Genome mapping and gene expression of NDP-sugar pathways in the giant duckweed Spirodela polyrhiza and its relevance for bioenergy

Debora Pagliuso  
University of Sao Paulo: Universidade de Sao Paulo

Bruno Viana Navarro  
University of Sao Paulo: Universidade de Sao Paulo

Adriana Grandis  
University of Sao Paulo: Universidade de Sao Paulo

Marcelo M. Zerillo  
University of Sao Paulo: Universidade de Sao Paulo

Eric Lam  
Rutgers University: Rutgers The State University of New Jersey

Marcos Silveira Buckeridge (✉️ msbuck@usp.br)  
University of Sao Paulo  https://orcid.org/0000-0002-5455-8136

Research Article

Keywords: Spirodela polyrhiza, cell wall, NDP-sugars metabolism, polysaccharide, biorefinery

DOI: https://doi.org/10.21203/rs.3.rs-776424/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Genome mapping and gene expression of NDP-sugar pathways in the giant duckweed *Spirodela polyrhiza* and its relevance for bioenergy

Débora Pagliuso¹, Bruno Viana Navarro¹, Adriana Grandis¹, Marcelo M. Zerillo², Eric Lam³, Marcos Silveira Buckeridge¹*

¹Laboratory of Plant Physiological Ecology, Department of Botany. Institute of Biosciences, University of São Paulo, Brazil.

²GaTE Lab, Department of Botany. Institute of Biosciences, University of São Paulo, Brazil.

³Department of Plant Biology and Pathology, Rutgers, The State University of New Jersey, New Brunswick, New Jersey, USA

*Correspondence:
Corresponding Author
msbuck@usp.br

ORCID
Débora Pagliuso - https://orcid.org/0000-0002-4691-6167
Bruno Viana Navarro - https://orcid.org/0000-0001-9289-4328
Adriana Grandis - https://orcid.org/0000-0002-8416-3733
Marcelo M. Zerillo - https://orcid.org/0000-0001-9671-2894
Eric Lam - https://orcid.org/0000-0001-8462-9794
Marcos Silveira Buckeridge - https://orcid.org/0000-0002-5455-8136

Abstract

Duckweeds are fast-growing aquatic plants suitable for bioenergy due to fermentable-rich biomass with low lignin. The duckweed sub-families Lemnoideae and Wolffioideae are also distinguished by the distribution of two pectin classes (apiogalacturonan and xylogalacturonan), which seem to be related to their growing capacity and the starch content. The plant cell wall is built from pathways of nucleotide sugars syntheses that culminate in cell wall synthesis and deposition. Therefore, understanding these pathways through mapping the genes involved and their expression would be important to develop tools to improve bioenergy production. Here we used the available information of NDP-sugar metabolism to search for orthologous genes involved in the synthesis of cell wall polysaccharides in *Spirodela polyrhiza*. We detected 190 genes and mapped them onto the plant chromosomes. The genes were roughly arranged in groups according to their category: "Starch and sucrose metabolism," "Pectins," "Hemicelluloses," and "Cellulose." We followed the expression of thirty-eight of the orthologues’ transcripts – the higher expression being starch (SBE), pectin (GAUT1, MUR, USP, and GER), and...
mannan (CSLA) syntheses - corroborating the chemical composition of S. polyrhiza cell wall. We further investigated the carbohydrate metabolism pathways and discussed the implications of altering the NDP pathways for bioenergy and biorefinery. We conclude that S. polyrhiza displays suitable features for future genetic transformations leading to the adaptation of its cell wall for biofuels. However, such strategies will have to consider the trade-offs between fermentation and ethanol production benefits and the potential adverse effects of genetic transformation on plant growth and development.

Keywords: Spirodela polyrhiza, cell wall, NDP-sugars metabolism, polysaccharide, biorefinery.

1 Introduction

All plant cells are surrounded by an encoded polymeric structure composed of polysaccharides, proteins, and phenolic compounds [1]. The cell walls are complex structures that can be classified as primary and secondary. Secondary cell walls are covalently linked polymers, mainly hemicellulose and lignin, that confer resistance and strength to the tissue [2–4]. On the other hand, the primary walls are expandable, consisting of 90% of their dry weight in polysaccharides arranged with a cellulose core surrounded by hemicelluloses immersed in a pectin matrix [5–8]. Primary cell wall components maintain the mechanical and functional cell properties during plant growth and development [9].

The primary walls are subdivided into three types according to the composition of hemicelluloses and the proportions of pectin, hemicellulose-cellulose, and proteins [8]. Type I cell walls are found in eudicots and the non-commelinoid monocots, with xyloglucans as the main hemicellulose [8] and similar pectins and hemicelluloses proportions. Type II walls are characteristic of grasses, with arabinoxylan, β-glucan, and low pectin content [8]. Alternatively, type III is found in ferns, with mannans being the main hemicellulose and reduced pectins [10].

In plants, besides the mechanical resistance and cell protection, the cell wall promotes cell adhesion, delimiting the cell size and volume. Cell walls regulate the conduction of water and solutes within the plant. They also determine the turgor pressure and act as a signaling molecule source [11–15]. This encrypted polysaccharide structure displays a glycomic code [1], whose understanding holds the potential to elucidate new mechanisms associated with plant physiology and creates a niche for its application.

The cell wall is one of the most significant biomass sources, making available sugars for feedstock and industrial applications such as bioenergy, cosmetology, pharmaceutical, and food inputs [16–19]. Likewise, the cell walls are also an important carbon sink, fixed photosynthetically [19]. The main product of carbon assimilated through photosynthesis is triose-phosphate, which, when exported from the chloroplast to the cytosol, are involved in the synthesis of soluble sugars (glucose and fructose), soluble oligosaccharides (sucrose and raffinose), sugar alcohols (inositol, sorbitol, and mannitol), and the cell wall polymers through UDP-glucose pathway [20–22]. Alternatively, triose-phosphate can be maintained inside chloroplasts (within the stroma) and used as a substrate for starch synthesis via the ADP-glucose pathway, functioning as carbon storage [23]. The transitory starch could be degraded during the night to maintain the metabolic processes [23–25].

For the cell wall synthesis, the triose-phosphate sent to the cytosol is condensed into hexose-phosphates, which will generate the substrate for the nucleotide diphosphate (NDP)-sugars pathway [19]. Also, the NDP-sugars can be generated from the catabolism of sugars released from storage polymers, glycoproteins, glycolipids, and polysaccharide's recycling
during plant growth and development (primary and secondary walls restructuring) [26]. The latter can be described as a salvage pathway in which free sugars are recovered to produce sugars-1-phosphate [27]. After polymers turnover, the free sugars from the cell wall must be imported to the cell again before its reactivation into NDP-sugars [27]. Here we will focus on cell wall synthesis and possible applications of this pathway for biorefinery.

The wall synthesis has UDP-glucose as the precursor, which is synthesized by UDP-glucose-pyrophosphorylase (UGP, EC 2.7.7.9), sucrose synthase (SUSY, EC 2.4.1.13), and invertase (INV, EC 3.2.1.26) [19, 28–31] (Figure 1). The NDP-sugars are high-energy monosaccharide donors that will serve as a substrate for biosynthesis of polysaccharides, glycoproteins, proteoglycans, glycolipids, and glycosylated secondary metabolites [26, 32, 33]. Polysaccharide biosynthesis involves linking monosaccharides to each other through the action of several glycosyltransferases associated with the Golgi apparatus [34]. These polymers are further exported to the membrane by vesicles, gaining extracellular space [35, 36].

Cellulose biosynthesis differs from pectins and hemicelluloses by their location of synthesis [37]. It occurs in the plasma membrane, where cellulose synthases are assembled as complexes named rosettes, composed of six proteins (CESA; EC 2.4.1.12). They use UDP-glucose to form β-1,4-D-glucose chains [37, 38]. UDP-glucose is also the precursor of UDP-rhamnose (UDP-Rha), UDP-galactose (UDP-Gal), and UDP-glucuronate (UDP-GlcA), hence involved in hemicelluloses and pectin synthesis [39]. The only NDP-sugars that are not derived from the UDP-glucose are GDP-mannose (GDP-Man) and GDP-fucose (GDP-Fuc) [34], which are synthesized from fructose-6-phosphate and are part of the mannan type of hemicellulose and side branching of xyloglucan and pectins (Figure 1).

Usually, the cell wall is conserved among species despite slight modifications in different tissues [40, 41]. Recently, we found that duckweeds have remarkable plasticity in their cell walls [42]. This suggests that this plant may be an interesting model to evaluate the carbon distribution, cell wall dynamics, and correlation with plant development. Duckweeds are the smallest flowering aquatic plants with fast growth [43]. As a non-graminaceous monocot species, duckweeds have a primary cell wall of type I, with a large proportion of pectins, fermentable sugars, and a lack of lignin [44, 45]. These plants display a unique cell wall with apiogalacturonan as the principal polymer in pectins [46]. The unusual chemical composition of duckweed cell walls and the low recalcitrance make it suitable for bioenergy production, such as ethanol [45, 47–49]. Besides, the properties of apiogalacturonan make it suitable for use as adjuvants [50] or as cosmetic and dermatological preparation for skincare [51]. Moreover, the fast-grow (up to 100-ton dry matter ha⁻¹ year⁻¹) [52, 53] and their capacity of water clean-up [54–58] makes duckweed a sustainable feedstock for several applications, including bioenergy.

The present work brings an overview of the NDP-sugar pathway in Spirodela polyrhiza, the giant duckweed, correlating genes and some transcripts' expression with cell wall precursors. We also provide cell wall chemical characterization to instigate and improve the potential applications of duckweed cell walls towards biorefinery.

S. polyrhiza 9509 has a genome of 138.6 Mbp distributed in 20 chromosomes, hosting 18,507 predicted genes. This genome has low heterozygosis, low methylation levels, and low contents of non-essential proteins, transposons, rDNA, and long terminal repeats [59]. The NDP-sugar pathways directly relate to the carbon flux from photosynthesis with the hexoses-phosphate distribution to synthesize the cell wall, soluble sugars, and starch [19]. Besides, critical points of the pentoses-
phosphate, involved in synthesizing organic acids, nucleotides, amino acids, lignin, and polyphenols, interconnect with these pathways [33, 60]. Therefore, understanding the NDP-sugar pathways enlightens the plant metabolism in its essential function in growth, impacting directly in biomass accumulation.

In this work, we mapped the genes associated with the NDP-sugar pathway (de novo and salvage) and correlated them to the chemical characterization of the giant duckweed (S. polyrhiza) cell wall. This plant biomass has been characterized as having 3% starch, 49% soluble sugars, 40% cell wall, and 8% non-measured compounds. The cell walls are synthesized by the NDP-sugar pathway and represent a significant carbon sink. This sink results from the action of proteins encoded by 155 orthologs of the 34 targets of the NDP-sugar pathway. The cell wall was evaluated as displaying 49% pectins, 23% hemicellulose, and 10% cellulose. These carbohydrates have the potential for biorefinery as adjuvants, cosmetics, food additives, stabilizers, and gelling agents, and principally as biofuels.

2 Material and methods

2.1 Plant material

The S. polyrhiza clones 9509 and 7498, originally from Germany (Lotschen, Stradtroda) and USA (North Carolina, Durham), respectively, were acquired from Rutgers Duckweed Stock Cooperative (RDS C). The plants were grown axenically in 250 mL of ½ Schenck-Hildebrandt (Sigma-Aldrich®) medium supplemented with 0.5% sucrose and maintained at 25 °C in a 16 h light/8 h dark photoperiod with a light intensity of 20 µmols.m⁻².s⁻¹.

2.2 Non-structural carbohydrates

Plants were harvested after 10-15 days of cultivation, freeze-dried, and ground in GenoGrinder2010 (Spex®, USA). Five hundred mg of each milled sample was extracted 4 times with 25 mL of 80% ethanol (v/v) at 80 °C for 20 min [40]. The supernatants containing soluble sugars and other soluble compounds were recovered, vacuum dried (ThermoScientific® Savant SC 250 EXP), and resuspended in 1 mL of water and 1 mL of chloroform. The residue (Alcohol Insoluble Residue-AIR) was dried at 40 °C and reserved. The soluble sugars were analyzed by High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) in a Dionex® system (ICS 5000), using a CarboPac PA1 column eluted with 150 µM sodium hydroxide in an isocratic run of 27 min [42].

2.3 Starch determination

The AIR dried-residue from the ethanolic extractions was treated with 120 U/mL of α-amylase (E.C. 3.2.1.1) (Megazyme® Inc., Australia) diluted in 10 mM MOPS buffer pH 6.5 at 75 °C for 1h for starch removal. For the starch determination, the reactions followed with the addition of 30 U.mL⁻¹ of amyloglucosidase (E.C. 3.2.1.3) (Megazyme® Inc., Australia) diluted in 100 mM sodium acetate pH 4.5 at 50 °C for 1h. The mixture was submitted to the colorimetric assay described by Amaral et al. (2007) [61] and Pagliuso et al. (2018) [42]. The final residue (de-starched AIR – cell wall) was recovered, washed in ethanol, frozen, and freeze-dried.

2.4 Cell wall fractionation

The cell walls were submitted to several extractions to solubilize each polysaccharide class [40]. First, the material was extracted three times with 25 mL of 0.5 M ammonium oxalate (pH 7.0) at 80 °C for 1 h each with continuous stirring.
to remove pectins. The supernatants were recovered by centrifugation, and the oxalate-extracted cell wall residues were extracted with 25 mL of 3% (w/v) of sodium chlorite in 0.3% (v/v) acetic acid for lignin removal [62]. The supernatants were also recovered, and the hemicelluloses from the sodium chlorite cell wall residues were extracted three times with 25 mL each of 0.1, 1, and 4 M NaOH supplemented with 3 mg.mL\(^{-1}\) with sodium borohydride at room temperature for 1 h. All the supernatants and the residue were recovered, neutralized, dialyzed, and freeze-dried. The mass balance and fractionation yield were calculated gravimetrically.

2.5 Monosaccharide analysis

The cell wall fractions were hydrolyzed with 1.5 mL of 2 M trifluoroacetic acid (TFA) for 1 h at 100 °C with continuous stirring. The vacuum-dried reactions were resuspended in 1 mL MiliQ water and filtrated on 0.22 µm (Merck Millipore®). The monosaccharides were analyzed by HPAEC-PAD into a CarboPac SA10 column (ICS 5.000 system, Dionex-Thermo®) eluted isocratically with 99.2% water and 0.8% (v/v) 150 mM NaOH with a flux 1 mL.min\(^{-1}\). The cell wall monosaccharides were detected using a 500 mM NaOH post-column base with a flux of 0.5 mL.min\(^{-1}\). A standard curve with each monosaccharide was prepared and injected into the HPAEC-PAD. The standard curve was used to calculate the concentration of each monosaccharide in the fractionation samples.

