Depletion of UDP-D-apiose/UDP-d-xylose Synthases Results in Rhamnogalacturonan-II Deficiency, Cell Wall Thickening, and Cell Death in Higher Plants\textsuperscript{*}\textsuperscript{S}

Joon-Woo Ahn\textsuperscript{1,2}, Rajeev Verma\textsuperscript{3}, Moonil Kim\textsuperscript{1}, Jae-Yong Lee\textsuperscript{1}, Yu-Kyung Kim\textsuperscript{1}, Jae-Wook Bang\textsuperscript{**}, Wolf-Dieter Reiter\textsuperscript{4,5}, and Hyun-Sook Pai\textsuperscript{1,3}

From the \textsuperscript{1}Laboratory of Plant Genomics, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Korea, \textsuperscript{2}Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269-3125, \textsuperscript{3}BioNanotechnology Center, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Korea, \textsuperscript{4}Department of Biology, Yonsei University, Seoul 120-749, Korea, and \textsuperscript{5}Department of Biology, Chongnam National University, Taejon 305-764, Korea

\textbf{D-Apiose serves as the binding site for borate cross-linking of rhamnogalacturonan II (RG-II) in the plant cell wall, and biosynthesis of D-apiose involves UDP-D-apiose/UDP-D-xylose synthase catalyzing the conversion of UDP-D-glucuronate to a mixture of UDP-D-apiose and UDP-D-xylose. In this study we have analyzed the cellular defects of depletion of UDP-D-apiose/UDP-D-xylose synthases in plants by using virus-induced gene silencing (VIGS) of NbAXS1 in Nicotiana benthamiana. The recombinant NbAXS1 protein exhibited UDP-D-apiose/UDP-D-xylose synthase activity in vitro. The NbAXS1 gene was expressed in all major plant organs, and an NbAXS1-green fluorescent protein fusion protein was mostly localized in the cytosol. VIGS of NbAXS1 resulted in growth arrest and leaf yellowing. Microscopic studies of the leaf cells of the NbAXS1 VIGS lines revealed cell death symptoms including cell lysis and disintegration of cellular organelles and compartments. The cell death was accompanied by excessive formation of reactive oxygen species and by induction of various protease genes. Furthermore, abnormal wall structure of the affected cells was evident including excessive cell wall thickening and wall gaps. The mutant cell walls contained significantly reduced levels of D-apiose as well as 2-O-methyl-L-fucose and 2-O-methyl-D-xylose, which serve as markers for the RG-II side chains B and A, respectively. These results suggest that VIGS of NbAXS1 caused a severe deficiency in the major side chains of RG-II and that the growth defect and cell death was likely caused by structural alterations in RG-II due to a D-apiose deficiency.

The primary wall of higher plants consists of a rigid cellulose-xylglucan network that is embedded in and interacts with a pectin network. Rhamnogalacturonan II (RG-II)\textsuperscript{4} is a structurally complex pectic polysaccharide found in primary walls of angiosperms and gymnosperms. RG-II is composed of at least 12 different glycosyl residues linked together by more than 20 different glycosidic linkages, but despite this complexity, the glycosyl-residue composition of RG-II is remarkably conserved among species (1, 2). RG-II exists predominantly as a dimer that is covalently cross-linked by a borate diester. Borate cross-linking of RG-II additionally results in cross-linking of the two homogalacturonan chains upon which the RG-II molecules are constructed (2). These processes of cross-linking are thought to result in a stable three-dimensional pectin network.

Although little is known about how the structural complexity of RG-II contributes to its biological function, evidence suggests that the structural integrity of the RG-II molecule is essential for normal borate cross-link formation. The \textit{mur1} mutant is dwarfed and has brittle stems (3), and these defects are caused by a mutation in \textit{GMD2} encoding a GDP-mannose 4,6-dehydratase, an enzyme required for the biosynthesis of L-fucose (4). RG-II synthesized by the \textit{mur1} mutant completely lacks L-Fuc residues, and only \textasciitilde50% of the RG-II is cross-linked by borate (5). Interestingly, the \textit{mur2} mutant, which is defective in a xyl glucan-specific fucosyltransferase, synthesizes xylglucan that contains little L-Fuc but grows normally (6), indicating that xylglucan fucosylation is not critical for plant growth regulation and wall strength. Taken together, these results suggest that the dwarfed phenotype of \textit{mur1} plants is caused by reduced cross-linking of RG-II due to lack of L-fucose residues. Supporting this, an exogenous supply of borate rescues the growth defect of the \textit{mur1} mutant (5). The \textit{nolac-H18} tobacco callus mutant shows reduced cell adhesion and defective shoot development, which is caused by altered RG-II structure (7). The RG-II of the mutant lacks the \textit{GMD2} encoding a putative glucuronosyltransferase in the mutant (8). RNAi knockdown of \textit{GMD2} expression results in a stable three-dimensional pectin network.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{6} Data Bank with accession number(s) AY391715.

\begin{thebibliography}{3}
\bibitem{1} This research was supported in part by grants from the Plant Diversity Research Center of the 21st Century Frontier Research Program, the Molecular and Cellular BioDiscovery Program, and the Plant Signaling Network Research Center (at Korea University) of the Science Research Center Program (to H.-S. Pai), all of which were funded by the Ministry of Science and Technology of Korea. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
\bibitem{2} The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.
\bibitem{3} The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{6}/EBI Data Bank with accession number(s) AY391715.
\bibitem{4} Present address: Dept. of Chemistry and Biomolecular Sciences, Macquarie University, NSW 2109, Australia.
\bibitem{5} Funded by United States Dept. of Energy Grant DE-FG02-99ER20203. To whom correspondence may be addressed. Tel.: 860-486-5733; Fax: 860-486-4331; E-mail: wdreiter@uconn.edu.
\bibitem{6} To whom correspondence may be addressed. Tel.: 82-2-2123-5653; Fax: 82-2-312-5657; E-mail: hspaill@yonsei.ac.kr.
\end{thebibliography}
Thus, reduced cross-linking of RG-II is likely responsible for the dwarfed phenotype of the *bor1* mutant.

