Journal of Bioscience and Bioengineering
VOL. 121 No. 5, 573–583, 2016

www.elsevier.com/locate/jbiosc

UDP-galactose transporter gene *hUGT1* expression in tobacco plants leads to hyper-galactosylated cell wall components

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Received 7 March 2015; accepted 29 September 2015
Available online 24 October 2015

We reported previously that tobacco plants transformed with the human UDP-galactose transporter 1 gene (*hUGT1*-transgenic plants) displayed morphological, architectural, and physiological alterations, such as enhanced growth, increased accumulation of chlorophyll and lignin, and a gibberellin-responsive phenotype. In the present study, we demonstrated that *hUGT1* expression altered the monosaccharide composition of cell wall matrix polysaccharides, such as pectic and hemicellulosic polysaccharides, which are biosynthesized in the Golgi lumen. An analysis of the monosaccharide composition of the cell wall matrix polysaccharides revealed that the ratio of galactose to total monosaccharides was significantly elevated in the hemicellulose II and pectin fractions of *hUGT1*-transgenic plants compared with that of control plants. A hyper-galactosylated xyloglucan structure was detected in hemicellulose II using oligosaccharide mass profiling. These results indicated that, because of the enhanced UDP-galactose transport from the cytosol to the Golgi apparatus by hUGT1, galactose incorporation in the cell wall matrix polysaccharides increased. This increased galactose incorporation may have contributed to increased galactose tolerance in *hUGT1*-transgenic plants.

[**Key words:** Transgenic plant; Nucleotide sugar transporter; Cell wall matrix; UDP-galactose; Monosaccharide]

Cell wall materials (CWMs) play essential roles not only in plant growth and development, but also in the response of plants to the environment and in their interactions with symbionts and pathogens (1). There are two types of cell walls, primary and secondary. Primary cell walls are deposited during cell growth and consist mainly of polysaccharides that can be broadly classified as cellulose, cellulose-binding hemicelluloses, and pectins. The latter two classes of cell wall components are often referred to as matrix polysaccharides, which are synthesized within Golgi cisternae, whereas cellulose is generated at the plasma membrane in the form of paracrystalline microfibrils (2,3). Secondary cell walls are deposited between the primary cell wall and the plasma membrane in confined locations where great mechanical strength and structural reinforcements are required, such as the xylem, and are produced after primary cell wall deposition and cell expansion are completed.

In the Golgi apparatus, glycosyltransferases catalyze the glycosidic linkages between a sugar moiety and specific polysaccharide acceptor, resulting in various sugar chain formations (4). Nucleotide sugar transporters (NSTs) are indispensable because they incorporate substrates used in the biosynthesis of matrix polysaccharides into the Golgi lumen as glycosyltransferase partners (5). Genes that encode NST family proteins are widely found in eukaryotic organisms and have been isolated from animal, plant, and yeast cells (6). Among the NST family of proteins, UDP-galactose (UDP-Gal) transporters are involved in transporting UDP-Gal into the Golgi lumen (6). UDP-Gal transporters have been described in mammals, *Drosophila*, *Caenorhabditis elegans*, *Entamoeba*, *Giardia*, *Leishmania*, yeast, and other organisms (7). Plant UDP-Gal transporters have been reported in *Arabidopsis* (8) and rice (9). The *Arabidopsis* UDP-Gal/UDP-glucose (UDP-Glc) transporter AtUTr1 and UDP-Gal transporter AtUTr2 have been isolated and characterized (10,11). Subsequently, two additional UDP-Gal transporters, AtUDP-GalT1 and AtUDP-GalT2, were identified (12), and the recombinant protein AtNST-KT1 was functionally characterized as a UDP-Gal transporter (13). Furthermore, three UDP-Gal transporters have been isolated from *Oryza sativa* (9).

Recently, Zhang et al. (14) and Song et al. (15) reported that an UDP-Glc transporter of rice, OsNST1, modulates cell wall biosynthesis. The *bc14* mutant harbors a mutation in *OsNST1*, which shows reduced mechanical strength owing to decreased cellulose content and altered wall structure. These findings indicate that absence of or a defect in NSTs strongly perturbs the supply of substrates, thus affecting polysaccharide biosynthesis and cell wall matrix composition. Although NSTs are likely to contribute to carbohydrate production outside the plasma membrane of plant cells, the machinery involved in this reaction is not fully understood.

In our previous work, we described the characteristics of tobacco plants transformed with the human UDP-Gal transporter 1 gene (*hUGT1*) (16), designated *hUGT1*-transgenic tobacco plants (17). *hUGT1* is the first mammalian nucleotide sugar transporter for which a cDNA sequence was identified (16), and the important

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.09.014

amino acid residues composing transmembrane domains and UDP-Gal recognition sites have been thoroughly analyzed (18). Although a number of plant UGTs have been identified (8–13), we chose *hUGT1*, as the best-studied UGT, for our investigations. In a previous report (17), we demonstrated that *hUGT1* was transcribed and translated in transgenic tobacco plants and that hUGT1, which showed UDP-Gal transporter activity, was mainly localized to the trans-Golgi network and endoplasmic reticulum in tobacco cells, similar to human cells (17). These transformants displayed enhanced growth during cultivation in soil and axillary shoots showed an altered determinate growth habit. Although the *hUGT1* expression level and UDP-Gal transport activity were not strongly correlated with growth and morphology among the *hUGT1*-transgenic tobacco plants examined, line 23 showed the strongest phenotype described above (17). Increased leaf thickness, caused by an increased amount of spongy tissue, increased numbers of xylem vessels in the stem, increased accumulation of lignin and chlorophyll, and hyper-galactosylated arabinogalactan proteins were observed in *hUGT1*-transgenic plants (17).

