Quantitative proteomic analysis to understand the role of Arabidopsis thaliana LEAFY COTYLEDON 2 promoting lipid accumulation in Chlorella sorokiniana by upregulating photosynthetic proteins and G3PDH

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Abstract

Microalgae attracted significant attention towards biofuel production and transcription factors that can effectively regulate lipid accumulation in higher plants. In this study, we expressed the transcription factor LEAFY COTYLEDON 2 from *Arabidopsis thaliana* on *Chlorella sorokiniana*. The electron transport rate (ETR) of *C. sorokiniana-AtLEC2* improved significantly under high-intensity light. As a result, the total lipid content and main fatty acid composition (C16, C18) increased by more than one fold. Differentially expressed proteins were analyzed through a label-free strategy to elucidate the regulation mechanism of AtLEC2. The results showed upregulation of TAG biosynthesis (G3PDH) and photosynthesis-related proteins (e.g., ATP synthase F0, Photosystem II, Photosystem I P700, Cytochrome b6, Cytochrome b6-f, Chlorophyll a-b binding protein, Photosystem I assembly protein). Furthermore, AtLEC2 expression in *C. sorokiniana* led to fixed carbon flow in photosynthesis to TAG through G3PDH upregulation. The current study will provide a new direction towards cultivating high-yield oil algae and reveal the regulation mechanism of AtLEC2 on TAG accumulation in *C. sorokiniana*.

Introduction

Microalgal biofuels are likely to replace fossil fuels due to their higher growth rate, photosynthetic efficiency, and high lipid content (Chen 2010, Juntila et al. 2015, Gouveia and Oliveira 2009, Ho et al. 2010). In addition, microalgae have minimum nutrient demand all year round and require less area than other land crops. For example, compared with oil palm, microalgae produce more than 10–20 times of oil, effectively reducing the land area to 1/20–1/10(Milano et al. 2016, Andruleviciute et al. 2014, Demirbas 2009).

Chlorella is a vital biofuel raw material because of its high biomass concentration obtained from heterotrophic and mixotrophic cultures (Juntila et al. 2015, Sharma et al. 2011, Gorgônio et al. 2013, Garnier et al. 2014). However, oil content remains one of the main blockages in biofuel production from *Chlorella*(Williams and Laurens 2010, Wu et al. 2012). Limiting nitrogen limitation and adding carbon sources are efficient ways of increasing lipid accumulation(Zhang et al. 2013, Ramanna et al. 2014, Li et al. 2015). In addition, different strategies have been adopted to improve oil content. These include upregulating the expression of rate-limiting enzymes (Bhowmick et al. 2015, Hsieh et al. 2012, Xue et al. 2016, Niu et al. 2013), downregulating competing pathways(Emily M. Trentacoste 2013), and systematically regulating the expression of specific transcription factors (Bhowmick et al. 2015, Wang et al. 2015). Transcription factors are known to regulate metabolic pathways through various checkpoints. Over-expressing transcription factors can effectively increase lipid accumulation in higher plants or some microalgae, like WRI1(To et al. 2012), LEC2(Santos Mendoza et al. 2005), LEC1(Tan et al. 2011), ABI3(Elahi et al. 2015), FUS3(Yun-Ting et al. 2018), Dof (Wang et al. 2007), and several others.

The LEC2 transcription factor, which encoded a B3 domain (Stone et al. 2001), directly triggered oil accumulation in *Arabidopsis* leaves (Santos Mendoza et al. 2005). Overexpression of LEC2 in *Arabidopsis*, tobacco, and soybean can significantly increase oil content (Andrianov et al. 2010, Angeles-

Nunez and Tiessen 2011, Manan et al. 2017). The *lec2* mutation decreases about 30% of oil content while accumulating 140% sucrose and five-fold starch in *Arabidopsis* seeds(Angeles-Nunez and Tiessen 2011). GmLEC2 upregulated the expression of downstream transcription factors like LEAFY COTYLEDON1(LEC1), FUS3, ABSCISIC ACID INSENSITIVE 3 (ABI3), DNA binging with one finger 11(Dof11), and wrinkled 1 (WRI1) in soybean (Manan et al. 2017).On the other hand, the proteins involved in carbohydrate biosynthesis and catabolism of TAG were downregulated. Thus, studies have shown that the LEC2 can regulate oil accumulation, but only a few reports have existed for algae until now.

To better understand the regulation mechanism of LEC2 from *Arabidopsis* (ATLEC2) in *C. sorokiniana*, differentially expressed proteins between *C. sorokiniana* and the recombinant *C. sorokiniana-AtLEC2* were analyzed through a label-free strategy. This study could provide new ideas to increase TAG accumulation in microalgae.

Methods

Culture of C. sorokiniana

C. sorokiniana cells were retrieved from a sewage treatment plant, and cultured in a BG11 medium (solid medium with 1.8% agarose), at 25°C and 40 μmol·m^{-2}·s^{-1} under a 12:12 h d^{-1} cycle.

Hygromycin inhibition test of C. sorokiniana

Chlorella is sensitive to hygromycin(Ming 2015, Zhibing 2012). Therefore, *C. sorokiniana* cells during the logarithmic phase were spread on a solid BG11 medium containing different hygromycin concentrations to find a suitable concentration of the correct antibiotic marker for its genetic modification. The optimum antibiotic concentration of hygromycin used to select transgenic *C. sorokiniana* was determined by observing algal growth for 20 days.

Construction of the AtLEC2 plant expression vector

The full-length cDNA of *LEC2* (Gene Bank No. NM_102595.2) was amplified with primers containing *Kpn* I (forward: GGGGTACCatggataacttcttaccctt) and *Xba* I (reverse: GCTCTAGAccaccactcaaagtcgttaa) restriction sites from the seeds of *A. thaliana*. Then, the amplicons were cloned into the vector PMD18-T and sequenced. Finally, the LEC2 was digested using *Kpn* I and *Xba* I restriction enzymes and inserted into the vector pCIMBIA1300–35s-GFP between *Kpn* I and *Xba* I.

