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

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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 polyrrhiza*, 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. polyrrhiza 9509 has a genome of 138.6 Mbp distributed in 20 chromosomes, hosting 18,507 predicted genes. This genome has low heterozygosity, 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 (RDSC). 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 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

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 $\cdot\text{mL}^{-1}$ 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 de 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 Phytozome v12.1.6 website (<https://phytozome.jgi.doe.gov/>), or by using local BlastP against our predicted-proteins database of the 9509 genome since the latter was not available on Phytozome. 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 Phytozome, 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 α 3/ α 5 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>), 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-Blast® (NCBI) tool (<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/analyser/>) was used to estimate the potential formation of secondary structures in those. Primers targeting *phosphoglucose isomerase (SpPGI)*, *mannose-6P-isomerase (SpMPI)*, *phosphomannomutase (SpPMM)*, *mannose-1P-guanlyltransferase (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 μ M, 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 μ g 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-apirose/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 μ L of cDNA (1:10), 7 μ L 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 $\mu\text{g.mg}^{-1}$ cell wall - 38%), arabinose (23.8 $\mu\text{g.mg}^{-1}$ cell wall - 16%), and galactose (28.9 $\mu\text{g.mg}^{-1}$ cell wall - 19.5%), with traces of fucose (2.9 $\mu\text{g.mg}^{-1}$ cell wall - 2%) (**Table 1**, AmnOx and Chlorite columns). Xylose (39.1 $\mu\text{g.mg}^{-1}$ cell wall - 32%), glucose (17.3 $\mu\text{g.mg}^{-1}$ cell wall - 14%), and galactose (17.2 $\mu\text{g.mg}^{-1}$ 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 $\mu\text{g.mg}^{-1}$ cell wall) and glucose (4.8 $\mu\text{g.mg}^{-1}$ cell wall) (**Table 1**). The residue comprised more than 80% cellulose (151.7 $\mu\text{g.mg}^{-1}$ cell wall) (see glucose levels on Residue II in **Table 1**). The residue II

fraction composition also showed small amounts of galactose (6.3 $\mu\text{g}\cdot\text{mg}^{-1}$ cell wall), xylose (11.8 $\mu\text{g}\cdot\text{mg}^{-1}$ cell wall), and apiose (5.8 $\mu\text{g}\cdot\text{mg}^{-1}$ 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*, *SpHXX*, *SpPGI*, *SpPGM*, *SpUGP*, *SpAPL*, *SpAPS*, *SpSS*, and *SpSBE* contemplating 49 orthologues), “pectin” (*SpRHM*, *SpUGD*, *SpAXS*, *SpGAE*, *SpGAUT*, *SpGALAK*, *SpGLCAK*, *SpUAE*, *SpUAM*, *SpARA1*, *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 (C_q) ≤ 35 , with reactions that reached the plateau phase. The amplitude of the C_q 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 C_q 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 *SpPPP2A* 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 (SpHXX)*, 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 *APL* and *APS*), *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. polyrrhiza* 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. polyrrhiza* 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. polyrrhiza* 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 polyrrhiza*' 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% ($\pm$ 1.9) galactose, 17.4% ($\pm$ 1.1) arabinose, 15.5% ($\pm$ 1.1) xylose, 21.9% ($\pm$ 2.9%) apiose, 5.4% ($\pm$ 1.7) mannose, 7.5% ($\pm$ 0.6) rhamnose, 9.3% ($\pm$ 1.9) glucose, and 2.4% ($\pm$ 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], *Lemna minor* under ammonium toxicity [72], *Lemna minor* 5500 under ionizing radiation [87], *Lemna aequinoctialis* 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 found 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 *CESA* 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-Galacturonate 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-Galacturonate 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 $\mu\text{g}\cdot\text{mg}^{-1}$) of AIR, being the main monosaccharide found in ammonium oxalate (44.3 $\mu\text{g}\cdot\text{mg}^{-1}$) and sodium chlorite (12.2 $\mu\text{g}\cdot\text{mg}^{-1}$) fractions (**Table 1**), even with a *Low* relative transcript expression of 0.76 (**Table 2**). Otherwise, xylose represents 16.1% (51.9 $\mu\text{g}\cdot\text{mg}^{-1}$) of AIR and 5.8% of the ammonium oxalate (6.2 $\mu\text{g}\cdot\text{mg}^{-1}$) and sodium chlorite (5.4 $\mu\text{g}\cdot\text{mg}^{-1}$) 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. polyrrhiza* 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 $\mu\text{g.mg}^{-1}$ 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 $\mu\text{g.mg}^{-1}$ AIR) and was found mainly on the pectin-rich fractions (AmnOX - 10.1 $\mu\text{g.mg}^{-1}$ and Chlorite - 13.7 $\mu\text{g.mg}^{-1}$) and 0.1 NaOH 1M fractions (5.35 $\mu\text{g.mg}^{-1}$) (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 uridylyltransferase (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 *GalSI* (*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 $\mu\text{g.mg}^{-1}$ AIR – **Table 1**). The fractionation process demonstrated that the galactose was mainly galactans (AmnOx – 17.4 $\mu\text{g.mg}^{-1}$ - **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 mannan 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 $\mu\text{g.mg}^{-1}$ 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 $\mu\text{g.mg}^{-1}$) and sodium chlorite fractions (3.40 $\mu\text{g.mg}^{-1}$) (**Table 1**). Besides that, the mannan-related transcripts *mannose-6-phosphate-isomerase* (*SpMPI*), *phosphomannomutase* (*SpPMM*), *mannose-1P-guanylyltransferase* (*SpMGP*), and *glucomannan-4 β -mannosyltransferase* (*SpCSLA*) levels were the highest levels identified (**Table 2**). The pathway to mannan synthesis has a low number of gene copies, except for the *glucomannan synthase* (*MSR*) and *glucomannan-4 β -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., BNV, and D.P. analyzed the data. A.G., BNV, D.P., E.L., and MSB wrote the manuscript.