2.6 Spirodela polyrhiza 9509 gene prediction

No gene annotation was available for S. polyrhiza 9509 in public databases. We performed gene prediction analysis on its twenty chromosomal sequences (GenBank assembly accession GCA_001981405.1, loci CP019093.1 - CP019112.1). As a first attempt, we used the Augustus gene prediction tool (version 3.3.2) [63] with the default parameters and trained on the Arabidopsis thaliana dataset. The predicted genes associated with sugar metabolism were validated in further analyses, as described below.

2.7 NDP-sugar genes screening and query selection

Based on information of amino- and NDP-sugar metabolisms available at the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway (map00520) and data from Verbančič et al. (2017) [19], it was established a comprehensive pathway depicting the enzymes, target genes, and sugar products related to cell wall metabolism (Figure 1). The sequences of related genes and predicted proteins from A. thaliana were used as queries on a sequence similarity search [64] against the genomes of S. polyrhiza 9509 and 7498 (Supplemental Table 1). Comparison with the 7498 genomes was performed by using the tBlastN tool (E-value > e\(^{-10}\)) on the Phytozone v12.1.6 website (https://phytozone.jgi.doe.gov/), or by using local BlastP against our predicted-proteins database of the 9509 genome since the latter was not available on Phytozone. Sequences with coverage ≥ 70% and a similarity ≥ 80% were retrieved and considered orthologs (Supplemental Table 2, 3, and 4). The homologous protein sequences obtained were annotated and classified using HMMER Scan (https://www.ebi.ac.uk/Tools/hmmer/) (Supplemental Table 5, 6, and 7). Multiple alignments were performed using Clustal Omega Program (Bioedit®) to identify possible introns and conserved regions, and the latter were selected for primer design. The physical distribution among the 20 chromosomes was determined based on data retrieved from Phytozone, and the chromosomal ideogram of the NDP-sugar pathway genes evaluated in the present study was designed by PhenoGram tool software (https://ritchielab.org/software/phenogram-downloads), available online [65].
The sugar and cell wall-related genes of the 9509 genome were further compared with the Reference Sequence (RefSeq) database [66] of NCBI by BlastX (E-value > e^{-10}) and with the InterPro database [67] for the search of functional domains with protein families associated with sugar metabolism.

2.8 Candidate reference genes selection

Thirteen reference genes were selected as normalizers candidates: 60S ribosomal protein L18A-A (60S), Actin-related protein (ARP7), cyclophilin (CYP), Elongation factor 1-α (EF1), Translational initiation factor 4B (ELF4B), F-box family protein (FBOX), protein phosphatase 2A (PP2A), D1 subunit (PSAB), S24 ribosomal protein (S24), Tubulin α3/α5 chain (TUA), Ubiquitin-conjugating enzyme (UBQ9), Ubiquitin 7 (UBQ7), and Ribosomal RNA small subunit methyltransferase NEP 1 (18S) [68–80] (Supplemental Table 8). The annotated genome of S. polyrhiza (7498) was analyzed using a blasting program (NCBI®) ([https://blast.ncbi.nlm.nih.gov/Blast.cgi](https://blast.ncbi.nlm.nih.gov/Blast.cgi)), by which the sequences of A. thaliana and Lemna minor were used as queries as described above for primer design (Supplemental Table 9).

2.8 Primers design

Primers were designed for 38 genes of the NDP-sugar pathway and all the reference gene candidates (Supplemental Table 9), by using Primer-Blat® (NCBI) tool ([https://www.ncbi.nlm.nih.gov/tools / primer-blast/](https://www.ncbi.nlm.nih.gov/tools / primer-blast/)), according to MIQE guidelines [81]. Oligo Analyzer Tool® program (Integrated DNA Technologies®) ([https://www.idtdna.com/calc/analyzer/](https://www.idtdna.com/calc/analyzer/)) was used to estimate the potential formation of secondary structures in those. Primers targeting phosphoglucone isomerase (SpPGI), mannose-6P-isomerase (SpMPI), phosphomannomutase (SpPMM), mannose-1P-guanylyltransferase (SpGMD), and UDP-arabinose-4-epimerase (SpUAE) were designed based exclusively on the S. polyrhiza 7498 transcripts. The lyophilized oligonucleotides (DNA Express Brasil® and Exxtend®) were resuspended in Tris 10 mM EDTA 1 mM pH 7.0 (ThermoScientific®), to reach the final concentration of 100 µM, and stored at -20 °C.

2.9 RNA extraction, DNase treatment, and cDNA synthesis

Plant material was harvested after 10 days of cultivation and homogenized in liquid nitrogen. The RNA was extracted using ReliaPrep™ RNA Tissue Miniprep System (Z6112, Promega®, USA) and followed the DNase treatment as the manufacturer's instructions. RNA quantification and purity were assured using a Nanodrop ND-100 spectrophotometer (Thermo Fischer Scientific), and samples with 260/280 ratios between 1.8 and 2.2 were taken as sufficiently pure. The samples' integrity was also secured by electrophoresis on a 1% agarose gel and stained with SYBR Safe DNA gel stain (1771581, Thermo Fischer Scientific, USA). Approximately 1 ug of each RNA sample was reverse transcribed with random hexamers by SuperScript III Reverse Transcriptase (Thermo Fischer Scientific, USA). The cDNA acquired were tested for the absence of genomic DNA with UPD-apiose/UDP-xylose synthase (AXS) primers (Forward: 5'-GCATCCAGTTCCACCGTCTC -3'; Reverse: 5'-GCAGGGCGTTTCATCTTCTTT -3').

2.10 qRT-PCR analysis

The relative abundance of target transcripts in S. polyrhiza 9505 and 7498 was measured by qRT-PCR analysis using a QuantiStudio 6 Flex Real-Time PCR system (Applied Biosystems by Life Technologies, NY, USA). The PCR reactions were performed with 1.4 µL of cDNA (1:10), 7 µL 2X SYBR Green Master Mix (Applied Biosystems by Life
Technologies, NY, USA), and the following cycling conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The amplification of a single product was confirmed by a melting curve analysis (data not shown). After testing the concentrations of 200 nM, 400 nM, and 800 nM of the primer set, we chose 800 nM as the optimal concentration based on the lowest quantification cycle (Cq) values, highest primer efficiency values, and the absence of primer dimers. The Cq values and the efficiency of each primer were determined using LinRegPCR software [82], and only the genes with transcripts holding Cq values ≤ 35 followed the further analysis (Supplementary Figure 1).

### 2.11 Consistency and validation of reference genes

The consistency of the candidate reference genes was determined by the statistical approaches of GeNorm v.3.5 software (http://medgen.ugent.be/~jvdesomp/genorm/) (Supplemental Table 10) and NormFinder software (http://mdl.dk/publicationsnormfinder.htm) (Supplemental Table 11). The relative expression level of the gene SpAXS was evaluated in both S. polyrhiza lineages to confirm and validate the selected reference genes. The relative expression of SpAXS was quantified compared to the average expression, normalized by the highest Cq value of the tested sample, and the Cq values of the target genes were normalized by the geometric average of the reference genes combination (Supplementary Figure 2).

### 2.12 Statistical analysis

Five replicates were used for the experiments. Data were analyzed by ANOVA one-way followed by Tukey's test (p<0.05). The analysis was carried out with R version 3.6.1.

### 3 Results

#### 3.1 Non-structural and structural carbohydrates

S. polyrhiza carbohydrates were analyzed as soluble sugars, starch, and cell wall polysaccharides. The soluble sugars and starch constitute the non-structural carbohydrates representing more than half of the biomass (Figure 2). The S. polyrhiza biomass was determined as 48.9% soluble sugars, 3.3% starch, 39.5% structural carbohydrates (cell walls), and 8.3% other compounds (Figure 2). When calculated as the percentage of the biomass, the soluble sugars were mainly fructose (20%), followed by sucrose (17%) and glucose (11.7%), and low amounts of raffinose (0.3%).

Regarding the cell walls, 59% were pectins (fractions from extraction with ammonium oxalate and sodium chlorite), 28% hemicelluloses (fractions from extraction with 0.1, 1, and 4 M sodium hydroxide), and 13% cellulose (residue) (Table 1). The cell wall fractionation will be described as the mentioned classes (pectin, hemicellulose, and cellulose) within the corresponding fractions summed. The majority of the pectins were composed of apiose (56.5 µg.mg⁻¹ cell wall - 38%), arabinose (23.8 µg.mg⁻¹ cell wall- 16%), and galactose (28.9 µg.mg⁻¹ cell wall - 19.5%), with traces of fucose (2.9 µg.mg⁻¹ cell wall - 2%) (Table 1, AmnOx and Chlorite columns). Xylose (39.1 µg.mg⁻¹ cell wall - 32%), glucose (17.3 µg.mg⁻¹ cell wall - 14%), and galactose (17.2 µg.mg⁻¹ cell wall - 14%) were the main monosaccharides of the hemicellulose fractions, suggesting xylan as the principal hemicellulose followed by xyloglucan in S. polyrhiza (Table 1, 0.1 M, 1 M, and 4 M NaOH fractions). The soluble hemicellulose glucomannan was found on the fraction of ammonium oxalate with significant proportions of mannose (6.1 µg.mg⁻¹ cell wall) and glucose (4.8 µg.mg⁻¹ cell wall) (Table 1). The residue comprised more than 80% cellulose (151.7 µg.mg⁻¹ cell wall) (see glucose levels on Residue II in Table 1). The residue II
fraction composition also showed small amounts of galactose (6.3 µg.mg⁻¹ cell wall), xylose (11.8 µg.mg⁻¹ cell wall), and apiose (5.8 µg.mg⁻¹ cell wall), suggesting possible crosslinks among apiogalacturonans, galactans, and xyloglucans with the cellulose microfibrils (Table 1, residue II fraction).

3.2 Mapping of orthologous sequences throughout the chromosomes of *S. polyrhiza*

Using thirty-seven target genes of *de novo* and salvage NDP-sugars pathways and starch synthesis (Figure 1) of *A. thaliana*, we retrieved 190 orthologous sequences from *S. polyrhiza* genome (Figures 3 and 4, Supplemental Table 1). The recovered sequences had similarities above 70% with queries in public databases (Supplemental Tables 2, 3, and 4) and showed conserved protein domains (Supplemental Tables 5, 6, and 7).

The recovered genes were divided into the categories that we named as: “starch and sucrose metabolism” (*SpSUSY, SpINV, SpFK, SpHXK, SpPGI, SpPGM, SpUGP, SpAPL, SpAPS, SpSS, and SpSBE* contemplating 49 orthologues), “pectin” (*SpRHM, SpUGD, SpAXS, SpGAE, SpGAUT, SpGALAK, SpGLCAK, SpUAE, SpUAM, SpARAI, SpUSP, SpUGE, SpGALT, and SpGALK* -54 orthologues), “hemicellulose” (*SpUXS, SpXYL4, SpMPI, SpPMM, SpMGP, SpGMD, SpGER, SpMSR, SpCSLA, SpFKGP, and SpASD* -65 orthologues), and “cellulose” (*SpCESA, 23 orthologues*) (Figures 1 and 3). The distribution of the selected genes of this work, which focuses on the carbon pathways that lead to storage (starch and sucrose) or structure (cell walls), which tends to have a higher number of genes in the chromosomes 1 and 2 (24 and 22 genes, respectively) that are the largest ones (14.77 and 11.37 Mb) (Figures 3 and 4). The other chromosomes (3, 4, 5, 8, 9, 13, and 15 – that have ~7.7 Mb) display a range between 7 and 15 genes compared to the rest (chromosomes 6, 7, 10, 12, 14, 16, 17, 18, 19 and 20 – that have ~ 5.3 Mb). Thus, it can be said that some carbon pathways genes could be differentially partitioned in the genome of *S. polyrhiza*. The size of the chromosomes is not related to the occurrence of genes, suggesting that other factors are associated with the topology of the carbon pathways genes in the genome of *S. polyrhiza*.

When analyzed according to metabolic pathways (Figure 1) that lead to the formation of non-structural sugars (starch, sucrose, and monosaccharides) and structural cell wall carbohydrate polymers (pectin, hemicellulose, and cellulose), the gene distribution varies according to the category (Figures 3 and 4). Starch-related genes are more randomly distributed throughout the chromosomes 1-3, 5, 9, 11-13, 15, and 19. Pectin-related genes (a total of 53 orthologues) display a more concentrated group in chromosomes 1-5, 8, and 13, with more than 70% of the category's genes. The rest of the chromosomes, 4 (8.6 Mb), 6 (8 Mb), 7 (7.9 Mb), 10 (6.6 Mb), 14 (5.6 Mb), 16 (4.45 Mb), and 18 (4.13 Mb), showed a lower number of genes. For hemicelluloses (65 orthologues), some chromosomes display many genes (1, 2, 13, and 15), with 50% of them in the category. Besides, hemicellulose-related orthologues are not found in chromosomes 10, 11, 16, and 20. Regarding cellulose, approximately 50% of the genes are in chromosomes 7, 10, and 11 (Figures 3 and 4).

3.3 Efficiency and specificity of primers and qRT-PCR amplification

The efficiency and specificity of the primers were tested by qRT-PCR to quantify the relative expression levels of the target genes. They presented values of quantification cycle (Cq) ≤ 35, with reactions that reached the plateau phase. The amplitude of the Cq of the evaluated genes is shown in Supplemental Figure 1, ranging from 14 to 35 cycles. Among the genes involved in cell wall synthesis, the Cq values in more than 50% of the tested genes were greater than 30 cycles,
with UDP-apiose/UDP-xylose synthase (SpAXS) (22.5 ± 0.1) having the lower Cq-value and UDP-glucuronate decarboxylase (UXS3) (35.0 ± 0.1) the higher Cq-value (Supplemental Figure 1B).

3.4 Expression levels of NDP-sugars and related genes

The relative gene expression levels of the NDP-sugar pathways targets were normalized to that of the SpARF7 and SpPP2A genes (Supplemental Tables 8, 10, 11, and Supplemental Figure 2). The expression range of the evaluated targets was 0.5-1.4. Therefore, three levels were established, from 0.5-0.8 as Low, 0.81-1.1 as Medium, and 1.11-1.4 as High.

Out of the 190 NDP-sugar orthologs, 29 targets and 38 orthologues were selected for expression evaluation by qRT-PCR analysis (Table 2). These were the ones that successfully amplified the expected target genes. The expression of sucrose synthase (SpSUSY), invertase (SpINV), hexokinase (SpHXK), and fructokinase (SpFK) denote anabolic activities that lead to the synthesis of starch and monosaccharides (Figure 1 and Table 2). Among these, SpINV showed the lowest expression value, while other target genes of sugar metabolism ranged from 0.62 to 0.74 (Table 2). The synthesis of starch occurs through the ADP-Glucose pathway (Figure 1), which involves the ADP-Glucose pyrophosphorylase (encoded by APS and APL), starch synthase (SS), α-glucan-branching enzyme (SBE), granule-bound starch synthase (GBSS). These targets showed a medium expression level when compared to sucrose-related genes (Table 2). The high level of 1,6-α-glucan branching enzyme (SpSBE - 0.9) might indicate that the starch present in S. polyrhiza is highly branched. The starch synthesis pathway in duckweeds has particular importance due to the modulation's flexibility for starch accumulation depending on photosynthesis activity.