Genetic transformation of C. sorokiniana

The *atlec2* gene was transformed into *C. sorokiniana* by bombardment, according to the process described by Jin et al.(Liu et al. 2014) and Ahmad et al.(Talebi et al. 2013). The bombarded cells were

recovered after 24 h and then spread on the BG11 solid medium containing 15 μg/mL hygromycin for selection. Colonies appearing after one week were picked up and inoculated again into the BG11 culture.

Primary identification of transgenic algae strains

Genomic DNA, RNA, and proteins were extracted to identify transgenic algae strains by PCR, RT-PCR, and Western blotting.

Lipid Content analysis

Cells were harvested and dried at 80°C for lipid content analysis. The lipid was extracted using the following : measured algal powder was mixed sufficiently with 4 mol/L hydrochloric acid at room temperature for 30 minutes. After bathing in boiling water for 3 mins and cooling in a − 20°C refrigerator for 30 mins, the residue was recovered using centrifugation and extracted with chloroform-methanol treatment (2:1, v/v) for 24 h. The process was repeated three times. The residue was dried at 80°C to obtain constant weight. Oil content was calculated using the following equation: $Y\ (\%) = (W_p - W_r)/W_p$, where W_p and W_r were the weights of algal powder and residue, respectively.

Fatty acid composition analysis

Fatty acid analysis was carried out by Qingdao Sci-tech Innovation Testing Co., LTD using a gas chromatography-mass spectrometer. 100 mg of *C. sorokiniana* powder was added with 2 mL 5% methanol hydrochloride solution and 3 mL chloroform-methanol (1:1, V/V). 100 μL methyl nonadecanoate was used as the internal standard. After an 85°C water bath for one hour, 1 mL hexane was added and later extracted for 2 mins. Then after standing for an hour, 100 μL of supernatant was absorbed and was made constant to 1 mL using hexane. The test was performed after filtration with a 0.45 μm membrane.

Label-Free Proteomic Analysis

C. sorokiniana cells were harvested at the end of the logarithmic phase and cultured for two days in a nitrogen-limiting BG11 liquid medium. Bangfei bioscience Co Ltd (Beijing) performed the proteomic analysis label-free.

The collected cells were ground with liquid nitrogen and added to the extracting solution. After centrifugation, the supernatant was absorbed and added to the precooled acetone/methanol volume three times, kept at − 20°C for an hour. The protein precipitates were collected by centrifugation and rinsed with precooled acetone two times. The protein was dried at room temperature, and lysis buffer was added for quantitative detection using the Bradford kit. After being reduced and alkylated with dithiothreitol and iodoacetamide, the proteins were trypsin digested.

Samples were separated using a Nano-HPLC, after which MS level measurements were performed. The qualitative and quantitative analysis of MS data was done using the Mascot and Proteome Discoverer (Thermo Scientific) and uni_taxonomy_chlor.fasta data bank.

Photosynthesis and Growth Analysis

Transgenic and control algal strains were inoculated into a fresh medium and continuously cultured for eight days. The growth curve was portrayed according to the results of cell counting every day.

Cells were harvested at the end of the logarithmic phase to induce oil accumulation and cultured for seven days in a nitrogen-limiting BG11 liquid medium.

The fluorescence yield and the chlorophyll light response curves were measured with a multi-color-PAM (HeinzWalz, Germany) (Shin et al. 2016, Schreiber and Klughammer 2013). Briefly, algal cells were extracted to 200 g/L after six days and dark-adapted for 20 min. Then, a 440 nm LED light was used to measure the fluorescence yield, and Fv/Fm was automatically calculated. The effective quantum yield of PSII (Y (II)) was recorded as increasing light intensities of 440 nm LED with a step width of 30 s.

Results

Obtain of LEC2 transgenic strains

Different concentrations of the hygromycin inhibition test indicated that *C. sorokiniana* was inhibited up to 15 µg/mL. Therefore, hygromycin was used as the screening marker for transgenic algae in this study. *Lec2* from *Arabidopsis* was cloned and inserted into the pCIMBIA1300–35s-GFP using the CaMV 35S as the promoter and *sgfp* gene as the selection marker. The selected clones were verified using PCR (Fig. 1A), RT-PCR (Fig. 1B), and western blot (Fig. 1C). AtLEC2-specific bands from transgenic strains *C. sorokiniana-AtLEC2* were obtained, but not from wild-type (WT). It indicated the successful integration and expression of *AtLEC2* into the genome of *C. sorokiniana*.

Effect of AtLEC2 expression on the growth and photosynthetic activity of C. sorokiniana-AtLEC2

The growth curve and chlorophyll fluorescence were measured to investigate the growth and photosynthetic activity of the transgenic *C. sorokiniana*. They both grew slowly in the first two days, proliferated rapidly in the next 3–7 days, and slowed down on the 7th day (Fig. 2A). The growth curves of *C. sorokiniana-AtLEC2* and WT were highly coincident and inferred that AtLEC2 expression did not significantly change the growth cycle of *Chlorella sp*.

Chlorophyll fluorescence is a sensitive bioindicator of early damage in algae and plants (Lin et al. 2015). The maximum quantum yield of PS II (Fv/Fm) and light response curves of electron transport rate through PS II (ETR) were measured to reflect the changes of photosynthesis induced by AtLEC2 expression in *C. sorokiniana-AtLEC2*. Fv/Fm values have only a few significant differences between *C. sorokiniana-AtLEC2* and WT as measured on the 6th day (Fig. 2B). As an indicator of stress effects(Schreiber et al. 2007), the Fv/Fm values showed that the expression of AtLEC2 did not cause

stress effects in cells. ETR increased linearly in both *C. sorokiniana-AtLEC2* and WT, and the curves almost overlapped when light intensity was less than 50 μmol·m^{-2}·s^{-1} (Fig. 2C). ETR increased gradually with enhanced PAR. When light intensity was up to 100 μmol·m^{-2}·s^{-1}, the ETR of *C. sorokiniana-AtLEC2* was significantly higher than WT, with a maximum of 17.1/13 μmol electrons m^{-2} s^{-1} for *C. sorokiniana-AtLEC2* and around 400 μmol photons m^{-2} s^{-1} for WT. All these results showed that *C. sorokiniana-AtLEC2* had higher photosynthetic efficiency with enhancing light intensities.

