Photosynthetic activity influences cellulose biosynthesis and phosphorylation of proteins involved therein in Arabidopsis leaves

Edouard Boex-Fontvieille, Marlène Davanture, Mathieu Jossier, Michel Zivy, Michael Hodges and Guillaume Tcherkez

1 Institut de Biologie des Plantes, CNRS UMR 8618, Saclay Plant Sciences, Université Paris Sud, 91405 Orsay cedex, France
2 Plateforme PAPPSO, UMR de Génétique Végétale, Ferme du Moulon, 91190 Gif sur Yvette, France
3 Institut Universitaire de France, 103 Boulevard Saint-Michel, 75005 Paris, France

* To whom correspondence should be addressed. E-mail: guillaume.tcherkez@u-psud.fr

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Abstract

Cellulose is one of the most important organic compounds in terrestrial ecosystems and represents a major plant structural polymer. However, knowledge of the regulation of cellulose biosynthesis is still rather limited. Recent studies have shown that the phosphorylation of cellulose synthases (CESAs) may represent a key regulatory event in cellulose production. However, the impact of environmental conditions on the carbon flux of cellulose deposition and on phosphorylation levels of CESAs has not been fully elucidated. Here, we took advantage of gas exchange measurements, isotopic techniques, metabolomics, and quantitative phosphoproteomics to investigate the regulation of cellulose production in Arabidopsis rosette leaves in different photosynthetic contexts (different CO$_2$ mole fractions) or upon light/dark transition. We show that the carbon flux to cellulose production increased with photosynthesis, but not proportionally. The phosphorylation level of several phosphopeptides associated with CESA1 and 3, and several enzymes of sugar metabolism was higher in the light and/or increased with photosynthesis. By contrast, a phosphopeptide (Ser126) associated with CESA5 seemed to be more phosphorylated in the dark. Our data suggest that photosynthetic activity affects cellulose deposition through the control of both sucrose metabolism and cellulose synthesis complexes themselves by protein phosphorylation.

Key words: Arabidopsis, cellulose, phosphoproteomics, photosynthesis, protein phosphorylation.

Introduction

Cellulose biosynthesis by land plants probably represents a flux of ≈3.6 gigatons of carbon a year and is thus a huge carbon sink in terrestrial ecosystems. In fact, the lattice of cellulose microfibrils forms the fundamental component of plant cell walls and, as such, cellulose represents about 5–10% of plant dry matter. Despite this considerable importance, a full picture of cellulose biosynthesis is currently missing and, quite critically, the regulation of cellulose biosynthesis in response to photosynthetic carbon provision is virtually unknown (Joshi and Mansfield, 2007). In fact, cellulose production might respond to photosynthetic activity owing to changes in the availability of precursors for biosynthesis (e.g. glucose phosphate) or changes in the demand for cell wall deposition caused by growth fluctuations arising from variations in organic carbon availability.

Isotopic carbon labelling studies that examined the metabolic commitment into leaf cellulose synthesis are scarce. In cotton (Gossypium hirsutum) and sugar cane (Saccharum officinarum) leaves, labelling with $^{14}$CO$_2$ showed that insoluble glucan material (which includes cellulose) represented about 4% of fixed $^{14}$C (Hatch and Slack, 1966; Dickson and Larson, 1975). Using labelling with $^{14}$C-glucose on broad bean (Vicia
Populus alba
Vigna radiata
Pillonel
Loader
Prunus
Nicotiana tabacum

μ

Furthermore, phosphorylation at Thr166, whereas it is inhibited by phosphorylation at other positions (such as Ser167). Furthermore, phosphorylation of CESA1 influences the interaction with microtubules and thus the geometry of cellulose deposition (Chen et al., 2010). Wide

examination of gene expression with microarrays has suggested that genes associated with cell wall synthesis peak during the night (Harmer et al., 2000). Specific gene expression studies (i.e. focused on cellulose biosynthesis) then showed that several CESA genes have a circadian expression pattern (CESA1, 4 and 7) and that CESA5 transcription is inhibited by illumination (Hamann et al., 2004).

Taken as a whole, owing to multiple effects of the light/dark transition on individual CESA isoforms, the net response of the metabolic flux of cellulose biosynthesis to light/dark, or more generally to photosynthetic conditions, is not straightforward. Intuitively, cellulose synthesis in leaves should be orchestrated by three forces, namely, the availability of carbon sources (glucose or UDP–Glc) from photosynthesis, the demand in cellulose synthesis associated with growth, and the competition for UDP–Glc between cellulose synthesis and sucrose production. However, the mechanisms and even the direction in which these forces push cellulose synthesis are still unknown. As an aid in clarifying these aspects, we investigated the influence of different photosynthetic contexts (CO2 mole fraction, light/dark) on cellulose biosynthesis by intact rosettes of Arabidopsis thaliana. We took advantage of gas-exchange coupled to instant sampling with liquid nitrogen to examine 13C-labelling of cellulose (upon 13CO2 feeding), metabolic profiling, and phosphopeptides associated with sucrose and cellulose metabolism. Our results show an augmentation of the carbon flux into cellulose synthesis as photosynthesis increases, and coordinated changes in the phosphorylation of CESAs as well as in other enzymes involved in sugar metabolism.

Material and methods

Plant material

After sowing on potting mix, Arabidopsis (Col-0 ecotype) plants were transplanted to individual pots and grown in a controlled environment (growth chamber) under 8:16h light/dark (short days) at an irradiance of approximately 100 μmol m–2 s–1, 20/18 °C day/night temperature, 65% humidity and nutrient solution (1 g 1–1 PP14-12–32, [Plant-Prod, Puteaux, France] supplemented with 20 μl 1–1 fertilogo L [Fertil, Boulogne-Billancourt, France]) twice a week.

