Proteomics Insight into Imbalanced Down-Regulation of Photosynthetic Components during Blueberry Fruit Maturation

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Abstract

Background: Blueberries are admired for both their delicious flavor and extensive health benefits. In blueberry, fruit photosynthesis provides a large part of the carbon requirements, and is associated with fruit quality. To explore mechanism underlying fruit photosynthesis, photosynthetic ability was determined at three maturation phases (green, pink, and blue) by CO2 gas exchange and chlorophyll fluorescence, and proteomics was conducted by MS/MS shotgun and PRM strategies. Results: The fruit photosynthetic ability gradually decreased as fruit maturation. The gross photosynthesis rate in green fruit accounted for 26.36% of that in the leaf, followed by 16.73% in pink fruit, and 9.11% in blue fruit. Fv/Fm (0.76) was observed in green fruit, comparable to that in leaves (0.78), and followed by 0.66 in pink fruit (no data was generated for blue fruit). Degeneration of fruit photosynthesis was started with imbalances within down-regulation of PS core/LHC, and PSI/PSII. In green/pink, PS core proteins were more down-regulated than LHC proteins. A decreasing ratio of PSII core/LHCII was reflected in the decreasing ratios of Chl a/b and carotenoids/Chl. KEGG analyses also indicated that the down-regulated LHC proteins were enriched in pink/blue, but not in green/pink. In green/pink, PSII was more down-regulated than PSI, while in blue/pink, PSI was more down-regulated than PSII. GO also suggested that down-regulated PSII proteins were enriched in green/pink, whereas down-regulated PSI proteins were enriched in pink/blue. Moreover, down-regulation of FNR and ATP synthase resulted in a decreasing yield of NADPH and ATP, while the down-regulation of both RuBisCO and RCA severely hindered Calvin cycle, and thereby led to a decreased efficiency of carbon fixation.

Conclusions: Dynamic imbalances in degeneration of photosynthetic components occurred during fruit maturation; this may be related to physiological attributes e.g. carbon contribution and attractiveness to frugivores.

Background

Blueberry is one of the most desirable and nutritious fruits. During fruit development, the blueberry’s organoleptic properties and phytonutrient composition are ever-changing [1]. Five typical phases (pads, cups, green, pink, and blue) have been characterized [2]. The former two (pads and cups) are divided into “expansion”, while the latter three are
into “maturation” [3]. Fruit maturation are typically characterized by fruit softening, coloring, and sweetening [4].

The ability of fruit photosynthesis changes with the conversion of chloroplasts to chromoplasts, and conversion is acquisition of organoleptic traits that make the fruit attractive and palatable [5, 6]. Fruit photosynthesis assists in meeting the plant’s carbon requirements to some extent, although the contributions are usually low compared with that in leaves [7]. In non-foliar plants, chlorophyllous reproductive structures can contribute up to 60% of the total carbon requirement [8]. In blueberry, fruit photosynthesis contributes up to 85% of the total carbon requirement [9]. Additionally, fruit photosynthesis reduces CO$_2$ concentration to avoid acidification while supplies O$_2$ to alleviate the adverse effects of hypoxia on developing seed [10]. In a study on transgenic tomato, the suppression of photosynthesis delayed seed development [11]. Recent studies have revealed some links between photosynthesis in the developing fruit and quality of the ripe fruit, e.g. chloroplast enhancement leads to a higher content of the fruit-specialized metabolites [5, 12-15]. The high links between carbon contribution and fruit quality emphasizes the importance of studying the mechanism underlying alterations in fruit photosynthesis.

During fruit maturation, the down-regulation of photosynthesis-related genes is pronounced not only at transcriptional level but also at translational level [6]. A transcriptomic study has been conducted with a commercial highbush blueberry (V. corymbosum) i.e. “Bluecrop” cultivar to identify genes associated with cold acclimation and fruit development [16].
In addition, an RNA-based blueberry draft-genome has been assembled, based on five fruit developmental phases [2]. Although the study of transcription and its regulation have provided important information regarding early stage transmission from genome to cellular machinery, mRNA abundance is not always consistent with the cognate proteins [17]. This is because the expression of proteins is affected by a series of posttranscriptional and posttranslational processes (e.g. mRNA alternative splicing and protein phosphorylation) [18].

Parallel reaction monitoring (PRM) is a method based on targeted mass spectrometry. Targeted mass spectrometry circumvents the necessity of antibodies for immunoblotting to confirm the presence of specific proteins. PRM data is typically highly consistent with immunoblot analyses and shotgun proteomics data [19]. PRM based on quadrupole-orbitrap (Q-orbitrap) has the advantage of a high resolution/accurate mass (HR/AM) because of a more flexible selection of fragments post-acquisition. Compared to conventional selected reaction monitoring (SRM), PRM is more convenient in assay development for absolute quantification of proteins and peptides. Combinative analysis of PRM and TMT-label shotgun-MS provides highly accurate quantitative information while requiring much less effort in assay development for targeted proteomics applications. In this study, we analyzed the fruit proteomes during maturation in order to explore the potential mechanism underlying fruit photosynthesis. To confirm the shotgun proteomics result, we applied the PRM technique to quantitatively measure twelve proteins involved in photosynthesis. This information regarding the dynamic variation of enzymes will be helpful in developing an understanding of fruit photosystem degeneration and the corresponding carbon contribution to fruit.