D-Apiose is only present in RG-II in the cell walls of most higher plants, and it serves as an attachment point for two highly complex side chains to the homogalacturonan backbone (10). The two vicinal hydroxyl groups in the furanose ring of D-apiose form a cyclic diester with borate to result in RG-II cross-linking. Biosynthesis of D-apiose has been mostly studied in the duckweed *Lemma minor* and in parsley, where D-apiose is an abundant component of apiogalacturan and apiin, respectively (11, 12). In both duckweed and parsley, highly purified fractions of UDP-D-apiose synthase activity still contained UDP-D-xylose synthase activity, indicating that both reactions are catalyzed by a single enzyme, termed UDP-D-apiose/UDP-D-xylose synthase. Recently, Molhoj et al. (13) cloned the *AXS1* gene encoding this synthase from *Arabidopsis* and demonstrated that the recombinant AXS1 protein indeed has both UDP-D-apiose synthase and UDP-D-xylose synthase activities. The *Arabidopsis* genome has a second gene (*AXS2*) that encodes a protein with 96% sequence identity to *AXS1*, and both genes are expressed in all *Arabidopsis* tissues. These results indicate that *Arabidopsis* has at least two UDP-D-apiose/UDP-D-xylose synthases. Consequently, an *Arabidopsis* line carrying a T-DNA within the *AXS1* gene did not show any visible defects.5

In this study we show that depletion of UDP-D-apiose/UDP-D-xylose synthases results in RG-II deficiency in the cell walls by using VIGS of a *Nicotiana benthamiana* homolog of the *AXS* genes, designated *NbAXS1*. Cell wall abnormalities including incomplete walls and wall thickening were correlated with reduced levels of the RG-II-specific monosaccharides D-apiose, 2-O-methyl-L-fucose, and 2-O-methyl-D-xylose. Thus, the apparent dwarfism and cell death of the *NbAXS1* VIGS plants were likely caused by RG-II deficiency due to a defect in the biosynthesis of UDP-D-apiose.

**EXPERIMENTAL PROCEDURES**

*Viruses-induced Gene Silencing—N. benthamiana* plants were grown in a growth room at 24 °C under a regime of 16 h of light and 8 h of dark. cDNA segments from *NbAXS1* were PCR-amplified and cloned into the pTV000 vector containing a part of the tobacco rattle virus (TRV) genome (14) using BamHI and Apal sites. Virus-induced gene silencing was carried out as described (15). For RT-PCR analyses, the fourth leaf above the infiltrated leaf was used.

**DNA and RNA Gel Blot Analysis**—For DNA gel blot analysis, the genomic DNA isolated from leaves of *N. benthamiana* was digested with EcoRI, HindIII, and EcoRV, electrophoresed on a 0.8% agarose gel, and blotted onto Hybond-N nylon membrane (Amersham Biosciences). Prehybridization and hybridization was carried out as described (16). For RNA gel blot analysis using plant tissues, total RNA was prepared by using TRizol™ reagent (Invitrogen) following the manufacturer’s instructions. Approximately 30 µg of total RNA was electrophoresed on an agarose gel containing 5.1% (w/v) formaldehyde and blotted onto Hybond-N nylon membrane. Prehybridization and hybridization was carried out as described (16). For DNA and RNA gel blot analyses, the probe was the 0.6-kb PCR fragment corresponding to the C-terminal region of the *NbAXS1* cDNA.

**Semiquantitative RT-PCR**—Semiquantitative RT-PCR was carried out using 5 µg of total RNA as described (16). Primers for RT-PCR were made according to published cDNA sequences as described (15).

**Subcellular Localization of the *NbAXS1* Protein**—The *NbAXS1* cDNA corresponding to the entire coding region was cloned into the 326-GFP plasmid (17) using XbaI sites to generate the *NbAXS1*-GFP fusion protein. The fusion constructs were introduced into *Arabidopsis* protoplasts prepared from seedlings by polyethylene glycol-mediated transformation (18). Expression of the fusion constructs was monitored by a confocal laser scanning microscope (Carl Zeiss LSM 510) at 24 h after transformation. The filter sets were BP505–530 (excitation 488 nm, emission 505–530 nm) and LP650 (excitation 488 nm, emission 650 nm) (Carl Zeiss) for green fluorescent protein and autofluorescence of chlorophyll, respectively.