α-cellulose extraction and isotopic composition

Samples from isotopic labelling experiments (see below) were lyophilized and ground to a fine powder. α-cellulose was extracted using a hot solvent/chemical bleaching protocol, which has been shown to be quantitative and thus to yield an accurate isotopic composition (i.e. avoiding isotopic adulteration) (Loader et al., 1997, Boettger et al., 2007). Briefly, 50mg leaf powder was placed in a borosilicate thimble and extracted with a Soxhlet using an ethanol/toluene mixture (1:2 v/v) at 90 °C for at least 6h to yield a pigment-, isoprenoid- and resin-free sample material. The sample was then bleached with sodium hypochlorite (2% w/w) for 2h and purified with NaOH (10% w/w) at 80 °C for 45min under ultrasonication. The (perfectly white) cellulose extract was rinsed twice with deionized water and lyophilized. Cellulose was weighed in tin capsules and analysed by elemental analysis coupled to isotope ratio mass spectrometry (EA-IRMS Pyrocube-Isoprime, Elementar, France), using glutamic acid (USGS-40 of the IAEA, Vienna) as an isotopic standard (δ13C = −26.39‰).
Metabolomic analyses

Metabolomic analyses were performed as in Bathellier et al. (2009). Briefly, 20 mg of leaf powder from lyophilized leaf samples was extracted with 2 ml methanol:water (70:30 v/v). GC-MS metabolomics analyses were carried out on supernatants chemically derivatized with methoxyamine and MSTFA in pyridine, with a Pegasus III GC-TOF-MS system (Leco, France).

Gas exchange and sampling

Photosynthesis and respiration rates were monitored with the gas exchange open system Li-Cor 6400 xt (Li-Cor, Austin, USA), under a controlled humidity of 80% fixed with a dew-point generator (Li-Cor 610). Net photosynthesis (A) was measured in typical conditions (desired CO₂ mole fraction, 21% O₂, 22 °C, 250 μmol m⁻² s⁻¹ PAR [photosynthetically active radiation], 10% blue). CO₂ mole fraction was either 100 (LC), 380 (NC), or 1000 (HC) μmol mol⁻¹. Gas-exchange was carried out with a purpose-built cuvette adapted to three Arabidopsis rosettes connected to the sample channel of the Li-Cor 6400 xt. Air temperature in the chamber was maintained with a water-bath. Leaf rosettes were separated from the below-ground part and soil of the pot by a Plexiglass wall (with specific holes for collars) so as to avoid alteration of gas-exchange by soil and root respiration. The upper wall of the leaf cuvette was made of a tight polyvinyl chloride film allowing instant sampling by liquid N₂ spraying. Photosynthesis was allowed to stabilize under the desired CO₂ mole fraction (at 250 μmol m⁻² s⁻¹ PAR) and after 4 h rosettes were instantly frozen and stored at −80 °C for further analyses. Rosettes sampled in darkness were collected after 4 h at 380 ppm CO₂ and 2 h of dark-adaptation.

13CO₂ labelling

Separate experiments were carried out for labelling to avoid any 13C-enrichment in either metabolites or proteins detected in metabolomics or proteomics, respectively (i.e. 13C would compromise mass spectrometry analyses). The 13C label was fed as 13CO₂ (99.9% 13C, 20 bars cylinder, Eurisotop, France) monitored by the Li-Cor 6400 xt (see above for gas exchange techniques). The total amount of 13C assimilated was thus followed by gas exchange. Photosynthesis was allowed to stabilize for 1 h with ordinary CO₂ (13C at natural abundance, 1.1%) and inlet carbon dioxide was then abruptly changed to 13CO₂. Rosettes were sampled (liquid N₂ spray) after 3 h in the light. As an isotopic control (no 13C-enrichment expected in cellulose, unless anaplerotic CO₂ fixation occurred), 13CO₂ labelling was also carried out in darkness (right bar in Fig. 1B).

General protocol for phosphoproteomics

Arabidopsis rosettes were placed in the cuvette and after 4 h in a photosynthetic steady-state at the desired CO₂ mole fraction, leaf rosettes were sprayed with liquid nitrogen and sampled. Sampling in the dark was carried out on dark-adapted leaves (that is, after 2 h in darkness following 4 h of steady photosynthesis at ordinary CO₂, 380 μmol mol⁻¹). For proteomic characterization, protein samples were digested by trypsin. Peptides were then methylated via formylation with either labelled (deuterated) or non-labelled formaldehyde and reduction with cyanoborohydride. Non-labelled and labelled peptides were mixed (the condition of interest (non-labelled) and the mix of all samples as a reference (labelled) mixed with a 1:1 mixing ratio), and underwent a SCX (strong cation exchange) chromatography. Collected fractions were enriched in phosphopeptides by IMAC (immobilized ion metal affinity chromatography) and then analysed by nanoLC-MS/MS. The quantity of proteins was determined by direct analysis (no SCX and labelling). Detailed protocols associated with phosphoproteomics are already described in Bonhomme et al. (2012) and Boex-Fontvieille et al., (2013), therefore methods are only briefly described below.

Protein extraction, digestion, and stable isotope dimethyl labelling

Leaf proteins were extracted and denatured with TCA/acetone and resolubilized using urea/thiourea. Total protein content was determined using the 2-D Quant-kit (GE Healthcare), with bovine serum albumin as a standard. For each biological replicate, 2 mg of proteins were collected and reduced with 10 mM DTT. Samples were subsequently alkylated with iodoacetamide and diluted with ammonium bicarbonate. Protein digestion (Trypsin Gold, Promega) was performed at an enzyme/substrate ratio of 1:30 (w/w) by overnight
incubation at 37 °C, and stopped with formic acid 1% (v/v). Tryptic peptides were spin-dried and re-suspended in formic acid 5%. Stable isotope dimethyl labelling was performed according to the on-column procedure described by Boersema et al. (2009) using formaldehyde or [H3]formaldehyde (labelling). Each sample was loaded on a separate SepPak C18 cartridge column (3cc, Waters) and washed with 0.6% acetic acid. SepPak columns were flushed ten times with 1 ml of the respective labelling reductive reagent (50 mM sodium phosphate buffer pH 7.5, 30 mM NaBH4-CN and 0.2% CH3OH or C2H5OCH3/v/v). Samples were eluted with 500 µL of 0.6% acetic acid and 80% acetonitrile. All heavy dimethyl-labelled peptides were homogenized to form a reference sample, before being mixed with the light dimethyl-labelled peptides in a 1:1 abundance ratio.