**Methods**

**Sample collection**

Blueberry fruits were collected from three plants of the O’Neal variety of southern highbush blueberry (*V. corymbosum*) at green, pink and blue phases during the growing season (May, 2017) at Yangdu,
Zhejiang (Figure 1A). These fruit were from three phases of maturation as described above [2], i.e. green, pink, and blue.

**Protein Extraction, Trypsin Digestion and TMT Labeling**

Fruit tissues were frozen in liquid nitrogen to be ground, and proteins were extracted in TCA-acetone extraction method as described by Li et al. [20]. The protein concentration was determined using a BCA protein assay kit (TermoFisher) as its protocol. The extracted proteins were reduced, and then digested by trypsin as described by Li et al. [20]. After trypsin digestion, peptides were desalted and processed as TMT10plex™ Isobaric Label Reagent (TermoFisher).

**HPLC Fractionation, LC-MS/MS Analysis and PRM Assay**

The labeled peptides were fractionated by HPLC, and the peptides were divided into 18 fractions. The peptides were loaded into tandem mass spectrometry (MS/MS), Orbitrap Fusion™ Tribrid™ (ThermoFisher). These processes were conducted as described by Li et al. [20]. The threshold of the differentially expressed proteins was set up (change fold $>1.5$ or $<0.67$ and $P <0.05$).

The PRM analyses were performed using a Q-Exactive Plus mass spectrometer (ThermoFisher). Proteins were extracted, digested into peptides and the peptides were separated as described by Li et al. [20].

**MS/MS Database Search and Analysis**

The resulting shotgun MS/MS data were processed using a Maxquant search engine (v.1.5.2.8), and searched against a blueberry database concatenated with a reverse decoy database [2]. The resulting PRM MS data was processed using Skyline (v.3.6). The parameters were set up as described by Li et al. [20]. The proteomic data was deposited in PRIDE (www.ebi.ac.uk/pride/) (Accession: PXD011815).
GO and KEGG Annotation, and Functional Enrichment

Gene Ontology (GO) and KEGG annotation for proteomic data were performed as described by Li et al. [20]. Enrichment of the differentially expressed proteins was conducted by a two-tailed Fisher’s exact test. The significant threshold was set up (p-value < 0.05) for both GO and KEGG enrichment.

Chlorophyll and Carotenoid determination

Approximately 2 g of the sample were weighed then ground with 1mL ddH₂O and 50mg calcium carbonate. The resulting slurry was combined with 50ml extractive solvent of ethyl alcohol and acetone (v:v=1:2). The extraction was performed in darkness for 3 hr. The absorbance of the extractive solvent was measured at 663nm and 645nm. The samples were dried at 60 °C, ground, and then sifted through a mesh screen. Approximately 0.1g of powder was weighed and combined with 2ml petroleum ether and acetone (v:v=2:1). The mixture was vortexed for 360 min. The absorbance of the extractive solvent was 440nm.

CO₂ gas exchange

CO₂ gas exchange was analyzed by a spectroradiometer (Model LI-6800, Li-Cor, Inc). Excised fruits were placed in the measuring chamber of the instrument and the difference between internal and external CO₂ values was stabilized in darkness. Dark respiration was monitored for several mins and subsequently the samples were exposed to the highest light (2000 mmol photons m⁻²s⁻¹) for net photosynthesis determinations. Gas exchange was determined on basis of a surface area unit.

Modulated chlorophyll fluorescence

A pulse-amplitude modulation (PAM) fluorometer (PAM 2500, Heinz Walz GmbH) was used for in vivo measurements of chlorophyll fluorescence as described by Ferroni et al. [21]. Fluorescence measurements were performed in excised fruits on the leaf clip with a filter paper on the floor of the clip. After the fruits adapted to darkness for at least 30 min, the background fluorescence level F0 was measured. After the fruits were exposed to a saturating pulse
of white light (0.8 s), the maximum fluorescence level FM was obtained.

Results

Chlorophyll content and photosynthetic activity of the berry

The content and proportions of Chl, carotenoids and anthocyanins determine fruit color and appearance, and serves as markers of quality. Chl and carotenoids, located mainly in the chloroplast, are involved in photosynthesis. During maturation, the content of Chl and carotenoids both declined. Total chlorophyll content dropped by 38.08% in green/pink, and by 64.52% in pink/blue, accompanied by a decrease in two types of Chl, Chla and Chlb (Fig. 1B). Chl a/b ratio decreased by 35.75% in green/pink and by 32.27% in pink/blue. Carotenoids also showed a sharp decline by 86.49% in green/pink and by 69.43% in pink/blue. Carotenoids/Chls ratio decreased by 78.87% and 13.84% in the two transitions, respectively.