Polysaccharides in the plant cell wall not only provide physical support, but are also signaling substances. For example, oligosaccharides derived from some Gal-containing polysaccharides, such as xyloglucan and galactoglucomannan, have biological activities (19–23). In addition, arabinogalactan proteins play a crucial role in various physiological functions in plant cells (24). Thus, changes in Gal-containing cell wall polysaccharides might have an important impact on plant growth and development.

Hyper-galactosylation of cell wall matrix polysaccharides has been successfully achieved by introduction of an UDP-Glc/Gal epimerase gene into potato (25) and a galactosyltransferase gene into *Arabidopsis* (26). However, introduction of an UDP-Gal transporter has not been reported previously. Given that plant cell wall matrix polysaccharides are synthesized in the Golgi apparatus, an UDP-Gal transporter must play a key role in linking UDP-Gal biosynthesis in the cytosol and galactosylation of polysaccharides in the Golgi apparatus. We hypothesized that increased UDP-Gal transport activity will drastically affect cell wall matrix polysaccharide structure. In this study, we revealed that enhanced UDP-Gal transport activity caused by *hUGT1* expression altered the monosaccharide composition of the cell wall matrix components of *hUGT1*-transgenic plants. In particular, we focused on the increase in the ratio of galactose to total monosaccharide residues, so called hyper-galactosylation, in the cell wall matrix of transgenic plants. We also showed that the additional UDP-Gal transport activity increased the tolerance to galactose, which may be toxic to plant cells. The dynamic change in cell wall polymer composition caused by hyper-galactosylation of cell wall matrix polysaccharides is discussed.

MATERIALS AND METHODS

Plant materials and growth conditions As reported previously (17), tobacco (*Nicotiana tabacum* cv. SR-1) plants were transformed with *hUGT1* (16), together with a hemagglutinin tag inserted between the CaMV *35S* promoter and a nopaline synthase gene (*nos*) terminator, designated pBIN-hUGT1. Transformation was mediated by *Agrobacterium tumefaciens* strain LBA4404. As a control, tobacco plants were transformed with the empty vector pBIN19. The pBIN19- and *hUGT1*-transgenic tobacco plants were cultured *in vitro* for 1 month, then transferred to soil in pots and cultivated with the addition of 1:1000 diluted Hyponex fertilizer (Hyponex Japan, Osaka, Japan) at 25 °C with a 16-h photoperiod under a fluorescent daylight lamp (50 μmol/m^2/s) in a climate-controlled room. After growth in soil for 2 months, the pBIN19- and *hUGT1*-transgenic tobacco plants were harvested and used as material for CWM extraction.

Extraction of CWM To extract CWM from a large number of plant samples, a simplified method devised by Foster et al. (27) was employed with several modifications. The eleventh leaf or tenth stem internode from the shoot apex of harvested plants were used for CWM extraction. Air-dried plant material (60 mg) was ground with 5-mm stainless balls in a 2-mL screw cap tube (Watson, Tokyo, Japan) using a bead crusher μT-12 (Taitec Corp., Saitama, Japan) at 2600 rpm for 1 min. The homogenate was washed in 1.5 mL of 70% ethanol, centrifuged at 9700 ×*g* for 10 min, and the supernatant was removed. The pellet was resuspended in 1.5 mL chloroform/methanol [1:1 (v:v)], centrifuged at 9700 ×*g* for 10 min, and the supernatant was removed. The pellet was resuspended in 500 μL acetone, and the solvent was evaporated. To remove starch, the sample was resuspended in 1.5 mL of 0.1 M sodium acetate buffer, pH 5.0, and incubated for 20 min at 80 °C. The sample was mixed with a reagent mixture comprising 35 μL of 0.01% NaN_3, 35 μL α-amylase (50 μg/mL H_2O; from *Bacillus* species; Sigma–Aldrich Corp., St Louis, MO, USA), and 17 μL pullulanase (18.7 units from *Bacillus acidopullulyticus*, Sigma–Aldrich Corp.), and incubated overnight at 37 °C with moderate mixing. After incubation, the sample was heated for 10 min at 100 °C, centrifuged at 9700 ×*g* for 10 min, and the supernatant was discarded. The pellet was washed three times with 1.5 mL distilled water, resuspended in 500 μL acetone, and air dried. The residue was defined as the CWM.