Cation exchange chromatography and phosphopeptide enrichment

Before SCX, dimethyl-labelled peptides were spin-dried and resuspended in 500 µl of solvent A (30% acetonitrile, 5% formic acid, pH 2.5). SCX was performed at 200 µl min−1 using Zorbax BioSCX-Series II columns isocratically at 100% solvent A and then with increasing pH gradient using solvent B (30% acetonitrile, 5% formic acid, 540 mM ammonium formate, pH 4.7). SCX fractions were dried and resuspended in 300 µl of solvent C (250 mM acetic acid, 30% acetonitrile). Peptides were gently mixed with 80 µl of Phos-Select iron affinity gel (Sigma-Aldrich) and incubated for 1.5 h using a tube rotator, as described in Nühse et al. (2007). The mixture was transferred to SepPak spin columns (Sigma-Aldrich) and the flow-through fractions containing the non-phosphorylated peptides were collected. Iron affinity gel with bound phosphopeptides was rinsed twice with 200 µl of solvent C, then once with double distilled water. The elution of bound phosphopeptides was achieved with 100 µl of solvent D (400 mM NH4OH, 30% acetonitrile) by centrifugation at 8200 g. Flow-through fractions and eluted phosphopeptides were dried and kept at −20 °C until LC-MS/MS analysis.

LC-MS/MS analysis

On-line liquid chromatography was performed using a NanoLC-Ultra system (Eksigent). A 4 µl sample was loaded at 7.5 µl min−1 on a pre-column cartridge (stationary phase: C18 PepMap 100, particles of 5 µm; column: 100 µm i.d., 1 cm length; Dionex) and desalinated with 0.1% formic acid in water. After 3 min, the pre-column cartridge was connected to the separating PepMap C18 column (Dionex). Buffers were 0.1% formic acid in water (solvent E) and 0.1% formic acid in acetonitrile (solvent F). Peptide separation was achieved using a linear gradient from 5–30% F at 300 nl min−1. Eluted peptides were analysed with a Q-Exactive mass spectrometer (Thermo Electron) using a nano-electrospray interface. Ionization (1.5 kV ionization potential) was performed with liquid junction and a non-coated capillary probe (10 µm i.d.; New Objective). Peptide ions were analysed using Xcalibur 2.1. Under our conditions, the mass spectrum repeatability of all of the phosphopeptides considered (i.e. number of occurrences of the mass spectrum considered within each sample) was always satisfactory (between 4 and 206).

Identification of peptides and phosphorylation sites and quantification

Database searches were performed using X!Tandem Cyclone (http://www.thegpm.org/TANDEM). Cys carboxymethylidethylation and light and heavy dimethylation of peptide N-termini and lysine residues were set as static modifications, whereas Met oxidation and phosphorylation of tyrosine, serine, or threonine residues were set as variable modifications. Mass error tolerance was 10 ppm for both precursor and fragments. Identiﬁcations were performed using the TAIR release 8 database (http://www.Arabidopsis.org). Identified proteins were filtered and grouped using the X!Tandem pipeline v3.2.0 (http://pappso.inra.fr/bioinfo/xtandempipeline/). Relative quantification of non-phosphorylated peptides and phosphopeptides was performed using the MassChroQ software (Valot et al., 2011) by extracting ion chromatograms (XICs) of all identiﬁed peptides within a 0.3 Th window and by integrating the area of the XIC peak at their corresponding retention time, after LC-MS/MS chromatogram alignment.

Statistics

Phosphoproteomics analyses were carried out three times for each condition (i.e. three biological replicates). Phosphopeptides considered to vary signiﬁcantly between photosynthetic (CO2 mole fraction and/or light/dark) conditions were those with P<0.05 using crossed Student-Welch tests (phosphopeptides reported in Table 1). This value ensured an acceptable false discovery rate (FDR), estimated as in Tan and Xu (2014), including the Hochberg-Benjamini correction (Hochberg and Benjamini, 1990). FDR was of 4.6% (light/dark effect) and 4.8% (CO2 effect), over the whole dataset (of 2500 phosphopeptides). The relationship between phosphopeptides (phosphoproteome) and metabolites (metabolome) was examined in three steps. An analysis of variance (one-way ANOVA, four conditions) was conducted separately on phosphopeptides and metabolites, and those with P<0.01 were kept for the second step. Note that the use of P<0.05 instead of 0.01 would have weakened the statistical model and yielded spurious correlations, owing to cumulative errors within single principal components. The relationship (X-Y correlation) was analysed through O2PLS® (phosphopeptides as X variables and metabolites as Y variables) carried out with Simca® (MKS Umetrics, Sweden). The effect of each phosphopeptide in explaining the linear X-Y relationship was quantified using the pc score (X loading p and Y loading weight c combined to one vector) (supplemental Fig. S1). Phosphopeptides associated with enzymes (photosynthesis, sugar metabolism and cellulose synthesis) with the best 10% pc scores (and those with the lower 90% pc scores) were tabulated (supplemental Table S1). The cosine correlation coefficient between phosphopeptides and metabolites was calculated (correlation matrix). Correlation coefficients were rescaled via generalized Gauss-transformation and a hierarchical clustering was carried out to group phosphopeptides with similar metabolic correlations (supplemental Fig. S2).

Results

Photosynthesis and 13C-labelling

The net CO2 exchange of rosettes is shown in Fig. 1A. Three gas exchange conditions in the light were used: ordinary [CO2] (380 µmol mol−1, NC), high [CO2] (1000 µmol mol−1, HC), and low [CO2] (100 µmol mol−1, LC). There was a clear effect of CO2 mole fraction on photosynthesis, with a 5-fold change of net assimilation between LC and HC conditions. In darkness, net CO2 exchange was negative owing to dark respiration. Cellulose seemed to be labelled upon 13CO2 feeding in the light, demonstrating the consumption of current photosynthates to fuel cellulose biosynthesis (Fig. 1B). As expected, the control experiment with 13CO2 in the dark did not change the isotopic composition of cellulose from natural abundance. The absolute 13C-flux into cellulose synthesis increased in the different photosynthetic contexts, from 0.10±0.01 µmol 13C m−2 s−1 at low CO2 to 0.15±0.01 µmol 13C m−2 s−1 at high CO2. The relative 13C-flux in cellulose (expressed in % of fixed 13C) declined with increasing CO2 mole fraction used during illumination, from 4% at low CO2...
**Table 1. List of phosphopeptides associated with proteins involved in sugar metabolism**

Phosphopeptides are considered to be ‘significantly affected’ if the phosphopattern changed significantly \((P<0.05)\) when \(\text{CO}_2\) or light conditions varied (as indicated). No. Int. Pep. (right column) indicates the number of other phosphopeptides of the given protein but detected intermittently, with no exploitable phosphopattern.

