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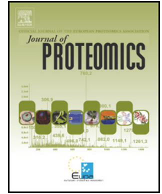

Differences in properties and proteomes of the midribs contribute to the size of the leaf angle in two near-isogenic maize lines

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ABSTRACT

The midrib of maize leaves provides the primary support for the blade and is largely associated with leaf angle size. To elucidate the role of the midrib in leaf angle formation, the maize line Shen137 (larger leaf angle) and a near isogenic line (NIL, smaller leaf angle) were used in the present study. The results of the analysis showed that both the puncture forces and proximal collenchyma number of the midribs of the first and second leaves above the ear were higher in NIL than in Shen137. Comparative proteomic analysis was performed to reveal protein profile differences in the midribs of the 5th, 10th and 19th newly expanded leaves between Shen137 and NIL. Quantitative analysis of 24 identified midrib proteins indicated that the maximum changes in abundance of 22 proteins between Shen137 and NIL appeared at the 10th leaf stage, of which phosphoglycerate kinase, adenosine kinase, fructose-bisphosphate aldolase and adenylate kinase were implicated in glycometabolism. Thus, glycometabolism might be associated with leaf angle formation and the physical and mechanical properties of the midribs. These results provide insight into the mechanism underlying maize leaf angle formation.

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1. Introduction

Food security is globally an important issue, particularly in developing countries, such as China and India, which have large populations. As a C4 plant, maize has a higher yield potential than wheat and rice; thus, maize has become increasingly important in sustainable food production. However, maize productivity has reached a plateau, as hybrid technology has been fully exploited. Increasing maize yield per area with limited arable land in China might be a promising strategy to break through the bottleneck of maize production. Therefore, density tolerance characters, such as smaller leaf angle and larger leaf orientation, in maize have received much in maize improvement programs for the development of mechanized large-scale maize production in China.

High density planting can significantly increase aboveground biomass, thereby increasing maize yield [1,2]. However, with increasing plant population in a fixed area, leaf overlap between individuals within populations occurs; thus, dense maize planting might encounter two defects: shade avoidance syndrome (SAS) and excess humidity [3]. Prolonged SAS induces early flowering and insufficient premature reproductive growth in plants, which affects nutrient storage and seed development [4]. As light is a critical environmental factor in the regulation of maize growth and development, unpredictable yield loss might result from SAS. High humidity within the maize leaf canopy is another problem resulting from dense planting. Leaf photosynthesis efficiency is reduced when gas exchange is poor. High plant density also affects disease severity [5] and host-pathogen interactions [6]. Compared with maize genotypes with large leaf angles, maize genotypes with small leaf angles are more suitable for dense planting, thereby avoiding SAS and high humidity. Thus, a better understanding of the mechanism of leaf angle formation is extremely important for maize breeding programs.

Leaf angle is a quantitative trait controlled through several genes, regulated through complex molecular signaling and transduction networks. Several genes associated with leaf angle, such as *OsDWARF4*, *OsLAZY1*, *OsBRI1*, *BU1*, *OsGRS1*, *ILI1*, *LC2*, *ILA1*, and *OsLIC* have been cloned in rice [7–15]. Specifically, the upregulation of *brd1*, *dwf1*, *Osdwarf4*, *d61-7* and *LC2* has been associated with the metabolism of hormones, including brassinosteroid and gibberellin (GA), and the interrelated links between these genes have been demonstrated as important for leaf initiation and architecture in rice [7,11,13,16–20]. Brassinosteroid is a crucial regulation factor [21] in the crosstalk with light, GA and auxin pathways associated with photomorphogenesis in Arabidopsis [22]. In maize, QTLs associated with leaf angle determination are distributed on chromosomes 1–5 and 7–10 [23–27]. Using the F2:3 populations constructed using compact maize line Yu82 and plate inbred line Yu87-1, we previously located five QTLs associated with leaf angle on chromosomes 1, 2, 5 and 7, cloned the main effect QTLqLA2 (*ZmTAC1*) and demonstrated that qLA2 *ZmTAC1* positively regulates the size of the leaf angle [28,29].

In addition, there is evidence for the involvement of mechanical factors as non-primary regulators of plant development [30]. A T-DNA

Abbreviations: SAS, shade avoidance syndrome; NIL, near isogenic line.

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insertion in the *ILA1* gene, a nuclear localized group C MAPKKK family member, resulted in a smaller mechanical tissue area through a reduction of the cell wall cellulose and xylan content. The increased expression of ILA1 in the bundle sheath of the lumina joint of maize leaves reduced mechanical strength and increased leaf angle [31].

In the present study, the maize inbred line Shen137 (larger leaf angle) and a near isogenic line (NIL, smaller leaf angle) were used to explore the role of the midrib in leaf angle formation. We compared the differences in the midribs between the two genotypes at physical, morphological and proteomic levels. The results showed that differences in properties and proteomes of the midribs contribute to the size of the leaf angle in maize.

2. Materials and methods

2.1. Plant materials

The maize line Yu82 is a compact inbred line and Shen137 is an expanded inbred line. The qLA1 for leaf angle is located on chromosome 1 bin 1.02–1.03. Shen137 (recurrent parent) was crossed with Yu82 (non-recurrent parent) and the F1 hybrid was backcrossed five times to Shen137. One BC5F1 plant was self-pollinated to develop the NIL, in which the donor size was estimated at 30.26 kb. We obtained an NIL (Shen-NIL68) displaying a semi-compact plant type, which harbored one Yu82 chromosomal segment on chromosome 1 bin 1.02–1.03.