Pectins have the most complex structure among the cell wall polysaccharides, being composed of several monosaccharides. The evaluated expression levels likely associated with pectins should be analyzed carefully due to the co-existence of the same sugars in pectin and hemicelluloses. Pectins' scaffold has the highest transcript levels (α-1,4-galacturonosyltransferase (GAUT) and UDP-glucose-4-epimerase (UGE)), indicating a high carbon sink for pectin (Table 2), as can be seen in the fractionation process in which this class of polysaccharides is half of the S. polyrhiza cell wall (Figure 2 and Table 1). The transcript level of SpAXS, which encodes the enzyme responsible for apiose biosynthesis, was 1.8 times lower than another pectin-related gene (SpGAUT1), while apiose was one of the most significant neutral monosaccharides found in the S. polyrhiza cell wall (Tables 2 and 1).

We evaluated the expression of twelve genes related to hemicellulose biosynthesis pathways. Genes mainly related to xyloglucan, xylan, and mannan biosynthesis are found in the Medium and Low categories (Table 2). A glucomannan synthesis transcript was the second more highly expressed among the transcripts evaluated (glucomannan-4-β-mannosyltransferase – SpCSLA – 1.3) (Table 2).

3.5 Monosaccharide variation in Spirodela polyrhiza' cell wall under distinct cultivation and hydrolysis methods

Spirodela 9509 grown under the same conditions at different developmental stages may have some quantitative variation in the monosaccharide levels (Supplemental Table 13). Therefore, we decided to evaluate the AIR composition published and sets of data from our lab for the species. The recovered data was normalized, and the growth media, microclimate conditions, time of cultivation, and hydrolysis method were considered for comparison. The normalized
monosaccharide levels are consistent and reveal a cell wall characterization of 20.6% (± 1.9) galactose, 17.4% (± 1.1) arabinose, 15.5% (± 1.1) xylose, 21.9% (± 2.9%) apiose, 5.4% (± 1.7) mannose, 7.5% (± 0.6) rhamnose, 9.3% (± 1.9) glucose, and 2.4% (± 0.2) fucose (Supplemental Table 13).

4 Discussion

The cell wall matrix is responsible for plant structure and protection. In this way, most of the carbon efflux in a plant is directed to its synthesis [19]. Tavares and Buckeridge [83] hypothesized that the cell walls form a Glycomic Code [83] and implied that mapping monosaccharides perform its encryption onto the cell wall polymers. The theory was later expanded to the extracellular matrix of all living organisms [1]. In this theory, the original code present in the DNA is considered the signs (primary metabolites) mapped through adaptors (the mechanisms that operate on the signs), leading to a meaning (an output, i.e., the cell wall). The plant cell wall is represented by the polysaccharides assembly that forms the walls with their architecture. The metabolic pathways that lead to the wall's formation are the adaptors, which is the focus of the present work.

Here we examined the genes related to carbohydrate metabolism that lead to the cell wall's formation to assess the adaptors of the Glycomic Code of the cell wall in the Giant Duckweed S. polyrhiza. Other related studies were performed, as the transcriptomic analysis of Landoltia punctata under nutrient starvation [84, 85], Spirodela polyrhiza 7498 under abscisic acid treatment [86], Lemma minor under ammonium toxicity [72], Lemma minor 5500 under ionizing radiation [87], Lemma equinoclitis 6000 under nitrogen starvation [88], and Landoltia punctata 6000 under cadmium exposure [89]. However, most of the data are not available in public databases. The only available RNA-seq data from S. polyrhiza was obtained from a response to treatment that induces the turions formation (dormancy state), which is known to be lower and distinct in S. polyrhiza strain 9509 [90, 91]. However, even with these transcript databases defined for duckweeds, exploring these data aiming at the application for bioenergy is poorly unexplored.

A set of 190 genes related to known reactions that lead to cell wall polysaccharides synthesis in plants (mainly NDP-sugars) were searched and mapped onto the chromosomes of S. polyrhiza. The NDP-sugar-related genes were clustered in groups on different chromosomes according to the pathways that lead to the formation of non-structural carbohydrates (starch and sucrose), pectins, hemicelluloses, and cellulose. Clustering-like patterns were detected in categories of "sucrose metabolism," "pectins," and "hemicelluloses." However, for starch and cellulose, the distribution appears to be more arbitrary.

The mapping of candidate genes for decreasing recalcitrance of cell walls to hydrolysis has usually been performed using QTL analyses [92, 93]. Endogenous metabolic processes could be used to improve cell walls for bioenergy use [94, 95]. Nawaz et al. (2017) performed a genome-wide analysis of cell wall-related genes to find candidates associated with endogenous wall loosening and degradation, in which 505 sequences were mapped on the chromosomes of soybean. The authors evaluated expression in different organs [93]. Here we used a similar approach for S. polyrhiza but focusing mainly on the NDP-sugars and synthases that lead to the cell walls' production and decoration.

Carpita et al. (2001) [60] divided the building process to complete the cell wall into six stages: (1) synthesis of NDP-sugar and monolignols. The latter was not studied here because lignin is negligible in duckweeds [45]; (2) synthesis
of oligomers and polysaccharides at the plasma membrane and Endoplasmic Reticulum-Golgi apparatus; (3) the targeting and secretion of the Golgi-derivate materials; (4) the assembly and architectural patterning of polymers; (5) dynamic rearrangement during cell growth and differentiation; (6) wall disassembly and catabolism of the polymers. Each of these stages is fundamental to address the final matrix, and each of them is complex and lacks a complete understanding.

In this work, we focused on stages 1, 2, and 6. Stage 1 is crucial for monosaccharides synthesis, the building blocks of the polysaccharides. Therefore, understanding the carbon partition to the cell wall may enhance modulation sets to improve industrial applications. Stage 1 is also crucial because the reactions in its pathways determine the fate of sugars towards their transformation into polymers that will be part of a given plant's cell wall. Also, knowing the recycling of the sugars (salvage pathway - Stage 6) during plant growth and development may enhance bioenergy applications to deciphering the cell wall recalcitrance. From the biotechnological viewpoint, Stage 1 would supposedly determine cell wall composition, and the control of those reactions could be fundamental for cell wall engineering. For instance, at the Stage 1 level, the balance among expression and enzyme activities related to the NDP-sugars is expected to determine the final balance of the polysaccharides deposited in the walls.

The cell wall core, cellulose, is synthesized on the plasma membrane by cellulose synthases (CESA; EC 2.4.1.12) complexes [96]. In S. polyrhiza gene screening, we identified 23 CES4 genes (Figure 4), distributed among 13 chromosomes (1,4,7-11,13,15,17-20) (Figures 3 and 4). Cellulose is composed of β-1,4-D-glucose chains that, in S. polyrhiza, represent only 13% of the cell wall (Figure 2 and Table 1). This confirms previous reports [48, 97]. However, some studies reported duckweed with cellulose contents ranging from 25 to 55% [46, 98], suggesting that cellulose contents may vary up to fivefold. This polymer displays some recalcitrance to hydrolysis, which interferes with biofuel production, but could be bypassed by modifying cellulose crystallinity [99–101]. The low proportion of cellulose in duckweeds compared to the 40% found in land plants [48] may indicate the higher importance of pectins and hemicelluloses for duckweed walls. The higher proportion of pentoses in duckweeds is due to the high levels of apiose and xylose (pectin and hemicellulose, respectively) (Table 1 and Supplemental Table 13). Also, their negative aspect for fermentation makes the increase of cellulose suitable for bioelectricity and bioenergy. However, for this purpose, a deep study in the multigenic family of cellulose synthases is needed, along with evaluating these polymer crosslinks within duckweeds. Despite detecting the scarcity of cellulose in S. polyrhiza cell walls and fully understanding cellulose application of this polymer to bioenergy, more accurate analysis needs to be performed, focusing on this polysaccharide synthesis.

Pectins are responsible for holding together the cellulose-hemicellulose portions in the cell wall and are also found in the middle lamella, junction zone, xylem, and fiber cells [35]. Pectins are galacturonic acid-rich polysaccharides such as homogalacturonans (HG), rhamnogalacturonans (RG), apiogalacturonans (API), and xylogalacturonans (XGA) [102].

The pectin scaffold (α-1,4 linked galacturonic acids) is built from the derivation of UDP-glucuronate, which is synthesized by the oxidation of UDP-Glucose by UDP-glucose dehydrogenase (UGD; EC 1.1.1.22), followed by the epimerization to UDP-Galacturionate by UDP-Glucuronate-4-epimerase (GAE, EC 5.1.3.6) [103, 104] (Figure 1). Galacturonosyltransferase (GAUT; EC 2.4.1.43) catalyzes the transfer from UDP-Galacturionate to homogalacturonans acceptors, generating the polysaccharide chains [105–107]. Duckweeds biomass is pectin-rich [45, 47–49, 108, 109], and here it is reported that pectins represent 49% of the S. polyrhiza cell wall (Figure 2 and Table 1). Some of these pectins
are likely to be linked to the cellulose microfibrils since pectin monosaccharides (apiose and rhamnose) were found on the cellulose-containing residue fractions (Table 1). Similar results were reported by Yadav et al., (2016) [98]; Ge et al., (2012) [48]; Soda et al. (2015) [49]; and Zhao et al. (2014) [44]. This high pectin (and pentoses) content and its possible interlinks to cellulose negatively impact bioethanol production from biomass due to the barrier to hydrolytic enzymes from accessing fermentable sugars. Lionetti et al. (2010) [110] found that the reduction of homogalacturonan's methyl esterification increased the saccharification efficiency of Arabidopsis biomass. Latarullo et al. (2017) [111] reviewed the importance of pectins in cell wall recalcitrance and pointed out the importance of this class of cell wall polysaccharides for bioenergy. These authors proposed that more pectinases should be employed in enzymatic cocktails to provide fermentable sugars for bioethanol production. Because duckweeds display proportionally high amounts of pectins, this group of plants could benefit from pectin modifications to reach higher saccharification levels. However, it is essential to remember that duckweeds are aquatic plants and the pectins they synthesize are probably crucial for the maintenance of growth in this kind of environment. Thus, any strategy to decrease the proportion of pectin in duckweeds will need to consider their possible effects on growth.

UDP-glucuronate also serves as the substrate for the synthesis of UDP-Xylose and UDP-Apiose by UDP-glucuronate decarboxylase and UDP-apiose/UDP-xylose synthase (UXS and AXS, EC 4.1.1.35), which are required for the biosynthesis of the pectins xylogalacturonan and apiogalacturonan [46, 112, 113] (Figure 1). UDP-Xylose also is a substrate for part of xyloglucan and xylans polymers. Xylogalacturonan and apiogalacturonan are derived from HG with the substitution of O-3 or O-2 and O-3 linked with xylose and apiose, respectively [35]. These two pectin classes are the trade-off between Lemnoideae and Wolffioideae subfamilies of duckweed [42, 114], highlighting this as an interesting point for analysis of duckweed cell wall plasticity. The apiose represents 15.4% (49.8 µg.mg⁻¹) of AIR, being the main monosaccharide found in ammonium oxalate (44.3 µg.mg⁻¹) and sodium chlorite (12.2 µg.mg⁻¹) fractions (Table 1), even with a Low relative transcript expression of 0.76 (Table 2). Otherwise, xylose represents 16.1% (51.9 µg.mg⁻¹) of AIR and 5.8% of the ammonium oxalate (6.2 µg.mg⁻¹) and sodium chlorite (5.4 µg.mg⁻¹) fractions (Table 1). The pentoses apiose and xylose are not fermentable by typical Saccharomyces cerevisiae [115]. Currently, genetically modified S. cerevisiae strains can ferment xylose [116–119] but still are not fully employed in industries. Therefore, high levels of apiose and xylose are not yet considered suitable for biofuel production. Taking depletion mutants of A. thaliana AtUXS as an example, it is possible to improve glucose release for saccharification by 18%, together with greater accessibility to xylan's enzymatic breakdown [120]. Xylan contributes to recalcitrance reduction by the polysaccharide length and arrangement with other cell wall polymers, consequently improving biofuel production [120]. On the other hand, apiose is essential for plant development [121, 122]. In duckweeds, apiose is positively related to fast growth and negatively for starch accumulation [42]. Thus, reducing the apiose content hypothetically increases starch levels, which would be beneficial for first-generation bioethanol. The starch content in S. polyrhiza was 3.3% (Figure 1). However, this non-structural carbohydrate modulation can be increased by up to 70% through alterations in growth conditions [123, 124], with no need for biomolecular approaches.

Aiming at high-value cosmetics and drug delivery instead of biofuel, these potential applications of apiogalacturonan could be an alternative for biorefinery [50, 51]. Pectins are used as a gelling and stabilizing agent in the food and cosmetic industries. Besides, some pectin-related molecules can improve human health by reducing cancer [125–128], cholesterol [129], blood glucose levels [130], and stimulate the immune response [131]. Thus, apiogalacturonan
properties should also be investigated for these alternative purposes, and as its biosynthesis is dependent on a unique copy of the *S. polyrhiza* genome, molecular approaches should be interesting.

Fucose is a monosaccharide present in rhamnogalacturonan type-II (RG-II) and xyloglucan. It is derived from GDP-mannose by the action of GDP-mannose-4,6-dehydratase (GMD, EC 4.2.1.47) and GDP-mannose-3,5-epimerase-4-reductase (GER, EC 1.1.1.271) [132] (Figure 1). Duckweeds display a small proportion of fucosylated polysaccharides (Table 2). However, this sugar is essential for RG-II's maintenance [133] and growth [134]. RG-II is the most complex pectin subclass of cell wall polysaccharides, containing an α-1,4-galacturonic acid backbone with several side branches consisting of 12 sugars and over 20 linkages [102, 135]. Otherwise, RG-I consists of a backbone repeat of [α-D-galacturonic acid-1-2-α-1-rhamnose-1,4]ₙ with side chains of α-L-arabinose and β-D-galactose residues linked to the rhamnosyl sugars units [35, 102]. Rhamnose is an exclusive sugar from rhamnogalacturonan. It is synthesized from UDP-Rhamnose through a three-stage reaction involving dehydration, epimerization, and reduction of UDP-Glucose into UDP-Rhamnose by UDP-glucose-4,6-epimerase (RHM, EC. 4.2.1.76) [136] (Figure 1). *SpRHM* relative expression level was Medium (0.91) (Table 2), and rhamnose represented 8.8% of the cell wall monosaccharides (28.36 µg.mg⁻¹ AIR – Table 1). *AtRHM1* overexpression also increased galactose content with a concomitant reduction of glucose in *Arabidopsis* [137]. Notwithstanding, the silencing of *AtRHM1* did not alter the cell wall pattern [137], which may not impact biofuel production. However, a mutation on the *RHM* gene in *A. thaliana* suppressed *leucine-rich repeat extensin* (*Irx*) genes that alter root hair formation [138] and also rhamnose-polysaccharides coordinated some helical distribution of leaves during plant growth [139]. Therefore, altering the *RHM* gene in *S. polyrhiza* would probably change frond (leaf) morphology and affect plant development. Rhamnose is widely used in cosmetics [140], and it may also be responsible for anti-inflammatory [141, 142], antiviral [143], and anti-cancer activity [144]. Thus, rhamnose could also be a niche for applications through an *SpRHM* overexpression focusing on biorefinery.

