Apiose is a branched monosaccharide that is present in the cell wall pectic polysaccharides hrganogalacturonan II and apiogalacturanon and in numerous plant secondary metabolites. These apiose-containing glycans are synthesized using UDP-apiose as the donor. UDP-apiose (UDP-Api) together with UDP-xyllose is formed from UDP-glucuronic acid (UDP-GlcA) by UDP-Api synthase (UAS). It was hypothesized that the ability to form Api distinguishes vascular plants from the avascular plants and green algae. UAS from several dicotyledonous plants has been characterized; however, it is not known if avascular plants or green algae produce this enzyme. Here we report the identification and functional characterization of UAS homologs from green algae and from a monocot (duckweed). The recombinant UAS homologs all form UDP-Api from UDP-glucuronic acid albeit in different amounts. Apiose was detected in aqueous methanolic extracts of these plants. Apiose was detected in duckweed cell walls but not in the walls of the avascular plants and green algae. Overexpressing duckweed UAS in the moss Physcomitrella patens led to an increase in the amounts of aqueous methanol-acetonitrile-soluble apiose but did not result in discernable amounts of cell wall-associated apiose. Thus, bryophytes and algae likely lack the glycosyltransferase machinery required to synthesize apiose-containing cell wall glycans. Nevertheless, these plants may have the ability to form apiosylated secondary metabolites. Our data are the first to provide evidence that the ability to form apiose existed prior to the appearance of hrganogalacturanon II and apiogalacturanon and provide new insights into the evolution of apiose-containing glycans.

Apiose (3-C-[hydroxymethyl]-D-erythrofuranose; Api) is a branched-chain monosaccharide that is present in many plant secondary metabolites and in the primary cell walls of vascular plants (1). To date, only two cell wall polysaccharides, namely hrganogalacturanon II (RG-II) and apiogalacturanon (ApiGalA), have been shown to contain Api (2). ApiGalAs may have a limited taxonomic distribution as they have only been detected in the cell walls of seagrasses and duckweeds (3–5). By contrast, RG-II is present in the primary walls of all vascular plants examined to date (2, 6). Apiose links two side chains (A and B) to the galacturanon backbone of RG-II. The apiosyl residues of side chain A in two RG-II molecules are cross-linked by a borate diester to form the RG-II dimer (7–10). At least 90% of the RG-II in primary walls exists as a dimer (11), and a reduction in the extent of RG-II cross-linking typically results in the formation of abnormal cell walls (12). Plants carrying mutations that affect Api metabolism as well as RG-II structure and cross-linking are dwarfed or fail to develop normally (13–16).

Early studies of the biosynthesis of the plant flavonoid apin (apigenin::7-(2-O-apiosylglucoside)) in parsley led Grisebach and Döbereiner (17) to propose that UDP-apiose (UDP-Api) and UDP-xylose (UDP-Xyl) are formed from UDP-glucuronic acid (UDP-GlcA) by UDP-apiose synthase (UAS). Subsequent studies identified UDP-Api in parsley and in Lemma (18). It was then proposed that UDP-Api is the activated nucleotide sugar used by apiosyltransferases that catalyze the incorporation of apiose into ApiGalA and into apiin (3, 19). No apiosyltransferase has been purified to homogeneity nor have the genes encoding this glycosyltransferase been identified.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) KX344124, KX344125, KX344126, KX344127, KX344128, KX344129, KX344130, KX344131, and KX344132.

The abbreviations used are: Api, apiose; RG-II, hrganogalacturanon II; ApiGalA, apiogalacturanon; Xyl, xylose; GlcA, glucuronic acid; UAS, UDP-apiose synthase; HILIC, hydrophilic interaction chromatography; ESI, electrospray ionization; MeOH, methanol; ACN, acetonitrile; XIC, extracted ion chromatogram.
UDP-apiose Synthases of Avascular Plants and Green Algae

Results

Identification of UDP-apiose Synthase Homologs in Avascular Plants and Green Algae—UAS-like homologs with >70% amino acid sequence identity to Arabidopsis AXS1/UAS1 were identified in monocots, mosses, liverworts, hornworts, and streptophyte green algae (Table 1) using publically available transcriptomic data from The 1,000 Plants (1KP) Project (22) and the Phytosome genomics portal. No UAS-like homologs were detected in the available transcripome data from Marchantia paleacea (20). The recent availability of the sequenced genome of the hornwort, a hornwort, and green algae produced by the 1,000 Plants (1KP) Project (22) allowed us to re-examine this hypothesis.

Here we report the identification and functional characterization of UAS homologs from the monocot Spirodea polyrhiza (a duckweed), four mosses (P. patens, Dicranum scoparium, Hedwigia ciliata, and Sphagnum lescurii), a liverwort (Marchantia paleacea), a hornwort (Megaceros vincentianus), and two green algae (Mougeotia spp. and Netrium digitus). Our results provide evidence that UDP-Api appeared prior to the appearance of wall-associated apiose and that bryophytes and green algae likely synthesize apiose-containing secondary metabolites but lack the biosynthetic machinery required for the synthesis of apiose-containing wall polysaccharides.

In Microbe Formation of UDP-apiose—The coding sequences of the selected UAS homologs were cloned into a modified pET28b E. coli expression vector (28). The UAS-containing plasmids or empty plasmid (negative control) was then individually transformed into E. coli together with a pCDFDuet plasmid containing the UDP-Glc dehydrogenase coding sequence (29) from Bacillus thuringiensis (BtBDH) to ensure the production of UDP-GlcA. Nucleotide sugar-containing extracts from the isopropyl β-D-thiogalactoside (IPTG)-induced E. coli cells were shown by hydrophilic interaction liquid chromatography with electrospray mass spectrometry (HILIC-ESI-MS/MS) to contain two product peaks eluting at 11.3 and 12.2 min (Fig. 2). These peaks were not detected in the comparable extract of E. coli cells harboring the empty plasmid (Fig. 2). The ESI mass spectra of both components contained an ion at m/z 535.00 (Fig. 2), which corresponds to [M − H]− for a UDP-pentose. MS/MS analysis (Fig. 3) of each product peak gave a fragment ion at m/z 323.00 that is consistent with [UMP − H]−. The peak eluting at 12.2 min has the same elution time and MS fragmentation pattern as authentic UDP-Xyl. Proton NMR (1H NMR) analyses confirmed that the UDP-pentose eluting at 11.3 min was UDP-Api. These data suggest that the UAS-like enzymes do synthesize UDP-Api.

