Starch biosynthesis is crucial for maintaining photosynthesis and leaf growth under drought stress

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Abstract

To understand the growth response to drought, we performed a proteomics study in the leaf growth zone of maize (*Zea mays* L.) seedlings and functionally characterized the role of starch biosynthesis in the regulation of growth, photosynthesis and antioxidant capacity, using the *shrunken2* mutant (*sh2*), defective in ADP-glucose pyrophosphorylase. Drought induced differential expression of 284 proteins overrepresented for photosynthesis, amino acids, sugar and starch metabolism, and redox-regulation. Changes in protein levels correlated with enzyme activities (increased ATP synthase, cysteine synthase, starch synthase, RuBisCo, peroxiredoxin, glutaredoxin, thioredoxin and decreased triosephosphate isomerase, ferredoxin, cellulose synthase activities, respectively) and metabolite concentrations (increased ATP, cysteine, glycine, serine, starch, proline and decreased cellulose levels). The *sh2* mutant had a reduced ability to increase starch levels under drought conditions, causing soluble sugar starvation at the end of the night and impaired leaf growth. Increased RuBisCo activity and pigment concentrations observed in WT in response to drought were lacking in the mutant, which suffered more oxidative damage and recovered more slowly after re-watering. These results demonstrate that starch biosynthesis plays a crucial role in maintaining leaf growth under drought stress and facilitates enhanced carbon acquisition upon recovery.

Introduction

One of the first responses of plants to drought is reduced leaf growth in order to limit transpiration and conserve water (Ahmad et al., 2016; Avramova et al., 2016). In crops, this contributes to a loss of up to 60% of the potential yield, even during mild drought conditions when visual signs of wilting are absent (Ribaut et al., 1997). Leaf growth responses are mediated by cell division and expansion (Rymen, 2010), regulated by molecular networks of integrated signals, including hormones (Nelissen et al., 2012; Pacifici et al., 2015), reactive oxygen species (ROS, Tsukagoshi et al., 2010) and sugar signals (Gibson et al., 2005). Drought stress affects physiological parameters such as stomatal aperture, water relations and photosynthesis and causes changes in carbon metabolism (Farooq et al., 2009). Starch plays a key role in balancing growth and carbon assimilation (Thalmann and Santelia, 2017). It accumulates during the photoperiod and is degraded during the night to support respiration and growth. Drought increases starch concentrations during the photoperiod (Foyer et al., 1998). In mutants unable to synthesize or degrade starch, growth is impaired (Stitt and Zeeman, 2012). Starch reserves are remobilized to release soluble sugars, which function as osmolites, support growth and serve as signal molecules (Thalmann and Santelia, 2017).

To obtain an integrated mechanistic understanding of the growth response to drought, we combined cellular, physiological and molecular analyses of the maize (*Zea mays* L.) leaf growth zone (Avramova et al., 2015a;

Avramova et al., 2017). The large size of this growth zone (Avramova et al., 2015b) provides enough tissue for analyses at the transcriptome (Avramova et al., 2015a; Czedik-Eysenberg et al., 2016; Kravchik and Bernstein, 2013; Li et al., 2010; Wang et al., 2014), epigenome (Candaele et al., 2014), proteome (Bonhomme et al., 2012; Facette et al., 2013; Majeran et al., 2010; Riccardi et al., 1998) and metabolome levels (Avramova et al., 2015a, 2017; Czedik-Eysenberg et al., 2016; Nelissen et al., 2012, 2018; Pick et al., 2011; Wang et al., 2014) along the developmental gradient and allows to study responses to environmental stress (Avramova et al., 2015a, 2017; Kravchik and Bernstein, 2013; Nelissen et al., 2018; Rymen et al., 2007; Walter et al., 2009).

The effect of drought stress on the maize leaf proteome has been studied by gel-based approaches (Benešová et al., 2012; Riccardi et al., 1998; Xin et al., 2018), which are limited to detect only the most-abundant proteins in mature leaves. LC-MS/MS based techniques, which have a higher sensitivity, are increasingly used to study proteome and phosphoproteome changes, including responses to drought stress in mature leaves (Zhao et al., 2016) and growth zone of growing leaves (Dai Vu et al., 2016). Currently, the only large-scale studies comparing the effect of drought in dividing, expanding and mature tissues of maize leaves have been performed at the transcriptome (Avramova et al., 2015a), the phosphoproteome (Bonhomme et al., 2012) and the metabolome (Avramova et al., 2017; Nelissen et al., 2018) levels. In addition to transcriptional regulation, molecular adaptation could be regulated at the post-transcriptional, posttranslational and metabolite levels (Ghatak et al., 2017; Nägele and Weckwerth, 2014). Therefore, integrated studies combining data from different regulatory levels are needed to obtain a mechanistic understanding of the growth response to drought.

In this study, we investigated the drought response in the leaf growth zone at the proteome level, using a mass spectrometry-based protein quantification, complemented with high-resolution metabolite and biochemical measurements. We compared wild type and *sh2* mutant plants, defective in starch biosynthesis under drought conditions and demonstrate the importance of increased starch synthesis in maintaining growth and facilitating recovery during and upon recovery from drought.

Materials and Methods

Plant material

The inbred line B73 (Iowa Stiff Stalk Synthetic) was used for proteomic, metabolite and biochemical measurements. The *shrunken2* mutant and its wild type (W22), were obtained from MaizeGDB (http://www.maizegdb.org/stock_catalog).

Growth experiments

Growth conditions were identical to those previously described in more detail (Avramova et al, 2015a, 2016). Briefly, maize seedlings were grown in a growth chamber (16h day/8h night, 25°C/18°C day/night [d/n], 300-400µmol.m^{-2} s^{-1}Photosynthetically Active Radiation, provided by high pressure sodium lamps). Control pots were re-watered daily to a Soil Water Content (SWC) of 54%. For drought treatments, water contents were allowed to drop after sowing to 43% SWC (mild stress, no wilting), and 34% SWC (severe stress, leaves are wilting during the day), respectively, where they were maintained (Figure S1). Three days after emergence of the fifth leaf, the plants were harvested at the middle of photoperiod (14:00 h, MD), end of the day (22:00 h, ED), two hours after beginning of the night (24:00 h, 2hN), end of the night (6:00 h, EN). The first 10 cm from the base of leaf five of each plant were cut in ten segments of 1 cm and the samples were immediately frozen in liquid nitrogen and kept at -80°C until used for measurements (protein and metabolite quantification, enzyme activities).