<table>
<thead>
<tr>
<th>Name</th>
<th>#</th>
<th>Peptide</th>
<th>Position</th>
<th>No. Int. Pep.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peptides significantly affected by light/dark:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cNSPase</td>
<td>A1g74910</td>
<td>RV(s)(s)FEALQPATR</td>
<td>217, 218</td>
<td>0</td>
</tr>
<tr>
<td>cF2,6BPase</td>
<td>A1g07110</td>
<td>AAQRN(pS)EDSOVGTVDGPSAK</td>
<td>234</td>
<td>1</td>
</tr>
<tr>
<td>cF2,6BPase</td>
<td>A1g07110</td>
<td>N(s)EDSOVGTVDG(s)PSAK</td>
<td>234, 244</td>
<td>1</td>
</tr>
<tr>
<td>cINV 1</td>
<td>A1g35580</td>
<td>SVLD(s)pP(s)PL(s)AR</td>
<td>70, 73, 74</td>
<td>3</td>
</tr>
<tr>
<td>MiPase 13</td>
<td>A1g05630</td>
<td>K(pS)LPSLDVPR</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>pPFK like</td>
<td>A1g69200</td>
<td>AAAA(s)EDEVK</td>
<td>132, 133</td>
<td>0</td>
</tr>
<tr>
<td>TPS 7</td>
<td>A1g06410</td>
<td>(s)Y(pS)NLLELNGFSFTSF</td>
<td>5, 7</td>
<td>0</td>
</tr>
<tr>
<td>TPS 6</td>
<td>A1g068020</td>
<td>(s)Y(s)NLLELNGDFSFTSF</td>
<td>5, 7</td>
<td>0</td>
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<tr>
<td>SPS A1</td>
<td>At4g10120</td>
<td>IR(pS)EMQWSEDK</td>
<td>180</td>
<td>0</td>
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<tr>
<td>SPS C</td>
<td>At4g10120</td>
<td>VPEELTS(pS)LR</td>
<td>717</td>
<td>0</td>
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<td><strong>Peptides significantly affected by ([\text{CO}_2]):</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cINV 1</td>
<td>A1g35580</td>
<td>(s)M(s)EL(s)TGYSR</td>
<td>44, 46, 49</td>
<td>3</td>
</tr>
<tr>
<td>GlcTase</td>
<td>A3g07020</td>
<td>VW(pT)MLEQQSSSDKCESSTTNQPR</td>
<td>82</td>
<td>2</td>
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<tr>
<td>MiPase 1</td>
<td>A1g34120</td>
<td>IK(s)H(s)DPP(s)PSK</td>
<td>185, 187, 191</td>
<td>Y</td>
</tr>
<tr>
<td>TPS 5</td>
<td>A4g177700</td>
<td>(s)Y(s)NLLELNGFSFTSF</td>
<td>5, 7</td>
<td>1</td>
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<tr>
<td>RS 5 (SIP1)</td>
<td>A4g403900</td>
<td>SD(pS)GINGVDFTEK</td>
<td>11</td>
<td>0</td>
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<tr>
<td><strong>Peptides significantly affected by both light/dark and ([\text{CO}_2]):</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>cNSPase</td>
<td>A1g74910</td>
<td>V(s)(s)FEALQPATR</td>
<td>217, 218</td>
<td>0</td>
</tr>
<tr>
<td>CESA1 (1)</td>
<td>A4g32410</td>
<td>NAS(pS)PYPIDPR</td>
<td>180</td>
<td>0</td>
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<tr>
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<td>TT(pS)GPLGPSDR</td>
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<td>0</td>
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<tr>
<td>CESA3 (1)</td>
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<td>QDT(s)GEFSAA(s)PER</td>
<td>144, 151</td>
<td>1</td>
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<tr>
<td>CESA3 (2)</td>
<td>A5g05170</td>
<td>LPPSVDDNQ(s)PSNR</td>
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<td>0</td>
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<tr>
<td>CESA5</td>
<td>A5g09870</td>
<td>SGLESETF(s)R</td>
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<td>1</td>
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<td>cF2,6BPase</td>
<td>A1g07110</td>
<td>SVETL(pS)PFQQK</td>
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<td>CS1</td>
<td>A2g22125</td>
<td>MPHSEPP(t)PHE(t)Y(t)IK</td>
<td>37, 40, 42</td>
<td>0</td>
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<td>pPGM</td>
<td>A5g51820</td>
<td>ANGGFM(s)A(s)HNPGGEYDWGI</td>
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<td>0</td>
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<tr>
<td>SPS A1</td>
<td>A5g02280</td>
<td>IN(s)AE(s)MELWASOQK</td>
<td>152, 155</td>
<td>3</td>
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<td><strong>Peptides with no significant phosphopattern:</strong></td>
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<td>βAMY 5</td>
<td>A4g15210</td>
<td>(t)(s)N(s)QLTLEDIAADACP</td>
<td>466, 467, 469</td>
<td>0</td>
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<tr>
<td>βAMY 7</td>
<td>A3g23920</td>
<td>SGEYDSSLLIS(pS)PPSAR</td>
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<td>1</td>
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<tr>
<td>βAMY 7</td>
<td>A3g23920</td>
<td>AHQTDPP(s)PPM(s)PILGATR</td>
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<td>1</td>
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<td>GGVDDASAPDILDAEALLNDEAR</td>
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<td>CESA3 (4)</td>
<td>A5g05170</td>
<td>NTGPVSTOA(pS)SER</td>
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<td>SL(pS)ASSFLIDTK</td>
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<td>cF2,6BPase</td>
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<td>218, 220</td>
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<td>cPGM</td>
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<td>ATGAFIL(t)A(s)HNPGPTEDFGIK</td>
<td>122, 124</td>
<td>0</td>
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<tr>
<td>CSL C04</td>
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<td>S(s)ESLAFAAK</td>
<td>562, 564</td>
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to 1.5% at high CO₂. In other words, the absolute carbon flux to cellulose increased but the commitment of fixed carbon to cellulose biosynthesis (that is, the apparent partitioning of photosynthetically fixed carbon to cellulose) decreased as net CO₂ assimilation increased.