Purified Recombinant UAS from S. polyrhiza, Mosses, a Liverwort, a Hornwort, and Green Algae Convert UDP-GlcA to UDP-Api and UDP-Xyl—To obtain additional evidence that the monocot, avascular plant, and green algal UAs form UDP-Api, the recombinant His6-tagged proteins were solubilized from E. coli cells and purified using nickel affinity columns. Each recombinant UAS gave one major band on SDS-PAGE with a predicted mass of between 45 and 48 kDa (Fig. 4). Each purified UAS was shown by HILIC-ESI-MS/MS to convert UDP-GlcA to two UDP-pentose products. MS/MS analysis

| Organism ID | ID  | Percentage of sequence identity (ID) |
|-------------|-----|-------------------------------------|
| Green algae |     |                                     |
| M. endlicherianum | 75  |                                     |
| R. obtusa | 75  |                                     |
| Mougeotia spp. | 73  |                                     |
| N. digitus | 72  |                                     |
| C. brebissonii M2213 | 72  |                                     |
| C. cushionae | 73  |                                     |
| P. margaritaceum | 78  |                                     |
| M. viride | 61  |                                     |
| Hornworts |     |                                     |
| N. aenigmaticus | 77  |                                     |
| M. vincentianus | 76  |                                     |
| Liverworts |     |                                     |
| T. lacunosa | 83  |                                     |
| M. paleacea | 65, 82 |                                   |
| Mosses |     |                                     |
| N. elongatum | 80  |                                     |
| P. fontana | 80  |                                     |
| D. scoparium | 81  |                                     |
| H. ciliata | 78  |                                     |
| S. lescurii | 79  |                                     |
| S. fallax | 79  |                                     |
| P. patens | 74  |                                     |

* Two partial sequences obtained.
UDP-apiose Synthases of Avascular Plants and Green Algae

FIGURE 1. Unrooted phylogenetic tree of UDP-sugar decarboxylases from plants, bacteria, and human. Amino acid sequences used are human UXS1 chain A (NP_079352.2), Arabidopsis UX53 and UX52 (NP_001078768.1 and NP_191842.1), E. coli ArnA (WP_032205568.1), and R. solanacearum UDP-4-keto-pentose/UDP-xylene synthase (RsuApxs; WP_011002168.1). The UAS-like sequences used are from green algae (Mesostigma viride, Cylindrocystis brebissonii, Mougeotia spp., Cylindrocystis cusculeakea, N. digitus, Raya obtusa, Penium margaritaceum, and Mesotaeniurn endlicherianum), from hornwort (M. vinentianus and N. aenigmaticus), from liverwort (Treubia lacunose), from moss (P. patens, Niphophhium elongatum, H. ciliata, Philonotis fontana, S. lescuri, and Sphagnum fallax), from lycophytes (Selaginella acanthonota, Selaginella moellendorffii, and Selaginella apoda), and from angiosperms (Amborella trichopoda, Oryza sativa, Zea mays, Musa acuminate, S. polyrrhiza, Zostera marina, Arabidopsis thaliana, Populus trichocarpa, Medicago truncatula, and Solanum tuberosum). The alignment was made using Clustal Omega (61–63), and the tree was generated using Dendroscope (64).

(Fig. 5) of these product peaks (11.3 and 12.2 min) also gave a fragment ion at m/z 323.00 that is consistent with [UMP − H]−. Signals consistent with the presence of UDP-Api and UDP-Xyl were detected in all the 1H NMR spectra when the recombinant enzyme assays were performed in deuterated buffer (Fig. 6).

S. polyrrhiza UAS was the most highly expressed protein and was thus selected for further characterization. Real time 1H NMR spectroscopic analysis of the products formed when SpUAS reacts with UDP-GlcA (Fig. 7 and supplemental Table S1) confirmed that UDP-Api is the first product formed. SpUAS produces UDP-Api and UDP-Xyl in a ratio of ∼1.7:1.0, which is similar to potato UAS (30). Our studies with SpUAS also confirm that some of the UDP-Api is converted to the apiofuranosyl-1,2-cyclic phosphate during the in vivo reaction (Fig. 7). No degradation of UDP-Xyl is discernible over the course of the reaction.

Real time NMR-based assays provide the opportunity to detect transient intermediates (30). Our real time 1H NMR data using recombinant SpUAS confirm that UDP-4-keto-xylene is an intermediate formed during the conversion of UDP-GlcA to UDP-Api (Fig. 7). Grisebach and Dobereiner (17) and subsequently Choi et al. (31) proposed that during UAS enzymatic catalysis a ring contraction step occurs through a retroaldol mechanism. We detected no signals indicative of the formation of the proposed enediol intermediate. Nonetheless, if this intermediate is formed it may exist for such a short time or remain secured in the catalytic site of the enzyme and thus be “invisi-
liverworts, and Mougeotia (Table 2). No additional apiose was
detected in the subsequent solvent extracts (Fractions II–IX;
see “Experimental Procedures”) or in the cell walls generated
from these plants (Table 3). No Api was detected in any frac-
tions from Netrium. By contrast, Api was abundant in the
methanolic fraction and cell walls of S. polyrhiza. Such results
are not unexpected as the walls of this duckweed are known to
contain large amounts ApiGalA (3, 33).
Overexpression of SpUAS in P. patens and Detection of
UDP-apiose—We detected small amounts of Api in the metha-
nolic extract of P. patens, but no discernible amounts of Api
were present in its cell wall. Moreover, no UDP-API was
detected in the nucleotide sugar-containing extracts of P. patens
wild-type gametophyte tissue (Fig. 9B) or any of the other
avascular plants and green algae. Thus, we wondered whether
UDP-API is being degraded or rapidly metabolized. We
hypothesized that increasing the level of UDP-API in P. patens
gametophytes would generate a pool of activated Api that was
sufficient to allow this to be incorporated into glycans of the cell
wall. To this end, SpUAS was overexpressed in P. patens. The
SpUAS transcript was detected in five independent transgenic
lines (Fig. 9A). None of the transformed lines had a visibly
altered growth phenotype. The overexpressing lines readily
formed detectable amounts of UDP-API (Fig. 9B). Increased
amounts of Api were also present in the methanolic fractions
from the overexpressing lines (Fig. 9C). None of the lines con-
tained discernible amounts of Api in their cell walls. Thus, we
conclude that UDP-API is not appreciably degraded and is
likely not a limiting factor for the incorporation of Api residues
into walls of P. patens gametophyte.

Discussion
Our study is the first to identify functional genes encoding
UDP-apiose synthase in avascular plants and green algae. The
apiose in these plants was detected in aqueous methanolic
extracts. However, we found no discernible amounts of apiose-
containing glycans in the walls of any of the avascular plants or the algae. Thus, the Api detected in these plants is likely to be associated with a secondary metabolite. This contrasts with vascular plants where apiose is present in the cell wall polysaccharides RG-II and ApiGalA and in secondary metabolites (1, 34–36).

Plants, many animals, fungi, Bacteria, and Archaea produce enzymes (UXS) that convert UDP-GlcA to UDP-Xyl (27, 37–40), whereas UAS forms both UDP-Api and UDP-Xyl in a ratio of 2:1. It is not known whether UXS or UAS is the ancestral gene. To date, no UAS genes have been identified in prokaryotes, whereas UXS is present in many Bacteria and Archaea. Thus, it is likely that UAS first appeared in the plant kingdom, possibly from UXS (40).

The mechanism whereby UAS converts UDP-GlcA into two different UDP-sugars is not known. Plant UXS and UAS and the bacterial ArnA are all enzymes that decarboxylate UDP-GlcA via 4,6-dehydration and a UDP-4-keto-pentose intermediate. X-ray analyses of UXS and ArnA identified domains (supplemental Fig. S1) involved in catalysis and cofactor binding (41, 42). Protein sequence alignment (supplemental Fig. S1) shows that residues implicated in nucleotide sugar binding and catalysis are conserved among UXSs from diverse organisms but are different in the UASs. Additionally, regions of unique amino acid insertions are present in UAS but not in UXS or ArnA (Regions 1–5). These distinct regions may facilitate or necessitate the subsequent ring cleavage and rearrangement. Site-directed mutagenesis of these regions in combination with crystallographic and enzymatic activity studies are required to elucidate the mechanism of UAS activity and determine whether it occurs via an enediol intermediate.