**Western Blot Analysis of *NbAXS1*-GFP**—For *NbAXS1*-GFP fusion constructs were introduced into *Arabidopsis* protoplasts by polyethylene glycol-mediated transformation. At 24 h after transformation proteins were extracted from the protoplasts. As a control, proteins from untreated protoplasts were also prepared. Western blot analysis was carried out as described (16). Thirty µg of proteins were electrophoresed on a 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and probed with a polyclonal antibody raised against green fluorescent protein (GFP) (1:2000 dilution; Clontech). They were then reacted with anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:5000 dilution; Amersham Biosciences) and with ECL reagent (Amersham Biosciences) for detection.

**Measurement of the Mitochondrial Membrane Potential**—Tetramethylrhodamine methyl ester (Molecular Probes) was added to protoplasts isolated from leaves of the VIGS lines at a final concentration of 200 nM. After incubation for 1–2 min at 37 °C, the protoplasts were transferred to wells on microscope slides and examined under a confocal microscope (Carl Zeiss LSM 510) with optical filters (543-nm excitation, 585-nm emission) to visualize the red fluorescent probe. Quantitative images were captured, and the data were analyzed using the LSM 510 program (Version 2.8).

**Measurement of H2O2 Production in Vivo**—Protoplasts isolated from leaves of the VIGS lines were incubated in 2 mM 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular Probes) for 30, 60, 90, 120, and 150 s. Protoplasts were transferred to wells on microscope slides and examined under a confocal microscope (Carl Zeiss LSM 510) with optical filters (488 nm excitation, 505 nm emission) to visualize the oxidized green fluorescent probes.

**Measurement of Ion Leakage and Chlorophyll Contents, Detection of Autofluorescence, and Evans Blue Staining**—After Agrobacterium infiltration, the fourth leaf above the infiltrated leaf from the TRV control and TRV:*NbAXS1* lines was collected and analyzed. Measurement of membrane leakage, detection of autofluorescence, and Evans blue staining were performed as described (15). Total chlorophyll contents were measured as described (19).

**Analysis of Starch Content**—Leaves were harvested from the VIGS lines at 20 days after infiltration and bleached in 80% (v/v) ethanol. After rinsing with double-distilled water, the leaves were stained with Lugol’s iodine staining reagent (Sigma) and briefly destained with water.

**Measurement of Peroxidase and Ascorbate Peroxidase Activity**—Peroxidase and ascorbate peroxidase activities were measured as described (15).

**Electron Microscopy**—The fourth leaf above the infiltrated leaf from the TRV and TRV:*NbAXS1* lines was fixed, processed, and embedded in Spurr’s epoxy resin as described (20). Leaves were sectioned (1-µm thick sections) with an ultramicrotome (model MT-600; Sorvall) and stained in 1% toluidine blue. Ultrathin leaf sections were then generated and placed on Formvar-coated copper grids, where they were stained with lead citrate and uranyl acetate. The sections were observed in a JEOL electron microscope (model 100CX II) at 80 kV, and pictures were taken using Kodak Electron Microscope film no. 4489.

---

5 R. Verma and W.-D. Reiter, unpublished results.
Production of the Recombinant NbAXS1 Protein and Measurement of Its Enzyme Activity—The coding region of the NbAXS1 cDNA was PCR-amplified and cloned into the pET29a vector (Invitrogen) using NdeI and Xhol sites. Conditions for the induction of gene expression and the purification of recombinant NbAXS1 and AXS1 via nickel nitriiotriacetic acid affinity chromatography were as described (13). Assays for UDP-d-apiose/UDP-d-xylose synthase activity were conducted with 3 μg of the purified recombinant protein, and the formation of products was monitored by gas liquid chromatography of alditol acetates as described (13).

Monosaccharide Composition Analysis of the Cell Walls—A polysaccharide fraction containing predominantly cell wall material was prepared from the leaves of the VIGS lines by repeated extractions with 70% (v/v) ethanol as described (3). The polysaccharide was hydrolyzed with sulfuric acid, and the monosaccharide composition was determined as alditol acetates by gas-liquid chromatography as described (3). Because variable amounts of starch were found in the polysaccharide fraction, glucose was omitted from the calculation of the relative amounts of monosaccharide components.

RESULTS

Virus-induced Gene Silencing of NbAXS1 Putatively Encoding UDP-d-apiose/UDP-d-xylose Synthase—Previously, we used TRV-based VIGS in N. benthamiana to assess the functions of various signaling genes and genes whose suppressed expression may be lethal in embryos or seedlings (15, 21, 22). Approximately 15,000 ESTs were sequenced from three cDNA libraries constructed from various tissues of N. benthamiana, and >2,000 selected cDNAs were subjected to VIGS. The screening revealed that gene silencing of a homolog of Arabidopsis AXS genes encoding UDP-d-apiose/UDP-d-xylose synthase (13) severely inhibited plant growth and caused premature leaf yellowing. The screening vector contained the full-length cDNA, which was designated NbAXS1. The NbAXS1 cDNA is 1656 bp in length and encodes a polypeptide of 387 amino acids corresponding to a molecular mass of 43,345 Da.

The amino acid sequence of NbAXS1 was aligned with closely related sequences from Arabidopsis, tomato, potato, and rice (supplemental Fig. 1). NbAXS1 showed an 87–88% amino acid sequence identity to Arabidopsis AXS1 and AXS2 (13) and 94, 93, and 83% sequence identity to homologs of potato, tomato, and rice, respectively.