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541 **10 References**

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11 Legends

Figure 1. 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-apirose. The oxidation of UDP-Glc forms UDP-GlcA by UDP-Glucose dehydrogenase (UGD 1.1.1.22). UDP-xylose and UDP-apirose are synthesized by UDP-glucuronate decarboxylase and UDP-apirose/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-Ara^f 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 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-guanlyltransferase (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 glucomannan is built from 4-beta-mannosyltransferase (CSLA, EC 2.4.1.32) 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 (xylose), Api (apiose), GalA (galacturonic acid), HG (homogalacturonan), Ara (arabinose), Ara^f (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."

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 $\pm$ 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 themselves. 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.

1037 **Supplemental Table 5. *Arabidopsis thaliana* nucleotide sugar pathway-related genes sequences and its domain**
1038 **annotation in HMMERscan.**

1039 **Supplemental Table 6. *Spirodela polyrhiza* 7498 nucleotide sugar pathway-related genes sequences and its domain**
1040 **annotation in HMMERscan.**

1041 **Supplemental Table 7. *Spirodela polyrhiza*_V2 nucleotide sugar pathway-related genes sequences and its domain**
1042 **annotation in HMMERscan.**

1043 **Supplemental Table 8. Selected candidate reference genes.** The candidates of reference genes for *Spirodela polyrhiza*
1044 were recovered from the orthologs sequences of *Arabidopsis thaliana*. Two genomes of *S. polyrhiza* were used (lineage
1045 7498 and 9509).

1046 **Supplemental Table 9. Candidate reference genes and nucleotide sugar pathway genes for cell wall synthesis were**
1047 **used to design specific oligonucleotides.**

1048 **Supplemental Table 10. Ranking of *Spirodela polyrhiza* candidate reference genes based on analysis of the geNorm**
1049 **software.** M refers to the stability coefficient of the average variation of two internal control genes. The most stable genes
1050 have lower M values (cut-off <1.5). V corresponds to the $V_n / n + 1$ pair-wise variation values < 0.15 means that the use
1051 of the two most stable genes is sufficient to normalize the expression of a test gene in the corresponding set of samples. n:
1052 number of genes.

1053 **Supplemental Table 11. Ranking of *Spirodela polyrhiza* candidate reference genes based on the analysis of**
1054 **NormFinder software.** Genes ranked according to the slightest intragroup variation. Genes with the lowest stability values
1055 are considered to have the most stable expression.

1056 **Supplemental Table 12 - Representative monosaccharides data from diverse analysis of *Spirodela polyrhiza* (Sp) AIR.**
1057 *Spirodela polyrhiza* AIR composition was retrieved by, Avci et al. (2018), Sowinski et al. (2019), Longland et al. (1989),
1058 Pagliuso et al. (2018), and internal laboratory data. The analyzed quantitative monosaccharide data were summed and
1059 normalized in percentage proportions based on the total sum for comparative purposes. The darker blue cells in the heatmap
1060 indicate higher values within columns. Abbreviations: SH: Schenck-Hildebrandt, suc: sucrose, TFA: trifluoroacetic acid,
1061 h: hour, chrom: chromatography, publish.: published.

