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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