CO₂ gas exchange measurements indicated that fruit photosynthetic activity was in parallel with Chl changes. In contrast to green fruit, no positive net photosynthesis could be detected either in pink or in blue fruit, although they were capable of active photosynthesis to some extent (Fig. 1C). No positive net photosynthesis denoted that gross photosynthesis did not compensate for respiration. The maximum gross photosynthesis rate was observed in green fruit accounting for 26.36% of that in the leaf, followed by pink fruit for 16.73% and blue fruit for 9.11% (Fig. 1C). Interestingly, the maximum dark respiration was detected in pink fruit, followed by blue and green fruit. PSII activity was estimated by FV/Fm. The maximum PSII activity was in green fruit, which could rival that in leaf (0.76 vs. 0.78), followed by 0.65 in pink fruit (Fig. 1D). Actual photosynthetic efficiency is determined by the relative distribution of absorbed photon energy to photochemical and non-photochemical pathways. Relative allocation of energy to these pathways was indicated by the quenching coefficients qP and qN. qN was 0.52 in green fruit, comparable to 0.53 in leaf, but it increased by 21.06% and up to 0.63 in pink fruit. qP was 0.29 in green fruit, much lower than 0.98 in leaf, and it deceased by 7.79% and down to 0.26 in pink fruit. Notably, no significant amounts of F0, FM, qN and qP could be detected in blue fruit.

Proteomic analysis of blueberry maturation

Protein identification and quantification
Nine quantitative proteomes from green, pink and blue fruits have been profiled including three biological replicates for each phase. The proteomic analysis resulted in the identification of 25,440 unique peptides corresponding to 5,785 proteins, as well as the quantification of 4,808 proteins. Analysis of reproducibility for each phase showed a high correlation between reps (Pearson coefficient mean=0.92, P<0.001), much higher than that between phases (mean=-0.31, P>0.001) (Additional file: Fig. S1A). The largest difference was observed in pink/blue, indicated by the minimum Pearson coefficient (-0.80) and the highest number of differently expressed proteins (1,429) on a threshold (change fold>1.5 or <0.67 and P <0.05) (Additional file: Fig. S1B). Interestingly, the correlation in pink/blue (-0.50) was much lower than that in green/pink (0.30). A higher number of differently expressed proteins were more found in pink/blue (825) than in green/pink (653). The result indicated that more changes occurred in pink/blue rather than in green/pink.

GO and KEGG enrichment of the differentially expressed proteins

To reveal specific functional categories represented in blueberry photosynthesis during fruit maturation, GO enrichment was analyzed for the differentially expressed proteins based on pair-wise comparison. Most of proteins enriched into terms of secondary metabolites were up-regulated while most of proteins enriched into terms related to photosynthesis were down-regulated (Fig. 2B). In green/pink, 8, 15, and 17 down-regulated proteins were enriched in PSII, photosynthetic membrane, and photosynthesis process respectively. In pink/blue, 8, 14, and 16 down-regulated proteins were enriched in PSI, photosynthetic membrane, and photosynthesis respectively. In green/blue, 8, 8, 18, 21 down-regulated proteins were enriched in PSI, PSII, photosynthetic membrane, and photosynthesis respectively. The GO enrichment analysis indicated that the down-regulation of PSII mainly occurred in green/pink while PSI mainly occurred in pink/blue.

Mapping the differently expressed proteins into the KEGG database produced 670 annotations. In homologous pathways of Arabidopsis, annotated genes were predicted to be involved in 118 KEGG pathways. To discern the multivariate pattern of up- and down-regulation, analyses of KEGG enrichment were performed for these proteins (Fig. 3). Few up-regulated proteins were involved in
photosynthesis-related pathways. In green/pink, 11 and 23 down-regulated proteins were involved in photosynthesis and carbon fixation respectively. In pink/blue, 6, 7, and 18 down-regulated proteins were associated with photosynthesis, photosynthesis-antenna proteins and carbon fixation in photosynthetic organisms, respectively. In green/blue, 7, 15, and 26 were enriched in photosynthesis-antenna proteins, carbon fixation in photosynthetic organisms, and photosynthesis respectively. KEGG pathway enrichment-based clustering analysis suggested that photosynthesis-related pathways were down-regulated through the entire fruit maturation process, especially in regards to the carbon fixation pathway. The pathway related to antenna proteins or light-harvesting complex (LHC) was significantly enriched in pink/blue but not in green/pink (Fig. 3).

**Differently expressed proteins involved in fruit photosynthesis**

In higher plants, green tissues have many chloroplasts with thylakoid membrane and stroma for light-dependent reactions and light-independent reactions respectively. The thylakoid membrane houses chlorophylls and different protein complexes, including PSI, PSII, Cyt b6f, ATP synthase, and an electron carrier. In each photosystem, the PS core complex is surrounded by a large light-harvesting complex (LHC) or antenna complex that captures sunlight and transfers the excitation energy to the core with extremely high efficiency. These complexes work together to ultimately produce ATP and NADPH, which can be used in light-independent reactions. Regarding light reactions, 39 proteins were down-regulated through fruit maturation (Table 1, Fig. 5A). All of these proteins were significantly down-regulated in green/blue, between two non-adjacent phases. Of them, 76.92% were significantly down-regulated in pink/blue while 64.10% were in green/pink. The amplitude of down-regulation in pink/blue (an average decrease of 46.99%) was larger than in green/pink (29.07%) (Fig. 4A). LHC proteins were down-regulated mainly in pink/blue (51.49%) rather than in green/pink (11.43%). In green/pink, PS core proteins were down-regulated by 37.01% more than LHC proteins by 11.43%. PSI and PSII were differently regulated. In green/pink, PSII was down-regulated more than PSI (PSII 33.04% vs. PSI 24.82%) while in blue/pink, PSI was down-regulated more than PSII (50.09% in PSI and 44.10% in PSII). Notably, in pink/blue, the different regulation between PSI and PSII concentrated
mainly on the PS core proteins (PSI core down-regulation 51.48% vs. PSII core 39.65%).