Proteome analysis

Protein extraction, digestion, and labeling

Total protein extracts were prepared following a modified protocol of trichloroacetic acid (TCA)-acetone extraction (Méchin et al., 2007). Protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). After trypsin digestion at 37°C, peptides were labeled with isobaric tags for relative and absolute quantification (ITRAQ, Wiese et al., 2007). For the reconstitution of the tags, they were dissolved in 50 µl of isopropanol according to the manufacturer's protocol (Applied biosystems, Foster City, CA, USA). Subsequently, peptides were incubated with the tags for 2 hours at ambient temperature. Pooled samples were prepared based on the labeled samples with a peptide concentration ratio of 1:1:1:1:1:1:1:1. Details about the labeling design of the 4 sample pools are shown in Table S1.

Nano reverse phase liquid chromatography and mass spectrometry

To reduce the overall complexity of the ITRAQ-labeled samples, a 2D-LC fractionation was performed. In a first dimension, performed offline, samples were separated on an Acquity UPLC system (Waters, Milford, MA, USA) with an X-bridge BEH C18 LC column (130 Å, 5 µm particles, 4.6 mm x 150 mm). The column was operated at 40°C and the following mobile phases were used: mobile phase A: 2% acetonitrile and 0.25% formic acid at pH 9 with H_5NO and mobile phase B: 98% acetonitrile, 0.25% formic acid at pH 9 with H_5NO. A linear gradient from 2% B to 60% B in 9.5 min followed by a steep increase to 90% B in 0.5 min at a flow rate of 1.5 ml/min was used to separate the samples in 10 fractions. Subsequently, these peptide fractions were vacuum dried.

The peptide mixture was further separated by reversed phase chromatography on a Waters nanoAqcuity-UPLC system using an ACQUITY UPLC Peptide BEH C18 nanoACQUITY Column, 130 Å, 1.7 µm, 100 µm X 100 mm (Waters, Milford, MA, USA). Before loading, the sample was dissolved in mobile phase A, containing 2% acetonitrile and 0.1% formic acid and spiked with 20 fmol Glu-1-fibrinopeptide B (Glu-fib, Protea biosciences, Morgantown, WV, USA). A linear gradient of mobile phase B (0.1% formic acid in 98% acetonitrile) in mobile phase A (0.1% formic acid in 2% acetonitrile) from 2 to 35% in 110 min followed by a steep increase to 95% mobile phase B in 2 min was used at a flow rate of 350 nl/min. The nano-LC was coupled online with the mass spectrometer using a PicoTip Emitter (New Objective, Woburn, MA, USA) coupled to a nanospray ion source (Thermo Scientific, San Jose, CA). The LTQ Orbitrap Velos (Thermo Scientific, San Jose, CA) was set up in a MS/MS mode where a full scan spectrum (350 - 5000 m/z, resolution 60 000) was followed by a maximum of five dual CID/HCD tandem mass spectra (100 - 2000 m/z). Peptide ions were selected for further interrogation by tandem MS as the five most intense peaks of a full scan mass spectrum. Collision Induced Dissociation (CID) scans were acquired in the linear ion trap of the mass spectrometer, High energy Collision activated Dissociation (HCD) scans in the Orbitrap, at a resolution of 7500. The normalized collision energy used was 35% in CID and 55% in HCD. We applied a dynamic exclusion list of 30 sec for data dependent acquisition. The entire wet lab and LC-MS procedures were controlled for confounding factors.

Proteome data analysis

Proteome discoverer software (version 1.3, Thermo Scientific, San Jose, CA) was used to perform database searching against the Uniprot Zea mays database using both Sequest and Mascot algorithms, and the following settings: precursor mass tolerance of 10 ppm, fragment mass tolerance of 0.5 Da. Trypsin was specified as digesting enzyme and 2 missed cleavages were allowed. Methylthio (C) and ITRAQ modifications (N-terminus and lysine residues) were defined as fixed modifications and methionine oxidation and phosphorylation (STY) were variable modifications. The results were filtered for confident peptide-to-spectrum matches (PSMs) based on a non-concatenated target-decoy approach. The decoy database is a reversed version of the target database. Only first ranked peptides with a global False Discovery Rate (FDR) smaller than 5% were included in the results. In the ITRAQ quantification workflow the most confident centroid method was used with an integration window of 20 ppm. The reporter ion intensities were corrected for isotope contamination by solving a system of linear equations and the known label purity values from the vendor's data sheet. The 10 raw datasets from the offline fraction of each sample were analyzed simultaneously in Proteome Discoverer. All the sequences and reporter ion intensities of the peptide spectrum matches (PSMs) that match the confidence requirements were retained for further data-analysis. Data were normalized within

each sample pool, but not between the pools and expression values of all the peptides matching to the same protein were averaged. All peptides listed with multiple accessions were removed and peptide sequences belonging to single protein identifications were kept for further statistical analysis, increasing the confidence of protein quantification (Bradshaw et al., 2006; Neilson et al., 2011). Hierarchical clustering was performed using MATLAB (version 9.0, MathWorks Inc., Natick, MA, USA). In each pool, proteins not identified in all biological replicate samples were excluded from the analysis (1 protein in the meristem, 5 proteins in the mature zone, and 5 proteins in the pool comparing the 3 zones). Data were then $\log_2$-transformed and a one-way ANOVA was performed within each sample using the software MeV (Multi Experiment Viewer, Saeed et al., 2003) and False Discovery Rate (FDR) was used as a multiple testing correction. Original and corrected P -values are listed in Table S2. A Quality Threshold clustering (QT, Heyer et al., 1999) was performed in MeV (Multi Experiment Viewer, Saeed et al., 2003) using Euclidian distance, cluster diameter 0.4 and minimum cluster population 4. Enrichment studies of the differential proteins levels across the developmental zones and in response to drought in each zone, based on 5% and 10% FDR, respectively, were carried out by PageMan (Usadel et al., 2006).