The AtLEC2 increases the lipid content of C. sorokiniana-AtLEC2

The total lipid content and fatty acid compositions were measured under nitrogen-limiting conditions. *C. sorokiniana-AtLEC2* had up to 51% lipid content, more than one fold than WT (23%).

GC-MS analysis of FA showed that all the components of *C. sorokiniana-AtLEC2* increased from 1.4 to 3.8 folds. Both *C. sorokiniana-AtLEC2* and WT had similar FA components, and their main constituents were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and alpha-linolenic acid (C18:3) (Table 1). The other fatty acids were barely detectable. The main fatty acid content of *C. sorokiniana-AtLEC2* increased significantly, but the proportion of C16 and C18 was similar to WT. Compared to WT, the proportion of C18:2 in *C. sorokiniana-AtLEC2* increased by 24%; however, C18:3 decreased by a significant 36% (Fig. 3).

Quantitative Proteomic Analysis

The proteomic analysis of AtLEC2 through label-free detection was performed. It showed that 209 proteins were regulated ($P < 0.05$, fold change≥1.5), of which 106 were upregulated, and 103 were downregulated compared with the wild type. Specifically, glycerol-3-phosphate dehydrogenase (G3PDH) (responsible for glycerol synthesis) and cytochrome b6-f complex subunit 4 (petD) (responsible for photosynthesis) were upregulated 8.8 and 7.8 folds, respectively. On the contrary, photosystem I subunit IV (psaE), triosephosphate isomerase (TPI) chloroplast type, and ribulose bisphosphate carboxylase/oxygenase activase (responsible for photosynthesis) were downregulated by 0.02, 0.09, and 0.2 folds, respectively.

The protein interaction network (PPI) was mapped using STRING and Cytoscape-3.6.0 software. About 116 differentially expressed proteins matched in *Chlorella variabilis*, of which 44 were upregulated, and 72 were downregulated (Fig. 4). The results showed the strong relationship between different functional proteins and their upregulation through carbon metabolism, photosynthesis, and electron-transferring.

Table 1
Fatty acid component of *Chlorella sorokiniana*

Fatty acid component (mg/kg)	D	LEC2	LEC2/D	FA composition (%) of WT	FA composition (%) of LEC2
C14:0 (myristic)	86.3	220.3	2.6	0.34%	0.41%
C15:0 (Pentadecanoic)	42.8	96.1	2.2	0.17%	0.18%
C16:0 (Palmitic)	8592	18217.2	2.1	33.51%	33.55%
C17:0 (margaric)	142.1	394.3	2.8	0.55%	0.73%
C17:1 (Margaroleic)	205.2	414.1	2.0	0.80%	0.76%
C18:0 (Stearic)	1296.4	2343.3	1.8	5.06%	4.32%
C18:1n9 (Oleic)	706.1	1245.3	1.8	2.75%	2.29%
C18:2n6 (Linoleic)	8794	23097.3	2.6	34.30%	42.54%
C18:3n3 (Linolenic)	5527.4	7483.7	1.4	21.56%	13.78%
C20:0 (Arachidic)	112.2	417.1	3.7	0.44%	0.77%
C20:1 (Eicosenic cis 11)	4.1	10.9	2.7	0.02%	0.02%
C20:2 (Eicosadienoic)	20.5	43	2.1	0.08%	0.08%
C20:3n6 (g-Eicosatrienoic)	13.4	19.1	1.4	0.05%	0.04%
C21:0 (Heneicosanoic)	9.8	20.2	2.1	0.04%	0.04%
C22:0 (Behenic)	28.4	107.4	3.8	0.11%	0.20%
C23:0 (Tricosanoic)	14.8	37.2	2.5	0.06%	0.07%
C24:0 (Lignoceric)	42.5	132.3	3.1	0.17%	0.24%
total	25638	54298.8	2.1		

PPI of differentially expressed proteins in *C. sorokiniana-AtLEC2* with nodes connected by directed edges indicating differentially expressed proteins and relationships. The upregulated proteins are shown in pink; the downregulated in green, and the node size is proportional to the regulatory amplitude of protein expression.

Discussion

AtLEC2 upregulated the photosynthetic elements and improved the photon capture capacity of C. sorokiniana-

AtLEC2

In recent years, microalgal genomics has made significant advances, and the lipid yield of microalgae has been improved through genetic engineering technologies (Talebi et al. 2013, Cecchin et al. 2019, Gao et al. 2014, Fan et al. 2015, Shang et al. 2016). However, lipid content is regulated by operating a single gene of key enzymes involved in lipid metabolism (Williams and Laurens 2010, Xue et al. 2016, Fan et al. 2014). In this study, AtLEC2 expression promoted the accumulation of lipid in *C. sorokiniana*, which increased from 23–51%. Growth curves and photosynthetic parameters showed that growth cycles and Fv/Fm values had no significant changes, while light response curves of ETR changed significantly.

ETR is an essential parameter of photosynthetic efficiency(Conde-álvarez et al. 2002). In this study, the ETR of *C. sorokiniana-AtLEC2* and WT were similar when light intensity was under 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$. However, when light intensity was increased to 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the ETR of *C. sorokiniana-AtLEC2* was significantly higher than WT (Fig. 2C). Thus, *C. sorokiniana-AtLEC2* could have higher photosynthetic efficiency. Furthermore, under low light intensities with limited photons, the harvest of photons between *C. sorokiniana-AtLEC2* and WT was consistent. However, with increasing light intensities, *C. sorokiniana-AtLEC2* showed a higher capture capacity of photons.