Regarding the electron transfer chain of light-dependent reactions, Cyt b6f complex, the ATP-synthase and other electron carriers make use of the electronic gradient to produce molecular energy, NADPH and ATP. Cyt b6f transfers electrons from PSII to PSI while protons transfer into the thylakoid space from the chloroplast stroma across the thylakoid membrane into the lumen. Electrons are transferred between two redox carriers, plastoquinone (QH\(_2\)) and plastocyanin (Pc or PETE). A proton gradient in the lumen can be used to drive ATP synthesis by ATP-synthase. The Cyt b6f is a dimer, with each monomer composed of eight subunits. The complexes consist of four large subunits: cytochrome f (PETA), cytochrome b\(_6\) (PETB), Rieske iron-sulfur protein (PETC), and subunit IV (PETD); along with four small subunits: PETG, PETL, PETM, and PETN [22, 23]. Ferredoxin (Fd1), accepts electrons primarily from PSI and donates them to Ferredoxin-NADP+ reductase (FNR) [24]. FNR then transfers an electron from each of two Fd1 molecules to a single molecule of the two-electron carrier NADPH. ATP synthase consists of two main large subunits, F\(_O\) and F\(_1\), and F\(_O\) causes rotation of F\(_1\) for ATP production [25, 26].

Here, 3, 3, 2 and 1 differentially expressed proteins were respectively identified as belonging to Cyt b6f complex, chloroplast-specific ATP-synthase, FNR and Fd1 (Table 1; Fig. 5A). Cyt b6f proteins were significantly down-regulated in green/pink but not in pink/blue, and the amplitude of down-regulation was higher in green/pink than in pink/blue (37.39% vs. 27.62%) (Fig. 4B). Two proteins of ATP synthase F\(_1\) large subunits were significantly down-regulated in green/pink but not in pink/blue, and the down-regulation was more pronounced in green/pink than in pink/blue (53.65% vs. 32.15%). Notably, two FNR proteins (CUFF.25314.1 and FNR2 gene.scaffold00413.path1.gene10.1) and ATP synthase subunit b of Fo (ATPG) (CUFF.20353.1) were successively and significantly down-regulated through fruit maturation.
Light-independent reactions are chemical reactions that convert CO$_2$ and other compounds into glucose by using ATP and NADPH derived from light-dependent reactions. There are three stages of the light-independent reactions, collectively referred to as *Calvin cycle*, which includes carbon fixation, reduction reactions, and ribulose 1,5-bisphosphate (RuBP) regeneration. Unlike in light-dependent reactions, 21 proteins were down-regulated through fruit maturation and their down-regulation amplitude in green/pink (a decrease of 33.85% on average) was comparable to that in pink/blue (32.17%) (Table 1; Fig. 5B). Photorespiration is the light-dependent consumption of O$_2$ and the coupled release of CO$_2$ that occurs simultaneously with photosynthetic CO$_2$ uptake and O$_2$ release in plants [27]. Regarding photorespiration, 15 proteins were down-regulated through the entire fruit maturation cycle and their down-regulation amplitude in green/pink (a decrease of 34.70% on average) could be comparable to that in pink/blue (34.35%)(Table 1; Fig. 5C). Notably, RuBisCO was a rate-limiting enzyme for both photorespiration and the Calvin cycle, and its two small subunits were significantly and successively down-regulated throughout the fruit maturation. Accordingly, two of the five RuBisCO activase (RCA) proteins were also successively and significantly down-regulated.

We developed a PRM assay for the quantification of target peptides. Target peptides were selected based on uniqueness and anticipated chemical stability. Peptides previously identified in the shotgun data set were preferentially selected and each one was monitored by more than two unique peptides. Here, ten represented proteins were successfully quantified in nine protein libraries from the three fruit maturation phases. A comparison of expression in PRM and shotgun indicated a very high correlation (average of 0.996, Pearson correlation coefficient) (Additional file: Table S1). Thus, the result from shotgun was confirmed by PRM assay, which supported overall reliability of the results.

**Discussion**

Fruit photosynthesis supplies additional carbon-acquisition and occurs predominantly in green fruit.