Quantitative Real-Time PCR

The expression of *Sh2* was measured in the fifth leaf, three days after emergence. The first, third and eighth centimeter from the leaf base were harvested in *sh2* as meristem, elongation and mature tissues, respectively, based on the cell length profile. In the wild type, these zones were harvested as the first, fifth and tenth centimeter from the leaf base, respectively. Total RNA was diluted to the same concentration (0.4 µg. µL-1) for all samples and first strand cDNA synthesis was performed with superscript II Reverse Transcriptase according to the manufacturer's protocol (Thermo Fisher Scientific, USA). Quantitative Real-Time PCR was performed with the Takyon qPCR Kits for SYBR Assay (Eurogentec, Belgium) using the *UBCP* gene (Zm00001d001913) as a housekeeping gene (Manoli et al. , 2012). The primers 5'-GTAAGGGCATCCAAGAGG-3' and 5' -AAGCGGCTCTT ACCATAC-3' were designed to amplify the *Sh2* gene.

Metabolite analyses

Antioxidants

Peroxiredoxin (PRX) activity was determined according to Horling et al., (2003). Glutaredoxin (GRX) activity was determined according to Lundberg et al., (2001). Thioredoxin (TRX) activity was determined according to Wolosiuk et al. (1979). Ferredoxin activity was determined as described previously (Smillie, 1962).

Amino acids and sugars

Cellulose synthase was determined according to Blanton et al., (2000). Amino acids and sugars as well as the activities of their related enzymes were measured according to AbdElgawad et al., (2014 and 2015).

Starch, soluble sugars and chlorophyll measurements

Soluble sugars and starch were determined by the anthrone method (Yemm and Willis 1954). Pigments were extracted and determined according to the method described by Markwell et al. (1986). The contents of chlorophylls a and b were calculated using the formulas described previously (Porra *et al* ., 1989) and expressed as µg pigment g^{-1} fresh weight (FW).

Biochemistry

RuBisCo activity was measured by non-radioactive microplate-based assay, which determines the product (3-phosphoglycerate; 3-PGA) in an enzymatic cycle between glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate oxidase (Sulpice et al., 2007). The activity of triosephosphate isomerase was measured according to the method of Benítez-Cardoza et al. (2001) by monitoring NADH consumption at 340 nm. ATP concentration was measured by using ATP Assay System Bioluminescence Detection Kit (FF2000) as described by Promega, Wisconsin, USA. ATP synthase was measured according to the method of Cross and

Kohlbrenner (1978). Phosphoenolpyruvate carboxylase activities were monitored as absorbance changes at 340 nm. The AGPase activity was assayed according to Nakamura et al. (1989) in a reaction mixture (5 mM ADPG, 50 mM MgCl2, 100 mM Hepes–NaOH). Production of NADPH was monitored at 340 nm. Total amylase activity was determined as described by Bhatia and Singh (2002).

Soluble proteins were determined according to (Lowry et al., 1951) with bovine serum albumin as a standard.

MDA was extracted in 2 mL of 80% (v/v) ethanol and measured using a thiobarbituric acid-MDA assay (Hodges et al., 1999). H_2O_2 determination, was done by using Xylenol Orange reagent (Avramova et al, 2015a).

Photosynthesis and stomatal conductance measurements

Net CO_2 assimilation rate (photosynthesis) and stomatal conductance were measured on the exposed/mature part of the fifth leaf using a portable photosynthesis system (LI-COR LI-6400, LI-COR Inc., Lincoln, NE, USA).

Sucrose feeding

Immediately after emerging, the fifth leaf of well-watered plants and plants subjected to mild and severe drought stress were fed with 0.3 M sucrose solution contained in a glass vial through the cut leaf tip as in Spoehr (1942). The treatment was initiated early in the beginning of the night (22:00h). After the treatment and at the end of the night (6:00 h, EN), 10 samples of 1 cm were collected along the base of the leaf for soluble sugar concentration determination. Leaf length was measured as described below for LER determination.

Kinematic Analysis

The kinematic analysis was performed according to Sprangers et al. (2016). It includes measurements of leaf length, cell length profile along the leaf axis, calculation of leaf elongation rate and estimation of the size of the leaf basal meristem. Leaf length was measured daily with a ruler on the fifth leaf from the moment it was visible among the older leaves. LER was calculated for the first 3 days of its growth as the difference in length divided by the time difference between successive measurements. For meristem measurements, samples harvested 3 days after leaf emergence were analyzed by fluorescence microscopy (AxioScope A1, Axiocam ICm1, Zeiss, Germany) at 20× magnification. The size of the meristematic zone of the leaf was estimated by locating the most distal mitosis in the cell files. Cell length was measured by light microscopy (AxioScope A1, Axiocam ICm1, Zeiss, Germany) using differential interference contrast at 40× magnification and the online measurement module in the Axiovision software (Zeiss, Germany). The leaf growth zone was split in ten 1cm-long- segments and cell length measurements were carried out at four locations in each segment: at the tip (0 cm), at one-third of the segment (0.3 cm), at two-thirds (0.6 cm), and at the end of the segment (0.99 cm). Around 20 cells were measured at each location. The raw data obtained for individual leaves were smoothed and interpolated at an interval of 50 mm using the kernel-smoothing function locpoly of the KernSmooth package (Wand and Jones, 1995) for the R statistical software environment (R Core Team, 2011; https://www.R-project.org), which allowed averaging between leaves and comparison between treatments. At least 5 plants were measured per line/treatment combination.