Plants synthesize numerous secondary metabolites. In vascular plants, at least 1,200 of these metabolites have been reported to contain an apiose (1). These apiose-containing compounds include flavonoids, terpenoids, and cyanogenic glucosides (1, 35, 36, 43). Apiosylated secondary metabolites may protect a plant from pathogens and herbivores. Several secondary metabolites, including flavonoids, provide such defense as well as protection against UV radiation and oxidative stress (44–46). These metabolites are normally water-insoluble. The addition of a glycose such as apiose to these compounds would enhance their water solubility and perhaps facilitate their transport within the plant. Interestingly, P. patens has been reported to lack functional borate exporters (47); thus, the possibility cannot be excluded that apiosides sequester borate, which may become toxic if accumulated to high amounts (48). The ability to form apiose has broadened the library of secondary metabolites available to plants, including avascular bryophytes and algae. Mechanisms to incorporate apiose into the cell wall polysaccharides RG-II and ApiGalA must also have develop.
UDP-apiose Synthases of Avascular Plants and Green Algae

FIGURE 5. MS/MS spectra of UDP-API and UDP-Xyl generated by the purified recombinant UAs from green algae and avascular plants. Second stage MS fragmentation data for the peaks at the indicated retention times are shown. Left column, 11.3 min; right column, 12.2 min. From top to bottom are MouqUAS (Moug), NdUAS (Nd), MvUAS (Mv), PpUAS (Pp), HcUAS (Hc), SIUAS (Sl), DsUAS (Ds), MpUAS (Mp), and SpUAS (Sp). The most abundant ion at m/z 323.0 is indicative of UDP-sugar fragmentation into [UMP − H]−. Fragments at m/z 211.0 are consistent with [Ura − 2H]−. AU, arbitrary units.

FIGURE 6. Selected regions of 1H NMR spectra diagnostic for the products and intermediates generated by the purified recombinant UAs from green algae and avascular plants. The anomeric region between 5.50 and 5.75 ppm for the H1 protons of UDP-GlcA (G) reactant, UDP-API (A), and UDP-Xyl (X) products and UDP-4-keto-Xyl (K) intermediate are shown (magnified by 3× for clarity). The NMR region (5.95 and 6.08 ppm) diagnostic for UDP, NAD+ cofactor, and apiofuranosyl-1,2-cyclic phosphate (Ac) degradation product is included. NMR spectral traces from top to bottom show UAS activity of MouqUAS (Moug), NdUAS (Nd), MvUAS (Mv), PpUAS (Pp), HcUAS (Hc), SIUAS (Sl), DsUAS (Ds), MpUAS (Mp), SpUAS (Sp), and empty vector control. Peaks labeled N correspond to NAD+ H5 and H6. For additional chemical shift assignments, see supplemental Table S1.

UDP-apiose Synthases of Avascular Plants and Green Algae

Determined whether the *P. patens* apiosyltransferases that utilize UDP-API as a donor have common motifs with the apiosyltransferases involved in secondary metabolite and pectin biosynthesis in vascular plants will provide insight into the evolutionary origins of Api-containing glycans.

The identification and functional characterization of UAs from green algae and bryophytes provide a valuable tool to study the role of apiose and apiosylated metabolites in these organisms. The use of comparative genomics and transcript analyses will reveal glycosyltransferases responsible for addition of apiose to secondary metabolites and to cell wall structures.

Experimental Procedures

Plant Material and Growth Conditions—Living cultures of the mosses *Dicranum*, *Sphagnum*, and *Polytrichum*; the liverworts *Marchantia* and *Conocephalum*; and the algae *Mougeotia* and *Netrium* were obtained from Carolina Biological (Burlington, NC), harvested, and kept at −80 °C. *P. patens* (var. **OCTOBER 7, 2016 • VOLUME 291 • NUMBER 41** JOURNAL OF BIOLOGICAL CHEMISTRY 21439
Gransden) was maintained in liquid routine basal medium (1 liter of BCDAT medium contains 10 ml each of medium B (0.1 mM MgSO₄), medium C (1.84 mM KH₂PO₄, pH 6.5) + medium D (1 mM KNO₃, 4.5 mM FeSO₄) + AT (0.5 mM ammonium tartrate) with 1 ml of trace elements (0.22 mM CuSO₄, 10 mM H₃BO₃, 0.23 mM CoCl₂, 0.1 mM Na₂MoO₄, 0.19 mM ZnSO₄, 2 mM MnCl₂, 0.17 mM KI) and 1 ml of 1 M CaCl₂; distilled deionized H₂O was added to 1 liter). Protonema were grown at 22 °C on cellophane-covered BCDAT agar plates (53) in a controlled environment growth chamber (Conviron, Manitoba, Canada).

**FIGURE 7. Proposed reaction mechanism for UAS and real time ¹H NMR analysis of recombinant SpUAS activity.** Structures for reactant, products, detectable UDP-4-keto-Xyl intermediate (solid brackets), undetected UDP-enediol intermediate (dashed brackets), and degradation product are shown. The reaction was carried out at 37 °C. The selected anemic region (between 5.3 and 6.1 ppm) for protons of reactant, intermediate, and products is shown. Only select time-resolved spectra are displayed to prevent overcrowding of peaks.
**UDP-apiose Synthases of Avascular Plants and Green Algae**

**FIGURE 8. Characteristics of recombinant SpUAS.** A, the effect of temperature on the relative activity of purified recombinant SpUAS. B, the effect of pH buffers on the relative activity of purified recombinant SpUAS. C, size exclusion chromatography of recombinant SpUAS suggests that the active enzyme exists in solution as a dimer. Purified recombinant SpUAS was fractionated on a Superdex-75 gel filtration column, and fractions were collected (every 30 s) and assayed for activity. The relative activity (indicated by closed diamonds) was determined by HPLC. The molecular weight of the enzyme in solution is based on the interpolation from the relative elution times of standard protein markers (indicated by open circles). Error bars represent S.E. of 3 replicates.

**TABLE 2**

**Glycosyl residue compositions of the aqueous acetonitrile-methanol-soluble material from green algae, avascular plants, and S. polyrhiza**

The glycosyl residue composition (mol %) was determined by GC-MS analysis of the alditol-acetate derivatives generated from the aqueous acetonitrile-MeOH-soluble fractions from *Conocephalum* (Cono), *Dicranum* (Dicran), *Marchantia* (March), *Mougeotia* (Moug), *Netrium* (Net), *P. patens* (Physco), *Polytrichum* (Poly), *Sphagnum* (Sphag), and *S. polyrhiza* (Spiro). Data are the average ± S.E. of three independent samples. ND indicates not detected. Fuc, fucose; Rha, rhamnose; Ara, arabinose.