Additional carbon-acquisition supplied through photosynthesis in fruit i.e. internal CO$_2$ re-fixation, has been extensively reported in many plants such as coffee, pea, soybean, avocado, orange, tomato and
apple. In barley, the re-fixation rate of ears comprised 55 to 75% of the respective gross photosynthesis [28], and in wheat, ears provided 63% [29]. In olive, fruit re-assimilation rates are between 40 and 80% (Proietti et al. 1999). In two species of orchid, mature capsules also recycle 10 and 60% of internal CO$_2$, respectively, although positive net uptake is not detected [30]. Also, some green-ripe fruits of tropical plants (Piper hispidum, Solanum rugosum) reach positive carbon balance at light levels above 300 μmol m$^{-2}$ s$^{-1}$ [31], however at these light levels, most fleshy fruits such as apple are typically not able to reach their CO$_2$ compensation point (Willmer & Johnston 1976). In tomato, fruits own-fixed carbon constitutes 10% to 15% of the carbon requirement [32], and fruit accounts for 15% of gross photosynthesis transport activity [7]. In Jatropha curcas [33] and Arum italicum [21], the photosynthetic contribution of immature/green fruits is around 25% of that in its leaves. In a previous report on blueberry, fruit photosynthesis contributions reach about 15% of its carbon requirement during development, and even up to 50% throughout the first 10 days after bloom and 85% in the 5 days after petal fall [9]. Here, the maximum gross photosynthesis rate was observed in green fruit, accounting for 26.36% of that taking place in the leaf, followed by pink fruit for 16.73%. Also, blue fruit exhibited some ability to photosynthesize t (Fig. 1B).

In contrast to dehiscent fruits where net photosynthesis does not occur until a distinct age [34], most green/young fruits are capable of net photosynthesis, as seen in young tomato fruit [35], developing peach fruit [36] and young, green blueberries [37].
In *Jatropha curcas* fruit, net photosynthesis is observed throughout the entire fruit development, but decreases as the fruit matures [33]. The successive net photosynthesis may be due to high photosynthetic activity taking place in evergreen fruit tissues. In contrast, positive net photosynthesis are not detected in *Arum italicum* throughout the fruit development; this may be due to its high respiration rates [21]. In blueberry, the net photosynthetic rate was only observed in green fruit as a response to saturating light. Despite the compensation of the respiratory CO₂ released in light being a positive net rate, respiratory CO₂ evolution in the dark usually exceeded the photosynthetic gain and caused a net CO₂ loss from the fruit in the diurnal cycle.

The photosynthetic efficiency of PSII in fleshy green fruits is generally reported to be high and comparable to that in the plant leaves. In *Jatropha curcas*, Fv/Fm in immature or mature fruit are 0.822 and 0.827 respectively, comparable to 0.828 in leaves [33]. The Fv/Fm of fruit comparable to leaf has been observed in mango [37] and in tomato [2]. Similar to other fruit crops, Fv/Fm of blueberry green fruits could be rivaled to that in leaves (0.76 vs 0.78). Although photosynthetic rate and PSII efficiency in green fruit are comparable to that in leaf tissue, the carbon fixing ability is much lower in green fruit than in leaves. This low ability in carbon fixation may be reflected by the low efficiency of photochemical energy conversion (qP: 0.28 in fruits vs. 0.98 in leaves) (Fig. 1D). This may be attributed to fruit anatomy and internal respiratory CO₂ re-fixation [8]. Fruit chloroplast density is much lower in fruit photosynthetic tissues than that in leaves. In apple, photosynthetic rates in green fruit and leaves are quite comparable with regard to chlorophyll units [38]. Refixation of internal CO₂ from respiration is predominant in fruit photosynthesis via an efficient phosphoenolpyruvate carboxylase (PEPC) in both light and darkness, rather than the direct fixation of ambient atmospheric CO₂ via RUBISCO [9].
Moreover, an increase of excitation energy dissipation was observed as fruit maturation (qN: 0.52 in green vs. 0.63 in pink) (Fig. 1D). An increase in non-photochemical quenching has also been reported in *Jatropha curcas* fruit [33], and peach [39] during the fruit maturation. An increase of qN indicates a rapid diversion of absorbed photon energy away from the photochemical pathway [40]; this could be caused by the dissociation of LHCII from PSII core [41].

