Physiology

Comparative proteomics of leaves found at different stem positions of maize seedlings

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ABSTRACT

To better understand the roles of leaves at different stem positions during plant development, we measured the physiological properties of leaves 1–4 on maize seedling stems, and performed a proteomics study to investigate the differences in protein expression in the four leaves using two-dimensional difference gel electrophoresis and tandem mass spectrometry in conjunction with database searching. A total of 167 significantly differentially expressed protein spots were found and identified. Of these, 35% are involved in photosynthesis. By further analysis of the data, we speculated that in leaf 1 the seedling has started to transition from a heterotroph to an autotroph, development of leaf 2 is the time at which the seedling fully transitions from a heterotroph to an autotroph, and leaf maturity was reached only with fully expanded leaves 3 and 4, although there were still some protein expression differences in the two leaves. These results suggest that the different leaves make different contributions to maize seedling growth via modulation of the expression of the photosynthetic proteins. Together, these results provide insight into the roles of the different maize leaves as the plant develops from a heterotroph to an autotroph.

1. Introduction

C4 plants have ∼50% greater photosynthesis efficiency than do C3 plants owing to the metabolic cooperation of leaf bundle sheath cells (BSCs) and mesophyll cells (MCs) with Kranz-type anatomy surrounding the venation (Long and Spence, 2013). The C4 photosynthesis pathway consists of three key steps. First, the initial CO_2 fixation step occurs in the MC cytosol by phosphoenolpyruvate carboxylase, which forms the C4 acid oxaloacetate (OAA) from phosphoenolpyruvate (PEP). Second, OAA is transported to the BSCs after being reduced by malate dehydrogenase to form malate by the NADP-malic enzyme (NADP-ME), the NAD-malic enzyme (NAD-ME), or phosphoenolpyruvate carboxykinase depending on the type of C4 plant involved. Released CO_2 is then used in the BSCs by ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO),

the first enzyme in the Calvin cycle, which catalyzes the fixation of CO_2 needed for sugar synthesis. Third, pyruvate is shuttled back to the MCs chloroplasts where it is then phosphorylated by orthophosphate dikinase (Hatch, 1987; Matsuoka et al., 2001). This CO_2-concentrating pathway, together with the leaf anatomy of C4 plants enables an elevated photosynthetic capacity particularly at higher temperatures and the efficient use of water and nitrogen in contrast to the ability of C3 plants (Brown, 1999; Hatch, 1987; Sage, 2004).

Maize is a C4 monocot and an important food crop. It displays a leaf developmental gradient (from tip to base) in a highly regular and continuous manner (Evert et al., 1996; Sharman, 1942). Previous studies have analyzed the transcriptome and proteome of maize at successive stages of photosynthetic development (Hibberd and Covshoff, 2010; Li et al., 2010; Majeran et al., 2010). Tausta and colleagues compared the transcriptomes of maize BSCs and MCs at three successive developmental stages: the sink-source transition, during maturation, and at maturation (Tausta et al., 2014). They discovered that certain differentially expressed genes, including those that encode key functions for C4 photosynthesis, are maximally expressed at a stage well before full maturity.

Whole-leaf transcriptomic and proteomic studies of sequential developmental stages from the base to the tip of the young maize leaf by Nelson revealed a developmentally dynamic process involving the appearance and disappearance of transcripts, transcription factors, and other proteins, with a significant number reduced to a low level or absent in mature tissue. Of the transcripts differentially expressed along the developmental gradient, 64% and 21% were found in BSCs and MCs, respectively (Nelson, 2011). In addition, these findings revealed a dynamic transcriptome with transcripts for primary cell wall and basic cellular metabolism at the leaf base that transitioned to transcripts for secondary cell wall biosynthesis and C4 photosynthetic development toward the tip. Furthermore, the contents of the maize-leaf proteome and phosphoproteome were analyzed in parallel by Facette and his colleagues as leaves transitioned through the sequential developmental stages—proliferative cell division, differentiation, and cell expansion—from the base to the tip of the young maize leaf and revealed that posttranslational regulation plays a key role in the individual leaf developmental transition, providing further insight into maize leaf development (Facette et al., 2013).

Many other studies have focused on leaf development of etiolated seedlings after exposure to light. Shen and colleagues analyzed the dynamic proteomic differences between green seedlings, etiolated seedlings held in the dark, and etiolated seedlings that were illuminated for 6 or 12 h during greening by label-free quantitative proteomics (Shen et al., 2009) The expression levels of 73 proteins were significantly altered during greening. Of the differentially expressed proteins, 51% were involved in chloroplast development, indicating that proteins involved in chlorophyll synthesis and photosynthesis, which occurs during greening, must be rapidly synthesized and accumulate after seed germination (Shen et al., 2009).