Results

Identification of 3013 proteins in the growth zone of the maize leaf

Maize seedlings (B73) were subjected to well-watered, mild and severe drought conditions that inhibit leaf elongation rates by ca 30 and 60%, respectively (Avramova et al., 2015a). Based on kinematic analyses (Avramova et al., 2015a), we sampled meristem, elongating and mature tissues from the 5^{th} leaf at three days after emergence. We prepared 4 ITRAQ labeled (Wiese et al., 2007) pools of samples, 3 of these directly comparing the three growth conditions (control, mild and severe drought) within each developmental zone (meristem, elongation, and mature zone) and one contrasting the three zones under control conditions to examine differences along the developmental gradient (Table S1). In total, we identified 7636 peptides which matched 3013 unique proteins.

Differences in protein levels along the developmental gradient

We identified 1194 proteins in ITRAQ pool 4 comparing the three zones (meristem, elongation zone, mature zone) under well-watered conditions, which we assigned to 35 Mapman (Thimm et al., 2004) functional categories (Figure S2) using Mercator (Lohse et al., 2014). In total, 230 proteins were differentially expressed between the zones (FDR $< 5\%$; Table S4) and clustered into 7 patterns (Figure 1). Two of the clusters contained the majority of proteins (118 in cluster 1 and 66 in cluster 2), showing opposite patterns of increasing or decreasing expression. To identify the biological processes represented by these two clusters, we performed an enrichment analysis using Pageman (Usadel et al., 2006; Figure S3). The biggest cluster (1) with gradually increasing levels from the meristem to the mature zone contained 118 proteins. The categories "cell", and "transport" were significantly enriched in this group, while "sucrose degradation", "protein synthesis", "redox", and "RNA processing" were underrepresented. The second largest cluster (2) of 66 proteins had the opposite pattern, gradually decreasing protein levels from the meristem to the mature zone. The categories "secondary metabolism", "RNA processing", "protein synthesis", and "transport" were enriched in this group and "Calvin cycle", "lipid metabolism", "redox", "RNA binding" and "DNA synthesis" were underrepresented.

The remaining clusters contained smaller numbers of proteins (3-15; Table S2). Clusters 3 and 4 contained 15 proteins each specifically up- or downregulated in the meristem, respectively. Although the majority of them was uncharacterized, proteins related to ribosomal protein synthesis and lipid metabolism were detected as upregulated in the meristem in cluster 3. Photosystem proteins such as chlorophyll a-b-binding protein, and proteins related to protein degradation were present in cluster 4. Proteins with specific up and down regulation in the mature zone were grouped in clusters 5 (7 proteins, among which RuBisCo's large chain, Eukaryotic translation initiation factor and proteasome alpha subunit) and 6 (6 proteins, among which histone H2A, Peroxiredoxin-5, Magnesium-chelatase subunit chll and nuclear transport factor-2), respectively. Taken together, the differential protein levels across the growth zone reflect the developmental gradient with cell proliferation (DNA and protein synthesis, ribosomal proteins) at the base and photosynthesis in the mature part of the leaf connected by active transport.

Differential protein levels in response to drought

Next, we compared control, mild and severe drought treatments in each of the developmental zones (meristem, elongation and mature). Hierarchical clustering resulted in two major clusters: one contained all control samples and the other- the severe stress samples (Figure S4). Two of the mild stress samples clustered with the control and the remaining 4 with the severe stress samples, consistent with the intermediate character of the treatment. Within the main clusters, the samples were largely grouped by zone (Figure S4). In total, we identified 1791 proteins in the meristem, 1554 in the elongation zone, and 1695 in the mature zone of the leaf (Figure 2a). Around 31% (871) of these proteins were common for the three zones.

In response to drought only 81 proteins were significantly affected in the meristem, 213 in the elongation zone and one in the mature zone, even when a relatively loose multiple testing correction was applied (FDR < 0.1; Figure 2b; Table S2).

Enrichment analysis (Figure S5) showed that in the meristem the categories, "protein degradation", and "transport" were overrepresented among upregulated genes, whereas "N-metabolism", "redox regulation" (thioredoxin, ascorbate and glutathione), were overrepresented among the downregulated proteins. In the elongation zone, the categories "photosynthesis", "glycolysis", "tricarboxylic acid (TCA)/organic transformation", "regulation of RNA transcription", "calcium signaling", and "cell organization and vesicle transport" were overrepresented in upregulated proteins, while the categories "transport" and "protein" were underrepresented. The categories "oxidases- copper, flavone", "protein amino acid activation", and "cell vesicle transport" were overrepresented in the downregulated proteins. In the mature zone, the only protein with significantly downregulated expression in drought was a putative cystatin (Table S2).

Protein levels correlate with enzyme activity and metabolite concentration patterns

The most prominently upregulated classes of proteins in response to drought include effects on photosynthesis, which we studied earlier (Avramova et al., 2015a). In addition, the proteome shows a prominent effect on carbon and nitrogen metabolism and redox regulation (Figure S5). To confirm the biological relevance, we measured the activity and related metabolite levels for several differentially expressed enzymes involved in photosynthesis, amino acid and carbohydrate metabolism and redox regulation.

Chlorophyll and photosynthesis-related proteins were upregulated towards the mature part of the leaf and in response to drought (Figures S3 and S5). One of these proteins was adenosine triphosphate (ATP) synthase (Table S3), which converts energy from the light reactions to ATP. Consistently, we found that enzyme activity and ATP metabolite levels increased along the leaf axis and in response to stress (Figure 3a). The protein level of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), one of the main carbon-fixing enzymes, also increased towards the mature zone (Table S3). Its small subunit was slightly downregulated by drought. We found that the corresponding enzyme activity indeed increased towards the mature part of the leaf and in response to drought (Figure 3a). An opposite pattern was found for respiratory electron transport (ferredoxin) and glycolysis (triosephosphate isomerase, TPI) protein levels and enzyme activity, which were highest in the meristem and decreased in response to drought (Table S3; Figure 3a).

In the carbon metabolism, starch synthase levels in the elongation zone increased in response to drought (Table S3). Starch synthase activity, as well as starch concentrations, were the highest in the mature part of the leaf (Figure 3b) and, in parallel with the protein levels, increased in response to drought throughout the growth zone. Sucrose synthase protein levels and activity poorly correlated. Protein levels were downregulated in the elongation zone in response to drought (Table S3), while the activity of the enzyme was increased in the mature zone in response to drought (Figure 3b).