1062 **Supplemental Figure 1. Amplification cycle values (Cq) of the target genes in this work. A.** Cq distribution of *S.*
1063 *polyrhiza* candidate reference genes. **B.** Cq distribution of genes from the sugar-nucleotide pathway for the synthesis of *S.*
1064 *polyrhiza* cell wall. The colors in **B** represent the sugars categories: blue, pectins; green, hemicellulose; pink, starch; red,
1065 mannans; and black the general sugar metabolism. The thick line represents the median. The white boxes and their markings
1066 indicate the interquartile and the Cq the variance of each gene.

1067 **Supplemental Figure 2. Reference genes validation.** The graph shows the relative expression levels of *AXS* in the samples
1069 of *Spirodela polyrhiza* lineages 7498 and 9509 normalized with fourteen combinations of reference gene candidates. The
1070 different letters above the bars indicate statistically significant ($p < 0.05$) on the lineage of *Spirodela* tested by Tukey's test.

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1079 **Table 1**

	AIR	AmnOX	Chlorite	0.1 M NaOH	1 M NaOH	4 M NaOH	Residue	Residue II
A.Yield (%)	-	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 carbohydartes (µg.mg⁻¹ DW)								
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

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	ID	Expression level	Gene	Gene function	
Starch and sucrose metabolism	<i>SBE</i>	0.90	Starch branching enzyme	Performs branching of starch	 0.5 1.4
	<i>SS2</i>	0.87	Starch synthase	Synthesizes the starch main chain	
	<i>APL</i>	0.86	Glucose-1P-adenyltransferase	Converts Glc-1P to ADP-Glc, which is the substrate for starch synthesis	
	<i>SUSY1</i>	0.74	Sucrose synthase	Produces UDP-Glucose, the basis of all cell wall polysaccharides except for mannans	
	<i>SSI</i>	0.74	Starch synthase	Synthesizes starch main chain	
	<i>HXK</i>	0.72	Hexokinase	Produces glucose-6P that is related to starch and cell wall synthesis	
	<i>UGP2</i>	0.72	UDP-glucose pyrophosphorylase	Converts UDP-Glc into Glc-1P, determining the pathways of starch or cell wall syntheses	
	<i>FK</i>	0.70	Fructokinase	Converts Fructose into Fru-6P which will be substrate for mannan or other cell wall/starch polymers syntheses	
	<i>UGP1</i>	0.69	UDP-glucose pyrophosphorylase	Converts UDP-Glc into Glc-1P, determining the pathways of starch or cell wall syntheses	
	<i>PGI</i>	0.63	Phosphoglucose-isomerase	Converts Fru-6P to Glc-6P, determining direction towards starch and other cell wall polymers except for mannan	
	<i>SUSY2</i>	0.62	Sucrose synthase	Produces UDP-Glucose, the basis of all cell wall polysaccharides except for mannans	
	<i>INV</i>	0.52	Invertase	Hydrolyzes sucrose into glucose and fructose, leading to Glc-6P and Fru-6P respectively	
Pectin	<i>GAUT1</i>	1.37	α -1,4-galacturonosyltransferase	Polymerises the main chain of pectins (homogalacturonan)	
	<i>UGE</i>	1.20	UDP-glucose-4-epimerase	Converts UDP-Glc into UDP-galactose, determining rhamnogalacturonan, galactomannan, xyloglucan, and galactans	
	<i>USP</i>	1.05	UDP-sugar-pyrophosphorylase	Converts UDP-GlcA into GlcA-1P, determining rhamnogalacturonan II and glucuronoarabinoxylan syntheses	
	<i>RHM</i>	0.92	Rhamnose biosynthesis enzyme	Converts UDP-Glc into UDP-Rham, determining the pathway towards pectin synthesis (Rhamnogalacturonans)	
	<i>GAUT2</i>	0.89	α -1,4-galacturonosyltransferase	Polymerises the main chain of pectins (homogalacturonan)	
	<i>UAE</i>	0.89	UDP-arabinose-4-epimerase	Converts UDP-Xyl into UDP-Arap, determining arabinan synthesis and contributing to arabinoxylan and xyloglucan syntheses	