Genomic Organization and Expression of NbAXS1—DNA gel blot analysis was performed with the genomic DNA from N. benthamiana digested with restriction enzymes (supplemental Fig. 2A). The probe was the 0.6-kb PCR fragment corresponding to the C-terminal region of the NbAXS1 cDNA. EcoRl digestion resulted in four hybridizing bands, whereas HindIII and EcoRV digestion showed three hybridizing bands. These results suggest that the N. benthamiana genome contains at least two NbAXS1-related genes. This result and our previous finding that the Arabidopsis T-DNA knock-out mutant of the AXS1 gene did not show any visible defects indicate that the VIGS phenotype observed in this study is likely to be caused by gene silencing of multiple NbAXS1-homologous genes.

We examined expression of the NbAXS1 mRNAs in different tissues of N. benthamiana plants using RNA gel blot analysis. The ~1.9-kb NbAXS1 transcripts were detected in roots, stems, leaves, and flowers at similar levels (supplemental Fig. 2B).

UDP-d-apiose/UDP-d-xylose Synthase Activity of the Recombinant NbAXS1 Protein—To confirm that NbAXS1 indeed encodes a UDP-d-apiose/UDP-d-xylose synthase in N. benthamiana, the enzymatic activity of the recombinant NbAXS1 protein was examined in comparison with that of AXS1 from Arabidopsis (13). The coding region of the full-length NbAXS1 was cloned into the pET29a expression vector and expressed in Escherichia coli. The recombinant NbAXS1 protein was affinity-purified using the C-terminal histidine tag, yielding a polypeptide ~44 kDa in size, which is consistent with the predicted size of NbAXS1 (data not shown). After incubation of the recombinant NbAXS1 and AXS1 proteins with UDP-d-glucuronate, the reaction products were hydrolyzed, and the resulting monosaccharides were separated and quantified by gas liquid chromatography of alditol acetates. NbAXS1 converted UDP-d-glucuronate to a mixture of UDP-d-apiose and UDP-d-xylose as did AXS1 (Fig. 1A). In contrast, heat-inactivated NbAXS1 did not catalyze the reaction (Fig. 1A). These results demonstrate that NbAXS1 encodes a functional UDP-d-apiose/UDP-d-xylose synthase. A comparison of the kinetic parameters of NbAXS1 and AXS1 from Arabidopsis (13) did not reveal any significant differences between the two enzymes. Similarly, the relative amounts of UDP-d-apiose and UDP-d-xylose produced by NbAXS1 and AXS1 were essentially the same (Fig. 1A).

Subcellular Localization of the NbAXS1-GFP Fusion Protein—Subcellular distribution of the NbAXS1 protein in plant cells was examined by expressing a fusion protein between NbAXS1 and GFP. DNA constructs encoding NbAXS1-GFP or GFP alone under the control of the CaMV35S promoter were introduced into protoplasts isolated from Arabidopsis seedlings. After incubation at 25 °C, expression of the introduced genes was examined under a confocal laser scanning microscope with different filters to capture the image of GFP and autofluorescence of chlorophyll. After 24 h of incubation, the green fluorescent signal of the NbAXS1-GFP fusion protein was mostly localized in the cytosol, similar to the localization of a GFP control (Fig. 1B).

To examine if the NbAXS1-GFP fusion protein is expressed intact in the protoplasts, the fusion protein was detected by Western blotting (Fig. 1C). The NbAXS1-GFP fusion construct or the GFP vector alone were transformed into Arabidopsis protoplasts. As a control, the protoplasts were mock-transformed. After 24 h of incubation, total proteins were prepared and fractionated on SDS-PAGE. Western blot analysis was performed using the anti-GFP polyclonal antibody to detect GFP or NbAXS1-GFP fusion protein. The antibody visualized a ~30-kDa protein band in the GFP-transformed fraction and a ~75-kDa band in the NbAXS1-GFP-transformed fraction but none in the mock-transformed fraction. The size of the protein bands is consistent with the expected size of the GFP and the NbAXS1-GFP proteins, indicating that the green fluorescent signal detected in the NbAXS1-GFP-transformed protoplasts accurately depicts the subcellular localization of NbAXS1-GFP.

VIGS Phenotypes and Suppression of the Endogenous NbAXS1 Transcripts—To confirm gene silencing of NbAXS1, four different fragments of the NbAXS1 cDNA were cloned into the TRV-based VIGS vector pTRV00 (14), and N. benthamiana plants were infiltrated with Agrobacterium containing each plasmid (Fig. 2A). TRV:NbAXS1(N) and TRV:NbAXS1(C) contained a 0.6-kb N- and 0.6-kb C-terminal half of the coding region, respectively, whereas TRV: NbAXS1(F) contained a full-length NbAXS1-coding region. TRV: NbAXS1(T) contained a 90-bp cDNA fragment encoding the C-terminal end of the coding region. VIGS with all four constructs resulted in the same phenotype of growth arrest and senescence-like leaf yellowing (Fig. 2B). Newly emerged leaves were small and yellowish and made a leaf cluster near the shoot apex, and the stem growth was severely inhibited. The effects of gene silencing on the endogenous amounts of the NbAXS1 mRNA were examined by semiquantitative RT-PCR (Fig. 2C). Primers for RT-PCR were designed to exclude the cDNA regions used in the VIGS constructs, and the transcript level for actin was measured as a control. RT-PCR using NbAXS1-N primers (Fig. 2A) that
detect the N-terminal region of the NbAXS1 cDNA produced significantly reduced amounts of PCR products in the VIGS lines of TRV:NbAXS1(C) and TRV:NbAXS1(T) compared with the TRV control, indicating that the endogenous level of the NbAXS1 transcripts was greatly reduced in those plants. The same primers detected high levels of viral genomic transcripts containing the N-terminal region of NbAXS1 in the TRV:NbAXS1(N) and TRV:NbAXS1(F) lines. In contrast, NbAXS1-C primers (Fig. 2A) that recognize the C-terminal region of the cDNA showed suppression of the endogenous NbAXS1 transcripts in the TRV:NbAXS1(N) and TRV:NbAXS1(T) lines, whereas they detected the viral genomic transcripts in the TRV:NbAXS1(C) and TRV:NbAXS1(F) lines. The transcript levels of actin remained constant. These results demonstrate that expression of NbAXS1 was significantly reduced in the VIGS lines.