Cellulose synthase protein levels were also upregulated with progressing development, which was confirmed by the enzyme activity and the cellulose concentrations, which additionally showed a decrease in response to drought (Table S3, Figure 3b).

Upregulation of the protein levels of enzymes involved in serine-glycine-cysteine biosynthesis from the base towards the mature leaf parts and in response to drought (Table S3, Figure 3b) were reflected in the profiles of serine, cysteine and glycine concentrations as well as the activity of cysteine synthase (Figure 3b). This could also be a result of increased RuBisCo activity in the mature zone in response to drought (Figure 3a), because it catalyzes not only carbon fixation in the maize bundle sheath cells, but also the process of photorespiration, during which glycine and serine synthesis represent an intermediate step of the synthesis of 3-phosphoglycerate (Sage et al., 2012).

Proline is a well-known stress defense molecule (AbdElgawad et al., 2015) that adjusts cellular osmotic potential, protects membranes and proteins, stabilizes photosystem II and protects plants against oxidative damage (Szabados and Savouré, 2010). DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1 (P5CS1) plays a key role in proline biosynthesis and was upregulated in response to stress in the elongation zone (Table S3). This correlates with the upregulation of proline levels predominantly in the dividing and expanding tissues (Figure 3b). Other defense mechanisms, involving redox-regulation, were also activated in response to drought. The protein levels of peroxiredoxin, glutaredoxin and thioredoxin were upregulated in the mature tissues and in response to drought stress (Table S3, Figure 3c), which closely matched their enzyme activity patterns (Figure 3c). The activation of the redox system in the mature zone (Figure 3c) could also contribute to the observed induction of the photosynthetic machinery. Peroxiredoxins, thioredoxins and glutaredoxins are not only efficient antioxidants, but also cellular signals and chaperones, protecting proteins from oxidation (Dietz, 2011; Hanschmann et al., 2013) and regulators of photosynthetic enzymes (Buchanan et al., 2002). Moreover, thioredoxins are known to activate different chloroplast enzymes, such as AGPase, a rate- limiting enzyme in starch biosynthesis (Geigenberger et al., 2005). Consistently, increases in thioredoxins correlate with starch accumulation in the mature zone of the leaves in response to drought (Figure 3b and c).

An important finding from these studies was that drought stress increased photosynthetic capacity and starch biosynthesis, resulting in elevated starch levels in the mature part of the leaf (Figure 3b). Starch biosynthesis

facilitates maintenance of high photosynthetic rates by triose phosphate utilization and thereby preventing accumulation of inorganic orthophosphate (Pi), a limiting factor for ribulose-1,5-bisphosphate regeneration (Galtier et al., 1995, Paul and Foyer et al., 2001). Moreover, starch biosynthesis prevents the accumulation of soluble sugars during the photoperiod. The increase in soluble sugar levels can induce a feedback inhibition of photosynthesis by decreasing the gene expression levels of photosynthetic enzymes (e.g., PEPC, malic enzyme and RuBisCo; Jeannette et al. 2000; Sheen, 1993). Therefore, we hypothesized that increased starch synthase activity and starch levels in the mature part of drought stressed leaves are essential to facilitate the observed induction of the photosynthetic machinery and increased CO_2 assimilation and growth upon re-watering (Avramova et al., 2015a).

The *sh2* starch-deficient mutant is hypersensitive to drought

To validate this hypothesis, we studied the response of the*shrunken2* mutant (*sh2*) to drought and subsequent re-watering. The *Sh2* gene encodes the large subunit of ADP-glucose pyrophosphorylase (AGPase), the rate-limiting enzyme, providing the substrate ADP glucose for starch biosynthesis. Consistently, the endosperm of *sh2* is deficient in starch (Dickinson and Preiss, 1969; Tsai and Nelson, 1966). In addition to the seed, *Sh2* is expressed in leaves (Hannah et al., 2012), making*sh2* suitable for studying the role of starch synthesis in the response of leaf development to drought. We expected the reduced AGPase activity in *sh2* to reduce starch accumulation under normal and drought stress conditions, inhibiting photosynthesis, growth, and recovery from drought. To test this, we first measured AGPase, starch synthase activities and starch levels in the leaf growth zone (Figure 4, Figure S6). Although in well-watered conditions *sh2* had significantly lower AGPase (Figure 4a) and starch synthase (Figure 4b) activities compared to WT across the leaf growth zone, starch levels where not affected (Figure 4c). Severe drought stress significantly increased the activity of these enzymes and led to starch accumulation in the mature zone of the wild-type. In the mutant, however, the induction of starch synthase activity was significantly reduced and absent for AGPase activity, virtually abolishing the drought-induced starch accumulation, with the exception of the region at 3 - 6 cm from the base (Figure 4a, b and c). Thus, starch synthesis in the mutant is compromised throughout the growth zone, preventing the induction of starch levels in response to drought.

According to our hypothesis, the reduced ability of the mutant*sh2* to synthesize starch would lead to accumulation of soluble sugars in the mature zone of the maize leaf, causing a feedback inhibition of the photosynthetic machinery. To test this idea, we analyzed sugar levels of *sh2* and its WT in the growth zone of plants grown in well-watered and drought conditions (Figure 5; Figure S7) during the photoperiod. Drought-stress induced soluble sugar accumulation along the leaf growth zone in WT. As expected, reduced starch synthesis induced soluble sugar accumulation in *sh2* , particularly in the mature zone (Figure 5a-d). The activities of invertase, catalyzing the sucrose breakdown to glucose and fructose, and sucrose phosphate synthase, catalyzing the opposite reaction, showed contrasting patterns. Invertase activity was the highest in the elongation zone, while the activity of sucrose phosphate synthase gradually increased towards the mature zone (Figure 5e and f), suggesting that sucrose synthesis occurred predominantly in the photosynthetic tissues, while sucrose degradation was localized in the growing tissues. The activities of both enzymes were increased by about 50% throughout the growth zone in drought conditions. Sucrose phosphate synthase activity was elevated in the mature part of the *sh2*leaves, consistent with enhanced export of the sugars that, cannot be stored as starch in the mutant. Nevertheless, soluble sugars accumulated in the mature part of *sh2* leaves to higher levels compared to WT.