UDP-Arabinose is the only NDP-sugar that is synthesized in the Golgi lumen in its pyranose (*p*) form by the epimerization of UDP-Xylose by UDP-arabinose-4-epimerase (UAE, EC 5.1.3.5) [34, 145]. UDP-arabinopyranose (*p*) is transported to the cytosol for conversion in the furanose (*f*) form by UDP-arabinose mutase (UAM, EC 5.4.99.30) and then transported back to the Golgi lumen via UDP-Araf transporters [146]. Arabinose is a hexose found in arabinans and as a branching of rhamnogalacturonans and xylans. Arabinans can be hydrolyzed by α-arabinofuranohydrolase (ASD, EC 2.7.1.52), and the free arabinose can be phosphorylated by arabinokinase-1 (ARA1, EC 2.7.1.46) [147]. The biochemical evaluation of *S. polyrhiza* cell wall showed that arabinose constituted 17.3% of the AIR (56 µg.mg⁻¹ AIR) and was found mainly on the pectin-rich fractions (AmnOX - 10.1 µg.mg⁻¹ and Chlorite - 13.7 µg.mg⁻¹) and 0.1 NaOH 1M fractions (5.35 µg.mg⁻¹) (part of hemicellulose fraction) (Table 1). Possibly, arabinose in *S. polyrhiza* is used to form arabinan chains. Hemicellulosic arabinoses are responsible for biomass recalcitrance [95] by interlinks with β-1,4-glucan [148]. Thus, the low incidence of hemicellulosic arabinose makes *S. polyrhiza* suitable for bioenergy purposes.

Hemicelluloses are polysaccharides containing β-1,4-pyranosyl glycosidic bonds in the main chain. They form heteromannans, xyloglucans, heteroxylans, and mixed-linkage glucan with different combinations of arabinose, mannose, xylose, fucose, and glucose monosaccharides [6, 36, 40]. As mentioned previously, xyloglucan is the main hemicellulose of duckweeds [42, 47]. This polysaccharide comprises repetitive units of four β-D-glycosyl residues, three of which containing α-D-xylosyl at the C-6 position, which can present in branches with galactose and fucose [149, 150]. Thus, for
its synthesis, the NDP-sugars UDP-glucose, UDP-fucose, UDP-galactose, and UDP-xylose are necessary. The UDP-
galactose is formed directly from UDP-glucose through UDP-glucose-4-epimerase (UGE, EC 5.1.3.2) [151], and its
phosphorylation can be catalyzed by UDP-glucose hexose 1-phosphate uridyltransferase (GALT, EC 2.7.7.12). The salvage
pathway of galactose involves galactokinase (GALK, EC 2.7.16), which incorporates a phosphate into the free galactose
[26] (Figure 1). UGE can be considered a vital gene for xyloglucan synthesis (branching) and galactans [152]. The
overexpression of AtUGE2 in A. thaliana increased up to 80% of the cell wall galactose levels combined with GalS1
(galactan β-1,4-galactosyltransferase), which is a potential source for fermentable sugar [152]. Zhang et al. (2020) [153]
found a relationship between the lack of galactans with cellulose microfibrils orientation and plant growth interference on
rice. Thus galactans are needed for biomass accumulation. The galactose level in S. polyrhiza was 22% of the cell wall
(74.8 µg.mg⁻¹ AIR – Table 1). The fractionation process demonstrated that the galactose was mainly galactans (AmmOx – 17.4 µg.mg⁻¹ - Table 1) and secondarily as xyloglucans (0.1-4M NaOH – Table 2). Therefore, overexpressing SpUGE
could be favorable for biofuel production. Moreover, the galactose residues are required on xyloglucan for mechanical
strength during growth in A. thaliana [154].

The heteromannans are the most ancient hemicellulose synthesized with the NDP-sugars GDP-Mannose, GDP-
Glucose, and UDP-Galactose [155, 156]. The pathway for mannans synthesis depends directly on fructose-6-phosphate and
involves the enzymes mannose-6-phosphate-isomerase (MPI, EC 5.3.1.8), phosphomannomutase (PMM, EC 5.4.2.8), and
mannose-1-phosphate-guanylyltransferase (MGP, EC 2.7.7.13) [157, 158]. Mannans can occur as linear chains of mannose
that may be interspaced with glucose (glucomannan) and branched with galactose (galactomannan) [7, 159]. Mannans
represent 13.5% of the S. polyrhiza cell wall (43.9 µg.mg⁻¹ AIR - Table 1). A chemical linkage evaluation study determined
that mannose in duckweed is found as glucomannan [47]. Due to this polymer's solubility [160, 161], mannose is found
mainly in ammonium oxalate (6.2 µg.mg⁻¹) and sodium chlorite fractions (3.40 µg.mg⁻¹) (Table 1). Besides that, the
mannan-related transcripts mannose-6-phosphate-isomerase (SpMPI), phosphomannomutase (SpPMM), mannose-1P-
guanylyltransferase (SpMGP), and glucomannan-4β-mannosyltransferase (SpCSLA) levels were the highest levels
identified (Table 2). The pathway to mannans synthesis has a low number of gene copies, except for the glucomannan
synthase (MSR) and glucomannan-4β-mannosyltransferase (CSLA) gene with 21 and 16 paralogs, respectively
(Supplemental Table 1). Hence, the overexpression of the mannose precursors mannose-6-phosphate-isomerase (MPI),
phosphomannomutase (PMM), and mannose-1P-guanylyltransferase (MGP), along with glycosyltransferases involved in
this polysaccharide assembly, may increase the content of mannose in S. polyrhiza cell wall, which could make this plant
a source for nutrition and human-health food supplement [132, 162, 163]. Additionally, for biofuel production, mannose's
high content could increase ethanol yield due to its relatively efficient fermentation by wild S. cerevisiae via the Embden-
Meyerhoff pathway [115].

As fast-growing plants, duckweeds clonal fragmentation and its growth and morphological responses to nutrient
availability and population density [164] might be a source of distinct cell wall compositions. The clonal propagation is
related to the new fronds generated asexually, in which parental fronds can release up to twenty offspring, and these
"daughter" fronds differ phenotypically [165–167]. The daughters' distinct phenotypes along with growth conditions and
nutrient availability could impact the cell wall composition. It is known that biotic and abiotic stresses can alter cell wall
composition and characteristics [168–170]. Thus, a different phenotype makes sense for the cell wall in the offspring due
to the change in the nutritional availability, the reflection of light, stalking, and shadow influence. Besides that, cell wall
phenotype traits and proportions vary according to plant genotype [171–174]. The *S. polyrhiza* cell wall-AIR composition from previous studies has been compared with the ones obtained in this work and unpublished lab data from our lab. They are presented in Supplemental Table 13. The quantitative proportions of the sugars vary according to growth conditions (light intensity, photoperiod, temperature). However, when the data is normalized for comparative purposes of the percentages, a pattern emerges with 20.6% galactose, 17.4% arabinose, 15.5% xylose, 21.9% apiose, 5.4% mannose, 7.5% rhamnose, 9.3% glucose, and 2.4% fucose (Supplemental Table 13 – heatmap average). Therefore, quantitative data must be evaluated individually in each study, considering the plant growth conditions and the cell wall composition. The fermented sugars make up 60.7%, which is suitable for bioenergy applications.

5 Conclusion

The present work contributes to the elucidation of polysaccharides' metabolism in the cell wall and their relevance for industrial applications. Additionally, the determination of the reference genes and family structures in *S. polyrhiza* can contribute to further studies of the duckweed plant family. We found that the distribution of genes throughout the chromosomes may not be random, with clusters of genes that differ according to their functional category.

Furthermore, 190 genes were found in the genome of *S. polyrhiza* associated with pathways related to the formation of the cell walls and starch. We selected 38 of these genes to study expression in developing plants of *S. polyrhiza*. Their expression corroborated the composition of the sugars found in the plant. We conclude that *S. polyrhiza* carbohydrates display potential applications as adjuvants, cosmetics, food supplemental, stabilizing and gelling agents, and biofuels. The non-structural carbohydrates can be quickly accumulated by growth conditions, not needing a biomolecular approach. On the other hand, the cell wall could be modified to produce more galactose, mannose, and glucose, hexoses that are more readily fermented. Simultaneously, some pectin-related genes could be depleted to facilitate their conversion into bioproducts.

According to the mapping performed in this work, *S. polyrhiza* could be suitable for future genetic modifications capable of turning biomass more ideal for bioenergy production. However, any change in cell wall polymer proportions should be carefully planned to assess the trade-offs between the benefits for industry and the plant's growth capacity, directly related to the composition of the cell walls in duckweeds.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

MSB, D.P., AG, and E.L. planned the work. D.P. performed the biochemical analysis. BVN, D.P., and M.Z. did the bioinformatics work. A.G., BVN, and D.P. analyzed the data. A.G., BVN, D.P., E.L., and MSB wrote the manuscript.

8 Funding

This work was supported by the Instituto Nacional de Ciência e Tecnologia do Bioetanol – INCT do Bioetanol (FAPESP 2014/50884-5 and CNPq 465319/2014-9). D.P. (CAPES 88882.377113/2019-1). A.G. (FAPESP 2019/13936-


The support by a travel grant to E.L. by the U.S. Fulbright-Brazil Scholar Mobility Program (2014) to travel to the laboratory of M.B. to jump-start this project in 2014-2015 is gratefully acknowledged. MJPF and MB are fellow researchers of CNPq. The authors thank the Fulbright Foundation for the fellowship to E.L.

9 Acknowledgments

We thank Dr. Eny lochevet Segal Floh for the use of laboratory facilities.

10 References

1. Buckeridge MS (2017) The evolution of the Glycomic Codes of extracellular matrices. BioSystems 164:112–120. https://doi.org/10.1016/j.biosystems.2017.10.003

2. Gibeaut DM, Carpita NC (1994) Biosynthesis of plant cell wall polysaccharides. FASEB J 8:904–915. https://doi.org/0892-663814008-0904101.50

3. Himmel ME (2009) Biomass Recalcitrance: Deconstructing the Plant Cell Wall for Bioenergy. Blackwell Publishing Ltd.

4. Pogorelko G, Lionetti V, Bellincampi D, Zabotina O (2013) Cell wall integrity: Targeted post-synthetic modifications to reveal its role in plant growth and defense against pathogens. Plant Signal Behav 8:e25435. https://doi.org/10.4161/psb.25435

5. Popper ZA, Fry SC (2004) Primary cell wall composition of pteridophytes and spermatophytes. New Phytol 164:165–174. https://doi.org/10.1111/j.1469-8137.2004.01146.x

6. O'Neill MA, York WS (2003) The Composition and Structure of Plant Primary Cell Walls. Annu Plant Rev online 8:1–54. https://doi.org/10.1002/9781119312994.apr0067

7. Buckeridge MS (2010) Seed Cell Wall Storage Polysaccharides: Models to Understand Cell Wall Biosynthesis and Degradation. Plant Physiol 154:1017–23. https://doi.org/10.1104/pp.110.158642

8. Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3:1–30. https://doi.org/10.1111/j.1365-313X.1993.tb00007.x

9. Somerville C, Bauer S, Brininstool G, et al (2004) Toward a systems approach to understanding plant cell walls. Science (80-) 306:2206–2211. https://doi.org/10.1126/science.1102765

10. Silva GB, Ionashiro M, Carrara TB, et al (2011) Cell wall polysaccharides from fern leaves: Evidence for a mannan-rich Type III cell wall in Adiantum raddianum. Phytochemistry 72:2352–2360. https://doi.org/10.1016/j.phytochem.2011.08.020

11. Mcqueen-mason S, Cosgrove DJ (1994) Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. 91:6574–6578

12. Burton RA, Gidley MJ, Fincher GB (2010) Heterogeneity in the chemistry, structure and function of plant cell walls. Nat Chem Biol 6:724–732. https://doi.org/10.1038/nchembio.439

13. Gilbert HJ (2010) The biochemistry and structural biology of plant cell wall deconstruction. Plant Physiol 153:444–55. https://doi.org/10.1104/pp.110.156646

14. Kalluri UC, Keller M (2010) Bioenergy research: a new paradigm in multidisciplinary research. J R Soc Interface
15. Cosgrove DJ (2018) Diffuse Growth of Plant Cell Walls I. 176:16–27. https://doi.org/10.1104/pp.17.01541

16. Waldron KW, Parker ML, Smith AC (2003) Plant Cell Walls and Food Quality. Compr Rev Food Sci Food Saf 2:128–146. https://doi.org/10.1111/j.1541-4337.2003.tb00019.x

17. Schürch C, Blum P, Zülli F (2008) Potential of plant cells in culture for cosmetic application. In: Phytochemistry Reviews. Springer, pp 599–605

18. Burton RA, Fincher GB (2014) Plant cell wall engineering: Applications in biofuel production and improved human health. Curr. Opin. Biotechnol. 26:79–84

19. Verbančič J, Lunn JE, Stitt M, Persson S (2017) Carbon supply and the regulation of cell wall synthesis. Mol Plant 11:75–94. https://doi.org/10.1016/j.molp.2017.10.004

20. Chapin FS, Schulze E-D, Mooney HA (1990) THE ECOLOGY AND ECONOMICS OF STORAGE IN PLANTS

21. Hoch G, Richter A, Körner C (2003) Non-structural carbon compounds in temperate forest trees. Cell 1067–1081

22. Quentin AG, Pinkard EA, Ryan MG, et al (2015) Non-structural carbohydrates in woody plants compared among laboratories. Tree Physiol 35:1146–1165. https://doi.org/10.1093/treephys/tpv073

23. Smith AM, Stitt M (2007) Coordination of carbon supply and plant growth. Plant, Cell Environ 30:1126–1149. https://doi.org/10.1111/j.1365-3040.2007.01708.x

24. Smith AM, Kruger NJ, Lunn JE (2012) Source of sugar nucleotides for starch and cellulose synthesis. Pnas 109:E776. https://doi.org/10.1073/pnas.1200878109

25. Zeeman SC, Smith SM, Smith AM (2007) The diurnal metabolism of leaf starch. Biochem J 401:13–28. https://doi.org/10.1042/BJ20061393

26. Bar-Peled M, O'Neill MA (2011) Plant Nucleotide Sugar Formation, Interconversion, and Salvage by Sugar Recycling*. Annu Rev Plant Biol 62:127–155. https://doi.org/10.1146/annurev-arplant-042110-103918

27. Barnes WJ, Anderson CT (2018) Release, Recycle, Rebuild: Cell-Wall Remodeling, Autodegradation, and Sugar Salvage for New Wall Biosynthesis during Plant Development. Mol. Plant 11:31–46

28. Jang JC, León P, Zhou L, Sheen J (1997) Hexokinase as a sugar sensor in higher plants. Plant Cell 9:5–19. https://doi.org/10.1105/tpc.9.1.5