The changes in ETR were consistent with protein expression in photosynthesis (Fig. 5). ATP synthase F0, Photosystem II (psbC, psbB, psbH), Photosystem I (psaA, psaB), Cytochrome b6 (petB), Cytochrome b6-f (petD), Chlorophyll a-b binding protein (LHCB2), and Photosystem I assembly proteins were upregulated in *C. sorokiniana-AtLEC2*. Moreover, Oxygen-evolving enhancer protein 3 (psbQ) downregulated 1.3 folds but other detected Oxygen-evolving enhancer proteins were upregulated 2.6 folds. According to Thomas et al.(Thomas et al. 2001), the *psaE*$^{-}$ mutant did not affect the rates of PSI cyclic electron transport despite significant psaE downregulation. In general, the *AtLEC2* improved the light capture and the electron transport of *C. sorokiniana-AtLEC2*.

AtLEC2 improved the TAG accumulation in C. sorokiniana-AtLEC2 by upregulating the expression of G3PDH

Based on the proteomic analysis, enzymes involved in carbon fixation like TPI chloroplast type and ribulose bisphosphate carboxylase/oxygenase activase were downregulated without any decline in photosynthetic efficiency. Maybe it was due to the upregulation of G3PDH. G3PDH catalyzes the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate (G3P), which provides the glycerol backbone for TAG synthesis (Herrera-Valencia et al. 2012, Yao et al. 2014). Thus, G3PDH, a significant intermediary, connects glycolysis and TAG synthesis (Wang et al. 2018, Zhao et al. 2019).

In this study, G3PDH expression was upregulated by 8.8 folds in *C. sorokiniana-AtLEC2*, with enhanced conversion capacity of G3P from dihydroxyacetone phosphate. Dihydroxyacetone phosphate was consumed in large quantities. TPI catalyzed the isomerization between dihydroxyacetone phosphate and

glyceraldehyde-3-phosphate(Beck 1956). The rapid nature results in the maintenance of equilibrium between dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. At the equilibrium state, the concentration of dihydroxyacetone phosphate is much higher than glyceraldehyde 3-phosphate. Glyceraldehyde-3-phosphate produced during photosynthesis in chloroplasts was directly released into the cytoplasm by Triose Phosphate/Phosphate Translocator (Flügge and Heldt 1984) and was further used in the synthesis of TAG (Fig. 6). The down-regulated expression of TPI chloroplast type (10.6 folds) and upregulated cytoplasmic type (1.6 folds) verified it. The consumption of glyceraldehyde-3-phosphate affected the replenishment of ribulose-1,5-biphosphate (RuBP) in the Calvin cycle. However, RuBP is more likely to bind to rubisco in its inactive state. RuBP is a potent inhibitor of rubisco(Spreitzer and Salvucci 2002, Jr 2003). The reduction of RuBP increased the activity of rubisco indirectly (Streusand and Portis 1987). Therefore, the total activity of rubisco and the photosynthetic efficiency was not decreased despite the downregulation of Ribulose bisphosphate carboxylase/oxygenase activase and rubisco, which was consistent with the test results of photosynthetic efficiency.

Upregulated proteins are illustrated in red; downregulated proteins are in blue. RUBP: ribulose-1,5-bisphosphate; Rubisco: ribulose bisphosphate carboxylase/oxygenase; PDK: phosphoglyceric kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TPT: Triose Phosphate/Phosphate Translocator; TPI: triose phosphate isomerase; G3PDH: glycerol-3-phosphate dehydrogenase; ACS: long-chain acyl-CoA synthetase; GPAT: glycerol-3-phosphate O-acyltransferase; LPAAT: lysophosphatidate acyltransferase; PAP: phosphatidate phosphatase; DGAT: diacylglycerol O-acyltransferase.

Conclusion

In this study, transcription factors LEC2 from *Arabidopsis* were transferred to *C. sorokiniana* for expression. Although certain photosynthetic elements like photosystem I reaction center subunit IV, ribulose bisphosphate carboxylase/oxygenase activase, and TPI chloroplast type were downregulated significantly, other elements (ATP synthase F0, Photosystem II, Photosystem I P700, Cytochrome b6, Cytochrome b6-f, Chlorophyll a-b binding protein, Photosystem I assembly protein) and proteins involved in TAG biosynthesis were upregulated, predominantly G3PDH, which was upregulated 8.8 folds. In addition, ETR was significantly increased under high light intensity, and the lipid content of transgenic *C. sorokiniana* was one folds more than the WT. These results inferred that ATLEC2 expression in *C. sorokiniana* increased photosynthetic efficiency, leading to the flow of fixed carbon towards TAG through the upregulation of G3PDH.

Declarations

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.

AUTHOR'S CONTRIBUTIONS

Yilin Liu conducted laboratory experiments. Shanshan Yu contributed to interpretation and writing of the paper. Wenhao Dong provided the field data. Yongchang Xue developed a theoretical approach. Changbin Liu conducted calculations of the absorption coefficients and wrote the paper.