To test if this sugar accumulation led to feedback inhibition of photosynthesis, we investigated the effect of the compromised starch synthesis on photosynthetic capacity by measuring chlorophyll content and the activities of the main CO_2-fixing enzymes (PEPC and RuBisCo) of *sh2* and WT. PEPC activity was very similar in WT and *sh2* in well-watered conditions, but in drought the enzyme activity was less increased in the mutant (Figure 4d). RuBisCo activity was lower in *sh2* compared to WT in control conditions and significantly induced by drought only in WT (Figure 4e). Chlorophyll levels were similar in *sh2* and WT in control conditions, but were also induced more strongly in WT by drought (Figure 4e and f). These results confirm our hypothesis that the reduced ability of the mutant to accumulate starch under drought stress

impairs the induction of CO_2 fixing enzymes and chlorophyll accumulation by drought (Avramova et al., 2015a).

We then tested if this reduced induction of photosynthetic capacity under drought would affect rates of photosynthesis and compromise the recovery of the mutant plants upon re-watering. Gas-exchange measurements showed that in well-watered condition net photosynthesis (A_{net}) was significantly lower in the mutant (Figure 6a), whereas stomatal conductance (g_s) was unaffected by the mutation. As expected, under mild and severe drought, A_{net} and g_s were severely reduced only in WT (Figures 6 and S8). However, the reduction in $A_{net}$was significantly stronger in *sh2* compared to WT (Figure 6a), while no significant difference between the genotypes was observed in the response of g_s (Figures 6b and S8b). Starch concentration in the leaves of these WT plants increased in response to drought, while in the mutant, no significant changes were detected (Figure 6c). One day after re-watering, WT plants increased the rate of photosynthesis and g_s to the level of the control plants, while starch content increased to levels that were even significantly higher than in the control plants. Photosynthesis of the*sh2* mutant, however, only returned to control levels a week after re-watering, despite g_s being fully restored on the first day after re-watering. Consistently, no change in its leaf starch levels was detected. In accordance with earlier findings (Avramova et al., 2015a), a week after re-watering, photosynthesis in WT plants that had been exposed to drought conditions was significantly higher than controls that remained well-watered throughout the experiment. This compensation was not observed for *sh2* , where photosynthesis of drought exposed plants barely recovered to the level of the control plants. These results show that photosynthesis during drought and upon recovery was compromised in *sh* 2 due to its reduced ability to upregulate starch synthesis, confirming our hypothesis that starch synthesis plays an important role in the induction of the photosynthetic machinery under drought and increased CO_2 assimilation and growth upon re-watering.

Starch is essential to maintain cell division rates under drought-stress conditions

In the growing tissues, soluble sugars are required to drive cell division and cell elongation. Under drought conditions, they are also utilized to increase the antioxidant activity that protects the dividing cells (Avramova et al., 2015a) and as an osmotic agent to maintain cell turgor. Consistently, we observed an induction of soluble sugar concentrations in the meristem. In contrast to the mature part of the leaf, the response at the leaf base of *sh2* was similar to WT (Figure 5a).

Increasing soluble sugar levels in the growth zone indicate that the growth response to drought is not due to sugar limitations, but involves an additional signal. Leaf growth is typically reduced by drought in an ABA-dependent manner (Dekkers et al., 2008). Consistently, transcriptome analysis showed that ABA biosynthesis and signaling in the maize leaf growth zone were induced by drought (Avramova et al., 2015a). To investigate the effect of the mutation, we measured ABA levels throughout the growth zone of WT and mutant plants under control and drought conditions. As expected, ABA levels were strongly induced by drought and this increase was significantly higher in the meristem of*sh2* compared to the WT (Figure 7a; Figure S9a).

Drought typically induces oxidative stress in the growth zone, which partly explains the growth inhibition (Avramova et al., 2015a). Therefore, we measured redox and oxidative stress parameters in the growth zone of *sh2* and WT under well-watered and drought conditions. Confirming earlier results (Avramova et al., 2015a), drought induced malondialdehyde (MDA), a marker for membrane damage, throughout the growth zone. In *sh2* , this response was enhanced (Figure 7b; Figure S9b), suggesting that the mutant is less able to neutralize oxidative radicals. To test this, we measured the reactive oxygen species H_2O_2. As expected, H_2O_2 levels were induced by drought throughout the growth zone and more strongly in the mutant (Figure 7c; Figure S9c). These results show that *sh2* experienced elevated oxidative stress in its growing tissues.

Both ABA and H_2O_2 can inhibit cell cycle progression (Humplík et al., 2017; Reichheld et al., 1999). Therefore, we expected that cell division in the mutant and thereby its leaf growth rate would be more sensitive to drought than WT. To investigate this, we performed a kinematic analysis (Sprangers et al., 2016) to quantify the response of the two genotypes to drought. The *sh2* mutation reduced leaf elongation rate (LER) and final length of the 5^{th} leaf (FLL) even under well-watered conditions. On top of that, the

inhibition by mild and severe drought was stronger in *sh2* than in WT plants (Table 1; Table S5). The reduced growth of the mutant under well-watered conditions was due to a reduced mature cell length. Consistent with previous observations in B73 maize plants (Avramova et al., 2015a), the decrease in LER under the stress conditions was mainly due to lower cell production rates in both *sh* 2 and its WT (W22). The decrease in cell production rate for the *sh* 2 mutant was 53% for plants grown under severe drought, while this reduction was only 31% for WT plants (Table 1). In turn, the reduced cell production was due to a strong inhibition of cell division rates. Curiously, and in contrast to B73, we observed an increase in the number of dividing cells in W22 that partly compensated for the inhibited cell division rates. This increased meristematic cell number was absent in the mutant. The increased number of cells in the meristem of the wild type was due to cells being smaller in the meristem under drought stress conditions, which compensated for a concurrent reduction of the size of the meristem as a whole. In the mutant, the size of the meristem was reduced to a greater extent than that of WT, whereas the cell size reduction of meristematic cells was similar. Taken together these results confirm the hypothesis that the mutant is more sensitive to drought than the wild type due to a stronger reduction of cell division.

