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Expression of the human UDP-galactose transporter gene *hUGT1* in tobacco plants' enhanced plant hardness

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We reported previously that tobacco plants transformed with the human UDP-galactose transporter 1 gene (*hUGT1*) had enhanced growth, displayed characteristic traits, and had an increased proportion of galactose (hyper-galactosylation) in the cell wall matrix polysaccharides. Here, we report that *hUGT1*-transgenic plants have an enhanced hardness. As determined by breaking and bending tests, the leaves and stems of *hUGT1*-transgenic plants were harder than those of control plants. Transmission electron microscopy revealed that the cell walls of palisade cells in leaves, and those of cortex cells and xylem fibers in stems of *hUGT1*-transgenic plants, were thicker than those of control plants. The increased amounts of total cell wall materials extracted from the leaves and stems of *hUGT1*-transgenic plants supported the increased cell wall thickness. In addition, the cell walls of the *hUGT1*-transgenic plants showed an increased lignin contents, which was supported by the up-regulation of lignin biosynthetic genes. Thus, the heterologous expression of *hUGT1* enhanced the accumulation of cell wall materials, which was accompanied by the increased lignin content, resulting in the increased hardness of the leaves and stems of *hUGT1*-trangenic plants. The enhanced accumulation of cell wall materials might be related to the hyper-galactosylation of cell wall matrix polysaccharides, most notably arabinogalactan, because of the enhanced UDP-galactose transport from the cytosol to the Golgi apparatus by hUGT1, as suggested in our previous report.

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[**Key words:** Transgenic plant; UDP-galactose transporter; Galactosylation; Transmission electron microscopy; Cell wall accumulation; Biomass]

The cell wall is the principal structural element responsible for plant forms. The deposition and modification of cell wall materials play essential roles not only in plant growth, development and support but also in plant responses to environmental and pathogen-induced stresses (1). Most photosynthetically fixed carbon is incorporated into polymers that construct plant cell walls, which are the most abundant renewable resource on earth. Furthermore, cell wall materials are vital dietary components for animals, including humans, because they are sources of nutrients. Additionally, they are of practical importance for humans as sources of natural fibers for textiles and paper products. Thus, the increased production of cell wall polymers in a plant body has the advantages of reducing CO_2 emissions, as well as accumulating a renewable resource that can be used for food and industrial purposes.

Plants have two types of cell walls. Primary cell walls are synthesized during cell growth and consist mainly of polysaccharides that can be broadly classified as cellulose, cellulose-binding hemicelluloses and pectins. The primary cell walls provide mechanical stability with sufficient extensibility to permit cell expansion during cell growth. Cellulose is synthesized at the plasma membrane in the form of paracrystalline microfibrils, whereas hemicelluloses and pectins are synthesized within Golgi cisternae as matrix polysaccharides (2,3). Secondary cell walls, which are composed of cellulose, hemicellulose and lignin, are deposited between the primary cell wall and the plasma membrane at specialized cell types, such as xylem elements and sclerenchyma cells. They are produced after the cessation of cell growth and confer mechanical stability to the plant body, so it can stand against gravitational forces (2).

In plant cells, as in animal cells, glycoproteins and glycolipids are synthesized in the Golgi apparatus. In addition, cell wall matrix polysaccharides, such as hemicellulose and pectin, are biosynthesized in the Golgi apparatus (2,3). To produce the matrix polysaccharides, glycosyltransferases carry out sequential additions of sugar residues in the Golgi apparatus (4). To biosynthesize matrix polysaccharides, nucleotide sugar transporters (NSTs) are

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indispensable for the translocation of substrates into the lumen of the Golgi apparatus and act as partners of the glycosyltransferases (5). Genes that encode proteins in the NST family have been isolated from animal, plant and yeast cells (6). UDP-galactose transporters are NST family members and are involved in the transport of UDP-galactose into the Golgi lumen (6). UDP-galactose transporters have been described in humans, rodents, *Drosophila*, *Caenorhabditis elegans*, *Entamoeba*, *Giardia*, *Leishmania*, yeast (7), *Arabidopsis* (8) and rice (*Oryza sativa*) (9). Consequently, as in animal cells, plant NSTs must play crucial roles in the import of nucleotide sugars into the Golgi apparatus. The *Arabidopsis* UDP-galactose/UDP-glucose transporters AtUTr1 (10), AtUTr2 (11), AtUDP-GalT1, AtUDP-GalT2 (12) and AtNST-KT1 (13) have been identified. Furthermore, three UDP-galactose transporters, OsUGT1, 2 and 3, have been isolated from rice (9).