Degeneration of photosynthetic capability in fruit was started with imbalances in down-regulation of PS core/antenna and PSI/PSII In plants, two major mechanisms are used to elaborately regulate photosynthesis [42]. One acts on state transitions, known as a short-term response (STR); while the other enables the plant to re-adjust photosystem stoichiometry, leading to photosystem rate-limiting, known as a long-term response (LTR). The LTR regulation results in a series of changes involving adjustments in the LHCII size, PSI/PSII stoichiometry, photosynthetic pigment pattern and thylakoid architecture [43-45]. During fruit maturation, the gradual descent of photosynthesis is mainly caused by disruption of the photosynthetic machinery i.e. transition from chloroplast to chromoplast [46], analogous to the LTR regulation. This disruption leads to a decrease in thylakoid proteins and depends on their resistance to degradation [47]. The thylakoid proteins are involved in two photosystems, PSI and PSII. Light energy capture and transfer to PSI and PSII reaction centers (core complex) is mediated by LHCl and LHCII accordingly. In blueberry fruit, the degeneration of photosynthetic proteins begins with a decrease of the PS core rather than LHC. Most proteins of the PS core e.g. PSA and PSB were successively and significantly down-regulated throughout the fruit maturation. However, the proteins of LHCl and LHCII peaked at green phase, and were rapidly and significantly down-regulated after pink phase. The results suggested that PS core proteins were preferentially down-regulated than the LHC proteins.
KEGG enrichment also indicated that antenna proteins (LHC) were significantly enriched in pink/blue but not in green/pink (Fig. 3B). Consequently, the initial decrease of photosynthesis efficiency was attributed to an inherent uncoupling of PSII reaction centers and antenna. The decreasing ratio of PSII cores to LHCII was also reflected in a decreasing ratio of Chl $a/b$. Chl $a/b$. because about 90% of the total chlorophyll $b$ is bound to LHCII [48, 49]. As chloroplast became disorganized, the relative abundance of PSII core to LHCII complexes changed. A similar result has been reported in rice leaf senescence where PSII core, i.e. CP43 and CP47 is down-regulated faster than LHCII [50]. Moreover, a steep decrease in the ratio of carotenoids/Chls (a decrease of 78.87% in green/pink and 13.84% in pink/blue) indicated a relative high LHCII content, because LHCII has a higher ratio of Chls/carotenoids than PS core [51]. The higher stability of LHCII has been observed in many different systems undergoing thylakoid dismantling, with only a few exceptions [21, 52, 53]. Besides light harvesting, LHCII is also involved in the regulation of the thylakoid membrane, i.e. in the energy distribution between PSI and PSII and in the thermal dissipation of excess light energy [54, 55]. The occurrence of a defect in PSII core stability partially impairs the energy transfer from LHCII to PSII units. For example, PSBP, a subunit of the oxygen evolving center of PSII, is an assembly factor not only of PSII core [56, 57], but also of LHCII and PSII supercomplex [58]. In PSBP knockdown plants, a supercomplex consisting of LHCII and PSII failed to assemble [59].
As PSBP was down-regulated, association of the LHCII trimers with PSII dimers was impaired. This imparity resulted in the increase of qN during fruit maturation (qN: 0.52 in green vs. 0.63 in pink).

Previous studies have reported that PSI and PSII exhibit differences in down-regulation during fruit maturation [21, 59]. In green/pink, PSII was down-regulated more than PSI (PSI 24.82% vs. PSII 33.04%) while in pink/blue, PSI was more down-regulated than PSII (PSI 50.09% and PSII 44.10%). In pink/blue, down-regulation of PS primarily occurred in the PS core rather than in LHC (PSI core 51.48% vs. PSII core 39.65%) (Fig. 2). GO analysis suggested that, in green/pink, down-regulated proteins were significantly enriched in PSII, while, in pink/blue, the down-regulated proteins were significantly enriched in PSI (Fig. 2B). The results implied that an imbalance in down-regulation of PSI and PSII was also associated with a degeneration of photosynthesis during fruit maturation.

The relative stability of LHC may be associated with its special role regarding zoidospore. LHCII acts as a temporary storage of chlorophyll and its relative stability can maintain color for a relatively long period of time. Fruit’s green color is a visual signal of fruit not mature for animals consumption [60]. In green fruit, LHCII is also involved in pigment complement, stacking promotion, and thermal dissipation by fruit-specific arrangements of LHCB proteins [21]. Besides Chl, plants also use carotenoid as an antenna pigment [61]. Carotenoids not only absorb light energy for use in photosynthesis, but also protect chlorophyll from photodamage by absorbing excess energy and dissipating it as heat [62]. Carotenoids absorb violet and blue-green light, and the brightly colored carotenoids can be found in fruit e.g. the red of tomato (lycopene), the yellow of corn seeds (zeaxanthin), or the orange of an orange peel (β-carotene). In blueberry, coinciding with chlorophyll, carotenoids diminished as fruit maturation (Fig. 1B). The diminishing of both photosynthetic pigments was also reflected by a change in pericarp color (Fig. 1A). Blue and pink color appeared as anthocyanin increased while green and yellow color faded as Chl and carotenoids deceased. These results
indicated that carotenoids were no longer required for light energy absorption or for Chl protection. Down-regulation of FNR and ATP synthase hindered transfer from electron energy to chemical energy.

FNR is the last enzyme active during the transfer of electrons from PSI to NADPH [24]; this is a rate limiting step of the photosynthetic linear electron transfer chain [63]. FNR has an induced-fit mechanism of catalysis, inhibited by the presence of oxidized Fd1 and stimulated by the presence of NADP+ [64]. Electron cycling from Fd1 to NADPH only occurs in light in part because FNR activity is inhibited in darkness [65]. Here, the two FNR proteins were down-regulated successively and significantly through fruit maturation. Two forms of FNR have also been identified in the pericarp of tomatoes [66]. FNR II had a lower apparent relative molecular weight, a slightly altered absorption spectrum, and a lower specific activity for cytochrome c reduction than FNR I. FNR II is a partially degraded form of FNR I. A significant decrease of FNR in response to drought stress has also been found in Populus cathayana [67].

sunflower (Helianthus annuus) [68]. The rate-limiting regulation of FNR is conducted through controlling the rate of interaction with Fd1 [70]. The successive and significant down-regulation of FNR obstructs the electron transfer chain and thereby suppresses the production of NADPH, which also coupled to down-regulation of the
PSI core (Fig. 5C).

