C₄ photosynthetic machinery: insights from maize chloroplast proteomics

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INTRODUCTION
Chloroplasts are organelles for photosynthesis. Chloroplasts also participate in the amino acid, vitamin, isoprenoid, and lipid biosynthesis, as well as reduction of nitrite and sulfate (van Wijk, 2000; Baginsky and Grunsem, 2004). A previous study has proposed that there are ~3,000 proteins in mature chloroplasts that have specialized distributions and functions (Leustier, 2003). Based on the primary product of carbon fixation, plants are classified as C₃ and C₄ species. Oxaloacetate (a four-carbon compound) and 3-phosphoglycerate (a three-carbon compound) are the primary products of carbon assimilation in the C₃ and C₄ plants, respectively. Under certain conditions, the CO₂ assimilation rate of C₄ plants is much higher than that of C₃ plants. In addition, C₄ photosynthesis enables higher nitrogen and water use efficiency than C₃ photosynthesis. maize (Zea mays) is a representative C₄ plant of C₄ photosynthesis. C₄ plants exhibit much higher CO₂ assimilation rates than C₃ plants under certain conditions. The specialized differentiation of mesophyll cell and bundle sheath cell type chloroplasts is unique to C₄ plants and improves photosynthetic efficiency. maize (Zea mays) is an important crop and model with C₄ photosynthetic machinery. 2DE and high-throughput proteomics approaches (e.g., isobaric tags for relative and absolute quantitation and shotgun proteomics) have been employed to investigate maize chloroplast structure and function. These proteomics studies have provided valuable information on C₄ chloroplast protein components, photosynthesis, and other metabolic mechanisms underlying chloroplast biogenesis, stromal, and membrane differentiation, as well as response to salinity, highflow temperature, and light stress. This review presents an overview of proteomics advances in maize chloroplast biology.

Keywords: maize, chloroplast, proteomics, C₄ plant, photosynthesis
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Cell type-specific distribution of many biochemical processes exist in the M and BS chloroplasts (Figures 1A,B). For instance, the reductive phase of the Calvin cycle, reversible pentose phosphate pathway (PPP), oxidative PPP, methylerythritol phosphate pathway, and amino acid metabolism (e.g., biosynthesis of arginine, branched amino acids, and aromatic amino acids) are more active in the M chloroplasts than that in BS (Majeran et al., 2005; Friso et al., 2010). Based on proteomics results, most of the enzymes involved in the above processes were preferentially expressed in the M chloroplasts, suggesting high demand for various metabolites in the M chloroplasts (Friso et al., 2010; Figure 1A). In contrast, most of the enzymes involved in starch metabolism were more abundant in the BS chloroplasts than in the M chloroplasts (Figure 1B). This is consistent with the fact that BS chloroplasts possess more starch particles. However, various proteins involved in fatty acid synthesis were equally distributed across M and BS chloroplasts (Figure 1E), indicating the similar demands for fatty acids in both M and BS chloroplasts (Majeran et al., 2005; Friso et al., 2010).

Reactive oxygen species (ROS) production and redox balance play important roles in regulating plastid functions (Baier and Dietz, 2005). Proteomics results revealed that the majority of ROS scavenging enzymes showed high abundance in the M chloroplasts (Figure 1A). This is proposed to be associated with high linear electron transport rate and water-splitting activity of PSII in the M chloroplasts (Majeran et al., 2005; Majeran and van Wijk, 2009). In addition, a great portion of nucleotide metabolism-related enzymes, such as adenylate monophosphate kinase 2, nucleoside diphosphate kinase 2, soluble inorganic pyrophosphatase, and membrane-bound adenosine triphosphate (ATP)/adenosine diphosphate (ADP) translocator, showed preferential accumulation in the M chloroplasts (Friso et al., 2010; Figure 1A). Since de novo biosynthesis of nucleotides is energy consuming, the M chloroplasts could generate adequate energy through linear and cyclic electron transport (Zrenner et al., 2006).

The components of M and BS chloroplast protein synthesis machineries show overlapping but different expression patterns.
in C₄ plants. Comparative proteomics analysis showed that the majority of initiation and elongation factors (involved in protein translation initiation), general chaperones (related to protein processing), and Clp proteases (participate in protein degradation) were equally distributed across M and BS chloroplasts (Figure 1E). In contrast, ribosomal proteins and RNA syntheses, involved in protein synthesis, were much higher in the M chloroplasts than in the BS (Figure 1A). This implies that in the M chloroplasts, there is more protein synthesis which is required for repairing the chloroplast-encoded reaction center protein D1 (Berna-Gonzalez and Arso, 2002). Additionally, assembly factors for PSI complexes showed higher abundance in the M chloroplasts (Figure 1A), while photosystem I (PSI) complex assembly factors were preferentially expressed in the BS chloroplasts (Figure 1B). The well-correlated expression of proteins in the M and BS chloroplasts suggests existence of well-developed regulatory networks in C₄ photosynthesis (Friso et al., 2010).

