactose and arabinose, increased significantly, while the number of the galacturonic acid residues, DE and *Mw* were reduced.

3.4. Activity of enzymes in cells transformed with rol genes

This study detected many changes in enzyme activity levels when the transgenic cultures were compared with the control: intracellular β-galactosidase and α-L-arabinofuranosidase in the SB transgenic cultures was 1.5-fold lower (Table 6); intracellular β-galactosidase and α-L-arabinofuranosidase in the *rolC* cultures was 1.2-fold lower; intracellular polygalacturonase in the transgenic SB and SC calluses decreased by 4.3 and 8.5-fold, respectively; intracellular 1,3-β-D-glucanase in the SB and SC cultures was 2.5 and 2.8-fold higher, respectively; pectinesterase activity increased by 1.1 and 2.6-fold for the rolB and rolC transgenic cultures, respectively; extracellular α-L-arabinofuranosidase in the SB and SC cultures was 1.6 and 2.7-fold lower, respectively (Table 6); extracellular pectinesterase in the SC culture increased 1.8-fold; and extracellular polygalacturonase activity increased 1.7-fold. In contrast, the extracellular β-galactosidase and 1,3-β-D-glucanase activity of the SB and SC cultures was unchanged relative to the control.

The yield of intracellular proteins in the *rolB* transgenic callus was higher than that of the control and *rolC* transgenic cultures (Table 6). However, the yield of extracellular proteins in the *rolB* and *rolC* transgenic callus was higher than that of the control. No clear relationship between yield of proteins and enzyme activities was found.

Findings indicate that the *rol* genes are involved in the regulation of glycanase and esterase activity, which causes the hydrolysis of polysaccharides of plant cells. This hydrolysis leads to the modification of their structure, specifically the side chains of pectin and AG, *Mw* and DE of pectin.

An increase in pectin arabinose residue appears to be connected with a decrease in intracellular and extracellular α-L-arabinofuranosidase activity in transgenic campion calluses. An increase in pectin galactose residue may be due to a decrease in β-galactosidase activity in the *rolB* and *rolC* transgenic cultures. A decrease in pectin D-galacturonic acid and *Mw* of pectin may be

Table 5
Characteristics of pectic fractions separated through the ultrafiltration membranes.

Line	Fraction**	Yield (%)	Contents (% of total amount)							
			Gal	Ara	Rha	Glc	Xyl	Man	GalA	Protein
S (control)	SVC-I	77.1	1.6	1.7	1.2	0.4	0.3	0.5	80.5	13.9
	SVC-II	0.8	13.4	6.3	3.3	2.9	1.4	1.4	69.3	2.1
SB	SVC-I	89.1*	4.2*	7.1*	1.3	0.9*	0.2*	1.1*	74.1*	11.1*
	SVC-II	4.1*	19.3*	8.1*	4.2*	2.5*	1.7	0.7*	57.2*	6.5*
SC	SVC-I	84.2*	6.4*	5.8*	1.7*	1.5*	0.6*	0.8	69.3*	13.9
	SVC-II	5.1*	18.0*	6.8	3.6	2.2*	6.5*	0.7*	56.2*	6.0*

Note: S—untransformed culture (control); SB—the culture transformed with the *rolB* gene; SC—the culture transformed with the *rolC* gene; control—untransformed cells; SVC-I—fraction with molecular weight more than 300 kDa; SVC-II—fraction with molecular weight of 100–300 kDa; data are presented as the mean ± S.D.
* $p < 0.05$ vs. the control.
** The yield from the initial pectin.

Table 6
The enzyme activity in *S. vulgaris* cultures transformed with *rol* genes**.

Line	Protein (mg/ml)	β-Galactosidase	α-L-Arabinofuranosidase	Polygalacturonase	Pectinesterase	1,3-β-D-Glucanase
Intracellular enzymes						
S (control)	0.047 ± 0.001	1672 ± 30	88.8 ± 4.5	2.8 ± 0.2	2.3 ± 0.2	78.7 ± 5.5
SB	0.051 ± 0.002*	1152 ± 50*	60.8 ± 7.2*	0.7 ± 0.03*	2.7 ± 0.2*	193.7 ± 19.3*
SC	0.046 ± 0.001	1458 ± 57*	77.3 ± 1.7*	0.3 ± 0.02*	6.2 ± 0.3*	221.8 ± 12.8*
Extracellular enzymes						
S (control)	0.048 ± 0.001	1280 ± 150	37.7 ± 12.8	0	0.7 ± 0.1	85.0 ± 9.0
SB	0.056 ± 0.001*	1215 ± 52	24.2 ± 2.5*	1.7 ± 0.2	0.7 ± 0.2	68.8 ± 9.3
SC	0.057 ± 0.004*	1073 ± 95	13.8 ± 1.8*	1.7 ± 0.2	1.3 ± 0.2*	89.7 ± 11.7

Note: S—untransformed culture (control); SB—the culture transformed with the *rolB* gene; SC—the culture transformed with the *rolC* gene; data are presented as the mean ± S.D.
* $p < 0.05$ vs. the control.
** Activity of enzymes (kat/mg protein × 10^8).

connected with increased extracellular polygalacturonase activity in the *rolB* and *rolC* transgenic cultures. In addition, a low level of linearity may hinder the binding of pectic polysaccharides with calcium ions (Houben et al., 2011), explaining why some pectins of the transgenic cultures are vulnerable to polygalacturonase. Finally, an increase in the pectinesterase activity in transgenic cells was accompanied by a decreased DE of pectin (3-fold).