In plant mitochondrion, ATP synthase couples with ATP synthesis during cellular respiration, while in plant chloroplast, ATP synthase couples with ATP synthesis during photosynthesis. In both processes, ATP synthesis is driven by a proton gradient which is involved with electron transportation. ATP synthase consists of two main large subunits, F\textsubscript{O} and F\textsubscript{1}. F\textsubscript{O} causes a rotation of F\textsubscript{1} for ATP production [25, 26]. F\textsubscript{O} consists of a c-ring with subunits, i.e. a, b, d, and F6, while F\textsubscript{1} includes subunits, i.e. α, β, γ, and δ. F\textsubscript{O} mainly consists of hydrophobic regions, while F\textsubscript{1} includes a water-soluble portion which can hydrolyze ATP. Both create a pathway for proton movement across the membrane [71].

Here, three differently expressed chloroplast-specific proteins may be involved with ATP synthesis in chloroplasts during photosynthesis. Two F\textsubscript{1} subunit proteins α (ATPC) and δ (ATPD) were down-regulated more in green/pink than in pink/blue (Table 1; Fig. 4B). In contrast, F\textsubscript{O} b subunit (ATPG) was less down-regulated in green/pink than in pink/blue. The rapid down-regulation of ATPC and ATPD may be attributed to their hydrophilic structure. The successive and significant down-regulation of enzymes involved in the production of NADPH and ATP likely impeded the transfer from electron energy to chemical energy.

**Down-regulation of RuBisCO inhibits both the Calvin cycle and photorespiration**

The reaction of CO\textsubscript{2} fixation catalyzed by RuBisCO is the primary rate-limiting step of the Calvin cycle, and thus strictly controls the plant photosynthesis [72]. Photosynthetic efficiency can be improved by modifying RuBisCO genes in plants to increase catalytic activity and/or decrease oxygenation rates [73]. Photorespiration crosses the Calvin cycle by RuBisCO. In photorespiration, approximately 25% of reactions by RuBisCO involve oxygenation, creating a product that cannot be used in the Calvin cycle [74]. Rubisco is usually comprised of two types of subunits, Rbc L (chloroplast encoding) and
RbcS (nucleus encoding) [75]. A total of eight large-chains (forming four dimers) and eight small chains assemble into a larger complex to perform catalytic activity. In blueberry fruit, one RbcL (CUFF.21043.2) and two RbcS proteins (CUFF.8671.2, and gene.g4868.t1.1) have been identified. Two RbcS proteins exhibited a successive and significant decrease throughout the fruit maturation. The dismantling of RbcS from a large RuBisCO complex could lead to a decreasing ability of CO$_2$ biosequestration. Also, the dismantling of RuBisCO results in a decreasing photorespiration. Low photorespiration has also been reported in the peel of apple [76]. Low photorespiration may be related to a much lower stomatal density, larger diffusion resistance, and higher internal CO$_2$ concentration in fruit [38]. A relatively high concentration of CO$_2$ is the result of low photosynthesis and high respiration rates in fruit. RuBisCO activase (RCA) is required to active RuBisCO via carbamate in the active site of RuBisCO. RCA competes with two types of inhibitors, RuBP substrate and a substrate analog 2-Carboxy-D-arabinitol 1-phosphate (CA1P) by binding the active site of RuBisCO [77-79]. The successive and significant down-regulation of RuBisCO directly resulted in a lowered efficiency in CO$_2$ fixation and also responded to the successive and significant down-regulation of two RCA proteins.

**Conclusions**
The enzymes associated with light-dependent reactions and light-independent reactions (Calvin cycle) were gradually down-regulated as fruit maturation. However, the down-regulation was orchestrated to occur during specific phases of the fruit maturation to support certain physiological roles e.g. carbon contribution and attraction to frugivores.

**Abbreviations**
Cyt b$_6$f, Cytochrome b$_6$/f complex; FBA, fructose-bisphosphate aldolase; FBP, fructose-1,6-bisphosphatase; Fd1, ferredoxin; FNR, ferredoxin-NADP+ reductase; Fv/Fm, maximum photochemical quantum yield of PSII; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (NADP+); GGAT, glutamate--glyoxylate aminotransferase; GLDC, glycine decarboxylase. glycerate dehydrogenase; GO,
gene ontology; HAO, hydroxy-acid oxidase; HPR, glycerate dehydrogenase; LHCl/II, photosystem I/II light-harvesting proteins or antenna; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PGK, phosphoglycerate kinase; PGLP, phosphoglycolate phosphatase; PPE, phosphopentose epimerase or RPE, ribulose 5-phosphate 3-epimerase; PRK, phosphoribulokinase; PRM, Parallel reaction monitoring, a method based on targeted mass spectrometry; PS, photosystem; PSI/II core, photosystem I/II reaction center; PSI/PSII, photosystem I/II including PS I/II core and LHCl/II; qN, non-photochemical quenching parameters; qP, quenching coefficients; RBCL, RUBISCO large subunit; RBCS, RUBISCO small subunit; RCA, RuBisCO activase; RPI, Rib5P Isomerase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; RUBISCO, ribulose-bisphosphate carboxylase; RuBP, ribulose 1,5-bisphosphate; SBP, sedoheptulose-bisphosphatase; SGXT, Serine--glyoxylate aminotransferase; TK, transketolase; TMT, tandem mass tags.