29. Sturm A, Tang G (1999) Plants Are Crucial for Development. October 4:401–407

30. Koch K (2004) Sucrose metabolism: Regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr Opin Plant Biol 7:235–246. https://doi.org/10.1016/j.pbi.2004.03.014

31. Henry Christopher J van R, Van den Ende W (2018) UDP-glucose: A potential signaling molecule in plants? Front Plant Sci 8:6–11. https://doi.org/10.3389/fpls.2017.02230

32. Kleczkowski LA, Kunz S, Wilczynska M (2010) Mechanisms of UDP-glucose synthesis in plants. CRC Crit Rev Plant Sci 29:191–203. https://doi.org/10.1080/07352689.2010.483578

33. Figueroa CM, Lunn JE, Iglesias AA (2021) Nucleotide-sugar metabolism in plants: the legacy of Luis F. Leloir. J Exp Bot 72:4053–4067. https://doi.org/10.1093/jxb/erab109

34. Seifert GJ (2004) Nucleotide sugar interconversions and cell wall biosynthesis: How to bring the inside to the
35. Mohnen D (2008) Pectin structure and biosynthesis. Curr Opin Plant Biol 11:266–277. https://doi.org/10.1016/j.pbi.2008.03.006

36. Pauly M, Gille S, Liu L, et al (2013) Hemicellulose biosynthesis. Planta 238:627–642. https://doi.org/10.1007/s00425-013-1921-1

37. Somerville C (2006) Cellulose Synthesis in Higher Plants. Annu Rev Cell Dev Biol 22:53–78. https://doi.org/10.1146/annurev.cellbio.22.022206.160206

38. Carpita NC (2011) Update on Mechanisms of Plant Cell Wall Biosynthesis: How Plants Make Cellulose and Other (1->4)-D-Glycans. Plant Physiol 155:171–184. https://doi.org/10.1104/pp.110.163360

39. Bar-Peled M, O’Neill MA (2011) Plant Nucleotide Sugar Formation, Interconversion, and Salvage by Sugar Recycling*. Annu Rev Plant Biol 62:127–155. https://doi.org/10.1146/annurev-arplant-042110-103918

40. de Souza AP, Leite DCCC, Pattathil S, et al (2013) Composition and Structure of Sugarcane Cell Wall Polysaccharides: Implications for Second-Generation Bioethanol Production. Bioenergy Res 6:564–579. https://doi.org/10.1007/s12155-012-9268-1

41. Houston K, Tucker MR, Chowdhury J, et al (2016) The plant cell wall: A complex and dynamic structure as revealed by the responses of genes under stress conditions. Front Plant Sci 7:1–18. https://doi.org/10.3389/fpls.2016.00984

42. Pagliuso D, Grandis A, Igarashi ESES, et al (2018) Correlation of apiose levels and growth rates in duckweeds. Front Chem 6:1–10. https://doi.org/10.3389/fchem.2018.00291

43. Landolt E (1992) Lemnaceae Duckweed Family. J. Arizona-Nevada Acad. Sci. 26:10–14

44. Zhao X, Moates GKK, Wellner N, et al (2014) Chemical characterisation and analysis of the cell wall polysaccharides of duckweed (Lemna minor). Carbohydr Polym 111:410–418. https://doi.org/10.1016/j.carbpol.2014.04.079

45. Pagliuso D, Grandis A, Lam E, Buckeridge MS (2020) High Saccharification, Low Lignin, and High Sustainability Potential Make Duckweeds Adequate as Bioenergy Feedstocks. Bioenergy Res 1–11. https://doi.org/10.1007/s12155-020-10211-x

46. Longland JM, Fry SC, Trewavas AJ (1989) Developmental Control of Apiogalacturonan Biosynthesis and UDP-Apiose Production in a Duckweed1

47. Sowinski EE, Gilbert S, Lam E, et al (2019) Linkage structure of cell-wall polysaccharides from three duckweed species. Carbohydr Polym 223:115119. https://doi.org/10.1016/j.carbpol.2019.115119

48. Ge X, Zhang N, Phillips GC, Xu J (2012) Growing Lemma minor in agricultural wastewater and converting the duckweed biomass to ethanol. Bioresour Technol 124:485–488. https://doi.org/10.1016/j.biortech.2012.08.050

49. Soda S, Ohchi T, Piradee J, et al (2015) Duckweed biomass as a renewable biorefinery feedstock: Ethanol and succinate production from Wolffia globosa. Biomass and Bioenergy 81:364–368. https://doi.org/10.1016/j.biombioe.2015.07.020

50. Popov S V, Günter EA, Markov PA, et al (2006) Adjuvant effect of lemmam, pectic polysaccharide of callus culture of Lemma minor L. at oral administration. Immunopharmacol Immunotoxicol 28:141–152. https://doi.org/10.1080/08923970600626098

51. Filbry A, Siefken W, Breitenbach U, et al (2007) Cosmetic or dermatological preparations for skin care and cleaning, containing apiogalacturonan compounds or extracts thereof from seaweed, together with emulsifiers
52. Ziegler P, Adelmann K, Zimmer S, et al (2014) Relative in vitro growth rates of duckweeds (Lemnaceae) - the most rapidly growing higher plants. Plant Biol (Stuttg) 17:1–9. https://doi.org/10.1111/plb.12184

53. Cao HX, Fourounjian P, Wang W (2018) The Importance and Potential of Duckweeds as a Model and Crop Plant for Biomass-Based Applications and Beyond. In: Handbook of Environmental Materials Management. Springer International Publishing, pp 1–16

54. Hillman WS, Culley Jr. DD (1978) The Uses of Duckweed: The rapid growth, nutritional value, and high biomass productivity of these floating plants suggest their use in water treatment, as feed crops, and in energy-efficient farming. Am Sci 66:442–451. https://doi.org/10.2307/27848752

55. Oron G, Wildschut LR, Porath D (1985) Waste water recycling by duckweed for protein production and effluent renovation. Water Sci Technol 17:803–817. https://doi.org/10.2166/wst.1985.0181

56. Zirschky J, Reed SC (1988) The Use of Duckweed for Wastewater Treatment. Wiley

57. Körner S, Vermaat JE, Veenstra S (2003) The Capacity of Duckweed to Treat Wastewater. J Environ Qual 32:1583–1590. https://doi.org/10.2134/jeq2003.1583

58. Ceschin S, Crescenzi M, Iannelli MA (2020) Phytoremediation potential of the duckweeds Lemna minuta and Lemma minor to remove nutrients from treated waters. Environ Sci Pollut Res 1–9. https://doi.org/10.1007/s11356-020-08045-3

59. Michael TP, Bryant D, Gutierrez R, et al (2017) Comprehensive definition of genome features in Spirodela polyrhiza by high-depth physical mapping and short-read DNA sequencing strategies. Plant J 89:617–635. https://doi.org/10.1111/tpj.13400

60. Carpita NC, Tierney M, Campbell M (2001) Molecular biology of the plant cell wall: searching for the genes that define structure, architecture and dynamics. Plant Cell Walls 47:1–5. https://doi.org/10.1007/978-94-010-0668-2_1

61. Amaral LIV do, Gaspar M, Felix Costa PM, et al (2007) Novo método enzimático rápido e sensível de extração e dosagem de amido em materiais vegetais

62. Carpita NC (1984) Fractionation of hemicelluloses from maize cell walls with increasing concentrations of alkali. Phytochemistry 23:1089–1093. https://doi.org/10.1016/S0031-9422(00)82615-1

63. Stanke M, Morgenstern B (2005) AUGUSTUS: A web server for gene prediction in eukaryotes that allows user-defined constraints. Nucleic Acids Res 33:465–467. https://doi.org/10.1093/nar/gki458

64. Altschul SF, Gish W, Miller W, et al (1990) Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

65. Wolfe D, Dudek S, Ritchie MD, Pendergrass SA (2013) Visualizing genomic information across chromosomes with PhenoGram. BioData Min 6:18. https://doi.org/10.1186/1756-0381-6-18

66. Pruitt KD, Tatusova T, Maglott DR (2005) NCBI Reference Sequence (RefSeq): A curated non-redundant sequence database of genomes, transcripts and proteins. Nucleic Acids Res 33:. https://doi.org/10.1093/nar/gki025

67. Apweiler R, Attwood TK, Bairoch A, et al (2000) InterPro-an integrated documentation resource for protein families, domains and functional sites. Bioinformatics 16:1145–1150. https://doi.org/10.1093/bioinformatics/16.12.1145

68. Brunner AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol 4:14. https://doi.org/10.1186/1471-2229-4-14

69. Iskandar HM, Simpson RS, Casu RE, et al (2004) Comparison of reference genes for quantitative real-time
polymerase chain reaction analysis of gene expression in sugarcane. Plant Mol. Biol. Report. 22:325–337

70. Perini P, Pasquali G, Margis-Pinheiro M, et al (2014) Reference genes for transcriptional analysis of flowering and fruit ripening stages in apple (Malus × domestica Borkh.). Mol Breed 34:829–842. https://doi.org/10.1007/s11032-014-0078-3

71. Elbl P, Navarro B V., De Oliveira LF, et al (2015) Identification and evaluation of reference genes for quantitative analysis of brazilian pine (Araucaria angustifolia Bertol. Kuntze) gene expression. PLoS One 10:1–15. https://doi.org/10.1371/journal.pone.0136714

72. Wang W, Li R, Zhu Q, et al (2016) Transcriptomic and physiological analysis of common duckweed Lemna minor responses to NH4+ toxicity. BMC Plant Biol 16:1–13. https://doi.org/10.1186/s12870-016-0774-8

73. Czechowski T, Stitt M, Altmann T, et al (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in arabidopsis. Plant Physiol. 139:5–17

74. Expósito-Rodríguez M, Borges AA, Borges-Pérez A, Pérez JA (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol 8:1–12. https://doi.org/10.1186/1471-2229-8-131

75. Cruz F, Kalaoun S, Nobile P, et al (2009) Evaluation of coffee reference genes for relative expression studies by quantitative real-time RT-PCR. Mol Breed 23:607–616. https://doi.org/10.1007/s11032-009-9259-x

76. Hu R, Fan C, Li H, et al (2009) Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. BMC Mol Biol 10:93. https://doi.org/10.1186/1471-2199-10-93

77. Artico S, Nardeli SM, Brilhante O, et al (2010) Identification and evaluation of new reference genes in Gossypium hirsutum for accurate normalization of real-time quantitative RT-PCR data. BMC Plant Biol 10:49. https://doi.org/10.1186/1471-2229-10-49

78. Narsai R, Ivanova A, Ng S, Whelan J (2010) Defining reference genes in Oryza sativa using organ, development, biotic and abiotic transcriptome datasets. BMC Plant Biol 10:56. https://doi.org/10.1186/1471-2229-10-56

79. Mafra V, Kubo KS, Alves-Ferreira M, et al (2012) Reference Genes for Accurate Transcript Normalization in Citrus Genotypes under Different Experimental Conditions. PLoS One 7:e31263. https://doi.org/10.1371/journal.pone.0031263

80. de Vega-Bartol JJ, Santos RR, Simões M, Miguel CM (2013) Normalizing gene expression by quantitative PCR during somatic embryogenesis in two representative conifer species: Pinus pinaster and Picea abies. Plant Cell Rep 32:715–729. https://doi.org/10.1007/s00299-013-1407-4

81. Bustin SA, Benes V, Garson JA, et al (2009) The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622. https://doi.org/10.1373/clinchem.2008.112797

82. Vandesompele J, De Preter K, Pattyn ilip, et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes

83. Tavares EQPP, Buckeridge MS (2015) Do plant cell walls have a code? Plant Sci 241:286–294. https://doi.org/10.1016/j.plantsci.2015.10.016

84. Tao X, Fang Y, Xiao Y, et al (2013) Comparative transcriptome analysis to investigate the high starch accumulation of duckweed (Landoltia punctata) under nutrient starvation. Biotechnol Biofuels 6:72. https://doi.org/10.1186/1754-6834-6-72

85. Tao X, Fang Y, Huang MJ, et al (2017) High flavonoid accompanied with high starch accumulation triggered by nutrient starvation in bioenergy crop duckweed (Landoltia punctata). BMC Genomics 18:166. https://doi.org/10.1186/s12864-017-3559-z
86. Wang W, Wu Y, Messing J (2014) RNA-Seq transcriptome analysis of Spirodela dormancy without reproduction. BMC Genomics 15:60. https://doi.org/10.1186/1471-2164-15-60

87. Van Hoeck A, Horemans N, Nauts R, et al (2017) Lemna minor plants chronically exposed to ionising radiation: RNA-seq analysis indicates a dose rate dependent shift from acclimation to survival strategies. Plant Sci 257:84–95. https://doi.org/10.1016/j.plantsci.2017.01.010

88. Yu C, Zhao X, Qi G, et al (2017) Integrated analysis of transcriptome and metabolites reveals an essential role of metabolic flux in starch accumulation under nitrogen starvation in duckweed. Biotechnol Biofuels 10:167. https://doi.org/10.1186/s13068-017-0851-8

89. Xu H, Yu C, Xia X, et al (2018) Comparative transcriptome analysis of duckweed (Landoltia punctata) in response to cadmium provides insights into molecular mechanisms underlying hyperaccumulation. Chemosphere 190:154–165. https://doi.org/10.1016/j.chemosphere.2017.09.146

90. Kuehdorf K, Jetschke G, Ballani L, Appenroth KJ (2014) The clonal dependence of turion formation in the duckweed Spirodela polyrhiza—an ecogeographical approach. Physiol Plant 150:46–54. https://doi.org/10.1111/ppl.12065

91. Michael TP, Bryant D, Gutierrez R, et al (2017) Comprehensive definition of genome features in Spirodela polyrhiza by high-depth physical mapping and short-read DNA sequencing strategies. Plant J 89:617–635. https://doi.org/10.1111/tpj.13400

92. Serba DD, Sykes RW, Gjersing EL, et al (2016) Cell Wall Composition and Underlying QTL in an F1 Pseudo-Testcross Population of Switchgrass. Bioenergy Res 9:836–850. https://doi.org/10.1007/s12155-016-9733-3

93. Nawaz MA, Rehman HM, Imtiaz M, et al (2017) Systems Identification and Characterization of Cell Wall Reassembly and Degradation Related Genes in Glycine max (L.) Merill, a Bioenergy Legume. Sci Rep 7:1–16. https://doi.org/10.1038/s41598-017-11495-4

94. Grandis A, de Souza AP, Tavares EQP, Buckeridge MS (2014) Using natural plant cell wall degradation mechanisms to improve second generation bioethanol. In: Plants and BioEnergy. Springer New York, pp 211–230

95. Tavares EQPP, De Souza AP, Buckeridge MS (2015) How endogenous plant cell-wall degradation mechanisms can help achieve higher efficiency in saccharification of biomass. J Exp Bot 66:4133–4143. https://doi.org/10.1093/jxb/erv171

96. Lei L, Li S, Gu Y (2012) Cellulose synthase complexes: Composition and regulation. Front Plant Sci 3:1–6. https://doi.org/10.3389/fpls.2012.00075