Sugar content

GC-MS based metabolomics were used to provide a relative quantitation (semi-quantitative measurement) of major sugars contained by rosette leaves (Fig. 2), as well as other metabolites (Fig. S2). There was a clear increasing trend of total sugars and Glc as photosynthesis increased (and fewer sugars in darkness and at low CO₂). Levogluconosan, which is formed by the degradation of polysaccharide polymers such as α-cellulose upon chemical derivatization (such a derivatization is required for GC-MS analyses) (Minor 1983; Botanga et al., 2012), did not decline significantly as photosynthesis increased and the levogluconosan-to-sucrose ratio was lower at high photosynthesis (Fig. 2, inset), with a relatively high value in darkness (similar to LC).

Phosphopeptides and phosphorylation sites

The phosphoproteomics analysis yielded nearly 2500 phosphopeptides, among which 71 were related to sugar and cellulose metabolism (Table 1 lists them, with peptide sequences). Three CESAs (1, 3, and 5) were detected, represented by 8 phosphopeptides. The analysis of the total content in each peptide indicated no difference between the CO₂ and dark treatments. That is, the changes in phosphopeptide abundance described below are strictly related to changes in the phosphorylation ratio and are not due to changes in total (i.e. phosphorylated + non-phosphorylated) peptide content. Significant (i.e. with statistically significant changes under photosynthetic/light conditions) and insignificant phosphopeptides are distinguished in Table 1. We also found 'intermittent' (i.e. phosphorylated with little repeatability) peptides for a number of proteins, including SGLESE(pT)FSR phosphorylated at Thr124 from CESA5 (see also below).

Phosphorylation sites were mapped using MS spectra and searching with X!Tandem, thus giving obvious phosphorylated residues in peptides. In some instances, however, ambiguous cases occurred, in which the nature of the phosphorylated site could not be determined (two or more indistinguishable possibilities). This was the case for CESA3 and Ser151 (Table 1). New phosphorylation sites (i.e. absent from PhosPhAt 4.0) were detected from PhosPhAt 4.0 (see also below).

Phosphorylation sites were differentiated with numbers between parentheses. The symbol “Y” on the right indicates phosphorylation sites absent from PhosPhAt 4.0. Phosphorylation sites are indicated between parentheses with a lower case letter (several position possibilities) or an upper case letter with a preceding “p” (position known with certainty).
the peptide SGLESETFSR was found to be in two forms under our conditions; phosphorylated either at Thr124 (Fig. 3A) or at Ser126 (Fig. 3B). Phosphorylated peptides associated with the CESA proteins 2, 4, 6, and 7 were not detected.

We further found phosphorylated peptides associated with enzymes involved in primary carbon metabolism. Most of them are already present in the PhosPhAt 4.0 database (Table 1), with the exception of myo-inositol polyphosphatase 13 (=phytase, abbreviated MIPase), cytosolic invertase 2 (cINV2), galactosyl transferase (GalTase), plastidic aldolase (pALD), Suc phosphate synthase (SPS A1), and sugar responsive 45 (SR45). Five phosphorylation sites were found in SPS A1. The N-terminal sites are Ser27, and an unresolved site at Ser152 or 155. The position 152 is nevertheless much more likely as it is homologous to Ser158 in spinach (Spinacia oleracea), which is the target of a well characterised regulatory phosphorylation. Other sites are Ser418, 684, and 700. Two phosphorylation sites were found in another SPS isoform (SPS C), at Ser180 and Ser717. Sequence alignment shows that the former is homologous to Ser152 in SPS A1.

Phosphorylation patterns

Twenty-six phosphorylated sites (26 phosphopeptides in Table 1) were significantly affected (P<0.05) by conditions: light/dark and/or photosynthetic activity (CO₂ mole fraction). Their phosphorylation patterns are displayed in Figs 4 and 5. Phosphorylation sites in cellulose synthases were affected by both light/dark and photosynthesis conditions (Fig. 4). Conditions that increased photosynthesis increased CESA phosphorylation (Ser167 and Ser180 in CESA1 and Ser144/151 and Ser176 in CESA3) to a modest extent (≈1.3-fold change) and phosphorylation was often less pronounced in the dark. The effect of CO₂ mole fraction in the light was not consistent for CESA5; by contrast, Ser126 in CESA5 was nearly three times more phosphorylated in the dark compared with the light. Fig. 5 shows all significant phosphopeptides within a common heat map, with phosphopeptide classification by hierarchical clustering (left). Two main groups are apparent: photosynthesis-stimulated peptides (i.e. phosphorylation correlated with photosynthesis and decreased in the dark) and photosynthesis-inhibited peptides (i.e. phosphorylation anti-correlated with photosynthesis and increased in the dark). The former included phosphopeptides associated with CSI1, CESAl, and CESAs, and also raffinose synthase (RS5) and MIPase. The latter included the phosphopeptide associated with CESA5 as well as cytosolic NDP-sugar pyrophosphorylase (cNSPase, see also the discussion below), plastidic phosphoglucomutase (pPGM), phosphofructokinase (pPFK), and three putative trehalose phosphate synthases (TPS 5, 6, and 7). Several proteins have several sites with opposite patterns. Such was the case of (i) Suc phosphate synthase (SPS A1) in which Ser152/155 phosphorylation was photosynthesis-stimulated and Ser700 phosphorylation was photosynthesis-inhibited; (ii) cytosolic Fru-2,6-bisphosphatase (cf2,6BPase) in which Ser234/244 phosphorylation was photosynthesis-stimulated and Ser276 phosphorylation was photosynthesis-inhibited; (iii) cytosolic invertase (cINV) in which Thr/Ser 70/73/74 phosphorylation was photosynthesis-stimulated and Ser44/46/49 phosphorylation was photosynthesis-inhibited. The phosphorylation pattern seemed to be significant for the peptide VPEELTS(pS)LR but not for the peptide VPEELTS(pS)LRDVDDISLR, although they both correspond to the same phosphorylation site (Ser717). This discrepancy probably came from the fact that the longer peptide (VPEELTS(pS)LRDVDDISLR) was present in lower quantities and yielded a more variable signal or that trypsin-catalysed digestion of the long peptide was limited.