Cell Wall Defects and Premature Cell Death—We examined cell morphology of the leaves from the TRV:NbAXS1 VIGS lines using light and transmission electron microscopy compared with a TRV control. Transverse leaf sections revealed that the TRV control leaves had the typical leaf structure of dicotyledonous plants with distinct adaxial and abaxial epidermal layers (Fig. 3, A and C). TRV:NbAXS1 lines showed swollen cells filled with dense granule-like structures (Fig. 3, B and D). Strikingly, some cells exhibited wall gaps (marked by the black arrows in Fig. 3, B and D), and a few seemed to be disintegrating with ruptured cell walls (marked by the white arrow in Fig. 3D). Despite these abnormalities, the number of cell layers and the typical dorsoventral organization of the palisade and mesophyll cells were mostly maintained in the TRV:NbAXS1 lines. The incomplete cell walls and cell rupturing observed in TRV:NbAXS1 lines indicate that depletion of NbAXS1 causes abnormal cell wall biogenesis.

Transmission electron microscopic analysis of the leaf cells of the TRV:NbAXS1 lines showed dramatic degeneration of organelles and cellular ultrastructure (Fig. 3F, cf. control in Fig. 3E). The cells contained reduced amounts of cytosol, apparently no or an abnormal nucleus, and reduced numbers of chloroplasts. Some of the chloroplasts were undergoing degradation (marked by the arrows in Fig. 3F). The cells accumulated a large number of electron-dense particles that are likely the remains of disintegrating chloroplasts and other cellular compartments (Fig. 3F).

Accumulation of Cell Death-related Markers and Excessive Reactive Oxygen Species (ROS) Production—The physiology of the cell death phenotype in NbAXS1 VIGS lines was examined. We stained the detached leaves of the VIGS lines with Evans blue, a dye that is excluded...
by the membranes of living cells but diffuses into dead cells. The TRV: NbAXS1 lines exhibited black staining in the leaves (marked by the arrows), indicating localized cell death, whereas a TRV control showed no staining (supplemental Fig. 3A). Plant cells undergoing hypersensitive cell death deposit autofluorescent secondary metabolites. The leaves of the TRV:NbAXS1 lines with symptoms of cell death accumulated substantial amounts of autofluorescent products, whereas the TRV control showed little accumulation of those materials (supplemental Fig. 3A). We also analyzed the changes in the chlorophyll content in the leaves of the TRV:NbAXS1 lines compared with the TRV control (supplemental Fig. 3B). The content of total chlorophyll was significantly reduced (<40% of TRV control) in leaves from three independent TRV:NbAXS1 plants. During programmed cell death, modification of the mitochondrial membrane permeability initiates the death execution pathway (23). Mitochondrial membrane potential of the protoplasts isolated from leaves of TRV control and TRV:NbAXS1 lines was monitored by tetramethylrhodamine methyl ester fluorescence (supplemental Fig. 3C). Tetramethylrhodamine methyl ester is a lipophilic cation that is accumulated in mitochondria in proportion to the mitochondrial membrane potential, and a drop in the membrane potential leads to a decrease in fluorescence (24). The average fluorescence of protoplasts from the TRV:NbAXS1 leaves was only slightly lower than that of the TRV control, indicating that disruption of the mitochondrial membrane potential was not involved in the cell death (supplemental Fig. 3C).

To test whether ROS are produced in the cells undergoing cell death in the VIGS lines, we prepared protoplasts from the leaves of the VIGS lines and incubated them with H$_2$DCFDA to visualize the green fluorescent signal that is indicative of the presence of H$_2$O$_2$ (Fig. 4A). H$_2$DCFDA is a cell-permeable indicator for ROS that is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell (25). The rate of accumulation of fluorescent H$_2$DCFDA in protoplasts from the TRV:NbAXS1 lines was significantly higher than that of the TRV control; the mean fluorescence for continually illuminated protoplasts from the TRV:NbAXS1 lines reached an ~5-fold higher level than that of the TRV control after onset of illumination (Fig. 4A). These results demonstrate that the cell death induced by depletion of NbAXS1 is correlated with excessive ROS formation. Consistent with the high ROS levels, the overall activity of peroxidase and ascorbate peroxidase, the scavenging enzymes for ROS, was about 3.3- and 2.5-fold higher in the TRV:NbAXS1 lines than the TRV control, respectively (Fig. 4B and C). It has been shown that plant cell death is associated with an increase in cellular membrane leakage (26), which can be measured by ion leakage. The TRV:NbAXS1 lines exhibited about 2.2-fold higher levels of ion leakage than leaves from the TRV control (Fig. 4D).