97. Bayrakci AG, Koçar G (2014) Second-generation bioethanol production from water hyacinth and duckweed in Izmir: A case study. Renew Sustain Energy Rev 30:306–316. https://doi.org/10.1016/j.rser.2013.10.011

98. Yadav D, Barbora L, Bora D, et al (2016) An assessment of duckweed as a potential lignocellulosic feedstock for biogas production International Biodeterioration & Biodegradation An assessment of duckweed as a potential lignocellulosic feedstock for biogas production. https://doi.org/10.1016/j.ibiod.2016.09.007

99. Dadi AP, Schall CA, Varanasi S (2007) Mitigation of cellulose recalcitrance to enzymatic hydrolysis by ionic liquid pretreatment. In: Applied Biochemistry and Biotechnology. pp 407–421

100. HARRIS D, STORK J, DEBOLT S (2009) Genetic modification in cellulose-synthase reduces crystallinity and improves biochemical conversion to fermentable sugar. GCB Bioenergy 1:51–61. https://doi.org/10.1111/j.1757-1707.2009.01000.x

101. Harris DM, Corbin K, Wang T, et al (2012) Cellulose microfibril crystallinity is reduced by mutating C-terminal transmembrane region residues CES1A903V and CES1A3 T942I of cellulose synthase. Proc Natl Acad Sci U S A 109:4098–4103. https://doi.org/10.1073/pnas.1200352109
102. Caffall KH, Mohnen D (2009) The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydr Res 344:1879–1900. https://doi.org/10.1016/j.carres.2009.05.021

103. Gu X, Bar-Peled M (2004) The biosynthesis of UDP-galacturonic acid in plants. Functional cloning and characterization of Arabidopsis UDP-D-glucuronic acid 4-epimerase. Plant Physiol 136:4256–4264. https://doi.org/10.1104/pp.104.052365

104. Mølhøj M, Verma R, Reiter WD (2004) The biosynthesis of D-galacturionate in plants. Functional cloning and characterization of a membrane-anchored UDP-D-glucuronate 4-epimerase from arabidopsis. Plant Physiol 135:1221–1230. https://doi.org/10.1104/pp.104.043745

105. Doong Ron-Lou, Liljebjelke K, Fralish G, et al (1995) Cell-free synthesis of pectin. Identification and partial characterization of polygalacturonate 4-alpha-galacturonosyltransferase and its products from membrane preparations of tobacco cell-suspension cultures. Plant Physiol 109:141–152. https://doi.org/10.1104/pp.109.1.141

106. Atmodjo MA, Sakuragi Y, Zhu X, et al (2011) Galacturonosyltransferase (GAUT)1 and GAUT7 are the core of a plant cell wall pectin biosynthetic homogalacturonan:galacturonosyltransferase complex. Proc Natl Acad Sci U S A 108:20225–20230. https://doi.org/10.1073/pnas.1112816108

107. Sterling JD, Atmodjo MA, Inwood SE, et al (2006) Functional identification of an Arabidopsis pectin biosynthetic homogalacturonan galacturonosyltransferase. Proc Natl Acad Sci U S A 103:5236–5241. https://doi.org/10.1073/pnas.0600120103

108. Oron G (1994) Duckweed culture for wastewater renovation and biomass production. Agric Water Manag 26:27–40. https://doi.org/10.1016/0378-3774(94)90022-1

109. Su H, Zhao Y, Jiang J, et al (2014) Use of duckweed (Landoltia punctata) as a fermentation substrate for the production of higher alcohols as biofuels. Energy and Fuels 28:3206–3216. https://doi.org/10.1021/ef500335h

110. Lionetti V, Francocci F, Ferrari S, et al (2010) Engineering the cell wall by reducing de-methyl-esterified homogalacturonan improves saccharification of plant tissues for bioconversion. Proc Natl Acad Sci 107:616–621. https://doi.org/10.1073/pnas.0907549107

111. Latarullo MBGG, Tavares EQPP, Maldonado GP, et al (2016) Pectins, Endopolygalacturonases, and Bioenergy. Front Plant Sci 7:1–7. https://doi.org/10.3389/fpls.2016.01401

112. Mølhøj M, Verma R, Reiter WD (2003) The biosynthesis of the branched-chain sugar D-apiose in plants: functional cloning and characterization of a UDP-D-apiose/UDP-D-xylose synthase from Arabidopsis. Plant J 35:1781–1791. https://doi.org/10.1046/j.1365-313X.2003.01841.x

113. Choi SH, Mansoorabadi SO, Liu YN, et al (2012) Analysis of UDP-D-apiose/UDP-D-xylose synthase-catalyzed conversion of UDP-D-apiose phosphoryl to UDP-D-xylose phosphonate: Implications for a retroaldol-aldol mechanism. J Am Chem Soc 134:13946–13949. https://doi.org/10.1021/ja305322x

114. Avci U, Peña MJ, O’Neill MA (2018) Changes in the abundance of cell wall apiogalacturonan and xylogalacturonan and conservation of rhamnogalacturonan II structure during the diversification of the Lemnoideae. Planta 247:953–971. https://doi.org/10.1007/s00425-017-2837-y

115. van Maris AJAA, Abbott DA, Bellissimi E, et al (2006) Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: Current status. Antonie van Leeuwenhoek, Int J Gen Mol Microbiol 90:391–418. https://doi.org/10.1007/s10482-006-9085-7

116. Kötter P, Ciriacy M (1993) Xylose fermentation by Saccharomyces cerevisiae. Appl Microbiol Biotechnol 38:776–783. https://doi.org/10.1007/BF00167144

117. Karhumaa K, Sanchez RG, Hahn-Hägerdal B, Gorwa-Grauslund MF (2007) Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant
818  Saccharomyces cerevisiae. Microb Cell Fact 6:1–10. https://doi.org/10.1186/1475-2859-6-5

819 118. Bettiga M, Bengtsson O, Hahn-Hägerdal B, Gorwa-Grauslund MF (2009) Arabinose and xylose fermentation by recombinant Saccharomyces cerevisiae expressing a fungal pentose utilization pathway. Microb Cell Fact 8:40. https://doi.org/10.1186/1475-2859-8-40

820 119. Moysés DN, Reis VCB, de Almeida JRM, et al (2016) Xylose fermentation by saccharomyces cerevisiae: Challenges and prospects. Int J Mol Sci 17:1–18. https://doi.org/10.3390/ijms17030207

821 120. Kuang B, Zhao X, Zhou C, et al (2016) Role of UDP-Glucuronic Acid Decarboxylase in Xylan Biosynthesis in Arabidopsis. Mol Plant 9:1119–1131. https://doi.org/10.1016/j.molp.2016.04.013

822 121. Ahn JW, Verma R, Kim M, et al (2006) Depletion of UDP-D-apioside/UDP-D-xylose synthases results in rhamnogalacturonan-II deficiency, cell wall thickening, and cell death in higher plants. J Biol Chem 281:13708–13716. https://doi.org/10.1074/jbc.M512403200

823 122. Zhao X, Ebert B, Zhang B, et al (2020) UDP-Api/UDP-Xyl synthases affect plant development by controlling the content of UDP-Api to regulate the RG-II-borate complex. Plant J 1–16. https://doi.org/10.1111/tjp.14921

824 123. Xiao Y, Fang Y, Jin Y, et al (2013) Culturing duckweed in the field for starch accumulation. Ind Crops Prod 48:183–190. https://doi.org/10.1016/j.indcrop.2013.04.017

825 124. Yin Y, Yu C, Yu L, et al (2015) The influence of light intensity and photoperiod on duckweed biomass and starch accumulation for bioethanol production. Bioresearch Technol 187:84–90. https://doi.org/10.1016/j.biortech.2015.03.097

826 125. Salman H, Bergman M, Djaldetti M, et al (2008) Citrus pectin affects cytokine production by human peripheral blood mononuclear cells. Biomed Pharmacother 62:579–582. https://doi.org/10.1016/j.biopharma.2008.07.058

827 126. Leclere L, Cutsem P Van, Michiels C (2013) Anti-cancer activities of pH- or heat-modified pectin. Front Pharmacol 4 OCT:128. https://doi.org/10.3389/fphar.2013.00128

828 127. Zhang W, Xu P, Zhang H (2015) Pectin in cancer therapy: A review. Trends Food Sci. Technol. 44:258–271

829 128. Jenkins DJA, Newton C, Leeds AR, Cummings JH (1975) EFFECT OF PECTIN, GUAR GUM, AND WHEAT FIBRE ON SERUM-CHOLESTEROL. Lancet 305:1116–1117. https://doi.org/10.1016/S0140-6736(75)92503-9

830 129. Jenkins DJA, Leeds AR, Gassull MA, et al (1977) Decrease in postprandial insulin and glucose concentration by guar and pectin. Ann Intern Med 86:20–23. https://doi.org/10.1036/0003-4819-86-1-20

831 130. Popov S V., Ovodov YS (2013) Polypotency of the immunomodulatory effect of pectins. Biochem 78:823–835. https://doi.org/10.1134/s0006297913070134

832 131. Nakayama KI, Maeda Y, Jigami Y (2003) Interaction of GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase with GDP-mannose-4,6-dehydratase stabilizes the enzyme activity for formation of GDP-fucose from GDP-mannose. Glycobiology 13:673–680. https://doi.org/10.1093/glycob/cwg099

833 132. Reuhs BL, Glenn J, Stephens SB, et al (2004) L-galactose replaces L-fucose in the pectic polysaccharide rhamnogalacturonan II synthesized by the L-fucose-deficient mru1 Arabidopsis mutant. Planta 219:147–157. https://doi.org/10.1007/s00026-004-1205-x

834 133. Reiter W-D, Chapple C, Somerville CR (1997) Mutants of Arabidopsis thaliana with altered cell wall polysaccharide composition. Plant J 12:335–345. https://doi.org/10.1046/j.1365-313X.1997.12020335.x

835 134. Bar-Peled M, Urbanowicz BR, O’Neill MA, O’Neill MA (2012) The synthesis and origin of the pectic wall polysaccharides in Arabidopsis.
polysaccharide rhamnogalacturonan II - Insights from nucleotide sugar formation and diversity. Front Plant Sci 3:1–12. https://doi.org/10.3389/fpls.2012.00092

136. Reiter W-D, Vanzin GF (2001) Molecular genetics of nucleotide sugar interconversion pathways in plants. In: Plant Cell Walls. Springer Netherlands, pp 95–113

137. Wang J, Ji Q, Jiang L, et al (2009) Overexpression of a cytosol-localized rhamnose biosynthesis protein encoded by Arabidopsis RHM1 gene increases rhamnose content in cell wall. Plant Physiol Biochem 47:86–93. https://doi.org/10.1016/j.plaphy.2008.10.011

138. Diet A, Link B, Seifert GI, et al (2006) The Arabidopsis root hair cell wall formation mutant lrx1 is suppressed by mutations in the RHM1 gene encoding a UDP-L-Rhamnose synthase. Plant Cell 18:1630–1641. https://doi.org/10.1105/tpc.105.038653

139. Saffer AM, Carpita NC, Irish VF (2017) Rhamnose-Containing Cell Wall Polymers Suppress Helical Plant Growth Independently of Microtubule Orientation. Curr Biol 27:2248-2259.e4. https://doi.org/10.1016/j.cub.2017.06.032

140. Pageon H, Azouaoui A, Zucchi H, et al (2019) Potentially beneficial effects of rhamnose on skin ageing: an in vitro and in vivo study. Int J Cosmet Sci 41:213–220. https://doi.org/10.1111/ics.12523

141. Rho HS, Ghimeray AK, Yoo DS, et al (2011) Kaempferol and kaempferol rhamnosides with depigmenting and anti-inflammatory properties. Molecules 16:3338–3344. https://doi.org/10.3390/molecules16043338

142. Lee SH, Ko CI, Ahn G, et al (2012) Molecular characteristics and anti-inflammatory activity of the fucoidan extracted from Ecklonia cava. Carbohydr Polym 89:599–606. https://doi.org/10.1016/j.carbpol.2012.03.056

143. Choi HJ, Song JH, Park KS, Kwon DH (2009) Inhibitory effects of quercetin 3-rhamnoside on influenza A virus replication. Eur J Pharm Sci 37:329–333. https://doi.org/10.1016/j.ejps.2009.03.002

144. Tomšík P, Soukup T, Cermakova E, et al (2011) L-rhamnose and L-fucose suppress cancer growth in mice. Cent Eur J Biol 6:1–9. https://doi.org/10.2478/s11535-010-0087-0

145. Burget EG, Verma R, Molhøj M, Reiter W (2003) The Biosynthesis of L -Arabinose in Plants : Molecular Cloning and Characterization of a Golgi-Localized UDP- D -Xylose 4-Epimerase Encoded by the MUR4 Gene of Arabidopsis. Plant Cell 15:523–531. https://doi.org/10.1105/tpc.008425.response

146. Rautengarten C, Birdseye D, Pattathil S, et al (2017) The elaborate route for UDP-arabinose delivery into the Golgi of plants. Proc Natl Acad Sci 114:4261–4266. https://doi.org/10.1073/pnas.1701894114

147. Behmüller R, Kavkova E, Düh S, et al (2016) The role of arabinokinase in arabinose toxicity in plants. Plant J 87:376–390. https://doi.org/10.1111/tpj.13206

148. Li F, Zhang M, Guo K, et al (2015) High-level hemicellulosic arabinose predominate affects lignocellulose crystallinity for genetically enhancing both plant lodging resistance and biomass enzymatic digestibility in rice mutants. Plant Biotechnol J 13:514–525. https://doi.org/10.1111/pbi.12276

149. Fry SC (1989) The structure and function of xyloglucan. J Exp Bot 40:1–11

150. Scheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol 61:263–289. https://doi.org/10.1146/annurev-arplant-042809-112315

151. Rösti J, Barton CJ, Albrecht S, et al (2007) UDP-glucose 4-epimerase isoforms UGE2 and UGE4 cooperate in providing UDP-galactose for cell wall biosynthesis and growth of Arabidopsis thaliana. Plant Cell 19:1565–1579. https://doi.org/10.1105/tpc.106.049619

152. Gondolf VM, Stoppel R, Ebert B, et al (2014) A gene stacking approach leads to engineered plants with highly increased galactan levels in Arabidopsis. BMC Plant Biol 14:344. https://doi.org/10.1186/s12870-014-0344-x
153. Zhang R, Hu H, Wang Y, et al (2020) A novel rice fragile culm 24 mutant encodes a UDP-glucose epimerase that affects cell wall properties and photosynthesis. J Exp Bot 1–14. https://doi.org/10.1093/jxb/eraa044

154. Peña MJ, Ryden P, Madson M, et al (2004) The Galactose Residues of Xyloglucan Are Essential to Maintain Mechanical Strength of the Primary Cell Walls in Arabidopsis during Growth. Plant Physiol 134:443–451. https://doi.org/10.1104/pp.103.027508