Starch breakdown and growth are tightly coordinated. Starch gradually accumulates in leaves during the photoperiod and it is remobilized at night to provide energy and carbon that maintain plant growth (Thalmann and Santelia, 2017). The fact that the *sh2* mutation caused a stronger growth reduction under drought conditions led us to the hypothesis that reduced starch accumulation in *sh2* during the photoperiod reduces carbon and energy availability for growth during the night.

To validate this hypothesis, we measured the change in LER, starch and soluble sugar concentrations as well as starch degradation by the enzyme amylase in the leaf growth zone of *sh2* and WT in response to drought at four different time points: midday (MD, 8h into the photoperiod), end of the day (ED, after 16h of light period), 2h into the night (2hN, after 2h of dark period) and at the end of the night (EN, after 8h of dark period; Figure 8; Figure S10). As expected, in the WT, starch concentrations increased until the end of the day and decreased during the night, being almost completely depleted at the end of the night (Figure 8a). Accordingly, amylase activity peaked early in the night, when starch degradation was induced (Figure 8b). Similarly, soluble sugar concentrations peaked at the end of the day and decreased during the night, due to their utilization as a carbon source for growth, but unlike starch they were not depleted by the end of the night (Figure 8c). In contrast, the *sh2* mutant accumulated highly reduced starch levels during the day (Figure 8a). Consequently, soluble sugars were depleted during the night (Figure 8c). In the WT, drought induced both starch and soluble sugar accumulation during the day. Starch levels dropped at similar rates as under control conditions (Figure 8a) and soluble sugar levels were very similar to those in well-watered plants during the night (Figure 8c). In the mutant, the increase of starch levels under drought conditions was strongly reduced, whereas soluble sugar accumulation was enhanced (Figure 8a and c). In the night, sugar levels were rapidly depleted in the mutant under drought conditions (Figure 8c).

Leaf growth rates broadly mirrored the starch levels, being highest in day-time and lower during the night and in the mutant. During the night the reduction in leaf growth of sh2 was much higher (35 and 61% for WT and *sh2* , respectively; Figure 8d). Although under drought stress conditions starch and soluble sugar levels were increased, growth was severely inhibited both during the day and during the night.

We reasoned that if sugar depletion caused hypersensitivity of leaf growth to drought in the mutant, supplementing the leaves with sucrose solution during the night should, at least partly, restore the mutant phenotype. Therefore, we fed sucrose through the tip of the growing leaf (Spoehr, 1942). Using this setup, we were able to reduce the decrease of the soluble sugar content in the growth zone during the dark period for both wild type and *sh2* under control and drought conditions (Figure 8e; difference between 2hN and EN). The difference was larger in the drought treated leaves (45% increase due to sucrose feeding) compared to the control conditions (16% increase) and in *sh2*(92% increase for the controls and 143% for the drought-stressed plants) compared to the wild type, so that in *sh2* under drought conditions soluble sugar levels remained essentially stable through the night (Figure 8e). In contrast to the wild type, increasing sugar content in the leaf growth zone resulted in a significant increase in leaf growth of *sh2* under control conditions. During

the night this fully restored leaf growth rates in *sh2* to wild type levels (Figure 8f). Moreover, sugar feeding reduced the effect of drought in the wild type and almost doubled the leaf elongation rate of *sh2*under drought conditions (Figure 8f). Therefore, our results show that under drought conditions and in *sh2* growth is limited by sugar levels in the growth zone. The mutant phenotype shows that starch accumulation during the day is crucial to sustain sugar supply to the growth zone and leaf growth both during the photoperiod and the night. Drought conditions induce starch and soluble sugar accumulation during the day and inhibit growth at the same time.

Discussion

Drought impacts energy generating processes in the maize leaf growth zone.

Leaf growth is one of the most drought sensitive developmental processes and a broad range of studies across a wide variety of species have addressed the effects of drought on growth rates, cell division and expansion, physiological and molecular levels (Avramova et al., 2015a, 2016; Walter et al., 2009). Because the results were obtained in different experimental systems, we still lack an integrated, systems level understanding of these responses. This would require integration of observations at multiple organizational levels in the same experimental system (Ghatak et al., 2017). The linear organization of the developmental processes, the size of the growth zone, availability of a fully sequenced genome and a wide collection of genetic tools make the maize leaf an ideal model system for such integrative studies (Avramova et al., 2015b). Therefore, to extend earlier cellular, transcriptome, biochemical and metabolite studies (Avramova et al, 2015a, 2017), we performed a proteome analysis of the effect of standardized drought treatments in the maize leaf growth zone.

In accordance with transcriptome data (Li et al., 2010; Avramova et al., 2015a), "protein synthesis", "RNA processing" and "transport", were enriched in the meristem. In contrast, the proteome of the mature part was dominated by energy generating processes, including photosynthesis and carbohydrate metabolism such as glycolysis and TCA cycle. Consistent with the protein levels, the enzyme activities and metabolite analyses showed the induction of processes related to the light reaction and the carbon fixation, as well as starch and sugar biosynthesis and redox regulation in the mature part of the growth zone. We confirmed the upregulation of the photosynthetic machinery by increased chlorophyll content (Avramova et al., 2015a), increased activities of RuBisCo and ATP synthase and ATP metabolite concentrations in the mature cells (current study). The consistency of the photosynthetic response between protein (Table S3), mRNA levels (Avramova et al., 2015a), enzyme activities and metabolites (Figure 3a), supports transcriptional regulation of this process (Ponnala et al., 2014).