Declarations

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Availability of data and materials

The datasets generated and analyzed during the current study was deposited in PRIDE (www.ebi.ac.uk/pride/) (Accession: PXD011815).

Authors’ contributions

XL conceived of the study, and performed experiment design and coordination, and manuscript draft.

Ethics approval and consent to
participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Tables
Due to technical limitations, Table 1 is only available as a download in the supplemental files section.

Additional File Legends
Additional file 1: Fig. S1 Pairwise comparison of the proteomic libraries (A) Nine libraries derived from the three proteomes including three replications by Pearson correlation, and (B) The differently expressed proteins in green/blue, green/pink, and pink/blue on a threshold (change fold>1.5 or <0.67 and P <0.05)

Additional file 2 Table S1 Expression comparison of ten proteins related to photosynthesis and photorespiration between two methods i.e. PRM and Shotgun

Figures
Figure 1

(A) Blueberry fruit in five ripening phases (green, pink and blue) (1) longitudinal section (2) side view (3) latitude section and (4) top view. (B) the changes of chlorophylls (Chl) (consisting of Chla and Chlb) and carotenoids in three blueberry fruit mature phases i.e. (green, pink and blue). photosynthetic activity and light-energy dissipation in blueberry maturing fruit, and leaf for comparison. (C) CO2 gas exchange per surface unit from green to blue phases, and leaf for comparison (D) qN, non-photochemical quenching coefficient, providing a measure of conversion and dissipation of the excess excitation energy into heat and changes within the light harvesting proteins of photosystem (PS) II. qP, photochemical quenching coefficient, providing a measure of the oxidative state of the primary acceptor of photosystem II. FV/FM, maximum photochemical quantum yield of PSII in the dark-adapted state, measuring the maximum amount of the light energy to take the fluorescence
pathway. Samples were dark-adapted for 30 min and then exposed to actinic light of 800 mmol photons m\(^{-2}\) s\(^{-1}\). Values are means with standard deviations (n = 3-5 in experiments).
Go enrichment of the differentially expressed proteins. Categorization of proteins was performed according to cellular component, molecular function and biological process in GoSlim set. (A) the up-regulated proteins between two phases, (B) the down-regulated proteins between two phases. Under the three main categories, the up-regulated and down-regulated proteins were enriched based on the P value (P<0.05) of Fisher’s exact test as -log10 scale. The photosynthesis-related terms were in green rectangles.
Enriched KEGG pathways of the differentially expressed proteins. The rows suggested (A) the up-regulated proteins between two phases, (B) the down-regulated proteins between two phases. The size of each point represents the number of genes enriched in a particular pathway. A larger enrichment factor value and lower Q-values indicates a greater degree of enrichment. The photosynthesis-related pathways were shown in rectangles (significant threshold p < 0.05).
Down-regulated amplitude of differentially expressed proteins in two photosystems (PSI and PSII) (A) and electron transport chain (ETC) (B) during blueberry fruit maturing. PSI included PSI core and LHCIs while PSII included PSII core and LHCIIIs. Each photosystem, the PS core is surrounded by a large light-harvesting complex (LHC) that captures sunlight and transfers the excitation energy to the core with extremely high efficiency. ETC is a series of complexes that transfer electrons from electron donors to electron acceptors for the synthesis of NAPDH and ATP.
Schematic representation of photosynthesis and carbon metabolism revealed from proteomics during blueberry fruit maturating. Only differentially expressed proteins were presented, and the identified enzymes were represented by the rectangles in deep red while the unidentified enzymes were represented by rectangles in violet. (A) Light-independent reaction. LHCA/B, light-harvesting complexes I/II; PSA/B, photosystem I/II reaction center subunit; OHP, light-harvesting-like; PETA, cytochrome f subunit; PETC, rieske iron-sulfur protein; PETE, plastocyanin; ATPC/D, ATP synthase gamma/delta chain in F1 region; ATPG,
ATP synthase subunit b in Fo; FNR, ferredoxin-NAD+ reductase; FD1, ferredoxin-1; (B) Calvin cycle. PGK, Phosphoglycerate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (NADP+); FBA, fructose-bisphosphate aldolase; FBP, Fructose-1,6-bisphosphatase; SBP, sedoheptulose-bisphosphatase; PPE, Phosphopentose epimerase or RPE, ribulose 5-phosphate 3-epimerase; RPI, Rib5P Isomerase; TK,Transketolase; PRK, phosphoribulokinase; RUBISCO, ribulose-bisphosphate carboxylase; RCA, RUBISCO ACTIVASE; (C) Photorespiration. RUBISCO, ribulose-bisphosphate carboxylase; RBCL, RUBISCO large subunit; RBCS, RUBISCO small subunit; RCA, RUBISCO activase; HPR, glycerate dehydrogenase; PGLP, phosphoglycolate phosphatase; HAO, hydroxy-acid oxidase; GGAT, Glutamate--glyoxylate aminotransferase; SGXT, Serine--glyoxylate aminotransferase; GLDC, glycine decarboxylase.

**Supplementary Files**

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