However, these studies only focused on individual maize leaf development, with attention paid to the function of leaves at different positions. After breaking through the soil surface and being exposed to light, seed cotyledons open and become green, forming the first photosynthetic organs of a young plant. Once a seedling is capable of photosynthesis, it is no longer dependent on the seed energy reserves. However, little is known about the physiology and development of leaves at different positions as the seedling changes from a heterotroph to an autotroph. For this study, we measured the lengths, widths, quantum yields of PSI and PSII, RuBisCO and PEPC activities, and net photosynthesis rate of maize leaves at stem positions 1–4, and performed a proteomic study to investigate their physiological properties and developmental dynamics. Our results indicate that the first four leaves make different contributions to maize seedling growth via modulation of the expression of components of the photosynthetic apparatus and provide a foundation for studies on regulatory mechanisms in leaves at different positions as the seedling changes from a heterotroph to an autotroph.

2. Materials and methods

2.1. Plant material and growth conditions

Maize (Zea mays L., ecotype B73) seeds were germinated in wet vermiculite and then planted in soil. The plants were grown in a phytotron chamber (Sai Fu) under a 28 °C day/25 °C night ($\pm$2 °C) cycle with a 14-h photoperiod. The light intensity in the growth chamber was 200 μmol m^{-2} s^{-1}, and the relative humidity was between 40 and 50%. Fully expanded leaves at the different stem positions were harvested from the seedlings for morphological, physiological, and proteomic studies. The leaves were numbered according to their positions on the stem, with leaf 1 corresponding to the lowest leaf (Crespo et al., 1979).

2.2. Quantification of lengths and widths of maize leaves

Length and width measurements were performed on fully expanded maize leaves at the different stem positions. Each experiment was repeated three times with ten plants per replication. Data are the mean $\pm$ standard deviation of three independent experiments.

2.3. Protein extraction and quantification

Total protein extracts were prepared for two-dimensional difference gel electrophoresis (2D-DIGE) as described (Hurkman and Tanaka, 1986). Briefly, leaves were individually ground in liquid nitrogen and then dissolved in a two-phase system consisting of chilled phenol buffered with Tris, pH 8.8, and chilled 0.1 M Tris-HCl, pH 8.8, 10 mM EDTA, 0.9 M sucrose, and 0.4% (v/v) β-mercaptoethanol. After centrifugation at 20,000g for 20 min at 4 °C, each phenol phase was removed and transferred to a new conical tube. The phenol-extracted proteins were precipitated by adding five volumes of cold methanolic 0.1 M ammonium acetate into the phenol phase and incubating the mixture at −20 °C overnight. After centrifugation at 20,000g for 20 min at 4 °C, each supernatant was removed and discarded in a non-chlorinated waste container. Each pellet was individually suspended in two volumes of chilled methanolic 0.1 M ammonium acetate (−20 °C) and held at −20 °C for at least 30 min. Next, each sample was centrifuged at 20,000g for 20 min at 4 °C. The methanolic 0.1 M ammonium acetate wash was repeated once more. Then, each pellet was washed one to two times with cold 80% (v/v) aqueous acetone, and finally once with cold 70% (v/v) aqueous methanol. Each pellet was dried under vacuum and then solubilized in 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 30 mM Tris-HCl, pH 8.5 at room temperature for 1–2 h. Insoluble material was removed by centrifugation at 100,000g and 4 °C for 1 h. Protein concentration was determined using 2-D Quant kit reagents (GE Healthcare).

2.4. 2D-DIGE

2D-DIGE was performed as described (Li et al., 2011; Yi et al., 2014). Proteins (50 μg) from the leaves were labeled with 400 pmol Cy3 or Cy5. An internal standard was prepared by pooling an equal quantity of protein from each sample and labeling it with Cy2. Three biological replicates of each sample were prepared. All labeled samples were merged according to the principle of randomization. The reactions were quenched with 1 μl of 10 mM lysine, mixed, and incubated on ice for 10 min in the dark. Three differently labeled samples (150 μg total), i.e., one labeled with Cys3 from leaf x, one labeled with Cys5 from leaf y, and 25 μg each from leaf x and leaf y labeled with Cys2, with x $\neq$ y and in all possible x/y and y/x combinations, were added into 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) immobilized pH gradient buffer at a final volume of 450 μl. Isoelectric focusing was performed on pH 4–7, 24-cm IPG strips (GE Healthcare). The second-dimension separation was SDS-PAGE (12.5% acrylamide).

The 2D-DIGE images were acquired using a Typhoon Trio scanner (GE Healthcare). The differential in-gel analysis module in the DeCyder 7.0 software package (GE Healthcare) was used for spot detection. The spots with 1.5-fold change at least one leaf in normalized spot density with p-values less than 0.05 were considered as significantly different. The biological variation analysis module was applied to the three biological repeats to identify the differentially expressed spots with >95% confidence.