| Monosaccharide | Moug. | Net | March | Cono | Poly | Physco | Dicran | Sphag | Spiro |
|----------------|------|-----|-------|------|------|--------|--------|-------|-------|
| Rha            | 0.3±0.1 | 0.9±0.1 | 0.8±0.1 | 0.1±0.1 | 0.1±0.1 | 0.3±0.3 | 0.6±0.1 | 0.3±0.1 | 0.1±0.1 |
| Fuc            | 5.2±0.1 | 12.7±2.1 | 2.8±0.1 | 0.2±0.2 | 0.4±0.1 | 2.0±0.2 | 1.7±0.8 | 1.7±0.1 | 3.7±0.3 |
| Rib            | 0.7±0.1 | 4.7±1.0 | 1.4±0.1 | 0.2±0.3 | 1.2±0.1 | 1.9±0.2 | 1.9±0.1 | 1.9±0.1 | 3.6±0.1 |
| Ara            | 5.8±0.3 | 10.4±0.6 | 8.8±0.1 | 3.5±0.7 | 2.6±0.1 | 11.4±1.1 | 6.0±0.2 | 11.1±1.0 | 9.8±0.2 |
| Xyl            | 3.1±0.1 | 10.3±1.7 | 2.9±0.3 | 1.5±0.5 | 0.5±0.1 | 3.9±0.3 | 5.9±1.0 | 4.2±0.7 | 3.9±0.2 |
| Api            | 0.8±0.1 | ND | 1.1±0.1 | 0.5±0.2 | 0.3±0.1 | 0.8±0.1 | 0.9±0.1 | 1.9±0.3 | 3.1±0.1 |
| Man            | 36.7±0.1 | 15.8±2.4 | 41.4±0.1 | 41.3±12.5 | 53.1±0.2 | 4.9±0.6 | 56.1±0.5 | 58.6±1.2 | 44.0±0.5 |
| Gal            | 30.5±0.1 | 39.3±2.4 | 16.9±0.1 | 4.0±0.9 | 4.7±0.1 | 54.3±6.9 | 5.4±0.6 | 5.0±0.2 | 6.0±0.2 |
| G1c            | 16.8±0.1 | 6.0±0.7 | 24.0±0.1 | 47.±9.7 | 37.2±0.2 | 20.5±9.3 | 22.5±1.2 | 15.3±1.6 | 26.0±0.4 |

with a 16/8-h photoperiod. *S. polyrhiza* was obtained from Joachim Messing (Waksman Institute of Microbiology, Rutgers University) and maintained on 0.8% agar (w/v) containing Schenk and Hildebrandt basal salts (1.6 g/liter) and 0.5% (w/v) sucrose, pH 5.8, in a Conviron growth chamber with a 14/10-h photoperiod at 19/15 °C. Between six and eight fronds of *S. polyrhiza* were transferred to sterile liquid medium (50 ml) containing 1% (w/v) sucrose in 250-ml Erlenmeyer flasks and grown under a 14/10-h photoperiod at 24/20 °C.

**Identification and Cloning of DsUAS, HcUAS, MougUAS, MpUAS, MvUAS, NdUAS, PpUAS, PtUAS, SIA1, and SpUAS**—The moss, liverwort, and hornwort transcriptomes in the 1,000 Plants (1KP) Project and annotated monocot proteins in the Phytozome genomic portal were probed for homologs to the amino acid sequence of *Arabidopsis* AXS1/UAS1 by BLAST analyses. Analysis of the top hits revealed proteins with high sequence identity to *Arabidopsis* AXS1/UAS1, including several green algae and moss proteins with >70% amino acid sequence identity, liverwort proteins from *Treubia lacunosa* (83% identity) and *M. paleacea* (two incomplete; 65 and 82% identity), and hornwort proteins from *Nothoceros aenigmaticus* and *M. vincentianus* with 77 and 76% identity, respectively. The top hit for *S. polyrhiza* (locus SpipoG0011110; annotated as “bifunctional polymyxin resistance ArmA protein”) has an amino acid sequence identity of 85% to *Arabidopsis* AXS1/UAS1. The nucleotide sequence cor-
**TABLE 3**

**Glycosyl residue compositions of the cell walls isolated from selected green algae, avascular plants, and S. polyrhiza**

The glycosyl residue composition (mol %) was determined by GC-MS analysis of alditol-acetate derivatives generated from the cell walls of each plant. Data are the average ± S.E. of three independent samples. ND indicates not detected. Fuc, fucose; Rha, rhamnose; Ara, arabinose; Cono, Conocephalum; Dicran, Dicranum; March, Marchantia; Moug, Mougeotia; Net, Netrium; Physco, P. patens; Poly, Polytrichum; Sphag, Sphagnum; Spiro, S. polyrhiza.

| Glycosyl residue composition (mol %) | Moug | Net | March | Cono | Poly | Physco | Dicran | Sphag | Spiro |
|-------------------------------------|------|-----|-------|------|------|--------|--------|-------|-------|
| Rha                                 | 0.3 ± 0.1 | 0.4 ± 0.1 | 1.0 ± 0.1 | 0.3 ± 0.1 | 1.2 ± 0.1 | 1.2 ± 0.1 | 1.5 ± 0.1 | 1.9 ± 0.1 | 0.9 ± 0.1 |
| Fuc                                 | 19.5 ± 3.1 | 21.4 ± 0.3 | 5.4 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 | 2.0 ± 0.1 | 4.1 ± 0.1 | 4.7 ± 0.1 | 2.8 ± 0.1 |
| Rib                                 | 0.3 ± 0.1 | 1.5 ± 0.1 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.1 | 2.1 ± 0.2 | 0.5 ± 0.1 | 0.5 ± 0.1 | 2.8 ± 0.1 |
| Ara                                 | 11.7 ± 1.8 | 9.5 ± 0.3 | 24.9 ± 0.1 | 20.8 ± 0.9 | 6.7 ± 0.1 | 15.0 ± 0.7 | 11.7 ± 0.2 | 10.7 ± 0.1 | 14.8 ± 0.2 |
| Xyl                                 | 32.9 ± 4.1 | 10.1 ± 0.6 | 11.2 ± 0.1 | 12.2 ± 0.3 | 8.2 ± 0.1 | 15.1 ± 1.0 | 12.0 ± 0.3 | 11.3 ± 0.1 | 15.8 ± 0.8 |
| Api                                 | 0.3 ± 0.1 | 0.8 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 |
| Man                                 | 1.3 ± 0.2 | 1.5 ± 0.1 | 12.3 ± 0.1 | 21.5 ± 0.6 | 58.3 ± 0.1 | 80.0 ± 0.5 | 30.5 ± 2.1 | 30.4 ± 0.1 | 2.0 ± 0.1 |
| Gal                                 | 28.0 ± 10.2 | 23.8 ± 0.7 | 42.7 ± 0.1 | 40.0 ± 1.1 | 20.6 ± 0.1 | 48.5 ± 3.5 | 35.7 ± 2.9 | 36.3 ± 0.1 | 31.4 ± 0.6 |
| Glc                                 | 6.0 ± 0.9 | 31.5 ± 0.3 | 2.2 ± 0.1 | 3.9 ± 0.1 | 3.8 ± 0.1 | 8.2 ± 3.3 | 4.1 ± 0.1 | 4.2 ± 0.1 | 17.9 ± 0.5 |

**FIGURE 9. Overexpression of SpUAS in P. patens resulting in increased amounts of UDP-Api but not the formation of apiose-containing cell wall polysaccharides.** A, transcript analysis of SpUAS. RNA was extracted, and cDNAs were generated from wild-type P. patens (WT), five lines transformed to overexpress SpUAS (SpUAS OE 1–5), and S. polyrhiza. SpUAS and Physcomitrella EF1α were amplified by PCR and run on 1% (w/v) agarose. B, LC-MS analysis of ACN/MeOH/H2O (40:40:20, v/v/v) extracts from WT P. patens, SpUAS OE 1, SpUAS OE 5, and S. polyrhiza. XICs for m/z 535.0, diagnostic for UDP-pentose, are shown. The standard (Std) contains UDP-Xyl and UDP-arabinopyranose (UDP-Arap). UDP-Api is detected in S. polyrhiza and in the P. patens SpUAS-overexpressing lines. In all but the standard (Std) trace, the m/z 535.0 signal has been amplified by a factor of 100. C, GC-MS analysis of alditol-acetate derivatives from ACN/MeOH/H2O (40:40:20, v/v/v) extracts. The region of the total ion count for xylitol (Xyl) and apiitol (Api) is expanded, and that for cell wall fractions is below.

responding to the *S. polyrhiza* protein was used for primer design and cloning.