155. Lechat H, Amat M, Mazoyer J, et al (2000) STRUCTURE AND DISTRIBUTION OF GLUCOMANNAN AND SULFATED GLUCAN IN THE CELL WALLS OF THE RED ALGA KAPPAPHYCUS ALVAREZII Two new polysaccharides were isolated from the cell walls of the carrageenan producing red seaweed Kap- paphycus alvarezii ( Doty ) Doty . 902:891–902

156. Liepman AH, Wilkerson CG, Keegstra K (2005) Expression of cellulose synthase-like (Csl) genes in insect cells reveals that CslA family members encode mannan synthases. Proc Natl Acad Sci U S A 102:2221–2226. https://doi.org/10.1073/pnas.0409179102

157. Bonin CP, Potter I, Vanzin GF, Reiter W-D (1997) The MUR1 Gene of Arabidopsis Thaliana Encodes an Isoform of GDP-D-mannose-4,6-dehydratase, Catalyzing the First Step in the de novo Synthesis of GDP-L-fucose. Proc. Natl. Acad. Sci. U. S. A. 94:2085–2090

158. Bonin CP, Reiter W-D (2000) A bifunctional epimerase-reductase acts downstream of the MUR1 gene product and completes the de novo synthesis of GDP-L-fucose in Arabidopsis. Plant J 21:445–454. https://doi.org/10.1046/j.1365-313x.2000.00698.x

159. Voiniciuc C, Dama M, Gawenda N, et al (2019) Mechanistic insights from plant heteromannan synthesis in yeast. Proc Natl Acad Sci U S A 116:522–527. https://doi.org/10.1073/pnas.1814003116

160. Fu Y, Jeong SH, Callihan J, et al (2006) Preparation of fast-dissolving tablets based on Mannose. In: ACS Symposium Series. pp 340–351

161. Zalewski BM, Chmielewska A, Szajewska H (2015) The effect of glucomannan on body weight in overweight or obese children and adults: A systematic review of randomized controlled trials. Nutrition 31:437-442.e2

162. Garti N, Madar Z, Aserin A, Sternheim B (1997) Fenugreek galactomannans as food emulsifiers. LWT - Food Sci Technol 30:305–311. https://doi.org/10.1006/fstl.1996.0179

163. Hu X, Shi Y, Zhang P, et al (2016) -Mannose: Properties, Production, and Applications: An Overview. Compr Rev Food Sci Food Saf 15:773–785. https://doi.org/10.1111/1541-4337.12211

164. Zhang LM, Jin Y, Yao SM, et al (2020) Growth and Morphological Responses of Duckweed to Clonal Fragmentation, Nutrient Availability, and Population Density. Front Plant Sci 11:.

165. Barks PM, Laird RA (2015) Senescence in duckweed: Age-related declines in survival, reproduction and offspring quality. Funct Ecol 29:540–548. https://doi.org/10.1111/1365-2435.12359

166. Barks PM, Laird RA (2016) A multigenerational effect of parental age on offspring size but not fitness in common duckweed (Lemma minor). J Evol Biol 29:748–756. https://doi.org/10.1111/jeb.12823

167. Mejbel HS, Simons AM (2018) Aberrant clones: Birth order generates life history diversity in Greater Duckweed, Spirodela polyrhiza. Ecol Evol 8:2021–2031. https://doi.org/10.1002/ece3.3822

168. Le Gall H, Philippe F, Domon JM, et al (2015) Cell wall metabolism in response to abiotic stress. Plants 4:112–166

169. Tenhaken R (2015) Cell wall remodeling under abiotic stress. Front Plant Sci 5:1–9. https://doi.org/10.3389/fpls.2014.00771
Sugars are shown in blue and their precursor in black. The genes encode the enzymes that synthesize the NDP-sugars. The ones in red had their expression measured in this work, whereas the black did not. Some cell wall polysaccharides are shown in green. Dashed lines in black indicate multi-steps to generate the product indicated by the arrowhead. Dashed lines in green indicate unresolved pathways. Dashed lines in orange-brown indicate transporters to the Golgi complex. Dashed blue lines indicate the reactions of the salvage pathway. The carbon flux is directed for the UDP-Glc pathway for the cell wall synthesis, while the ADP-Glc directs the starch from Glc-1P. The ADP-glucose pathway occurs in plastids, and it is responsible for the synthesis of starch, the primary storage polymer, by the action of the enzyme starch synthase (SS, EC 2.4.1.32). The precursor ADP-glucose is catalyzed by glucose-phosphate-adenyl transferase (AGPase - APL and APS subunits, EC 2.7.7.27) from Glc-1P. The substrate for the synthesis of the NDP-sugar involved in the synthesis of hemicelluloses, pectins, and cellulose (cell wall carbohydrates) is produced by the metabolism of fructose-6P (photosynthetic product) via Glc 6P and Glc 1P, culminating with the formation of UDP-Glc by UDP-glucose pyrophosphorylase (UGP, EC 2.7.7.9). UDP-Glc can be a substrate for four different enzymes, leading to the synthesis of cellulose, UDP-rhamnose, UDP-galactose, and UDP-glucuronic. The cellulose is synthesized in the plasma membrane by a synthase complex (CSC) that contains cellulose synthases (CESA, EC 2.4.1.12), while hemicellulose and pectins are synthesized in the Golgi and most of the NDP-sugars in the cytosol. UDP-Rha is produced using a multifunctional rhamnose biosynthesis enzyme (RHM-EC 4.2.1.76) from UDP-Glc. This NDP will form the polymers of rhamnogalacturonan.

Galactose, a constituent of rhamnogalacturonans, galactans, xylolignans, and galactomannans, is synthesized by the epimerization of glucose by the enzymes UDP-glucose-4-epimerase (UGE, EC, 5.1.3.2), UDP-sugar pyrophosphorylase (USP, EC 2.7.7.64), and UDP-glucose-hexose-1-phosphate-uridylyltransferase (GALT, EC 2.7.7.12). The galactose can be recycled to Gal-1P by galactokinase (GALK, EC 2.7.1.6) in the salvage pathway. UDP-galactose also acts as a precursor to galacturonic acids. The nucleotide sugar UDP-GlcA is essential in the pathway of pectin synthesis since it is involved in the production of UDP-xylene, UDP-arabinose, UDP-galacturonic acid, and UDP-apiose. The oxidation of UDP-Glc forms UDP-GlcA by UDP-Glucose dehydrogenase (UGD 1.1.1.22). UDP-xylene and UDP-apiose are synthesized by UDP-glucuronate decarboxylase and UDP-apiose/UDP-xylene synthase (UXS and AXS, EC 4.1.1.35). Xylose can be converted into Xyl-1P by β-xyloligosidase-4 in the salvage pathway. UDP-xylene can be converted to UDP-arabinose (precursor to arabinose). UDP-Ara is the only NDP-sugar that is synthesized in the Golgi lumen in its pyranose form by UDP-arabinose-4-epimerase (UAE, EC 5.1.3.5), which is transported back to the cytosol for conversion to furanose form by arabinose mutase (UAM, EC 5.4.99.30) then transported again to Golgi lumen via UDP-Ara/transporters. Arabinose is a constituent of rhamnogalacturonans, arabinans, and arabinoxylans. Arabinans can be a broken down in the salvage pathway by arabinofuranosidase (ASD, EC 2.7.1.52) to arabinose. In the salvage pathway of arabinose, this hexose can be phosphorylated by arabinokinase (ARA1, EC 2.7.1.46). Galacturonic acid, the main pectic component, results from the epimerization of UDP-glucurionate by UDP-glucuronate-4-epimerase (GAE, EC 5.1.3.6). Galacturonic acid can be catabolized by galacturonokinase (GALAK EC 2.7.1.44) in the salvage pathway.
EC 2.4.1.43) catalyzes the transfer of UDP-GalA into the pectic polysaccharide homogalacturonan. The mannan synthesis pathway descends directly from fructose-6P and involves the enzymes mannose-6-phosphate-isomerase (MPI, EC 5.3.1.8), phosphomannomutase (PMM, EC 5.4.2.8), and mannose-1-phosphate-guanylyltransferase (MGP, EC 2.7.7.13). Mannans, hemicelluloses of mannose chains, are synthesized by mannan synthase (MSR, EC 2.4.1-), and the backbone for glucoman nan is built from 4-beta-mannosyltransferase (CSLA, EC 2.4.3.[2]) activity on mannan using GDP-mannose. Fucose, a sugar found in pectins and xyloglucans, is derived from the precursor GDP-mannose by dehydration, epimerization, and reduction of UDP-Man in UDP-Fuc by the action of GDP-mannose-4,6-dehydratase (GMD, EC 4.2.1.47), and GDP-4-keto-deoxy-d-mannose-3,5-epimerase-4-reductase (GER, EC 1.1.1.271). The salvage pathway of fucose constitutes the phosphorylation of free fucose followed by GMP attachment which involves fucokinase (FKGP, EC 2.7.1.52).

Abbreviations: NDP-sugars (NDP-sugars), UDP (uridine diphosphate) CSC (cellulose synthesizing complex), Fru-BP (fructose biphosphate), Suc (sucrose), Glc (glucose), Rha (rhamnose), Gal (galactose), GlcA (glucuronic acid), Xyl (xylene), Api (apioside), GaIA (galacturonic acid), HG (homogalacturonan), Ara (arabinose), Araf (arabinose furanose), Arap (arabinose pyranose), Fru (fructose), Man (mansenose), and fuc (fucose). Adaptations of Alonso et al. (2010), Bar-Celed, O'Neill (2011), Verbančič et al. (2017) metabolic pathways KEGG 00051 (fructose and mannose metabolism), 00052 (galactose metabolism), 00500 (starch and sucrose metabolism), 00520 (amino sugar and nucleotide sugar metabolism).

**Figure 2. Spirodela polyrhiza biomass composition.** This plant biomass was analyzed regarding the content of soluble sugars (fructose, sucrose, glucose, and raffinose), starch levels, cell wall proportion, and others. Others represent the biomass lost during the processing, comprehending possibly to secondary metabolites and lipids.

**Figure 3. Chromosome's ideogram of the nucleotide sugar pathway genes evaluated in the present study.** *S. polyrhiza* has 20 chromosomes which are represented in the figure. The colors on the chromosomes represent each class of polysaccharides or its involvement on the pathway, being blue for cellulose, green hemicelluloses, red for pectin, and black for starch and sucrose metabolism. For gene names, see Figure 1 and Supplemental 1.

**Figure 4. Distribution of genes throughout the chromosomes in the genome of Spirodela polyrhiza.** Heatmap of the number of genes in each chromosome of *S. polyrhiza* divided into the categories "sucrose and starch metabolism," "pectin," "hemicellulose," and "cellulose."

**Table 1. Cell wall yield and composition of Spirodela polyrhiza.** A. Yield of cell wall extractions of de-starched alcohol insoluble residues of *S. polyrhiza*. B. The structural carbohydrates show the cell wall composition by monosaccharides hydrolysis with TFA. Despite the monosaccharide's hydrolysis, the residue fraction, mainly cellulose, was also hydrolyzed with sulfuric acid 72% to prove its nature since TFA cannot hydrolyze cellulose (shown as Residue II). AIR, alcohol insoluble residue; AmnOx, ammonium oxalate fraction; chlorite, sodium chlorite fraction; 0.1 -4 M NaOH, sodium hydroxide fractions, and residue. All data are represented by the average ± standard error of the sugar in ug.mg-1 dry weight (n=5).

**Table 2. Heatmap of selected NDP-sugars pathway associated genes in Spirodela polyrhiza.** The map was generated by a comparison of all evaluated sugars against Expression levels. Expression levels in all samples analyzed were normalized to the reference genes ARF7 and PP2A.

**Supplemental Table 1. NDP-sugar pathway-related genes from Arabidopsis thaliana and Spirodela polyrhiza.** The cell wall synthesis genes of *S. polyrhiza* were recovered from the orthologs of *A. thaliana*. Two genomes of *S. polyrhiza* were used (lineage 7498 and 9509). The *S. polyrhiza_V2* represents the annotated version generated in the present work. *a* is the selected transcript for primer design.

**Supplemental Table 2. Similarity between Spirodela polyrhiza 7498 and Arabidopsis thaliana orthologs.** The table summarizes the results of blastp.

**Supplemental Table 3. Similarity between Spirodela polyrhiza 7498 and Spirodela polyrhiza 9509 orthologs.** The table summarizes the results of blastn.

**Supplemental Table 4. Similarity between Spirodela polyrhiza 9509 and Spirodela polyrhiza_V2 annotation orthologs.** The table summarizes the results of blastn. The columns Start 9509 and End 9509 denote the gene range from the annotation bank generated in the present work.
Supplemental Table 5. *Arabidopsis thaliana* nucleotide sugar pathway-related genes sequences and its domain annotation in HMMERscan.

Supplemental Table 6. *Spirodela polyrhiza* 7498 nucleotide sugar pathway-related genes sequences and its domain annotation in HMMERscan.

Supplemental Table 7. *Spirodela polyrhiza* V2 nucleotide sugar pathway-related genes sequences and its domain annotation in HMMERscan.

Supplemental Table 8. Selected candidate reference genes. The candidates of reference genes for *Spirodela polyrhiza* were recovered from the orthologs sequences of *Arabidopsis thaliana*. Two genomes of *S. polyrhiza* were used (lineage 7498 and 9509).

Supplemental Table 9. Candidate reference genes and nucleotide sugar pathway genes for cell wall synthesis were used to design specific oligonucleotides.

Supplemental Table 10. Ranking of *Spirodela polyrhiza* candidate reference genes based on analysis of the geNorm software. M refers to the stability coefficient of the average variation of two internal control genes. The most stable genes have lower M values (cut-off <1.5). V corresponds to the Vn / n + 1 pair-wise variation values < 0.15 means that the use of the two most stable genes is sufficient to normalize the expression of a test gene in the corresponding set of samples. n: number of genes.

Supplemental Table 11. Ranking of *Spirodela polyrhiza* candidate reference genes based on the analysis of NormFinder software. Genes ranked according to the slightest intragroup variation. Genes with the lowest stability values are considered to have the most stable expression.

Supplemental Table 12 - Representative monosaccharides data from diverse analysis of *Spirodela polyrhiza* (Sp) AIR. *Spirodela polyrhiza* AIR composition was retrieved by, Avci et al. (2018), Sowinski et al. (2019), Longland et al. (1989), Pagliuso et al. (2018), and internal laboratory data. The analyzed quantitative monosaccharide data were summed and normalized in percentage proportions based on the total sum for comparative purposes. The darker blue cells in the heatmap indicate higher values within columns. Abbreviations: SH: Schenck-Hildebrandt, suc: sucrose, TFA: trifluoroacetic acid, h: hour, chrom: chromatography, publish.: published.

Supplemental Figure 1. Amplification cycle values (Cq) of the target genes in this work. A. Cq distribution of *S. polyrhiza* candidate reference genes. B. Cq distribution of genes from the sugar-nucleotide pathway for the synthesis of *S. polyrhiza* cell wall. The colors in B represent the sugars categories: blue, pectins; green, hemicellulose; pink, starch; red, mannans; and black the general sugar metabolism. The thick line represents the median. The white boxes and their markings indicate the interquartile and the Cq the variance of each gene.