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DATA AVAILABILITY

All data generated or analysed during this study are included in this published article

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Figures

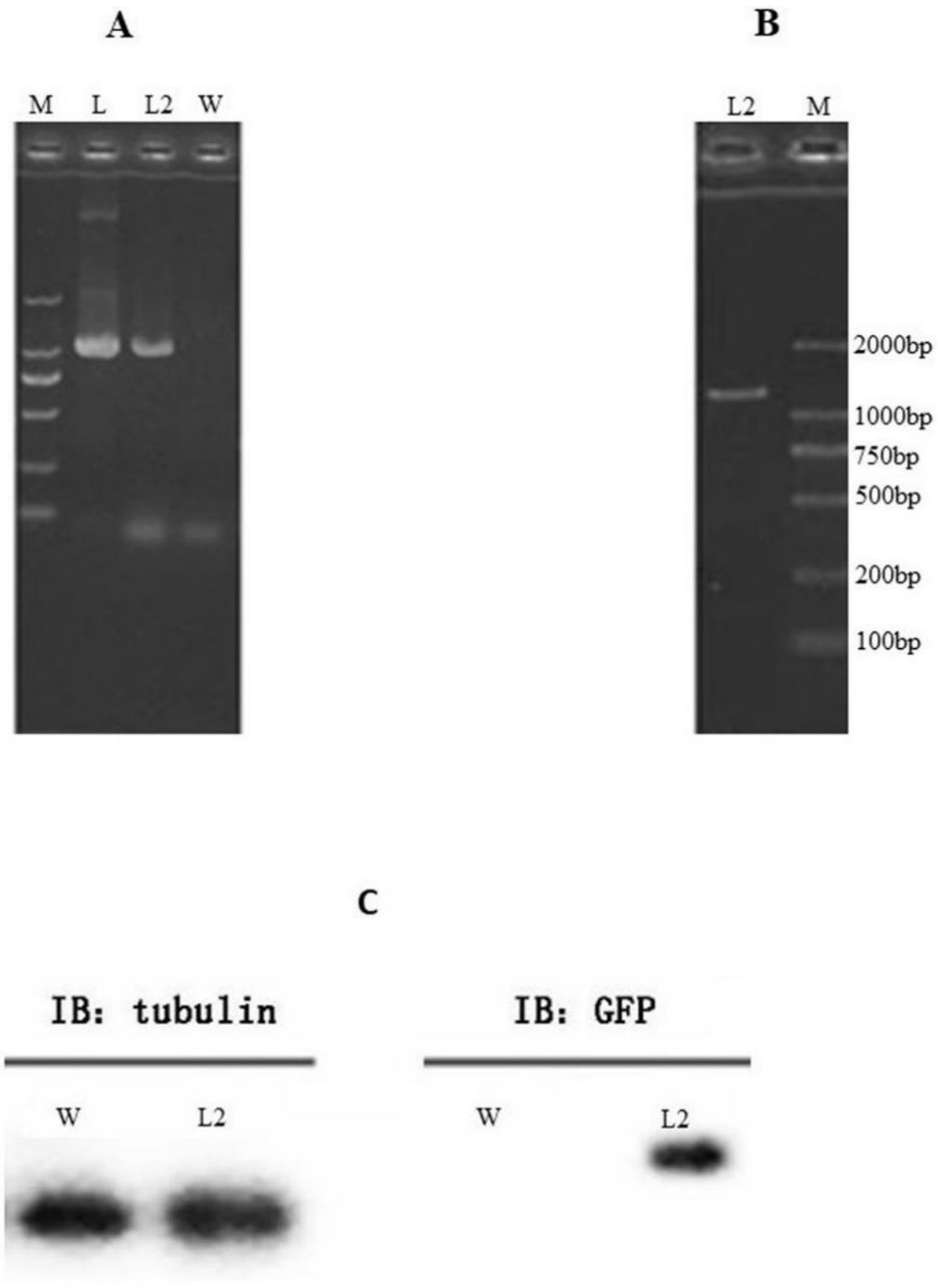

Figure 1

Identification of the transgenic *C. sorokiniana*

A: PCR, B: RT-PCR, C: Western blot; L: Plasmid, L2: The transgenic *C. sorokiniana* D: The wild transgenic *C. sorokiniana*.