Shen137 and the NIL were grown at the experimental farm of Henan Agricultural University. The planting density was 52,500 plants per hectare for both inbred lines. Standard cultural practices were performed until the crop was mature. The growth duration of the two lines was consistent, and the final leaf number of the mature plants was 22. The 17th leaf was the ear leaf for both lines. The midribs of the 5th, 10th and 19th expanded leaves of Shen137 and NIL were collected, immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Leaf angle and puncture force determination

The leaf angles (the angle between stem and blade) were measured when the leaves were fully expanded on healthy maize plants grown under field conditions. A minimum of 15 leaves were used in each set of measurements.

The punch test was applied to the first and the second leaves above ear to assess the midrib mechanical properties. The midribs were mounted onto a stainless steel plate. The force (N) required to puncture the midrib was determined using Stem Strength Tester YYD-1B. The puncture test was conducted using a conical rounded tip probe at room temperature. The measurements were obtained at three points (5, 15 and 25 cm from leaf lamina joint) on each midrib. A total of 12 midribs of each line were tested.

2.3. Microscopic observation

The leaf midribs were collected from the new fully expanded leaves at the 19th leaf stage and immediately fixed in FAA solution (10% formalin, 10% acetic acid, 45% ethanol) for at least 48 h prior to dehydration in an ethanol series and embedding in paraffin. Safranin was added to the dehydration step to mark the tissue for later orientation when sectioning. Successive sections, 10 μm thick, were cut on a rotary microtome (Leica RM2235 rotary microtome, Wetzlar, Germany), affixed to a glass slide using Haupt's adhesive and dried at room temperature for 24 h. The sections were deparaffinized with xylene and stained with Safranin and Fast Green FCF (Biotopped) according to Johansen's Safranin and Fast Green protocol [32]. Balsam neutral was used for permanent slide preparation. The specimens were observed using a Phoenix PH50 microscope fitted with 4× lenses. The stained specimens were digitized and recorded using ToupView x86.

2.4. Protein extraction and quantitation

The fresh midribs (1.0 g) were frozen in liquid nitrogen and ground to fine powder using a prechilled mortar and pestle prior to protein extraction. The dry powder was added directly to 10% TCA in acetone containing 5 mM DTT, homogenized using a mortar and pestle on ice and subsequently clarified at 12,000 rpm for 5 min. The precipitate was washed three times with 10% TCA/acetone, and each wash was followed by a cold acetone wash (containing 10 mM DTT). The final precipitate was air-dried at room temperature. The proteins were directly extracted using 2-D buffer containing 8 M urea, 2 M thiourea, 2% CHAPS and 20 mM DTT. The protein extract was clarified through centrifugation prior to 2-D electrophoresis. The protein was quantified using a Bradford assay with BSA as a standard [33]. The crude protein extracts were diluted to the linear range of the standard curve for an accurate assessment.

2.5. 2-D and image analysis

Approximately 800 μg proteins were applied to 11-cm linear pH 4–7 strips. Rehydration was performed at 20 °C overnight. Focusing was initiated at 250 V for 3 h, followed by increasing to 1000 V within 4 h, increasing to 8000 V within 4 h and remaining constant at 8000 V for 20 h (20 °C). The focused strips were equilibrated for 15 min in a buffer containing 0.1 M Tris–HCl (pH 8.8), 2% SDS, 6 M urea, 30% glycerol and 0.1 M DTT, followed by incubation for 15 min in the same buffer containing 0.25 M iodoacetamide instead of DTT. SDS-PAGE was performed on 13.5% gels (20 × 15 × 0.1 cm). After fixation with 40% methanol and 10% acetic acid for 30 min, the gels were stained with 0.1% (w/v) CBB G-250 and destained in 10% (v/v) acetic acid overnight. Digital images of the gels were processed using PDQUEST 7.1.0 (Bio-Rad). At least three biological independent experiments were acquired in the proteomic analysis. In each independent experiment, two technical repeats were conducted. The relative abundance of the identified proteins is represented as the mean of three replicates. Relative abundances of protein spots were calculated using PDQUEST software (Bio-Rad). For the data processing, the missing values were filled with zeros. In valid spots, the volumes were normalized to the total quantity. Logarithmic transformation was selected to calculate each data value [34].

2.6. MS/MS

Protein spots with abundance changes of at least two-fold in the leaf midribs were excised from the gels and digested using trypsin. The proteins were reduced (10 mM DTT), alkylated (50 mM iodoacetic acid) and subsequently digested with 10 mg/ml trypsin for 16 h at 37 °C in 50 mM ammonium bicarbonate. The supernatants were vacuum-dried and dissolved in 10 μl 0.1% trifluoroacetic acid, and 0.5 μl of the samples were added onto a matrix comprising 0.5 μl of 5 mg/ml 2,5-dihydroxybenzoic acid in water acetonitrile (2:1). The digested fragments were analyzed using a MALDI-TOF analyzer (Ultraflex III, Bruker, Germany). The MS/MS spectra were acquired in the positive ion mode and automatically submitted to Mascot 2.2 (http://www.matrixscience.com) Matrix Science for peptide mass fingerprintings against the NCBInr 20150117 database (57,412,064 sequences, http://www.ncbi.nlm.nih.gov/). The taxonomy was Viridiplantae (green plants) (2,874,303 sequences). The following search parameters were used: type of search MALDI-TOF ion search; enzyme: trypsin; variable modifications: Acetyl (Protein N-term), Deamidated (NQ), Dioxidation (W) and Oxidation (M); fixed modifications: Carbamidomethyl (C); mass values monoisotopic; protein mass: unrestricted; peptide mass tolerance: ±100 ppm; fragment mass tolerance ±0.5 Da; max missed cleavages: 1; and instrument type MALDI-TOF-TOF. Only significant scores greater than "identity," defined through the Mascot probability analysis, were considered for assigning protein identity. All of the positive protein identification scores were significant ($P < 0.05$, score >51).

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