Expression of Defense Genes—We next examined whether the affected cells in the TRV:NbAXS1 lines expressed defense-related
genes by using semiquantitative RT-PCR (Fig. 4E). PR1a, PR1b, PR1c, PR2, PR4, PR5, PR6, 630, HSR203J, HIN1, and SAR8.2a genes are all highly induced during HR cell death (27). NTCP-23 (cysteine protease) and p69d (serine protease) have been shown to be involved in pathogen-induced cell death, whereas the chloroplastic ClpP protease plays a role in chloroplast development but not in senescence or HR cell death (28). Among these genes, PR5, HSR203J, HIN1, ClpP, NTCP-23, and p69d genes were significantly induced in the leaves from the TRV:NbAXS1 lines, whereas expression of other PR genes, such as PR1a, PR1b, PR1c, PR2, PR4, S25-PR6, 630, and SAR8.2, was either slightly induced or not induced at all (Fig. 4E). Thus, progression of the cell death pathway caused by gene silencing of NbAXS1 resulted in expression patterns of defense genes that were different from HR cell death. Taken together, some features of HR cell death appeared to be conserved in the cell death of the NbAXS1 VIGS lines, but its gene expression profile was different from the HR.

FIGURE 4. ROS accumulation and defense gene expression. A, protoplasts isolated from leaves of the TRV control and TRV:NbAXS1 lines were incubated in 2 μM H2DCFDA for the indicated times (0–150 s). H2DCFDA is a ROS indicator that becomes fluorescent when oxidation occurs within the cell. Fluorescence of protoplasts from the TRV control and TRV:NbAXS1 lines was quantified as described under “Experimental Procedures.” Data points represent the means ± S.D. of 11–14 individual protoplasts. PIV, pixel intensity values. B, relative peroxidase activity. C, relative ascorbate peroxidase activity. D, relative ion leakage. E, semiquantitative RT-PCR analysis to examine the transcript levels of defense-related genes. RNA was extracted from the fourth leaf above the infiltrated leaf from several independent N. benthamiana plants infected with TRV or TRV:NbAXS1. As a control for RNA amount, actin mRNA levels were examined.
UDP-\(d\)-apiose/UDP-\(d\)-xylose Synthase and RG-II

**Cell Wall Thickening**—The wall structure of the leaf cells from the VIGS lines was examined by electron microscopy (Fig. 5). Scanning electron microscopy showed the jigsaw puzzle-shaped epidermal pavement cells with visible arrays of cellulose microfibrils in the TRV control (Fig. 5A, left). Compared with the TRV control, the epidermal layers of the TRV:NbAXS1 leaves exhibited thickened cell walls (marked by the arrow), less clear boundaries between cells, and less visible cellulose microfibrils, suggesting altered cell wall structure. Furthermore, the TRV:NbAXS1 leaf cells accumulated high levels of starch during the light period as shown by iodine staining (Fig. 5A, right). The intercalation of iodine molecules into starch yields an intense bluish-black color. This high starch accumulation may be caused by altered starch synthesis or breakdown or by altered carbon export (29).

Transmission electron microscopy revealed that the cells lacked most of their cytosol and organelles due to autolysis (Fig. 5B). Furthermore, the epidermal, palisade, and mesophyll cells of the TRV:NbAXS1 leaves all showed excessive cell-wall thickenings (about 3–4-fold thicker than the TRV control) (Fig. 5, B and C, data not shown). Taken together, these results indicate that depletion of NbAXS1 resulted in abnormal cell wall structure and altered starch metabolism.

**Defective RG-II Assembly**—The major structural features of primary wall RG-II are conserved between higher plant species (1). RG-II contains diagnostic monosaccharides such as \(d\)-apiose, 2-\(O\)-methyl-\(l\)-fucose, 2-\(O\)-methyl-\(d\)-xylose, and \(\alpha\)-apiose. 8, relative abundance of the major neutral monosaccharides in cell wall material except glucose.

**FIGURE 5. Cell wall thickening.** A, scanning electron micrographs (SEM) of the leaf surface (left) and iodine staining of the leaves (right) from TRV and TRV:NbAXS1 lines. The arrows indicate cell walls. Bars = 10 \(\mu\)m. B, transmission electron micrographs of the leaf mesophyll cells from TRV and TRV:NbAXS1 lines. The arrows indicate cell walls. Bars = 4 \(\mu\)m. C, transmission electron micrographs of the outer cell wall of a leaf epidermal cell from TRV and TRV:NbAXS1 lines. Bars = 1 \(\mu\)m.