![Figure 2](image-url)

**Fig. 2.** Metabolite content (relative to NC) in Arabidopsis rosettes either in the light under different photosynthetic conditions (LC, low CO₂; NC, ordinary CO₂; HC, high CO₂) or in darkness (D). AU, arbitrary units. Inset, levoglucosan-to-sucrose ratio. Letters indicate statistical classes across CO₂ or light conditions for a given metabolite or metabolite ratio (P<0.05). ‘Pentoses’ stands for the sum of arabinose, ribose and xylose.
Correlation between metabolites and phosphopeptides

The potential impact of phosphopeptide variations (content) on metabolism was examined with orthogonal projections to latent structures (OPLS) and calculation of the correlation matrix between phosphoproteome and metabolome, using best significant phosphopeptides and metabolites ($P<0.01$). The OPLS analysis yielded good correlation coefficients between datasets ($R^2_X=0.812$, $R^2_Y=0.7554$) and a good predictability of the statistical model ($Q^2=0.596$). Amongst phosphopeptides with the most significant correlation with metabolites were those associated with MIPase (phytase) and cNSPase, as well as other phosphopeptides associated with enzymes/proteins unrelated to sugar metabolism (such as phosphoenolpyruvate carboxylase [PEPC], Rubisco activase [RCA] and mitochondrial pyruvate dehydrogenase [PDH]) (Fig. S1 and Table S1). MIPase, PEPC, and PDH phosphopeptides were associated with a negative correlation with sugar content (hexoses, pentoses and sucrose) and photosynthetic/photorespiratory products (glycerate and glycolate) and a positive correlation with organic acids (ascorbic acid, succinate, pyruvate) and myo-inositol (Fig. S2). The reverse effect was observed for RCA and cNSPase phosphopeptides (i.e. positive correlation with sugar content and negative correlation with organic acids and myo-inositol). Phosphopeptides associated with CESA1, CESA3, and CSI1

Fig. 3. Identification of Thr 124 (A) and Ser 126 (B) in cellulose synthase 5 (CESA5) by mass spectrometric sequencing of two phosphorylated peptides. Spectra of phosphorylated peptides show b (N-terminal) and y (C-terminal) fragment-ions as displayed in the sequence (top of each spectrum). Lower case p indicates the phosphate group. Phosphorylation is localized according to the pattern of the fragment-ions containing phosphate and fragment-ions with phosphate loss. Ions showing a neutral loss of $\text{H}_3\text{PO}_4$ are labelled with “-1P”. Parental ion fragments are, as shown in insets: SGLESE(pT)FSR and SGLESETF(pS)R (both with $m/z$ 1224.545, $z = 2$). In sample runs, they were identified in 3 and 9 individual spectra, respectively.
Regulation of cellulose synthesis

Correlated positively with sugars, contrary to CESA5 (as expected from the data of Fig. 4). Our correlation and OPLS analysis seemed to be robust, as typical peptides followed the generally accepted pattern: (i) the reverse correlation between PDH phosphopeptide and sugar content reflects the inhibition of the enzyme by phosphorylation under photosynthetic conditions (Budde and Randall 1990); (ii) the increased phosphorylation of RCA and PEPC in the light matches...
photosynthetic metabolism (Boex-Fontvieille et al., 2013, Duff and Chollet, 1995).

Discussion

Taking advantage of phosphoproteomics, metabolomics, and gas exchange coupled to isotopic techniques, the possible regulation of cellulose biosynthesis and associated sugar metabolism under different photosynthetic contexts has been examined.

Metabolic flux to cellulose

Our results show that the absolute metabolic flux (in μmol C m⁻² s⁻¹) to cellulose increases as photosynthesis increases (Figs 1 and 2), but represents a smaller proportion of the fixed CO₂ (Fig. 1B). Increased photosynthesis is accompanied by an increase in sugar content (glucose, sucrose) in our metabolomics data (Fig. 2) and the analysis of phosphorylated compounds has further shown that it is also accompanied by an increase in glucose-6-phosphate, rather than a build-up in UDP-glucose (Stitt et al., 1984a, 1984b). Thus, cellulose production is probably not only driven by sugar availability, but also regulated by other mechanisms as photosynthesis increases. A number of phosphopeptides associated with proteins involved in cellulose synthesis (including CESAs) are differentially abundant under varying conditions (CO₂ mole fraction, light/dark) (Table 1). Other phosphopeptides associated with enzymes of sugar metabolism show concerted changes (Fig. 5) and correlation with metabolites (SI). This suggests that cellulose biosynthesis was controlled by two factors as photosynthesis changed: sugar metabolism and availability, and CSC activity. Consequently, when photosynthesis increases at high CO₂, the absolute carbon flux to cellulose deposition tends to increase. However, the fact that it represents a smaller proportion of fixed carbon allocated to cellulose biosynthesis suggests that CSC activity reaches a maximum or is down-regulated (e.g. by CESA phosphorylation) as CO₂ assimilation increases. In other words, at high photosynthesis, there is an increase in glucose and sucrose content, even though there is an increase of CESAs phosphorylation. The net effect is a moderate increase in the absolute flux and a decrease in the commitment of source carbon to cellulose deposition. It should also be noted that Arabidopsis rosettes are heterogeneous and this has to be considered when assessing changes in ¹³C-partitioning in Fig. 1. In fact, the allocation of ¹³C into cell wall synthesis is likely to differ between fully expanded (mature) and still-growing (immature) leaves, in which ¹³C enters metabolism mostly via photosynthetic CO₂ fixation and from imported ¹³C-sucrose, respectively. In addition, immature leaves in which cellulose synthesis is intense are not directly sustained by photosynthesis but rather by sucrose exported from mature leaves. That is, the pattern observed in this work is an average over the whole rosette, which may encompass source/sink relationships between leaves.