We previously reported on the characteristics of tobacco plants transformed with the human UDP-galactose transporter 1 gene (*hUGT1*; (14)), designated *hUGT1*-transgenic tobacco plants (15). hUGT1 is the first known mammalian nucleotide sugar transporter, and its structure and functions have been elucidated (14,16). Although a number of plant UGTs have been identified (8–13), we chose hUGT1, as the best-studied UGT, for our investigations. The hUGT1 expressed in tobacco plants was mainly localized to the trans-Golgi network and endoplasmic reticulum in tobacco cells, and it showed a UDP-galactose transporter activity, as in human cells (16). These *hUGT1*-trasgenic plants displayed enhanced growth during cultivation in soil.

Furthermore, we indicated that the enhanced UDP-galactose transport activity resulting from the expression of *hUGT1* altered the monosaccharide compositions of cell wall matrix components in the *hUGT1*-transgenic plants. There was an increase in the ratio of galactose to total monosaccharide residues in the *hUGT1*-transgenic plants (17), and the increased galactose ratio, so called hyper-galactosylation, was observed for xyloglucan in hemicellulose, rhamnogalacturonan I in pectin (17) and the arabinogalactan proteins (15). This increase in the galactose ratio was caused by an excess galactose transport activity from cytoplasm to Golgi lumen. This hyper-galactosylation of cell wall matrix polymers appeared to increase not only alterations in the cell wall structure but also the perception of environmental signals, such as phytohormones.

The *hUGT1*-transgenic plants were harder and more rigid to the touch than the pBIN19-transgenic plants. Increases in leaf thickness, caused by an enhanced amount of spongy tissue, greater numbers of xylem vessels in the stem, and an increased accumulation of lignin, were observed in the *hUGT1*-transgenic plants (15). Furthermore, the hyper-galactosylation of xyloglucan, which is a hemicellulose polysaccharide, was also presumed to enhance the hardness of *hUGT1*-transgenic tobacco (18). However, this hardness might result not only from these characteristics of *hUGT1*-transgenic plants but from other unknown reasons as well. Here, the practical hardness of *hUGT1*-transgenic plants was confirmed by physical strength measurements of leaves and stems using a tensile tester. Next, we determined whether the cell wall thickness in *hUGT1*-transgenic plants was altered. Finally, we demonstrated that the *hUGT1*-transgenic plants displayed an increased accumulation of cell wall materials, resulting in an increased biomass. The contribution of the heterologous expression of *hUGT1* to the increased accumulation of cell wall materials is discussed.

MATERIALS AND METHODS

Plant materials and growth conditions Tobacco plants (*Nicotiana tabacum* cv. SR-1) containing *hUGT1* (14) driven by the CaMV 35S promoter (pBIN-hUGT1) and the empty vector pBIN19 were used as *hUGT1*-transgenic plants and control pBIN19-transgenic plants, respectively, as described previously (15). T1 plants of each line were transplanted into soil in pots for cultivation. To compare these transgenic plants, wild type tobacco plants without any transgenes were also used. These plants were cultured *in vitro* for 1 month, 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. The leaves and stems of plants, prior to reproduction, which were growing in the soil (2.5 months after seeding), were used in the present work. The 11th and 16th leaf blades and the 7th, 10th and 15th stem internodes, counting from the smallest recognizable leaf (green blade of ~1–2 mm in length) at the top of the plants, were used as materials.