	<i>GAUT3</i>	0.84	α -1,4-galacturonosyltransferase	Polymerises the main chain of pectins (homogalacturonan)	
	<i>ARA1</i>	0.83	Arabinokinase	Converts Ara-1P into arabinose that is used for polymerization of chains of arabinan and branches of arabinoxylan and xyloglucan	
	<i>UAM</i>	0.82	UDP-arabinopyranose-mutase	Converts UDP-Arap into UDP-Araf, determining the pathway towards arabinan synthesis	
	<i>GALAK2</i>	0.81	Galacturonokinase	Converts UDP-GalA into GalA-1P determining the synthesis of homogalacturonan	
	<i>AXS</i>	0.76	UDP-apiose/UDP-xylose synthase	Converts UDP-GlcA into UDP-Api, determining the pathway of apiogalacturonan and rhamnogalacturonan II	
	<i>GALAK1</i>	0.67	Galacturonokinase	Converts UDP-GalA into GalA-1P determining the synthesis of homogalacturonan	
	<i>UGD</i>	0.65	UDP-glucose-6-dehydrogenase	Converts UDP-Glc into UDP-GlcA, determining the pathways of several pectin and hemicellulosic polymers	
Hemicellulose	<i>GLCAK</i>	0.54	Glucuronokinase	Converts UDP-GlcA into GlcA-1P, determining rhamnogalacturonan II and glucuronoarabinoxylan syntheses	
	<i>CSLA</i>	1.30	Glucomanan-4 β -mannosyltransferase	Polymerizes the main chain of mannan	
	<i>GER</i>	1.03	GDP-fucose synthase	Converts GDP-man into GDP-Fuc, contributing to the structure of xyloglucan and pectins	
	<i>MPI</i>	0.90	Mannose-6P-isomerase	Converts Fru-6P into Man-6P, determining the pathway towards mannan and glycoprotein synthesis	
	<i>UXS 2</i>	0.86	UDP-glucuronate-decarboxylase	Converts UDP-GlcA into UDP-Xyl determining the synthesis of xylans, arabinoxylans and xyloglucan	
	<i>UXS 4</i>	0.86	UDP-glucuronate-decarboxylase	Converts UDP-GlcA into UDP-Xyl determining the synthesis of xylans, arabinoxylans and xyloglucan	
	<i>XYLA</i>	0.84	1,4- β -xylanase	Converts UDP-Xyl into xylose that is incorporated by CSLD5 into the xylan polymer	
	<i>UXS 3</i>	0.76	UDP-glucuronate-decarboxylase	Converts UDP-GlcA into UDP-Xyl determining the synthesis of xylans, arabinoxylans and xyloglucan	
	<i>MGP</i>	0.74	Mannose-1P-guanylyltransferase	Converts Man-1P into GDP-Man, determining the pathway towards mannan and glycoprotein synthesis	
	<i>FKGP</i>	0.73	Fucokinase	Converts GDP-Fucose into fucose, contributing the branching of xyloglucan and pectins	
	<i>GMD</i>	0.72	GDP-mannose-4,6-dehydratase	Converts GDP-Man into mannose, determining the pathway towards mannan and glycoprotein synthesis	
	<i>UXS 1</i>	0.69	UDP-glucuronate-decarboxylase	Converts UDP-GlcA into UDP-Xyl determining the synthesis of xylans, arabinoxylans and xyloglucan	
	<i>PMM</i>	0.67	Phosphomannomutase	Converts Man-6P into Man-1P, determining the pathway towards mannan and glycoprotein synthesis	

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Figures

Figure 1

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 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 glucomannan is built from 4-beta-mannosyltransferase (CSLA, EC 2.4.1.32) 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 (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.

Chromosome number	Number of genes	Starch and sucrose metabolism	Pectin	Hemicellulose	Cellulose
1	24	8	6	9	1
2	22	5	6	11	0
3	10	4	4	2	0
4	14	4	5	4	1
5	11	3	3	5	0
6	7	3	2	2	0
7	10	2	1	4	3
8	13	0	10	2	1
9	7	3	0	3	1
10	6	0	1	0	5
11	9	2	3	0	4
12	6	3	1	2	0
13	15	0	6	7	2
14	6	2	1	3	0
15	14	5	0	8	1
16	3	1	2	0	0
17	3	1	0	1	1
18	5	2	1	1	1
19	4	1	1	1	1
20	1	0	0	0	1
TOTAL	190	49	53	65	23
	%	25.8	27.9	34.2	12.1

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

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