Previous research has shown that transformation with the *rolC* gene significantly increased the activity of some carbohydrases and the production of their molecular forms (Bulgakov, Kusaykin, Tchernoded, Zvyagintseva, & Zhuravlev, 2002). More specifically, α- and β-D-galactosidase (Bulgakov et al., 2002) and 1,3-β-D-glucanase (Kiselev et al., 2006) activities increased in *P. ginseng* cells transformed with the *rolC* gene. In addition, expression of the *rolC* gene in transformed ginseng cells correlated closely with the expression of a 1,3-β-D-glucanase gene and a corresponding increase in 1,3-β-D-glucanase activity (Kiselev et al., 2006). 1,3-β-D-glucanases comprise a PR-2 family of plant defence proteins. *RolC* and *rolB* genes significantly lowered intracellular ROS levels, thus acting as a powerful suppressor of ROS (Bulgakov et al., 2012). 1,3-β-D-glucanase activity increased in the *rolB* and *rolC* transgenic callus of *S. vulgaris* as well as in the *rolC* transgene of the *P. ginseng* cells (Kiselev et al., 2006). In contrast, the activity of β-galactosidase in the transgenic callus of madder (*R. cordifolia*) only increased after transformation with the *rolA* gene (Günter et al., 2013). The β-galactosidase activity in the transgenic callus of madder (lines RBH and RCL) (Günter et al., 2013) and campion decreased after their transformation with the *rolB* and *rolC* genes, which caused an increase in pectin galactose residues. Moreover, in contrast to the transgenic callus of *S. vulgaris*, an increase in α-L-arabinofuranosidase activity was observed in the madder transgenic cultures, which caused a decrease in pectin and AG arabinose residues (Günter et al., 2013). This phenomenon may be due to the use of different plant species as cell models. In addition, it has been previously shown that the change in carbohydrase activity in cells transformed with the *rolC* genes is most likely the result of the expression of new isoforms of enzymes and not the induction of the existing enzymes (Bulgakov et al., 2002). An increase was also observed in pectinesterase activity for madder (Günter et al., 2013) and campion transgenic cultures. It can be assumed that expression of the *rolB* and *rolC* genes in transformed campion and madder cells correlates with the expression of a pectinesterase gene and a corresponding increase in the pectinesterase activity. Moreover, the expression of *rolB* and *rolC* genes in transformed campion cells correlates with the repression of β-galactosidase and α-L-rabinofuranosidase genes and a corresponding decrease in their activity.

Although the mechanism of *rol* gene action in transgenic plants is not yet clear, some phenotypic changes indicate the biochemical effects of the genes on endogenous hormones, more specifically, through a change in the pool size of growth promoters or inhibitors (the hormone balance) or the sensitivity to free active hormones (Bettini et al., 2010). Previous studies have shown that *rolC* genes decrease the level of indoleacetic acid in cells and inhibit plant growth (Bettini et al., 2010).

The change in pectin arabinose and galactose residues in the transgenic campion cultures may be due to an increased sensitivity to auxin, which provides the transfer of arabinose from UDP-arabinose into pectin and AG (Rubery & Northcote, 1970) and participates in the control of channeling of UDP-galactose into the cell wall polymers (Seifert, Barber, Wells, & Keith, 2004).

4. Conclusion

This study has shown that the content and sugar composition of polysaccharides in callus cultures transformed with the *Agrobacterium rol* genes changes with respect to an untransformed culture. Pectins from the SB and SC transgenic cultures showed the lowest overall linearity and the greatest degree of RG-I branching. In cells transformed with the *rol* genes, a correlation between glycanase activity changes, sugar composition and DE of the synthesized polysaccharides has been shown. The mechanism of action for the *rol* genes may consist of regulating glycanase and esterase activities, thereby modifying polysaccharide structures, and having a regulatory effect on endogenous hormones, thus stimulating the transfer of UDP-sugars into the cell wall polymers. Therefore, the *Agrobacterium* transformation method using the *rol* genes allows changes to the polysaccharide structure, making it possible to obtain polysaccharides with modified compositions and new properties. This method may be employed as a tool for modifying the structural features of cell-wall polysaccharides. This study contributes valuable information toward the biosynthesis and production of polysaccharides by cell cultures and the elucidation of the biological function and action mechanism of the *rol* genes. While the mechanism underlying the action of the *rol* genes is currently emerging, unraveling the mystery of this mechanism would enable the engineering of plant cells with improved characteristics. The new data obtained would allow us to use this system as a possible means of modifying polysaccharides to obtain physiologically-active polysaccharides with desirable properties (immunomodulatory activity) and structural features, which would be extremely useful to both the pharmaceutical and food industries.

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