Extraction and fractionation of leaf CWM The eleventh leaf from the shoot apex of harvested plants was used for leaf CWM extraction. Leaves of the pBIN19- and *hUGT1*-transgenic plants (1 g) were ground to a powder in liquid nitrogen with a mortar and pestle. The powder was homogenized in ice-cold 250 mM potassium phosphate buffer (pH 7.0) with a Teflon homogenizer. The homogenate was centrifuged at 2000 ×*g* for 10 min at 4 °C. The pellet was resuspended in ice-cold buffer and centrifuged. This step was repeated twice. The pellet was resuspended in ice-cold water and centrifuged at 2000 ×*g* for 10 min at 4 °C, and then resuspended in ice-cold buffer and centrifuged. This step was repeated three times. Next, the pellet was suspended in a 10-fold volume of methanol and incubated for 30 min at 80 °C. After centrifugation at 2000 ×*g* for 10 min at 4 °C, the supernatant was discarded, and the pellet was resuspended in methanol and centrifuged again. The supernatant was discarded, and the pellet was resuspended in ice-cold water and centrifuged at 2000 ×*g* for 10 min at 4 °C. The supernatant was discarded. To remove starch, the pellet was incubated in 10 volumes of α-amylase solution (0.1 mL α-amylase Type I-A; Sigma–Aldrich Corp.) in 100 mL of 20 mM sodium phosphate buffer, pH 7.0, containing 6 mM NaCl for 24 h at 28 °C. After centrifugation at 2000 ×*g* for 10 min at 4 °C, the pellet was washed twice with ice-cold water. After the resuspension and centrifugation, the pellet, defined as a leaf CWM, was recovered.

To extract the pectin fraction, 50 mM EDTA solution (50 mM EDTA, 50 mM acetate-sodium acetate buffer, pH 4.5) was added to the leaf CWM and incubated for 4 h at 100 °C. After centrifugation at 1000 ×*g* for 10 min, the supernatant was recovered (supernatant1). Next, 20 mM EDTA solution (20 mM EDTA, 50 mM acetate-sodium acetate buffer, pH 4.5) was added to the pellet and homogenized with a Teflon homogenizer, and then the same incubation and centrifugation steps were carried out. The supernatant (supernatant2) was recovered and combined with supernatant1, and then dialyzed with water for 48 h at 4 °C to yield the pectin fraction. To extract the hemicellulose I fraction, the pellet obtained above was resuspended in 1 M KOH/20 mM $NaBH_4$ solution and incubated for 24 h at 28 °C. After centrifugation at 1000 ×*g* for 10 min, the supernatant was recovered and dialyzed with water for 48 h at 4 °C to yield the hemicellulose I fraction. To extract the hemicellulose II fraction, the pellet obtained above was resuspended in 4 M KOH/20 mM $NaBH_4$ solution and incubated for 24 h at 28 °C. After centrifugation at 1000 ×*g* for 10 min, the supernatant was recovered and dialyzed with water for 48 h at 4 °C to yield the hemicellulose II fraction.

To quantify each cell wall fraction, the orcinol–sulfuric acid method (28) was employed. A colorimetric analysis was carried out by measuring the absorbance at 600 nm.

Extraction and fractionation of stem CWM Isolation of CWM from stems of the pBIN19- and *hUGT1*-transgenic plants was performed as described by Selvendran and O'Neill (29) with a slight modification. The entire stem from harvested plants was used for stem CWM extraction. In total, 25 g (fresh weight) of stems were ground with a mortar and pestle in liquid nitrogen, blended with 100 mL (twice the volume of the material) of 1.5% SDS solution containing 5 mM $Na_2S_2O_5$ in a Waring blender, and subsequently homogenized with Ultraturrax (IKA Works GmbH & Co., Staufen, Germany). The homogenate was washed twice with a double volume of 0.5% (w/v) SDS solution containing 3 mM $Na_2S_2O_5$, and then crushed in the same solution in a pot mill containing ceramic balls at 60 rpm for 15 h at 4 °C. The crushed residue was recovered and washed twice in 100 mL distilled water by suspension and centrifugation. The pellet was resuspended in 90% DMSO and incubated overnight at room temperature to remove starch. After centrifugation, the pellet was resuspended in 90% DMSO and incubated for 1 h at room temperature, and then washed five times with distilled water. The pellet was resuspended in a small volume of distilled water and dialyzed overnight at 4 °C in distilled water. The pellet was recovered using centrifugation and stored at −20 °C as a CWM.

To obtain the pectin I fraction (the 1,2-diaminocyclohexanetetraacetic acid (CDTA)-soluble pectin), ~1 g of CWM was extracted using 50 mM CDTA solution, pH 6.5 (CDTA disodium salt, Sigma–Aldrich Corp.). After centrifugation, the supernatant was recovered and concentrated in an evaporator. The fraction was dialyzed overnight at 4 °C in distilled water and stored at −20 °C. To obtain the pectin II fraction (Na_2CO_3-soluble pectin), the CDTA-insoluble residue was extracted in 50 mM Na_2CO_3/20 mM $NaBH_4$ solution, pH 10.8. After centrifugation, the supernatant was recovered and concentrated in an evaporator. The fraction was dialyzed overnight at 4 °C in distilled water and frozen at −20 °C.