*S. polyrhiza* RNA was isolated from 10-day-old fronds. Fronds were collected, vacuum-filtered over nylon mesh, rinsed with deionized water, blotted dry, and then ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted using a Qiagen RNeasy Mini kit with an on-the-column DNase treatment to eliminate genomic DNA contamination. RNA (0.5 μg) was then reverse transcribed with an oligo(dT) primer using SuperScript III reverse transcriptase (Life Technologies). A portion of the reverse transcription (RT) reaction (2 μl), dNTPs, and 1 unit of Phusion® high fidelity DNA polymerase (New England Biolabs, Ipswich, MA) with a 0.2 mM concentration of each forward and reverse primer (Integrated DNA Technologies, Coralville, IA; supplemental Table S2) were used to amplify the SpUAS with the following thermal cycler conditions: one 98 °C denaturation cycle for 30 s followed by 25 cycles (each of 8-s denaturation at 98 °C, 25-s
annealing at 60 °C, and 30-s elongation at 72 °C) and finally termination at 4 °C. The PCR product was directly cloned into the E. coli expression vector pET28b (Novagen, Darmstadt, Germany) modified to contain an N-terminal His6 tag followed by a TEV cleavage site (28).

RNA was extracted from wild-type and transformed P. patens using 100 mg of 2-week-old gametophyte tissue. Tissue was harvested and immediately frozen in liquid nitrogen, and RNA was extracted and reverse transcribed with oligo(dT). No PpUAS transcript was detected on a 1% (w/v) agarose gel even after a second round of PCR amplification using a 0.2 μM concentration of each forward and reverse primer (supplemental Table S2) and an annealing temperature of 56 °C. The recently released P. patens Electronic Fluorescent Pictograph browser (54, 55) indicates that PpUAS (gene ID Pp1s379_19V6.1) is only released in P. patens archegonia. Thus, a synthetic ORF gene corresponding to an empty pET28b vector control were grown in LB medium (1.0% NaCl, 0.5% glucose, 0.5% tryptone, 0.5% yeast extract, 20 μg/ml kanamycin). After 7 days, the cells were centrifuged, and the cell pellets were suspended in 5 ml of liquid protoplast regeneration medium containing ampicillin (100 μg/ml) and 150 mM NaCl, transferred to Eppendorf tubes, centrifuged, and the protoplasts were suspended in 8.5% mannitol for 60 min at room temperature with 2% (w/v) Driselase (Sigma-Aldrich) and shaken at 60 rpm. The suspension was filtered through 40-μm nylon mesh, and the released protoplasts were suspended in 8.5% (w/v) mannitol. The suspension was centrifuged (250 × g, 5 min, 22 °C), the supernatant was discarded, and the pellet was resuspended in 8.5% mannitol. The filtration and resuspension were repeated two times. The number of protoplasts obtained was then determined using a hemocytometer. Protoplasts were centrifuged, and the protoplasts were suspended to a density of ~2 × 10^7 protoplasts/ml in 0.5 mM mannitol containing 15 mM MgCl_2 and 0.1% (w/v) MES, pH 5.6 (53). 0.3 ml of the protoplast suspension and 0.3 ml of PEG 8000 (Sigma-Aldrich) were added to 15 μl of BsaAI-linearized plasmid DNA, thoroughly mixed, and then kept at room temperature for 10 min. The mixture was heat-shocked for 3 min at 45 °C, immediately cooled to room temperature in a water bath, and kept for 10 min. The suspension was then centrifuged, the supernatant was discarded, and the protoplasts were suspended in 5 ml of liquid protoplast regeneration medium (=BCDAT + 8% (w/v) mannitol and 10 mM CaCl_2). A portion of this protoplast suspension (1.6 ml) was spread on cellophane layer over protoplast regeneration medium bottom layer (=BCDAT + 6% (w/v) mannitol, 10 mM CaCl_2, and 1% (w/v) agar) (53). The plates were kept for 5 days in a growth chamber at 22 °C.

The cellophane was then transferred onto BCDAT medium containing ampicillin (100 μg/ml) and kept for a further 7 days. The cellophane was then transferred onto BCDAT medium lacking antibiotic. After 7 days, the cellophane was transferred to BCDAT medium with ampicillin and kept for an additional 7 days to obtain stable transformants. Transformants were verified by PCR of locus 108 using the appropriate forward and reverse primers (supplemental Table S2) and transcript analysis.

In Microbe Assays—NDP-sugars from E. coli harboring the expression plasmids were harvested as described (57). BL21-derived E. coli cells (3 ml) were transferred with pCDFDuet-2BtBDH and either pET28b-TEV-DsUAS.2, pET28b-TEV-HcUAS.3, pET28b-TEV-MougaU.1, pET28b-TEV-MpUAS2.1, pET28b-TEV-MvUAS.6, pET28b-TEV-NdUAS.2, pET28b-TEV-PpUAS.1, pET28b-TEV-SiuA.1, and pET28b-TEV-SpUAS.1. Their amino acid sequences were deposited in GenBank under accession numbers KX344124, KX344125, KX344126, KX344127, KX344128, KX344129, KX344130, KX344131, and KX344132.

Transformation of P. patens—The ORF SpUAS was amplified by PCR using forward and reverse primers (supplemental Table S2), directly cloned into pENTR™/SD/D-TOPO®, and Gateway® cloned into pTHubiGate, a P. patens expression vector that has homologous recombination sites at P. patens locus 108 (56) with LR Clonase II (Life Technologies). The expression of SpUAS in pTHubiGate is driven by a ubiquitin promoter. For plant transformation, the binary plasmid pTHubiGate-SpUAS (50 μg) was linearized with BsaAI (New England Biolabs) and then precipitated by the addition of ethanol. The precipitate was dissolved in sterile water (1 μg/μl) and then used to transform P. patens protoplasts (53).
analysis or deuterium oxide (D₂O) for ¹H NMR analysis. 10 volumes of cold chloroform/methanol (1:1, v/v) or chloroform/d/methanol-d₄ (Cambridge Isotopes Laboratories, Tewksbury, MA) were added, and the samples were mixed for 20 min at 4 °C. The suspensions were centrifuged (18,000 × g, 5 min, 22 °C), and the upper aqueous phases were collected and recenterfuged. Portions of the aqueous phase were analyzed by HILIC-ESI-MS/MS and by ¹H NMR spectroscopy.