**C₄ CHLOROPLAST MEMBRANE PROTEOME IN M AND BS**

Maize thylakoid membrane proteins play key roles in C₄ photosynthesis. Thirty-four thylakoid membrane proteins were identified and quantified using shotgun proteomics approaches (Liu et al., 2011). The majority of the proteins (~76%) were involved in photosynthetic light reactions. Among them, only two PSI subunits were detected, suggesting that most of the PSI components accumulated at lower levels. In addition, a comparative proteomics study on the M chloroplast envelopes between maize and C₃ plant pea (Pisum sativum) revealed that C₄- and C₃-type chloroplasts contained qualitatively similar but quantitatively different membrane protein components (Brautigam et al., 2008).

For instance, several translocators (e.g., outer envelope porin, triosephosphate translocator, and phosphoenolpyruvate translocator) showed higher abundance in C₄ chloroplast envelopes than in C₃ plants. However, two protein import complex components, Tic55 and Clp/CmpH93, were found at lower expressional levels in C₄ chloroplast envelopes (Brautigam et al., 2008). These data imply that the C₄ chloroplast envelope transporters are adapted to meet the demand of high metabolic flux rates during C₄ photosynthesis. However, the small number of proteins identified in these studies provides limited information toward understanding the dynamics and functions of C₄ thylakoid membrane proteins.

Current quantitative proteomics approaches (e.g., isobaric tags for relative and absolute quantification (iTRAQ) and label-free quantification) provide more information for understanding the differentiation and oligomeric states of membrane proteins in the C₄ chloroplasts of BS and M (Majeran et al., 2008; Friso et al., 2010). For instance, the contents of PSI and PSII complexes were measured at lower levels. In addition, a comparative study on the M chloroplast membranes between maize and BS supported the following metabolic mechanisms: (1) The preferential M accumulation of ribosomal proteins suggests high translation rates in the M chloroplasts, which can contribute to the high abundance of PSI subunits and short lifetime of PSI reaction center proteins caused by light-induced damage (Friso et al., 2010; Figure 1C). (2) In terms of protein assembly, the preferential accumulation of low PSI subunit accumulation (i.e., “fpls-04-00085” — 2013/4/12 — 12:20 — page 3 — #3)
Additionally, the and cytochrome cates that the establishment of basic chloroplast functions takes the majority of the photosynthetic apparatus-related proteins started characteristics. The chloroplast biogenesis-related proteins accumulated expression data revealed obvious spatial differentiation charac-
terns highlighted the active transition and/or differentiation of C4 (Majeran et al., 2010). The changes of protein expression pat-
terns of wild-type thylakoids (Cowshoff et al., 2008). Additionally, the psbB-psbH-psbT-petB-petD polyprotein, encoding the components of PSII (e.g., psbB, psbH, psbN, and psbT) and cytochrome b6 (e.g., petB and petD), was misprocessed in the hcf136 mutant M (Cowshoff et al., 2008). These results prove that the mutation of hcf136 leads to disruption of PSI assembly or stability. Proteomics analysis found that hcf136 mutation led to differential accumulation of several proteins in the thylakoid membranes. These proteins were identified as FtsH1, CP47, oxygen evolving center 33-like protein, PSI-D1, LHC, pyruvate orthophosphate dikinase, heat shock protein 70 (HSP70), and Rubisco small subunit (Cowshoff et al., 2008; Figure 1G). How-
ever, the relative levels of the gene transcripts did not correlate with corresponding protein levels. This inconsistency between transcript accumulation and protein abundance suggests the involvement of transcriptional/translational regulations during C4 differentiation.

**CHANGES IN PROTEIN ABUNDANCE DURING C4 CHLOROPLAST BIOGENESIS**

Maize greening is accompanied by the differentiation of the M and BS chloroplasts for C4 photosynthesis. This process has long been considered as a model system to study the sophisticated mechanisms of chloroplast biogenesis. A large-scale proteomics analysis of the leaf and the BSs with their vascular bundle along the leaf developmental gradient has provided detailed dynamic information of more than 4,000 proteins for a systems-level understanding of maize leaf formation and differentiation (Majeran et al., 2010). The changes of protein expression patterns highlighted the active transition and/or differentiation of C4 malate-pyruvate shuttle, photosynthetic linear and cyclic electron flow, photosynthesis, protein translation, specific transporters, and other metabolic processes along the leaf developmental gradient (Majeran et al., 2010). Hierarchical clustering of protein expression data revealed obvious spatial differentiation characteris-
tics. The chloroplast biogenesis-related proteins accumulated to significant levels in the first 4 cm from the leaf base, and the majority of the photosynthetic apparatus-related proteins started to accumulate significantly beyond the 4 cm from ligule. This indi-
cates that the establishment of basic chloroplast functions takes place prior to the specific cell-type differentiation related to C4 functions (Majeran et al., 2010).