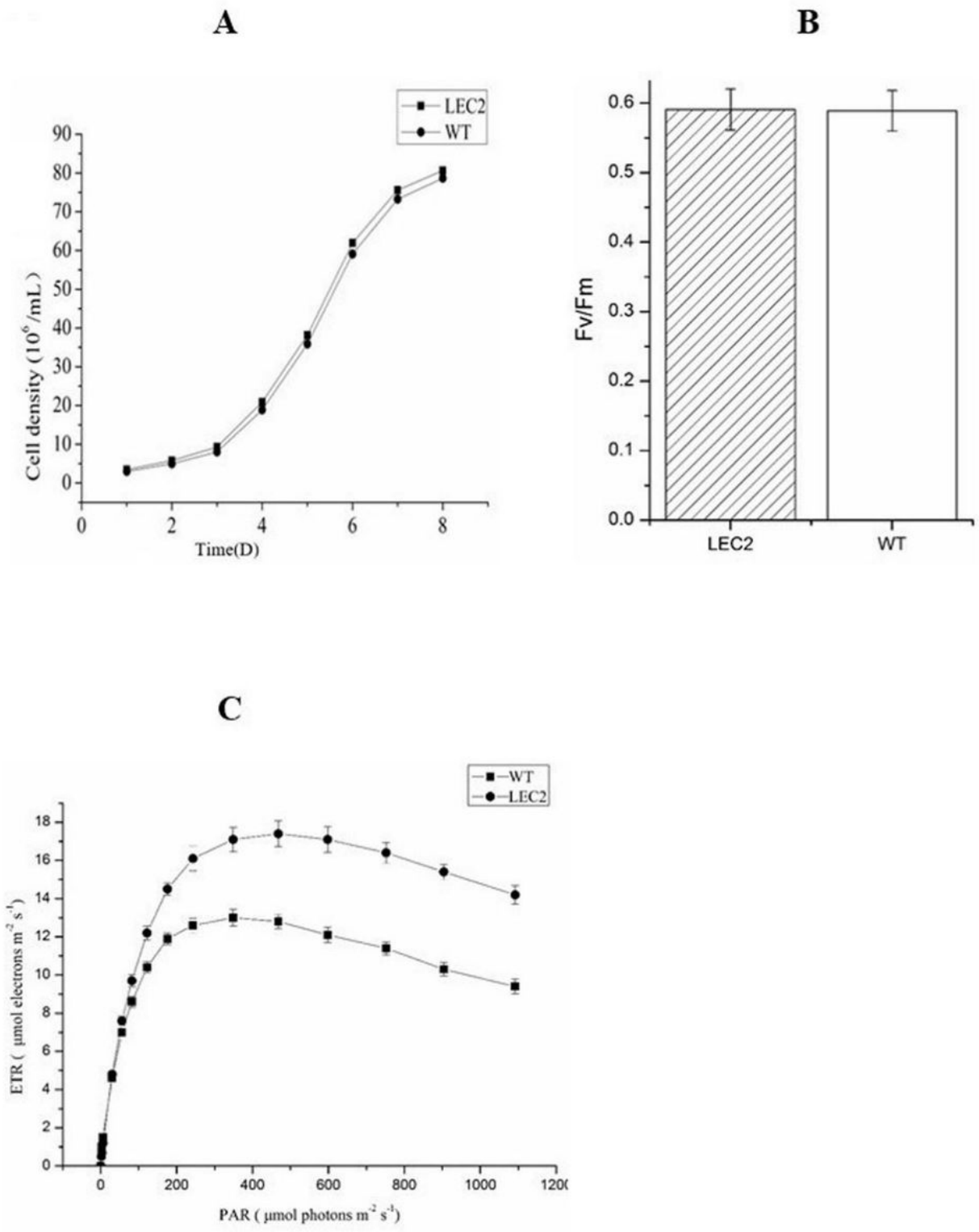

Figure 2

The detection of the growth curve and photosynthetic activity of transgenic *C. sorokiniana* and wild type.
LEC2: transgenic *C. sorokiniana*, WT: Wild type.

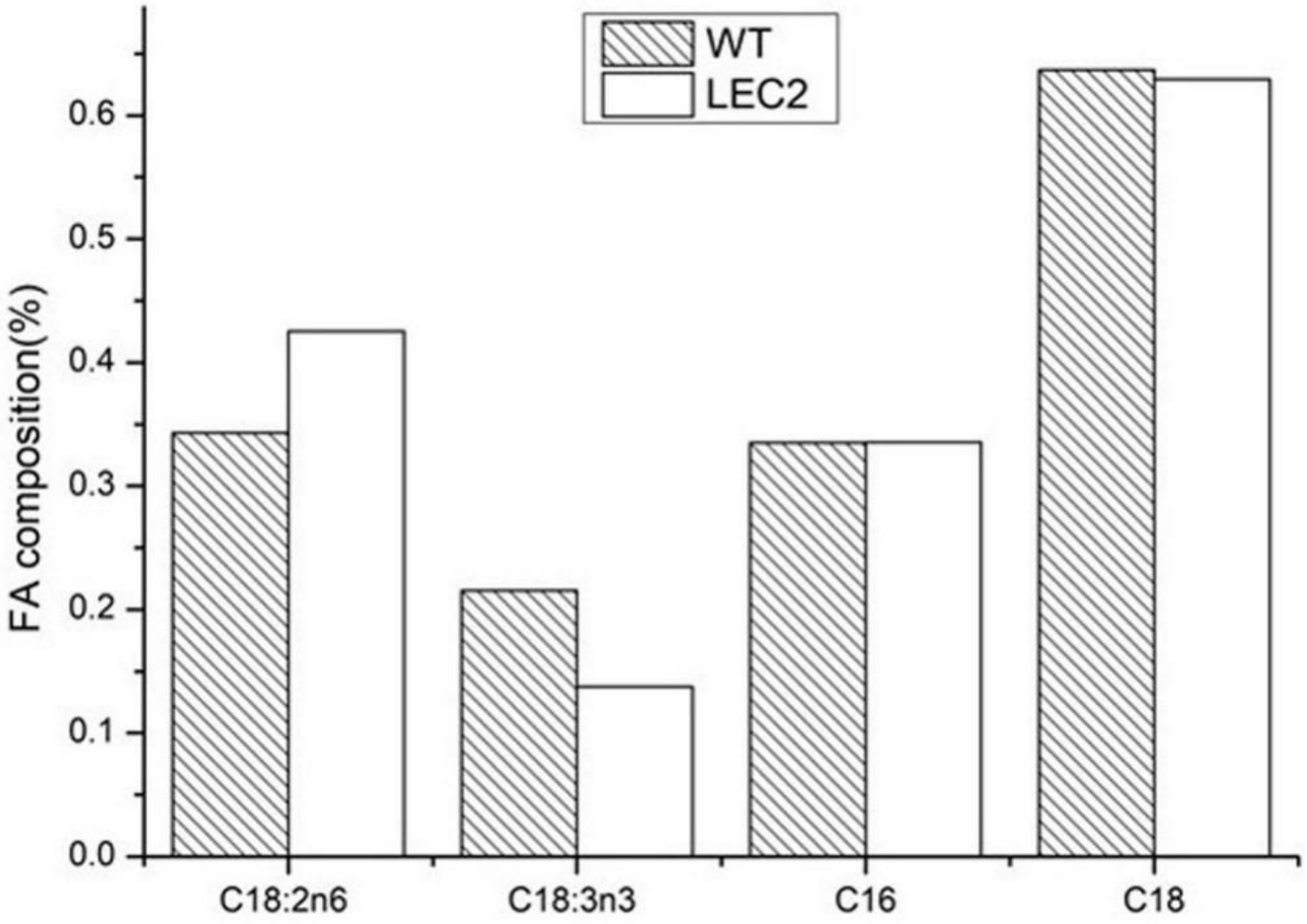

Figure 3

The proportion of critical fatty acids.