Expression and Purification—BL21-derived E. coli cells were transformed with pET28b-TEV-DsUAS.2, pET28b-TEV-HcUAS.3, pET28b-TEV-MougUAS.1, pET28b-TEV-MpUAS2.1, pET28b-TEV-MvUAS.6, pET28b-TEV-NdUAS.2, pET28b-TEV-PpUAS.1, pET28b-TEV-SI1UAS.1, pET28b-TEV-SpUAS.1, or the empty vector control. The cells were grown at 37 °C and 250 rpm for 16 h in LB medium (20 ml) containing kanamycin (50 μg/ml) and chloramphenicol (35 μg/ml). A portion (5 ml) of the culture was transferred to fresh LB plus antibiotics (245 ml) and grown under the same conditions until its A₆₀₀ nm was 0.8. IPTG was added to 0.5 mM to induce expression of UAS, and the culture then grown for an additional 4 h at 30 °C and 250 rpm. The induced cultures were cooled on ice and centri- fuged (6,000 × g, 10 min, 4 °C). The cell pellet was suspended in lysis buffer (50 mM Tris-HCl, pH 7.6, 10% (v/v) glycerol, 1 mM EDTA, 5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride; 10 ml). The cells were ruptured by sonication, and proteins were then isolated after centrifugation as described (28). The final soluble protein fraction (Fraction S20) was col- lected and kept on ice prior to purification.

The His₆-tagged proteins were purified using fast flow nick- el-Sepharose (GE Healthcare; 2 ml of resin packed in a 15 × 1-cm polypropylene column). Columns were washed and equilibrated with 50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl, and then Fraction S20 was added. Bound His₆-tagged proteins were eluted with the same buffer containing increasing concentrations of imidazole (10–250 mM). The active enzymes were eluted in 250 mM imidazole and then dialyzed (6,000–8,000 molecular weight cutoff; Spectrum Laborato- ries, Inc.) at 4 °C three times for a total of 2 h against 50 mM Tris-HCl, pH 7.6, containing 0.15 M NaCl, 10% (v/v) glycerol, 1 mM DTT, and 10 μM NAD⁺. The dialysates were divided into 150-μl aliquots, flash frozen in liquid nitrogen, and stored at −80 °C. Aliquots of purified protein were assayed for activity and run on SDS-PAGE.

SDS-PAGE was performed with 12% (w/w) polyacrylamide gels. Proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in aqueous 20% methanol (MeOH) containing 7% (v/v) acetic acid and destained with aqueous 20% methanol containing 7% (v/v) acetic acid. Protein concentrations were determined with the Bradford reagent (58) using bovine serum albumin (BSA) as the standard. The molecular mass of active recombinant SpUAS was estimated by size exclusion chromato- graphy. Purified, recombinant SpUAS was eluted with dialysis buffer as eluent over a Superdex-75 100/300 GL column (23) that had been calibrated with proteins of known molecular mass (Bio-Rad).

Recombinant UAS Enzyme Assays—Unless otherwise indi- cated, reactions were performed in 50 mM Tris-HCl, pH 7.9 (50 μl), containing 1 mM NAD⁺, 1 mM UDP-GlcA, and 10 μg of purified protein. The mixtures were kept at 37 °C for up to 45 min, and the reactions were terminated by placing the tubes in boiling water for 2 min followed by the addition of an equal volume of chloroform. The suspensions were vortexed and cen- trifuged (12,000 × g, 5 min, 22 °C), and the aqueous phase was analyzed for nucleotide sugars. ¹H NMR assays (180 μl) were performed in D₂O using 30 μg of purified protein.

Characterization of Recombinant SpUAS—SpUAS activity was assayed in different buffers, at different temperatures, and with various additives and nucleotide sugars. For pH studies, purified recombinant SpUAS (10 μg) was added to standard reactions (50 μl) containing various pH buffers (100 mM), 1 mM NAD⁺, and 1 mM UDP-GlcA and kept at 37 °C for 30 min. Inhibition assays were performed by first supplementing the standard reaction mixtures with various nucleotides and nucle- otide sugars, addition of purified protein, and incubation. The amounts of reactants and products were determined by UV spectroscopy and used to calculate enzyme activity as follows. The products from each recombinant enzyme assay were chromo- matographed over a Q-15 anion exchange column (200 × 1 mm; Amersham Biosciences) by elution with a linear gradient (5 mM to 0.6 M) of ammonium formate over 25 min at a flow rate of 0.25 ml/min using an Agilent (Santa Clara, CA) 1100 Series HPLC equipped with a G1313A autosampler, a G1315B diode array detector, and ChemStation software. Nucleotides and nucleotide sugars were detected by their A₂₆₁ nm (for UDP-sugars) and A₂₅₉ nm (for NAD⁺). The concentrations of reagents and products were determined by comparison of their peak areas with a calibration curve of standard UDP-GlcA (23).

Selected kinetic parameters of recombinant SpUAS (10 μg) were determined by varying the concentrations of UDP-GlcA in 50-μl reactions consisting of 1 mM NAD⁺ in 50 mM Tris- HCl, pH 7.9. Reactions were kept for 7 min at 37 °C, quenched with an equal volume of chloroform, and then vortex mixed. The reaction products in the aqueous phase were separated using a Q-15 anion exchange column as described above, and reaction rates were calculated from the depletion of the UDP-GlcA signal integral normalized to the NAD⁺ signal integral. Values from three independent replicates were used to generate a non-linear regression plot and resultant data using GraphPad Prism Version 6.04.

HILIC-ESI-MS/MS—ESI-MS/MS analysis was performed on a Shimadzu (Kyoto, Japan) LC-MS-IT-TOF operating in the negative ion mode. Plant nucleotide sugar extracts and in microbe and recombinant enzyme assay products were mixed with 5% volume aqueous 95% ACN containing 25 mM ammonium acetate, and an aliquot (10–20 μl) was chromatographed over an Accucore amide-HILIC column (150 × 4.6 mm; Thermo Fisher Scientific, Waltham, MA) eluted at 0.4 ml min⁻¹ with a linear gradient of aqueous 75% (v/v) acetonitrile containing 40 mM ammonium acetate, pH 4.4, to 50% (v/v) acetonitrile containing 40 mM ammonium acetate, pH 4.4, over 35 min using a Shimadzu LC-30AD HPLC. Mass spectra (mass range, 100–2,000 m/z) were collected every 1.3 s for 30 min. Second stage MS/MS data were collected by collision-induced dissociation with a collision energy of 35% and a nebulizing helium gas flow of 1.5 ml min⁻¹ (59).

UDP-apiose Synthases of Avascular Plants and Green Algae

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21444

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**Nucleotide Sugar Extraction of Plant Tissue**—Fresh tissues (100 mg wet weight) were ground in liquid nitrogen using a mortar and pestle and transferred to an Eppendorf tube, and ACN/MeOH/H$_2$O (40:40:20, v/v/v; 1 ml) was then added. The tubes were vortexed and rotated for 20 min at 4 °C. The tubes were centrifuged (18,000 × g, 5 min, 22 °C), and the supernatant was transferred to a clean tube. The solutions were concentrated to ~50% of their initial volume using a stream of nitrogen gas, and portions (800 μl) were analyzed by HILIC-ESI-MS/MS as stated above.