Hardness test The hardness levels of the leaves and stems of plants were measured as force (Newton, N) using a Compact Table-Top Tensile Tester EZ-SX 500N (Shimadzu Co., Kyoto, Japan) controlled by the RAPEZIUM X software for Windows (Shimadzu Co.). To determine the hardness of leaves and stems, penetration and bending tests, respectively, were employed. In the penetration test for leaf, a 3-mm diameter stainless-steel rod was used to make a hole in the 11th and 16th leaves by applying pressure from the adaxial side. The leaves were placed on a 52-mm diameter cylinder with a 5-mm diameter hole in the center and then were penetrated by the stainless-steel rod. In the three-point bending test for stem, the stem segments, which included 10th or 15th internodes, were laid sideways like a bridge on two acrylic boards (each 10 mm thick) vertically standing at intervals of 20 mm. The stems were folded until they were broken with a wedge (20-mm width, 8-mm depth and 14-mm height; cutting edge part, 20-mm width, 8-mm depth, 8-mm height, and 45° edge angle) at the distal end of the 5-mm diameter stainless-steel rod. To measure the hardness of the resilient seventh internode, the folding test without breaking was carried out. The stems, including seventh internodes, were pushed with the wedge described above until a 1-cm fold occurred. Plant materials derived from at least three independent plants were used for each measurement.

Transmission electron microscopy The 11th leaf blades and the 10th and 15th stem internodes of the pBIN19- and the *hUGT1*-transgenic tobacco plants cultured in soil were cut into small pieces. The pieces were immersed overnight in a prefixation solution [0.1 M sodium cacodylate buffer (pH 7.2), 2.5% (v/v) glutaraldehyde] under reduced pressure by vacuum-infiltration and subsequently washed with 0.1 M sodium cacodylate buffer. Samples were postfixed in 1.5% (w/v) OsO_4 for 2 h in 0.1 M sodium cacodylate buffer on ice and then dehydrated in a graded ethanol series [50%, 70%, 80%, 90%, 95% and 100% (v/v)]. The samples were substituted with an ethanol–propylene oxide mixture (1:1) once and with propylene oxide twice, and embedded in EPOK 812 resin (Oken Shoji, Tokyo, Japan) at 60 °C for 24 h. Ultra-thin sections (70–80 nm thick) were obtained with a diamond knife on an Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria), then stained with 2% uranyl acetate for 10 min and lead citrate for 5 min, and finally examined with a JEM 1200EX or JEM1400 transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

The cell wall thicknesses were determined by measuring at 10 different locations along the cell wall in each of the more than 100 electron microscopic images using ImageJ software (https://imagej.nih.gov/ij/). Then, the means and standard deviations were calculated.

Extraction of total cell wall materials Before the extraction of total cell wall materials (TCWMs), the fresh weights (FWs) of the 11th and 16th leaves, and the 10th and 15th stem internodes, of soil-cultured plants were measured. Then, after air-drying for 16 h at 65 °C in a dry oven, the dry weights (DWs) of these leaves and stem internodes were measured. The percentage DW/FW (%) was calculated as the ratio of DW to FW for each plant material. To extract TCWMs from plant samples, the method devised by Foster et al. (19) was employed with several modifications, as described in our previous report (17). In total, 60 mg of air-dried leaves and stem internodes was used for extraction. The final extracted weight of the TCWMs was calculated on a FW basis. Plant materials derived from at least three independent plants were used for each measurement.

Quantification of lignin and RT-PCR analysis of lignin biosynthetic genes For lignin extractions, 10 mg of air-dried samples harvested from the 11th and 16th leaves, and the 10th and 15th internodes, of the pBIN19- and the *hUGT1*-transgenic tobacco plants cultured in soil were used. These samples were 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 quantification of lignin was carried out by modifying the thioglycolic acid method described by Bonawitz et al. (20). Finally, based on the lignin standard (Nacalai Tesque Inc., Kyoto, Japan), the lignin content in each of the samples was calculated on a FW basis.

To determine the transcriptional levels of lignin biosynthetic genes by reverse transcription (RT)-PCR, total RNAs were isolated from the 5th leaves of the pBIN19- and the *hUGT1*-transgenic tobacco plants cultured in soil using a NucleoSpin RNA Kit (Macherey–Nagel GmbH & Co. KG., Duren, Germany) according to the manufacturer's instructions. Aliquots (1 μg) of the total RNA samples were used as templates for cDNA synthesis with ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) using an oligo-dT 17-mer primer. Six sets of primers, which were specific for five tobacco enzyme genes encoding phenylalanine ammonia-lyase (NtPAL), cinnamate 4-hydorxylase (NtC4H), 4-coumarate:CoA ligase (Nt4CL), cinnamoyl-CoA reductase (NtCCR), and cinnamyl alcohol dehydrogenase (NtCAD) in the lignin biosynthetic pathway (20)

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