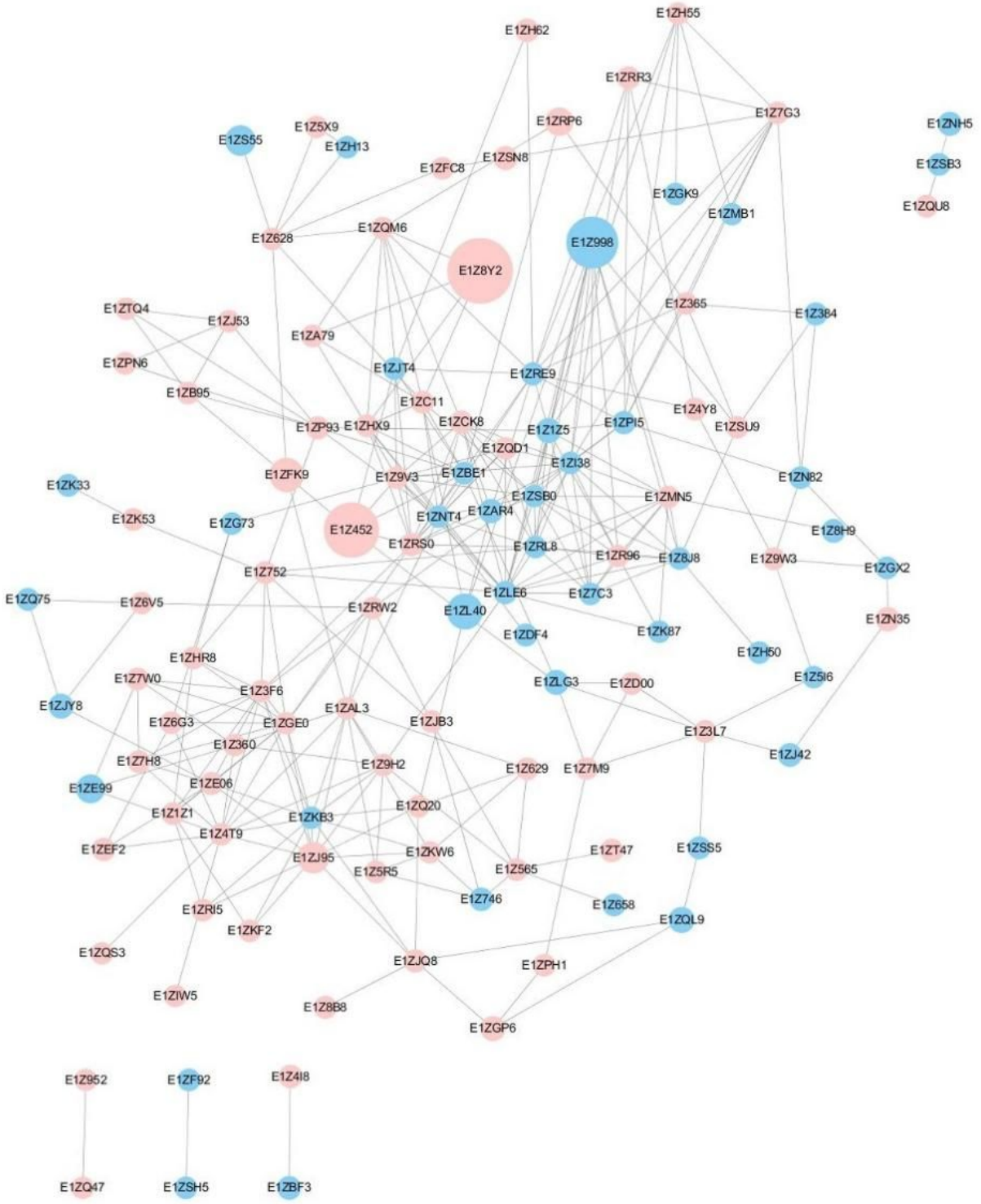

Figure 4

The PPI of differentially expressed proteins in *C. sorokiniana-AtLEC2*.