**Real Time $^1$H NMR Enzyme Assays**—All spectra were obtained using a Varian Inova 600-MHz spectrometer equipped with a 3-mm cryogenic probe. Continuous $^1$H NMR spectroscopic monitoring of reactions (180-μl volume) was carried out at 37 °C in a mixture of D$_2$O/H$_2$O (9:1, v/v) containing 0.83 mM 2,2-dimethyl-2-silapentane-5-sulfonate (internal reference), 50 mM Tris-HCl, pH 7.9, 1 mM UDP-GlcA, 1 mM NAD$^+$, and purified recombinant enzyme (30 μg). One-dimensional $^1$H NMR spectra with the water resonance signal referenced to 2,2-dimethyl-2-silapentane-5-sulfonate at 0.00 ppm (30).

**Fractionation and Cell Wall Polysaccharide Extraction**—Fresh plant tissue (0.1–1 g) was suspended in cold ACN/MeOH/H$_2$O (40:40:20, v/v/v; 10 volumes) and ground in a mortar and pestle on ice. The suspension was transferred to a 15-ml Falcon tube and kept for 30 min at 55 °C. The suspension was centrifuged (3,000 × g, 5 min, 22 °C), and the supernatant (Fraction I; “methanolic extract”) was saved. Aqueous 50% (v/v) EtOH (10 volumes) was then added to the pellet, and the suspension was vortexed and kept for 30 min at 55 °C. The suspension was centrifuged, and the supernatant (Fraction II) was saved. The procedure was repeated using aqueous 80 and 95% (v/v) EtOH to give soluble Fractions III and IV. The pellet was then suspended in water (5 volumes) and kept for 30 min 55 °C. An equal volume of ethyl acetate was added, and the suspension was vortexed and kept for 30 min at 55 °C. The suspension was centrifuged, and the top layer (Fraction V) was saved. The lower aqueous layer (Fraction VI) was transferred to a clean borosilicate tube. The pellet was suspended in CHCl$_3$/MeOH (1:1, v/v; 5 volumes) and vortexed. After centrifugation, the top aqueous layer (Fraction VII) was saved. The bottom organic layer (Fraction VIII) was also saved. The pellet was suspended in acetone (5 volumes), and after centrifugation the supernatant (Fraction IX) was saved. The final pellet, referred to as cell wall, was allowed to air-dry overnight in a fume hood.

**Glycosyl Residue Composition Analysis**—The aqueous and organic solvent extracts or cell wall (~1 mg) was supplemented with myo-inositol (10 μl of a 5 mM solution) as an internal standard, evaporated to dryness at room temperature using a stream of filtered air (REACTIVAP III, Thermo Fisher), and then hydrolyzed for 2 h at 120 °C with 2 M TFA (1 ml). TFA was removed by evaporation under a stream of filtered air (40 °C), and the residue was washed with isopropanol (3 × 500 μl). The released monosaccharides were then converted into their corresponding alditol-acetate derivatives according to York et al. (60), and the final residue was dissolved in acetone (100 μl).

The alditol-acetate derivatives were analyzed by gas-liquid chromatography (GLC; Agilent 7890A) equipped with a mass selective detector (EI-MS; Agilent 5975C). The sample (1 μl) was injected in the splitless mode using an Agilent 7693 autosampler onto a Restek RTx-2330 fused silica column (0.25-mm inner diameter × 30 m, 0.2-μm film thickness) with helium as carrier gas at a flow rate of 1.1 ml min$^{-1}$. The oven temperature was held at 80 °C for 2 min followed by an increase of 30 °C min$^{-1}$ to 170 °C and then at 4 °C min$^{-1}$ to 235 °C and a hold at 235 °C for 20 min. The column was then kept at 250 °C for 7 min, cooled to 80 °C, and kept at 80 °C for 1 min prior to the next injection. The injection port and the transfer line to the EI-MS were kept at 250 °C. Alditol-acetate derivatives of authentic apiose, rhamnose, fusose, ribose, arabinose, xylose, mannose, glucose, and galactose (50 μg each) were prepared under the same conditions as samples. Monosaccharides were identified based on their retention times and their EI mass spectra. Peak areas, obtained from the total ion chromatogram, were exported to Microsoft Excel and normalized using the amount of sample and the area of the internal standard. The amounts of each monosaccharide in a sample were calculated using the response factors of the monosaccharide standards.

**Author Contributions**—J. S. conducted most experiments and analyses. Y. Y. transformed P. patens. S. L. conducted fractionation and glycosyl residue composition analyses. O. O. A. generated constructs and performed in-microbe analyses. M. B.-P., M. A. O., and M. G. H. conceived the idea for the project. All authors reviewed the results, participated in writing the paper, and approved the final version of the manuscript.

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**References**

1. Pičmanová, M., and Möller, B. L. (2016) Apiose: one of nature’s witty games. *Glycobiology* 26, 430–442
2. O’Neill, M. A., Albersheim, P., and Darvill, A. G. (1990) The pectic polysaccharides of primary cell walls, in *Methods in Plant Biochemistry* (Dey, P. M., ed) Vol. 2, pp. 415–441, Academic Press, London
3. Hart, D. A., and Kindel, P. K. (1970) Isolation and partial characterization of apialgalacturonans from the cell wall of *Lemna minor*. *Biochem. J.* 116, 569–579
4. Golovenchenko, V. V., Ovodova, R. G., Shashkov, A. S., and Ovodov, Y. S. (2002) Structural studies of the pectic polysaccharide from duckweed *L. marina* L. *Phytochemistry* 60, 89–97
5. Glaougen, V., Brudieux, V., Closs, B., Barbat, A., Krausz, P., Sainte-Catherine, O., Kraemer, M., Maes, E., and Guerardel, Y. (2010) Structural characterization and cytotoxic properties of an apiose-rich pectic polysaccharide obtained from the cell wall of the marine phanerogam *Zostera marina*. *J. Nat. Prod.* 73, 1087–1092
6. Matoh, T., Kawaguchi, S., and Kobayashi, M. (1996) Ubiquity of a borate-polysaccharide complex from radish roots. *Plant Cell Physiol.* 37, 636–640
7. Matoh, T., Ishigaki, K., Ohno, K., and Azuma, J. (1993) Isolation and characterization of a boron-polysaccharide complex from radish roots. *Plant Cell Physiol.* 34, 639–642
UDP-apiose Synthases of Avascular Plants and Green Algae