Cellulose synthesis and CESA phosphorylation

We have found five phosphopeptides associated with CESAs as well as phosphopeptides associated with cellulose synthase-like proteins (CSL) (Table 1). All of these sites have already been tabulated in Nühse et al. (2007), Chen et al. (2010), or PhosPhAt 4.0, with the exception of Thr124 in CESAs (Figs. 4 and 5). We did not detect the three other phosphorylation sites (Ser162, Thr165, Thr166) in CESAs, or the phosphopeptides associated with CESAs in our analyses. However, we detected a phosphopeptide associated with cellulose synthase interacting protein 1 (CSI1), which has been found to participate in the function of CSCs by making a bridge between microtubules and CESAs (Gu et al., 2010; Bringmann et al., 2012; Li et al., 2012). Phosphorylation of CESAs is believed to exert a key role in CSC activity (Somerville, 2006). In CESAs, a point mutation leading to a Glu at Ser167 (S167E mutant), which mimics permanent phosphorylation, is detrimental to cellulose deposition, with lower growth and cell expansion; furthermore, the mutation causes a marked decrease in the downward cellulose-deposition velocity analysed by kymographs (Chen et al., 2010). Therefore, phosphorylation at Ser167 is believed to down-regulate CSC activity. Here, we provide evidence for a phosphopattern at this site, suggesting slightly less CESAs activity as photosynthesis increases (Figs. 4 and 5). Similarly, CESAs seemed to be slightly more phosphorylated at high photosynthesis. This phosphopattern was significant with P<0.05 for CESAs (Table 1) and P<0.01 for CESAs and CSI1 (Table S1).

The phosphopattern of CESAs at Ser126 was found to be rather different (Fig. 4). Using phosphomimetic mutations (Ser to Asp), phosphorylation of CESAs (at Ser230) has been suggested to be essential for CSCs in the absence of CESAs or in darkness (i.e. when CESAs presumably replaces CESAs in CSCs) and furthermore, the velocity of CESAs-containing CSCs seemed to be higher in phyB/cesa6 mutants than in single cesa6 mutants; that is, the activity of CESAs seems to be prevalent under dark conditions and stimulated by phosphorylation (Bischoff et al., 2011). This is consistent with the considerable increase in CESAs phosphorylation in darkness found here, and we propose that night-time cellulose deposition is catalysed by CESAs-containing CSCs. In other words, cellulose deposition in Arabidopsis rosettes probably does not stop along the day/night cycle, reflecting leaf growth pattern: leaf growth oscillates between day and night, with low (but non-zero) values at night and high values during the day (Wiese et al., 2007). However, CSC composition probably changes from light to dark conditions (Bischoff et al., 2011).

Phosphorylation of enzymes involved in sucrose, glucose, and myoinositol metabolism

The phosphoproteomic analysis detected phosphopeptides associated with several enzymes of sugar metabolism. Most of them are already present in the PhosPhAt 4.0 database (Table 1). To date, there is however little information on the physiological and metabolic role of most of these sites.
The most documented case is that of SPS, which has long been demonstrated to be inhibited by phosphorylation at Ser158 in spinach (Ser152 in Arabidopsis SPS A1) (Huber and Huber, 1992; Huber and Huber, 1996; Toroser and Huber, 1997). Phosphorylation at other sites has been observed in a number of cases, but it has been assumed to be unrelated to regulatory functions (Huber and Huber, 1996). Here, we show that SPS A1 phosphorylation at Ser152/155 increases under conditions that promote photosynthesis, whereas phosphorylation at Ser700 follows a reversed pattern (Fig. 5). On the other hand, both phosphorylation sites in SPS C (including at Ser180, homologous to Ser152) were anti-correlated with photosynthesis. It is plausible that leaf Suc synthesis is controlled by a balance between SPS isoforms with opposite phosphopatterns and not by (de)phosphorylation of a single SPS form. It is well-known that Suc synthesis occurs both in light and dark, from triose-phosphates and starch as a carbon source, respectively. When CO2 mole fraction increases, the main short-term effect on carbon allocation is a decrease of the Suc-to-starch ratio, that is, a relatively lower commitment to Suc compared with starch production (Sharkey et al., 1985). With our results, one may hypothesize that this balance is achieved with both SPS A1 (dark SPS activity) and SPS C (day SPS activity, regulated by CO2) via opposite phosphorylation tendencies.

Significant phosphopatterns in other key enzymes were observed here: F2,6BPase, INV, and plastidial PGM and PFK (Table 1, and a summary of the observed phosphorylated pattern is shown in Fig. 6). Despite the presence of associated phosphopeptides in PhosPhAt 4.0, the phosphorylation of these enzymes and the effect thereof is not well documented: (i) phosphorylation of F2,6BPase (by protein kinase A) has been found in mammals, with contrasting effects (stimulating either the phosphatase or the kinase activity, Hue and Rider 1987); (ii) phosphorylation of a cytosolic invertase isoform (cINV1, at4g34360, not found here) has been reported and suggested to decrease Suc cleavage activity under Fe deficiency (Lan et al., 2012); (iii) pPGM phosphorylation has been found in Arabidopsis (reported in PhosPhAt 4.0), poplar (Liu et al., 2013), and Chlamydomonas (Boesger et al., 2012). Although this enzyme has been found to be phosphorylated in mammals (thereby stimulating its activity for glycogen remobilization, Gururaj et al., 2004), the role of its phosphorylation is unknown in plants. Conversion of Glc-6-phosphate to Glc-1-phosphate by pPGM in the chloroplast is essential for starch synthesis in the light (Stitt and Zeeman, 2012). Here, we found that pPGM is more phosphorylated in the dark (Fig. 5), i.e. when starch is degraded. (iv) Fru-6-phosphate phosphorylation by PFK is believed to be a key point of glycolysis regulation in animals and yeast, but in plants, to our knowledge, pPKF phosphorylation has no known function.