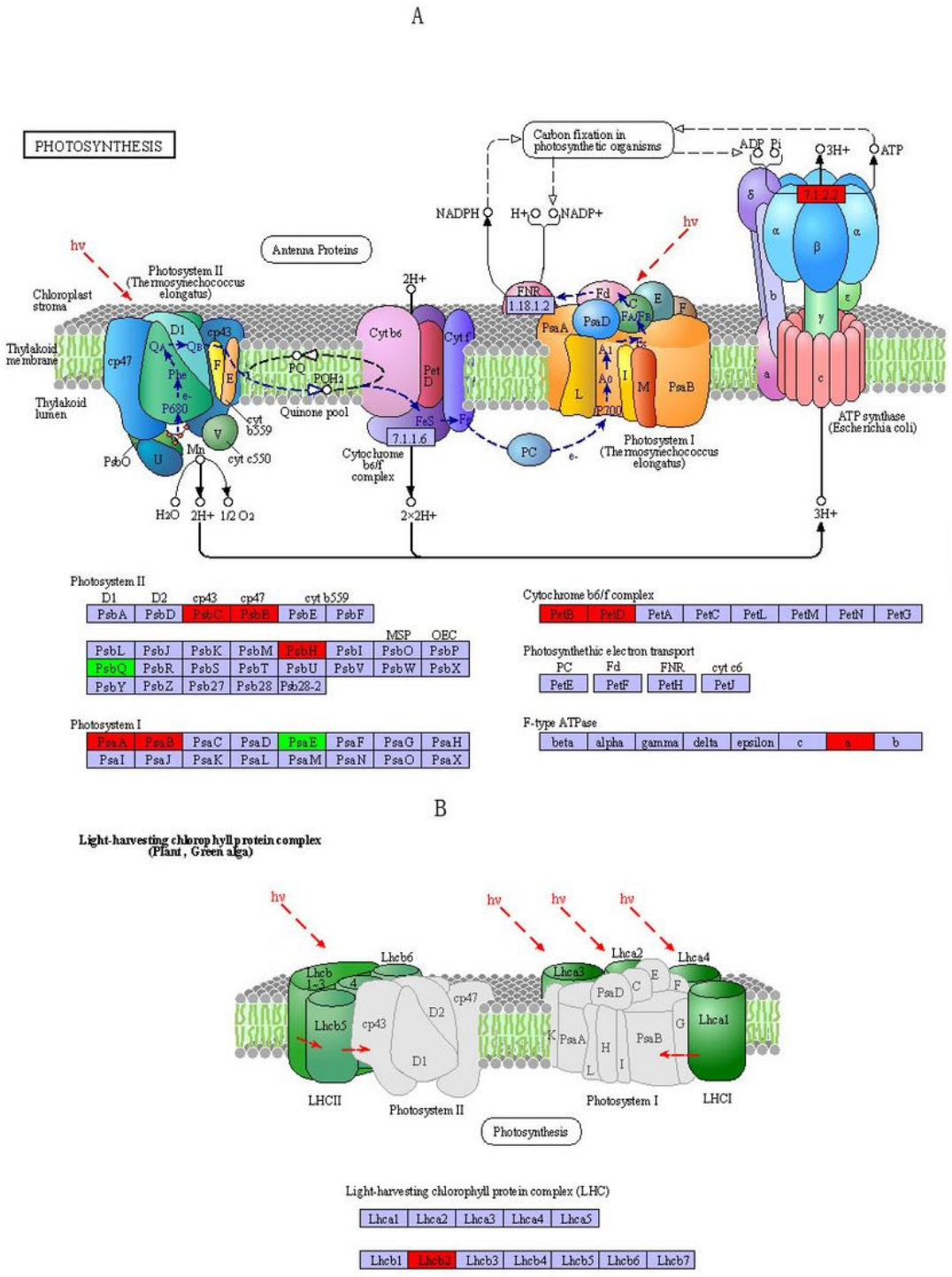

Figure 5

The regulation of ATLEC2 on photosynthesis and antenna proteins

Upregulated proteins are illustrated in red; downregulated proteins are in green.

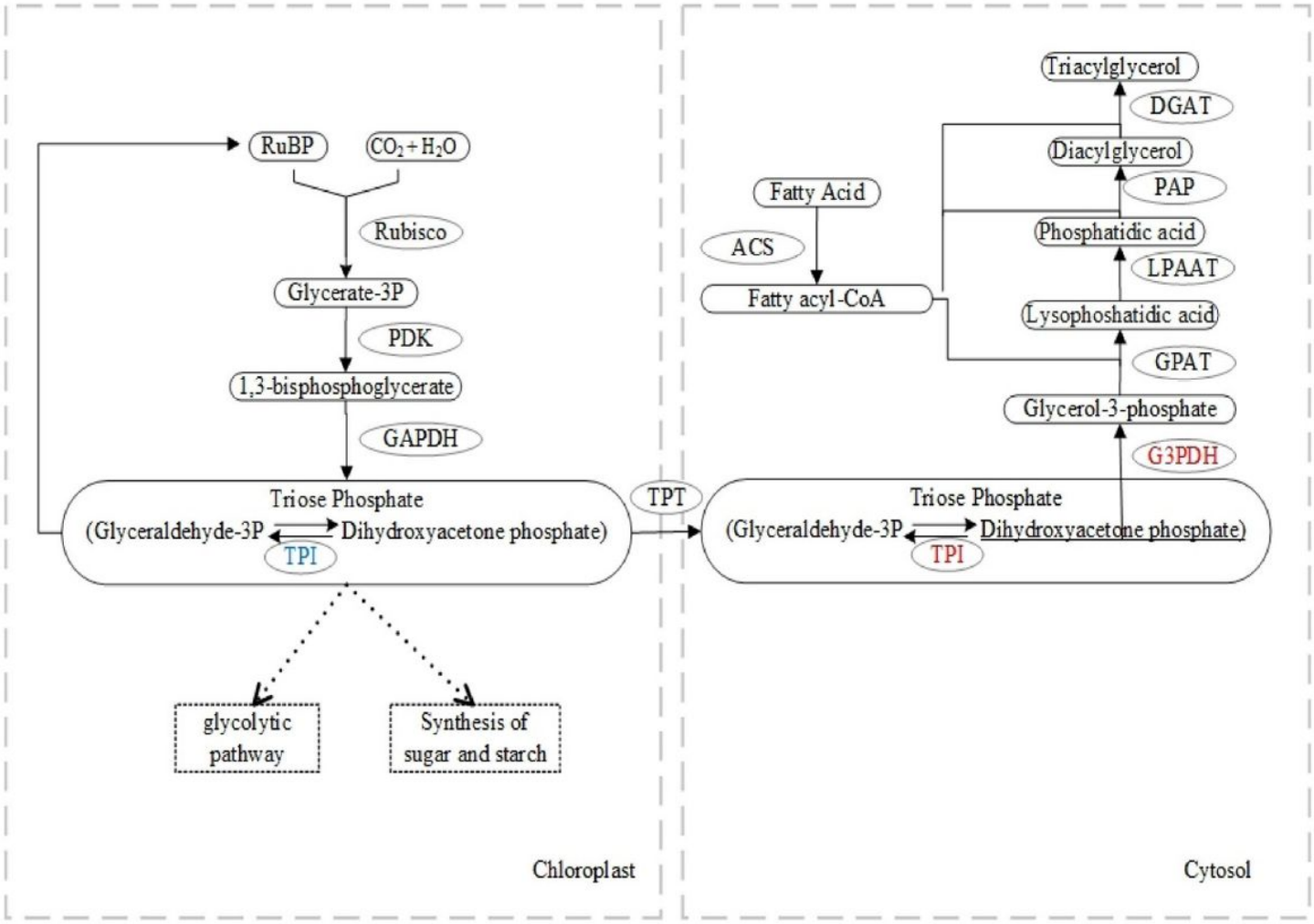

Figure 6

The regulation of ATLEC2 during lipid biosynthesis in *C. sorokiniana-AtLEC2*.