Supplemental Figure 2. Reference genes validation. The graph shows the relative expression levels of *AXS* in the samples of *Spirodela polyrhiza* lineages 7498 and 9509 normalized with fourteen combinations of reference gene candidates. The different letters above the bars indicate statistically significant (p<0.05) on the lineage of *Spirodela* tested by Tukey's test.
| A. Yield (%) | AIR | AmnOX | Chlorite | 0.1 M NaOH | 1 M NaOH | 4 M NaOH | Residue | Residue II |
|-------------|-----|--------|----------|------------|----------|----------|---------|------------|
|             | -   | 20.37 ± 0.44 | 38.33 ± 0.96 | 7.36 ± 0.40 | 8.71 ± 1.03 | 12.42 ± 0.78 | 12.85 ± 1.10 | -          |

B. Neutral carbohydrates (µg mg⁻¹ DW)

| Carbohydrate | AIR   | AmnOX | Chlorite | 0.1 M NaOH | 1 M NaOH | 4 M NaOH | Residue | Residue II |
|--------------|-------|--------|----------|------------|----------|----------|---------|------------|
| Galactose    | 74.85 ± 5.19 | 17.42 ± 0.09 | 11.53 ± 0.06 | 10.15 ± 0.04 | 4.47 ± 0.03 | 2.58 ± 0.01 | 7.37 ± 0.07 | 6.28 ± 0.00 |
| Arabinose    | 56.01 ± 5.09 | 10.12 ± 0.05 | 13.68 ± 0.05 | 5.35 ± 0.03 | 1.57 ± 0.02 | 0.75 ± 0.00 | 2.21 ± 0.02 | 1.50 ± 0.00 |
| Xylose       | 51.93 ± 2.06 | 6.11 ± 0.03 | 2.68 ± 0.02 | 11.35 ± 0.04 | 18.89 ± 0.17 | 8.87 ± 0.06 | 14.50 ± 0.10 | 11.84 ± 0.01 |
| Apiose       | 49.81 ± 4.15 | 44.28 ± 0.18 | 12.23 ± 0.08 | 20.80 ± 0.09 | 6.02 ± 0.03 | 4.97 ± 0.05 | 19.64 ± 0.21 | 5.86 ± 0.01 |
| Mannose      | 43.92 ± 3.11 | 6.18 ± 0.05 | 3.40 ± 0.02 | 0.90 ± 0.01 | 0.77 ± 0.00 | 0.63 ± 0.00 | 0.87 ± 0.00 | 3.12 ± 0.01 |
| Rhamnose     | 28.36 ± 1.70 | 8.00 ± 0.04 | 2.46 ± 0.02 | 3.97 ± 0.01 | 0.49 ± 0.01 | 0.57 ± 0.01 | 2.92 ± 0.03 | 0.00 ± 0.00 |
| Glucose      | 11.87 ± 1.34 | 4.79 ± 0.08 | 2.33 ± 0.01 | 2.52 ± 0.01 | 10.96 ± 0.17 | 3.81 ± 0.02 | 7.80 ± 0.07 | 151.7 ± 0.21 |
| Fucose       | 7.19 ± 0.55 | 2.14 ± 0.01 | 0.90 ± 0.00 | 1.02 ± 0.00 | 1.19 ± 0.01 | 0.62 ± 0.00 | 1.76 ± 0.02 | 1.08 ± 0.00 |
| ID   | Expression level | Gene       | Gene function                                                                 |
|------|------------------|------------|-------------------------------------------------------------------------------|
| SBE  | 0.96             | Starch branching enzyme | Performs branching of starch                                                  |
| SS2  | 0.87             | Starch synthase        | Synthesizes the starch main chain                                            |
| SPS1 | 0.86             | Glucose-1P-adenyltransferase | Converts Glc-1P to ADP-Glc, which is the substrate for starch synthesis       |
| SS1  | 0.74             | Sucrose synthase       | Produces UDP-Glucose, the basis of all cell wall polysaccharides except for mannans |
| SS2  | 0.74             | Starch synthase        | Synthesizes starch main chain                                                |
| SS3  | 0.69             | Hexokinase            | Produces glucose-6P that is related to starch and cell wall synthesis         |
| UGP2 | 0.72             | UDP-glucose pyrophosphorylase | Converts UDP-Glc into Glc-1P, determining the pathways of starch or cell wall synthesis |
| PK   | 0.70             | Fructokinase          | Converts Fru-6P into Fru-6P which will be substrate for mannose or other cell wall/sugar polymers syntheses |
| PGI1 | 0.69             | UDP-glucose pyrophosphorylase | Converts UDP-Glc into Glc-1P, determining the pathways of starch or cell wall synthesis |
| SUSY2| 0.62             | Sucrose synthase       | Produces UDP-Glucose, the basis of all cell wall polysaccharides except for mannans |
| INV2 | 0.62             | Invertase              | Hydrolyzes sucrose into glucose and fructose, leading to Glc-6P and Fru-6P respectively |
| GAUT1| 1.37             | α-1,4-galacturonosyltransferase | Polymerizes the main chain of pectins (homogalacturonan)                   |
| UGE  | 1.26             | UDP-glucose-4-epimerase | Converts UDP-Glc into UDP-GalA, determining rhamnogalacturonan and galactoglucomannan syntheses |
| USP  | 1.05             | UDP-glucose-6-dehydrogenase | Converts UDP-Glc into UDP-GalA, determining rhamnogalacturonan and galactoglucomannan syntheses |
| RDM  | 0.92             | Rhamnose biosynthesis enzyme | Converts UDP-Glc into UDP-Rham, determining the pathway towards pectin synthesis (Rhamnogalacturonans) |
| GAUT2| 0.89             | α-1,4-galacturonosyltransferase | Polymerizes the main chain of pectins (homogalacturonan)                   |
| UAM  | 0.89             | UDP-arabinose-4-epimerase | Converts UDP-Xyl into UDP-Arap, determining the pathway towards mannan synthesis and contributing to the structure of xylans, arabinoxylans and xyloglucans |
| GALAK1| 0.84             | α-1,4-galacturonosyltransferase | Converts UDP-Xyl into UDP-Arap, determining the pathway towards mannan synthesis and contributing to the structure of xylans, arabinoxylans and xyloglucans |
| GALAK2| 0.83             | Galacturonolactone kinase | Converts UDP-GlcA into UDP-GalA, determining the pathways of several pectin and hemicellulosic polymers |
| UGD  | 0.82             | UDP-arabinofuranose mutase | Converts UDP-Arap into UDP-Araf, determining the pathway towards mannan and glycoprotein synthesis and contributing to the structure of xylans, arabinoxylans and xyloglucans |
| GLCAK| 0.76             | Arabinokinase         | Converts UDP-GalA into UDP-GalA, determining the pathways of several pectin and hemicellulosic polymers |
| CSLA | 0.76             | 1,4-β-xylanase         | Converts UDP-Xyl into xylose, which is incorporated by CSLE1 into the xylan polymer |
| ARA1 | 0.76             | Arabinokinase         | Converts UDP-Xyl into xylose, which is incorporated by CSLE1 into the xylan polymer |
| UAM  | 0.76             | UDP-glucuronate-decarboxylase | Converts UDP-GlcA into UDP-Xyl, determining the pathway towards mannan and glycoprotein synthesis |
| PMM  | 0.67             | Phosphomannomutase     | Converts Man-6P into Man-1P, determining the pathway towards mannan and glycoprotein synthesis |
Starch synthesis and de novo and salvage pathways of NDP-sugars. Sugars are shown in blue and their precursor in black. The genes encode the enzymes that synthesize the NDP-sugars. The ones in red had their expression measured in this work, whereas the black did not. Some cell wall polysaccharides are shown in green. Dashed lines in black indicate multi-steps to generate the product indicated by the arrowhead. Dashed lines in green indicate unresolved pathways. Dashed lines in orange-brown indicate transporters to the Golgi complex. Dashed blue lines indicate the reactions of the salvage pathway. The carbon flux is directed for the UDP-Glc pathway for the cell wall synthesis, while the ADP-Glc directs the starch from Glc-1P. The ADP-glucose pathway occurs in plastids, and it is responsible for the synthesis of starch, the primary storage polymer, by the action of the enzyme starch synthase (SS, EC 2.4.1.32). The precursor ADP-glucose is catalyzed by glucose-phosphate-adenyl transferase (AGPase - APL and APS subunits, EC 2.7.7.27) from Glc-1P. The substrate for the synthesis of the NDP-sugar involved in the synthesis of hemicelluloses, pectins, and cellulose (cell wall carbohydrates) is produced by the metabolism of fructose-6P (photosynthetic product) via Glc 6P and Glc 1P, culminating with the formation of UDP-Glc by UDP-glucose pyrophosphorylase (UGP, EC 2.7.7.9). UDP-Glc can be a substrate for four different enzymes, leading to the synthesis of cellulose, UDP-rhamnose, UDP-galactose, and UDP-glucuronic. The cellulose is synthesized in the plasma membrane by a synthase complex (CSC) that
contains cellulose synthases (CESA, EC 2.4.1.12), while hemicellulose and pectins are synthesized in the Golgi and most of the NDP-sugars in the cytosol. UDP-Rha is produced using a multifunctional rhamnose biosynthesis enzyme (RHM-EC 4.2.1.76) from UDP-Glc. This NDP will form the polymers of rhamnogalacturonan. Galactose, a constituent of rhamnogalacturonans, galactans, xyloglucans, and galactomannans, is synthesized by the epimerization of glucose by the enzymes UDP-glucose-4-epimerase (UGE, EC 5.1.3.2), UDP-sugar pyrophosphorylase (USP, EC 2.7.7.64), and UDP-glucose-hexose-1-phosphate-uridylyltransferase (GALT, EC 2.7.7.12). The galactose can be recycled to Gal-1P by galactokinase (GALK, EC 2.7.1.6) in the salvage pathway. UDP-galactose also acts as a precursor to galacturonic acids. The nucleotide sugar UDP-GlcA is essential in the pathway of pectin synthesis since it is involved in the production of UDP-xylose, UDP-arabinose, UDP-galacturonic acid, and UDP-apiose. The oxidation of UDP-Glc forms UDP-GlcA by UDP-Glucose dehydrogenase (UGD 1.1.1.22). UDP-xylose and UDP-apiose are synthesized by UDP980 glucuronate decarboxylase and UDP-apiose/UDP-xylose synthase (UXS and AXS, EC 4.1.1.35). Xylose can be converted into Xyl-1P by β-xylosidase-4 in the salvage pathway. UDP-xylose can be converted to UDP-arabinose (precursor to arabinose). UDP-Ara is the only NDP-sugar that is synthesized in the Golgi lumen in its pyranose form by UDP-arabinose-4-epimerase (UAE, EC 5.1.3.5), which is transported back to the cytosol for conversion to furanose form by arabinose mutase (UAM, EC 5.4.99.30) then transported again to Golgi lumen via UDP-Araf transporters. Arabinose is a constituent of rhamnogalacturonans, arabinans, and arabinoxylans. Arabinans can be broken down in the salvage pathway by arabinofuranosidase (ASD, EC 2.7.1.52) to arabinose. In the salvage pathway of arabinose, this hexose can be phosphorylated by arabinokinase (ARA1, EC 2.7.1.46). Galacturonic acid, the main pectic component, results from the epimerization of UDP-glucuronate by UDP-glucuronate-4-epimerase (GAE, EC 5.1.3.6). Galacturonic acid can be catabolized by galacturonokinase (GALAK EC 2.7.1.44) in the salvage pathway. α-1,4-galacturonosyltransferase (GAUT, EC 2.4.1.43) catalyzes the transfer of UDP-GalA into the pectic polysaccharide homogalacturonan. The 990 mannansynthesis pathway descends directly from fructose-6P and involves the enzymes mannose-6-phosphate-isomerase (MPI, EC 5.3.1.8), phosphomannomutase (PMM, EC 5.4.2.8), and mannose-1-phosphate-guanylyltransferase (MGP, EC 2.7.7.13). Mannans, hemicelluloses of mannose chains, are synthesized by mannansynthase (MSR, EC 2.4.1-), and the backbone for glucomannan is built from 4-beta-mannosyltransferase (CSLA, EC 2.4.1.32) activity on mannans using GDP-mannose. Fucose, a sugar found in pectins and xyloglucans, is derived from the precursor GDP-mannose by dehydration, epimerization, and reduction of UDP-Man in UDP-Fuc by the action of GDP-mannose-4,6-dehydratase (GMD, EC 4.2.1.47), and GDP-4-keto-deoxy-d-mannose-3,5-epimerase-4-reductase (GER, EC 1.1.1.271). The salvage pathway of fucose constitutes the phosphorylation of free fucose followed by GMP attachment which involves fucokinase (FKGP, EC 2.7.1.52). Abbreviations: NDP-sugars (NDP-sugars), UDP (uridine diphosphate) CSC (cellulose synthesizing complex), Fru-BP (fructose biphosphate), Suc (sucrose), Glc (glucose), Rha (rhamnose), Gal (galactose), GlcA (glucuronic acid), Xyl (xylose), Api (apiose), GaLA (galacturonic acid), HG (homogalacturonan), Ara (arabinose), Araf (arabinose furanose), Arap (arabinose pyranose), Fru (fructose), Man (mannose), and fuc (fucose). Adaptations of Alonso et al. (2010), Bar-Peled, O'Neill (2011), Verbančič et al. (2017) metabolic pathways KEGG 00051 (fructose and
mannose metabolism), 00052 (galactose metabolism), 00500 (starch and sucrose metabolism), 00520 (amino sugar and nucleotide sugar metabolism).

Figure 2

Spirodela polyrhiza biomass composition. This plant biomass was analyzed regarding the content of soluble sugars (fructose, sucrose, glucose, and raffinose), starch levels, cell wall proportion, and others. Others represent the biomass lost during the processing, comprehending possibly to secondary metabolites and lipids.
Figure 3

Chromosome's ideogram of the nucleotide sugar pathway genes evaluated in the present study. S. polyrhiza has 20 chromosomes which are represented in the figure. The colors on the chromosomes represent each class of polysaccharide or its involvement on the pathway, being blue for cellulose, green hemicelluloses, red for pectin, and black for starch and sucrose metabolism. For gene names, see Figure 1 and Supplemental 1.
Figure 4

Distribution of genes throughout the chromosomes in the genome of Spirodela polyrhiza. Heatmap of the number of genes in each chromosome of S. polyrhiza divided into the categories "sucrose and starch metabolism," "pectin," "hemicellulose," and "cellulose."

### Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalTable1.xlsx
- SupplementalTable10.xlsx
- SupplementalTable11.xlsx
• SupplementalTable121.xlsx
• SupplementalTable2.xlsx
• SupplementalTable3.xlsx
• SupplementalTable4.xlsx
• SupplementalTable5.xlsx
• SupplementalTable6.xlsx
• SupplementalTable7.xlsx
• SupplementalTable8.xlsx
• SupplementalTable9.xlsx
• supplementalfig1.tiff
• supplementalfig2.tiff