Still, some phosphopeptides correlated tightly with metabolites, such as that associated with At1g74910, which is positively correlated to sugars (Fig. S2). This gene encodes a cytoplasmic protein putatively belonging to the AGPase family (ADP–glucose pyrophosphorylase family protein; nucleotidyltransferase activity). However, an AGPase activity is unlikely as leaf starch synthesis is believed to be strictly dependent on ADP–glucose synthesis by the chloroplastic enzyme (Tetlow et al., 2004; Stitt and Zeeman, 2012), whereas cytoplasmic AGPase seems to play a role in starch production in storage organs and grain endosperm (for a review, see Fernie and Willmitzer, 2004). That is, At1g74910 probably encodes a nucleotideyltransferase (NDP–sugar pyrophosphorylase) other than AGPase. Here, the correlation between At1g74910-associated phosphopeptides and metabolites simply suggests a role in carbon primary metabolism.

Two phosphopeptides associated with raffinose synthase (RS5) and phytase (MIPase 13) also showed a typical response to photosynthetic conditions (more phosphorylated in the light and at high photosynthesis, Fig. 5) and were amongst the best scored phosphopeptides correlating to metabolome, with a negative correlation with sugars (Suc, Glc, Fru) and a positive correlation with myo-inositol (Fig. S2). The role of the phosphorylation of these enzymes is unknown in plants. MIPases belong to the PTP (protein tyrosine phosphatase) family in which some human members have been shown to be regulated by phosphorylation (Garton and Tonks 1994, Echwald et al., 2002). It is plausible that phosphorylation RS5 and MIPase 13 plays a role in their activity, as myo-inositol is required to synthesise raffinose and thus export photosynthetic carbon. Here, we observed an increased phosphorylation of RS5 at light under high [CO2] (Fig. 5).

Conclusions and rationale

The results show that cellulose synthesis is influenced by photosynthesis, with a higher carbon flux but a proportionally lower metabolic commitment of photosynthetically fixed C to cellulose as net CO2 assimilation increases (Figs 1 and 2). Presumably, this effect is the result of two contrasting forces: substrate availability driven by carbon primary metabolism, and regulation of CSC activity, via CS11, CES1A1, and CES3A3 phosphorylation (Fig. 4). Cellulose production continues in the dark with an increased phosphorylation of CES5 (Fig. 5) (Bischoff et al., 2011). The stimulating photosynthetic effect on cellulose synthesis driven by sugar metabolism might also be underpinned by enzymatic phosphorylation (see discussion above) as well as metabolite contents (Figs S1 and S2). A tentative summary of the observed changes is presented in Fig. 6. In the light at ordinary or high [CO2], sugar metabolism is oriented towards starch and sucrose metabolism, thereby generating UGP–Glc, which is also consumed by CSCs (Fig. 6A). The competition between Suc (and raffinose) synthesis and cellulose production for UDP–Glc is probably minimal as Suc production in the illuminated leaf usually represents ≈40% of net fixed carbon (Stitt et al., 1983, Sharkey et al., 1985), whereas cellulose synthesis represents 1–4% (Fig. 1), that is, only 2.5–10% of the carbon flux to Suc. Furthermore, phytase (MIPase) activity sustains myo-inositol production (and thus raffinose synthesis), avoiding further consumption of Glc by myo-inositol neosynthesis. In the dark, starch remobilization feeds Glc metabolism and UDP–Glc
production. The competition between Suc and cellulose synthesis is also probably minimal owing to the Suc ‘futile cycle’ (simultaneous production and cleavage) and the down-regulation of raffinose synthesis (Fig. 6B).

We nevertheless recognize that our integrated picture in Fig. 6 may not be fully representative because (i) the effective consequence of phosphorylation on several proteins (e.g., RS5, phytase, TPS) is not documented yet with *in vitro* or molecular studies, and (ii) some uncertainty remains as to the metabolic role of several enzymes, such as TPS 5, 6, and 7, as in this case, no enzymatic activity has been found using heterologous expression in yeast (Ramon et al., 2009). That said, the phosphopatterns and metabolomics-phosphoproteomics correlations observed here for other documented phosphoproteins (such as CESA2) and CESA-associated proteins are not indicated for simplicity. SPS stands for SPS A1. For simplicity, the cytoplasmic NDP-sugar pyrophosphorylase (cNSPase, At1g74910) activity is not mentioned (see the Discussion).

Fig. 6. Tentative summary of plausible events involved in the regulation of cellulose synthesis by photosynthesis, as suggested by phosphopatterns reported in this work, for the photosynthetic (A) and the non-photosynthetic (B) case. Diamonds indicate phosphorylated (closed) or non-phosphorylated (open) status. Pathways that are likely to be down-regulated are indicated in grey. Enzymes abbreviations: see Table 1 legend. Metabolites abbreviations: Ino, inositol; MIP, polyphospho myo-inositol, TP, triose phosphates. In A, the curved dotted arrow stands for the RuBP-regenerating steps of the Calvin cycle. In B, for simplicity, starch degradation is only described by two paths: glucose and maltose production. In both A and B, other CESA proteins (such as CESA2) and CESA-associated proteins are not indicated for simplicity. SPS stands for SPS A1. For simplicity, the cytoplasmic NDP-sugar pyrophosphorylase (cNSPase, At1g74910) activity is not mentioned (see the Discussion).
Supplementary data

Supplementary data are available at JXB online.

Figure S1. X-Y correlation loadings of phosphopeptides in the O2PLS model between phosphoproteome and metabolome.

Figure S2. Heat map of correlation coefficient between significant phosphopeptides and metabolites.

Table S1. List of significant phosphopeptides associated with metabolism (encoding proteins associated with primary metabolism, photosynthesis, and cellulose synthesis).

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