8. Ishii, T., and Matsunaga, T. (1996) Isolation and characterization of a horon-rhamnogalacturonan-II complex from cell walls of sugar beet pulp. Carbohydr. Res. 284, 1–9
9. Kobayashi, M., Matoh, T., and Azuma, J. (1996) Two chains of rhamnogalacturonan-II are cross-linked by borate-diol ester bonds in higher plant cell walls. Plant Physiol. 110, 1017–1020
10. O’Neill, M. A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A. G., and Albersheim, P. (1996) Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester. In vitro conditions for the formation and hydrolysis of the dimer. J. Biol. Chem. 271, 22923–22930
11. Ishii, T., Matsunaga, T., Pellerin, P., O’Neill, M. A., Darvill, A., and Albersheim, P. (1999) The plant cell wall polysaccharide rhamnogalacturonan II self-assembles into a covalently cross-linked dimer. J. Biol. Chem. 274, 13098–13104
12. Fleischer, A., O’Neill, M. A., and Ehlwold, R. (1999) The pore size of non-granulacoseous plant cell walls is rapidly decreased by borate ester cross-linking of the pectic polysaccharide rhamnogalacturonan II. Plant Physiol. 121, 829–838
13. O’Neill, M. A., Eberhard, S., Albersheim, P., and Darvill, A. G. (2001) Requirement of borate cross-linking of cell wall rhamnogalacturonan II for Arabidopsis growth. Science 294, 846–849
14. Ahn, J. W., Verma, R., Kim, M., Lee, J. Y., Kim, Y. K., Bang, J. W., Reiter, W. D., and Pai, H. S. (2006) Depletion of UDP-α-apiose/UDP-α-xylene synthases results in rhamnogalacturonan-II deficiency, cell wall thickening, and cell death in higher plants. J. Biol. Chem. 281, 13708–13716
15. Pabst, M., Fischl, R. M., Brecker, L., Morelle, W., Fauland, A., Köfeler, H., Altman, F., and Léonard, R. (2013) Rhamnogalacturonan II structure shows variation in the side chains monosaccharide composition and methylation status within and across different plant species. Plant J. 76, 61–72
16. Dumont, M., Lehner, A., Bouton, S., Kiefer-Meyer, M. C., Voxeur, A., Pelloux, J., Lerouge, P., and Mollot, J. C. (2014) The cell wall pectic polymer rhamnogalacturonan-II is required for proper pollen tube elongation: implications of a putative sialyltransferase-like protein. Ann. Bot. 114, 1177–1188
17. Grisebach, H., and Döbereiner, U. (1964) The biosynthesis of apiose in parsley. Biochem. Biophys. Res. Commun. 17, 737–741
18. Sandermann, H. J., Tsue, G. T., and Grisebach, H. (1968) Biosynthesis of D-apiose. IV. Formation of UDP-apiose from UDP-D-glucuronic acid in cell-free extracts of parsley (Apium petroselinum L.) and Lemma minor. Biochim. Biophys. Acta 165, 550–552
19. Ortman, R., Sutter, A., and Grisebach, H. (1972) Purification and properties of apiose UDP-α-D-glucosyl]-flavone apiosyltransferase from cell suspension cultures of parsley. Biochem. Biophys. Acta 289, 293–302
20. Bar-Peled, M., Urbanowicz, B. R., and O’Neill, M. A. (2012) The synthesis and origin of the pectic polysaccharide rhamnogalacturonan II—insights from nucleotide-sugar formation and diversity. Front. Plant Sci. 3, 92
21. Rensing, S. A., Lang, D., Zimmer, A. D., Terry, A., Salamov, A., Shapiro, H., Nishiyama, T., Perroud, P. F., Lindquist, E. A., Kamisugi, Y., Tanahashi, T., Sakakibara, K., Fujita, T., Oishi, K., Shin-I, T., et al. (2008) The Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. Science 319, 64–69
22. Matsaci, N., Hung, L. H., Yan, Z., Carpenter, E. J., Wickett, N. J., Mirarab, S., Nguyen, N., Warnow, T., Ayyampalayam, S., Barker, M., Burleigh, J. G., Gitzendanner, M. A., Wafafa, E., Der, J. P., dePamphilis, C. W., et al. (2014) Data access for the 1,000 Plants (1KP) project. Gigascience 3, 17
23. Gu, X., Glushka, J., Yin, Y., Xu, Y., Denny, T., Smith, J., Jiang, Y., and Bar-Peled, M. (2010) Identification of a bifunctional UDP-4-keto-pentose/UDP-xylene synthase in the plant pathogenic bacterium Raistonia solanacearum strain GMI1000, a distinct member of the 4,6-dehydratase and decarboxylase family. J. Biol. Chem. 285, 9030–9040
24. Breazeale, S. D., Ribeiro, A. A., McClenner, A. L., and Raetz, C. R. (2005) A formyltransferase required for polymyxin resistance in Escherichia coli and the modification of lipid A with 4-Amino-4-deoxy-l-arabinose. Identification and function of UDP-4-deoxy-4-formamido-l-arabinose. J. Biol. Chem. 280, 14154–14167
25. Kavanagh, K. L., Jörnvall, H., Persson, B., and Oppermann, U. (2008) Medium- and short-chain dehydrogenase/reductase gene and protein fami-
45. Treutter, D. (2005) Significance of flavonoids in plant resistance and enhancement of their biosynthesis. *Plant Biol.* 7, 581–591
46. Neilson, E. H., Goodger, J. Q., Woodrow, I. E., and Møller, B. L. (2013) Plant chemical defense: at what cost? *Trends Plant Sci.* 18, 250–258
47. Wakuta, S., Mineta, K., Amano, T., Toyoda, A., Fujiwara, T., Naito, S., and Takano, J. (2015) Evolutionary divergence of plant borate exporters and critical amino acid residues for the polar localization and boron-dependent vacuolar sorting of AtBOR1. *Plant Cell Physiol.* 56, 852–862
48. Camacho-Cristóbal, J. J., Rexach, J., and González-Fontes, A. (2008) Boron in plants: deficiency and toxicity. *J. Integr. Plant Biol.* 50, 1247–1255
49. Baylson, F. A., Stevens, B. W., and Domozych, D. S. (2001) Composition and synthesis of the pectin and protein components of the cell wall of *Closterium acerosum* (Chlorophyta). *J. Phycol.* 37, 796–809
50. Popper, Z. A., and Fry, S. C. (2003) Primary cell wall composition of bryophytes and charophytes. *Ann. Bot.* 91, 1–12
51. Roberts, A. W., Roberts, E. M., and Haigler, C. H. (2012) Moss cell walls: structure and biosynthesis. *Front. Plant Sci.* 3, 166
52. McCarthy, T. W., Der, J. P., Honaas, L. A., de Pamphilis, C. W., and Anderson, C. T. (2014) Phylogenetic analysis of pectin-related gene families in *Physcomitrella patens* and nine other plant species yields evolutionary insights into cell walls. *BMC Plant Biol.* 14, 79
53. Ortiz-Ramírez, C., Hernandez-Coronado, M., Thamm, A., Catarino, B., Wang, M., Dolan, L., Feijó, J. A., and Becker, J. D. (2016) A transcriptome atlas of *Physcomitrella patens* provides insights into the evolution and development of land plants. *Mol. Plant* 9, 205–220
54. Vidali, L., Augustine, R. C., Kleinman, K. P., and Bezanilla, M. (2007) Profilin is essential for tip growth in the moss *Physcomitrella patens*. *Plant Cell* 19, 3705–3722
55. Yang, T., Bar-Peled, Y., Smith, J. A., Glushka, J., and Bar-Peled, M. (2012) In-microbe formation of nucleotide-sugars in engineered *Escherichia coli*. *Anal. Biochem.* 421, 691–698
56. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254
57. York, W., Darvill, A., McNeil, M., Stevenson, T., and Albersheim, P. (1986) Isolation and characterization of plant cell walls and cell wall components. *Methods Enzymol.* 118, 3–40
58. Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D., and Higgins, D. G. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7, 539
59. Li, W., Cowley, A., Uludag, M., Sizjuzato, S., Park, Y. M., Buso, N., Cowley, A. P., and Lopez, R. (2013) Analysis tool web services from the EMBL-EBI. *Nucleic Acids Res.* 41, W597–W600
60. Huson, D. H., and Scornavacca, C. (2012) Dendroscope 3: an interactive tool for rooted phylogenetic trees and networks. *Syst. Biol.* 61, 1061–1067