**DISCUSSION**

In this paper we demonstrated that NbAXS1 encodes a functional UDP-\(d\)-apiose/UDP-\(d\)-xylose synthase that catalyzes the conversion of UDP-\(d\)-glucuronate to a mixture of UDP-\(d\)-apiose and UDP-\(d\)-xylose. VIGS of NbAXS1 resulted in dwarfism and leaf yellowing accompanied by cell death phenotypes with disintegrating cellular structures and excessive ROS accumulation. Furthermore, the affected leaf cells exhibited cell wall thickening, wall gaps, and high accumulation of starch. The cell walls of the NbAXS1 VIGS lines contained severely reduced amounts of \(d\)-apiose, 2-\(O\)-methyl-\(l\)-fucose, and 2-\(O\)-methyl-\(d\)-xylose, indicating RG-II deficiency.
We found that apart from the alterations in starch content and RG-II side chains, the compositions and amounts of wall material did not significantly change in response to the NbAXS1 down-regulation. The lack of changes in the xylose level can be explained by the fact that almost all of the UDP-D-xylose is synthesized by UDP-D-glucuronate decarboxylase in higher plants (13). Thus, the UDP-D-apiose/UDP-D-xylose synthase, a bifunctional enzyme to form a mixture of UDP-D-apiose and UDP-D-xylose, is functionally redundant with UDP-D-glucuronate decarboxylases in regard to the formation of UDP-D-xylose. These results indicate that the cell walls of the NbAXS1 VIGS lines were RG-II-deficient due to a defect in the synthesis of UDP-D-apiose.

D-Apiose is only found in the pectic polysaccharide RG-II in the cell wall of most higher plants. RG-II is a highly complex molecule consisting of four characteristic side chains linked to a homogalacturonan backbone (31, 32). D-Apiose serves as an attachment point for the two highly complex side chains A and B to the backbone and is the binding site for borate in RG-II, which is critical for the formation of cross-links within the cell wall. The borate cross-link between apiose residues is essential for the structural integrity of the plant cell wall, consequently affecting plant growth and viability, as shown by the severe growth disruption in Arabidopsis mur1 (3, 5) and the nolac-H18 mutant of Nicotiana plumbaginifolia (7), which show a reduction in cross-link formation to 53 and 44% of wild-type, respectively. Furthermore, reduced cross-linking of RG-II is accompanied by formation of swollen cell walls in boron-deficient tobacco cell cultures (33). Boron deficiency in pumpkin plants causes growth inhibition, a decrease in borate cross-linking of RG-II, and cell wall thickening (34). An exogenous supply of borate to the boron-deficient pumpkin plants restores all of the defects including wall thickening. Thus, borate ester cross-linking of pectin may affect plant growth by controlling the mechanical properties of the primary wall.

NbAXS1 down-regulation most likely led to reduced cross-link formation between RG-II monomers and defects in RG-II synthesis and stability due to a decrease in D-apiose content. The mur1 RG-II with reduced borate cross-linking dimerizes at a lower rate than wild-type RG-II, and the dimeric form is less stable (5). The phenotypes of cell wall thickening and reduced wall strength, deduced from the observed rupturing of cells in TRV-NbAXS1 plants, are consistent with the phenotypes of other RG-II-defective mutants (3, 5, 7) and boron-deficient plants (33, 34). Because RG-II dimer formation results in the cross-linking of the two homogalacturonan chains on which RG-II molecules are constructed, leading to the formation of a three-dimensional pectic network, the reduced borate cross-linking may cause swelling of the cell walls. Reduced visibility of cellulose microfibrils in the epidermal cells of NbAXS1 VIGS lines (Fig. 5A) was likely caused by the loose, swollen wall structure.

The cell death phenotype in the NbAXS1 VIGS lines seemed to be a secondary effect of the alteration in wall synthesis and/or structure. Recently, the Arabidopsis cev1 mutant, which has reduced cellulose content due to a recessive mutation in the cellulose synthase gene CesA3, was shown to have constitutive expression of stress response genes and enhanced resistance to fungal pathogens (35). Surprisingly, the cev1 plants overproduced jasmonate and ethylene, which were largely responsible for the altered pattern of gene expression in this mutant. Thus, the cev1 plants displayed many of the physiological alterations that characterize wounded cells of the TRV:NbAXS1 line exhibiting strong induction of the genes regulated differently, at least in part, from HR cell death. Interestingly, the cells of the TRV:NbAXS1 line exhibited strong induction of the genes encoding various types of proteases, such as ClpP (chloroplastic protease), p69d (serine protease), and NTCP-23 (cysteine protease), indicating the involvement of these proteases in the cellular disintegration process observed in this study. It has been shown that both cysteine and serine proteases are also highly induced during the autolysis stage of xylemogenesis in Zinnia elegans (36, 37).

Using VIGS, we were able to demonstrate that silencing of UDP-D-apiose/UDP-D-xylose synthase genes severely affected plant growth and induced stress responses and cell death. These phenotypes are likely caused by defects in RG-II synthesis/stability due to depletion of D-apiose required for borate cross-linking. Detailed analysis of the chemical composition and structural modification of the cell wall of the VIGS plants would reveal how the lack of D-apiose causes structural changes in RG-II and other pectic components. Considering the phenotype we observed in this study, complete loss of UDP-D-apiose/UDP-D-xylose synthase function would likely cause embryo or seedling lethality, which might hamper detailed analyses of the phenotypes. For this reason, VIGS might be more suitable for the functional analysis of this type of genes than the conventional gene knock-out technology.

Acknowledgment—We thank Dr. Zee Won Lee (Korea Basic Science Institute, Taejon, Korea) for technical help for confocal microscopy.