In addition to leaf proteomics, a chloroplast proteomics study also revealed protein changes during maize greening (0–48 h) (Lonymsy et al., 2004; Figure 1H). (1) Proteins involved in light reactions changed during greening. For instance, ATPase is the most abundant protein identified in maize chloroplasts during greening. In general, the four subunits of ATPase increased continually with greening (0–48 h). However, some α and β subunits showed different expression patterns possibly due to the demands for different protein forms during specific chloro-
plant differentiation periods. (2) In general, photosynthetic carbon assimilation-related enzymes were increased during the early time (0–4 h) of greening. This is consistent with a previous notion that the plastid assembles the photosynthetic apparatus during early development. Afterward, some enzymes such as β-amylase, NADP malate dehydrogenase, and phosphoglyceric kinase (PGK) reached a plateau. Interestingly, two enzymes displayed opposite expression patterns. The levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) kept increasing, while isoamylase began to decrease after the early phase. Moreover, the expression patterns of PGK and GAPDH were consistent with their mRNA levels reported in previous studies (Dewdney et al., 1993; Wonghoy et al., 1996). (3) The plastid chaperonins and proteases also changed during maize greening. They are involved in protein processing and degradation, respectively. For instance, α subunit of the 60-kD and 20-kD chaperonins displayed moderate increases in the early (0–4 h) and mid-
dle (12 h) phases but decreased at 48 h. In addition, both HSP70 and ClpC increased during the initial phase of green-
ing and reached a plateau at 48 h. These data imply that the active protein folding and degradation takes place, which con-
tribute to active alterations of protein activity and turnover in various signaling and metabolic changes during chloroplast biogenesis. (4) Proteins involved in various plastid metabolic processes (e.g., acetyl-coA carboxylase, beta-D-glucosidase, nuclic acid-binding protein) showed complex patterns of protein abun-
dances during greening. All the above findings have provided valuable insights into the mechanisms underlying chloroplast biogenesis.

**STRESS-RESPONSIVE PROTEINS IN C4 CHLOROPLASTS**

Salinity is thought to have a strong influence on plant chloro-
plant protein composition. Several salt-responsive proteins have been identified in maize chloroplasts undergoing 25 mM NaCl treatment for 4 h using 2DE-based proteomics approaches (Zorb et al., 2009; Figure 1I). In the salt-stressed maize plants, three photosynthesis-related proteins (i.e., ferredoxin NADPH reductase, 23 kDa polypeptide of PSI, and PsH-like protein) were increased under NaCl stress. This would help to attenuate the severe effects of Na⁺ on the photosynthetic machinery. Addi-
tionally, the enhanced abundance of protoporphyrinogen IX oxidase was detected in salt-treated maize chloroplasts. This enzyme is involved in heme and chlorophyll biosynthesis, and its substrates are the targets of salt toxicity leading to massive oxidative stress. The increment of protoporphyrinogen IX oxidase would contribute to alleviate oxidative stress in salt-stressed maize
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CONCLUSION

The maize chloroplast is a good model for studying the C4 photosynthetic mechanism. The development of large-scale quantitative proteomics approaches together with the availability of maize genome sequences has provided a high-throughput platform with high resolution and sensitivity for analyzing protein expression patterns in the M and BS chloroplasts of maize. The conclusions drawn from the presented large-scale quantitative proteomics information acquired to date provides new insights into the specific C4 chloroplast biogenesis, M and BS differentiation, and stress response. However, the photosynthetic machinery and metabolic mechanisms are too complicated to be interpreted by just using quantitative protein profiles. Specialized protein complexes, protein-protein interaction, and post-translational modifications have been proposed to play key roles in photosynthesis. Thus, further proteomics studies should focus on the analysis of large-scale protein modifications and interactions to enhance our understanding of the protein networks in C4 photosynthesis.

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Caffarri, S., Frigerio, S., Olivieri, E., Bringloe, D. H., Rao, S. K., and Dyer, T. (2005). Low/low temperature and light effects on maize photosynthetic apparatus have been investigated (Caffarri et al., 2005). Low temperature led to decreases of chlorophyll contents and Fv/Fm in maize indicating that low temperature could cause photoinhibitory damage to the PSII reaction center. In addition, maize plants grown under low light and high temperature conditions exhibited an increased value of non-photochemical quenching. Under multiple temperature and light conditions, low temperature is the principal factor that affects protein expression in maize thylakoid membranes. For instance, LHCII contents in maize plants grown under low temperature were higher than under high temperature. Minor antenna proteins were decreased compared to the LHClI proteins in maize plants grown under low temperature (Figure 1). In addition, nine LHCII, two LHCIIb, and three LHCIIc protein spots were positively detected by corresponding antibodies on 2DE gels (Caffarri et al., 2005. Figure 1). They presented diverse expression patterns under different temperature/light conditions. This suggests that different genes were translated into proteins of thylakoid membranes in response to environmental stress, which might be a basic mechanism in the C4 photosynthetic apparatus for environmental adaptation.
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