REFERENCES
1. Pérez, S., Rodríguez-Carvaial, M. A., and Doco, T. (2003) Biochimie (Paris) 85, 109–121
2. O’Neill, M. A., Ishii, T., Albersheim, P., and Darvill, A. G. (2004) Annu. Rev. Plant Biol. 55, 109–139
3. Reiter, W.-D., Chapple, C. C. S., and Somerville, C. R. (1993) Science 261, 1032–1035
4. Bonin, C. P., Potter, I., Vanzin, G. F., and Reiter, W.-D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2083–2089
5. O’Neill, M. A., Eberhard, S., Albersheim, P., and Darvill, A. G. (2001) Science 294, 846–849
6. Vanzin, G. F., Madson, M., Carpita, N. C., Raikhel, N. V., Keegstra, K., and Reiter, W. D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3340–3345
7. Iwai, H., Masoko, M., Ishii, T., and Satoh, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16319–16324
8. Takano, J., Noguchi, K., Yasumori, M., Kobayashi, M., Gajdov, Z., Miwa, K., Hayashi, H., Yoneyama, T., and Fujitara, T. (2002) Nature 420, 337–340
9. Noguchi, K., Ishii, T., Matsunaga, T., Kagekawa, K., Hayashi, H., and Fujitara, T. (2003) J. Plant Nutr. Soil Sci. 166, 175–178
10. Stevenson, T. T., Darvill, A. G., and Albersheim, P. (1988) Carbohydr. Res. 182, 207–226
11. Golovchenko, V. V., Ovodova, R. G., Shashkov, A. S., and Ovodov, Y. S. (2002) Phytomethody 60, 89–97
12. Hahlbrock, K., Knobloch, K. H., Kreuzaler, F., Potts, J. R., and Wellmann, E. (1976) Eur. J. Biochem. 61, 199–206
13. Molbeig, M., Verma, R., and Reiter, W.-D. (2003) Plant J. 35, 693–703
14. Ratcliff, F., Martin-Hernandez, A. M., and Baulcombe, D. C. (2001) Plant J. 25, 237–245
15. Kim, M., Ahn, J.-W., Jin, O., Paek, K.-H., and Pai, H.-S. (2003) J. Biol. Chem. 278, 19406–19415
16. Lee, S. S., Cho, H. S., Yoon, G. M., Ahn, J.-W., Kim, H. H., and Pai, H.-S. (2003) Plant J. 33, 825–840
17. Lee, Y. J., Kim, D. H., Kim, Y.-W., and Hwang, I. (2001) Plant Cell 13, 2175–2190
UDP-<i>d</i>-apiose/UDP-<i>d</i>-xylose Synthase and RG-II

18. Abel, S., and Theologis, A. (1998) *Methods Mol. Biol.* 82, 209–217
19. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) *Biochim. Biophys. Acta* 975, 384–394
20. Lee, H.-S., Karunanandaa, B., McCubbin, A., Gilroy, S., and Kao, T.-H. (1996) *Plant J.* 9, 613–624
21. Ahn, J.-W., Kim, M., Lim, J. H., Kim, G.-T., and Pai, H.-S. (2004) *Plant J.* 38, 969–981
22. Park, J.-A., Ahn, J.-W., Kim, Y.-K., Kim, S. J., Kim, J.-G., Kim, W. T., and Pai, H.-S. (2005) *Plant J.* 42, 153–163
23. Vaux, D. L., and Korsmeyer S. J. (1999) *Cell* 96, 245–254
24. Zhang, H., Huang, H. M., Carson, R. C., Mahmood, J., Thomas, H. M., and Gibson, G. E. (2001) *Anal. Biochem.* 298, 170–180
25. Bethke, P. C., and Jones, R. L. (2001) *Plant J.* 25, 19–29
26. Pontier, D., Tronchet, M., Rogowsky, P., Lam, E., and Roby, D. (1998) *Mol. Plant-Microbe Interact.* 11, 544–554
27. Heath, M. C. (2000) *Plant Mol. Biol.* 44, 321–324
28. Beers, E. P., Woffenden, B. J., and Zhao, C. (2000) *Plant Mol. Biol.* 44, 399–415
29. Dörmann, P., and Benning, C. (1998) *Plant J.* 13, 641–652
30. Pellerin, P., Doco, T., Vidal, S., Williams, P., Brillouet, J. M., and O’Neill, M. A. (1996) *Carbohydr. Res.* 290, 183–197
31. Kobayashi, M., Matoh, T., and Azuma, J. (1996) *Plant Physiol.* 110, 1017–1020
32. Willats, W. G. T., McCartney, L., Mackie, W., and Knox, J. P. (2001) *Plant Mol. Biol.* 47, 9–27
33. Matoh, T., Takahashi, M., Kobayashi, M., and Takabe, K. (2000) *Plant Cell Physiol.* 41, 363–366
34. Ishii, T., Matsunaga, T., and Hayashi, N. (2001) *Plant Physiol.* 126, 1698–1705
35. Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J. G. (2002) *Plant Cell* 14, 1557–1566
36. Ye, Z. H., and Varner, J. E. (1996) *Plant Mol. Biol.* 30, 1233–1246
37. Demura, T., Tashiro, G., Horiguchi, G., Kishimoto, N., Kubo, M., Matsuoka, N., Minami, A., Nagata-Hiwatashi, M., Nakamura, K., Okamura, Y., Sasa, N., Suzuki, S., Yazaki, J., Kikuchi, S., and Fukuda, H. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 15794–15799