A mechanistic model for the role of starch biosynthesis in the regulation of leaf growth under drought stress

Our proteome analysis suggested a role for starch synthesis in the response to drought. We tested this hypothesis by studying *sh2* , a knockout allele of AGPase, a rate-limiting enzyme for starch biosynthesis. It was previously shown that *Sh2* is expressed in leaves (Hannah et al., 2012), but its mutation has only a minor effect on starch content of leaves (Boehlein et al., 2018). However, these observations were made in mature leaves and in well-watered conditions. We showed that *Sh2* expression is significantly higher in expanding tissues than in the mature part of the leaf. In addition, drought stress induces *Sh2* expression predominantly in the meristem (Figure S11), suggesting that the gene plays a role in the drought response of leaf growth. Consistent with the hypothesis, the mutation strongly reduced starch accumulation in the meristem, but to a higher extent in the mature zone. Moreover, the mutation increased sensitivity of leaf growth to drought and suppressed the growth compensation normally observed during recovery.

We integrated our results in a mechanistic model describing the effect of drought in mature and meristematic cells (Figure 9, black arrows). In the mature cells, drought stress inhibits photosynthesis, however

chlorophyll content and photosynthetic potential increase, which facilitates a superior CO_2 acquisition upon re-watering (Avramova et. al., 2015a). While net CO_2 assimilation (A_{net}) is reduced due to stomatal closure, presumably driven by increased ABA levels, an induction of the two photosystems, chlorophyll levels, and CO_2 fixation capacity by upregulation of RuBisCo and PEPC is observed in the mature cells under drought stress conditions. Increased protein levels of ATP synthase facilitate increased conversion of light energy into ATP. Additionally, increased thioredoxin activity stimulates the Calvin cycle (Buchanan et al., 2002), which leads to higher biosynthesis of glucose and fructose. At the transcript level, we observed an upregulation of sucrose synthesis (Avramova et al., 2015a), which is consistent with the increased activities of the enzymes sucrose phosphate synthase (SPS), AGPase (additionally activated by the induced thioredoxin activity; Geigenberger et al., 2005) and starch synthase (SSY), which convert the hexoses to sucrose and starch, respectively. When CO_2uptake decreases due to stomatal closure, starch in the mature part of the leaf is degraded to sucrose and transported to the meristem. Consistently, sucrose transporter genes were upregulated under drought (Figure S12), which contributes to the accumulation of sugars in the growing tissues. In the meristematic cells, sucrose is converted into hexoses by cytoplasmic invertase (INV), whose activity was induced by drought (Figure 9). Hexoses function as osmoprotectants and signals to induce plant stress responses and reduce ROS damage (Thalmann and Santelia, 2017). They contribute to maintenance of turgor pressure, required for cell expansion, and to synthesize cell-wall components, including cellulose (Thalmann and Santelia, 2017). However, the elevated levels of hexoses in the growth zone were possibly localized in the vacuole for osmoregulation and therefore unavailable for growth (Clifford et al., 1998). This could explain the downregulation of cellulose synthase activity and lower cellulose levels, particularly in the meristem in response to drought. Increased ABA levels most likely also mediate the growth inhibition under stress conditions (Kempa et al., 2008). ABA induces the cyclin-dependent kinase (CDK) inhibitors kip-related proteins (KRPs) and inhibits the expression of the mitotic B-type cyclins (CYCB; Humplík et al., 2017). Consistently, our transcriptome analysis showed induced expression of *krp2* and downregulation of *cdk* and *cycb* in the meristem, explaining the cell cycle inhibition under drought conditions (Avramova et al., 2015a). ABA induces ROS production by plasma membrane-associated NADPH oxdixases (Kwak et al., 2003) and mitochondria (He et al., 2012). Drought induced ROS accumulation could be an additional factor contributing to cell cycle inhibition (Reichheld et al., 1999, Waszczak et al., 2018).

By inhibiting AGPase activity, *sh2* limits starch biosynthesis from hexoses resulting from photosynthesis and largely prevents starch accumulation in the mature part of the leaves normally occurring under drought conditions (Figure 9, red arrows), which leads to increased accumulation of soluble sugars. The ratio soluble sugars/starch increases significantly in the mature parts of the leaf in response to drought (Figure S13), inducing a feedback inhibition of photosynthetic genes (Figure 9). Such a feedback was shown for glucose and sucrose, which negatively regulates photosynthetic gene expression (Carvalho et al., 2010; Paul and Foyer, 2001; Sheen, 1993). Soluble sugars, including glucose and sucrose exert specific repression of the promoter activity of several maize photosynthetic genes e.g., RuBisCo, chlorophyll-binding protein and PEP carboxylase (Sheen, 1990). The reduced ability of*sh2* to accumulate starch results in accumulation of triose-6-phosphate (Figure 9), which inhibits RuBisCo and ATP synthase activities (Paul and Foyer, 2001; Yang et al., 2016) and therefore reduces photosynthetic capacity. Due to lower CO_2fixation capacity by PEPC and RuBisCo, A_{net} decreases in the mutant in absence of a difference in ABA levels and stomatal conductance. This prevents the induction of the photosynthetic machinery (also chlorophyll contents in the two photosystems) during leaf development in stress conditions and the recovery of CO_2assimilation upon re-watering, despite the stomatal re-opening (Figure 5).

Similarly, in *Arabidopsis thaliana* a positive correlation between the rate of photosynthesis and starch biosynthesis was observed in the*tl25* mutant, with a mutation in the gene encoding the small subunit of AGPase, (Sun et al., 1999). Unlike the wild type, the*tl25* mutant was unable to increase photosynthesis in response to in high light, high CO_2 and low O_2, demonstrating the requirement for starch synthesis in adjusting photosynthetic capacity to environmental conditions. Moreover, overexpression of an altered maize AGPase large subunit (Sh2r6hs) in wheat (*Triticum aestivum L.*) increased CO_2assimilation rate in source tissue and consequently carbon metabolites in sink tissue and seed, which led to an increased grain yield (Smidansky

et al., 2007).

In the meristematic cells, the *sh2* mutation increased sensitivity to drought by increasing ABA levels and ROS accumulation, but no increase in hexose-induced osmoprotection (Figure 9). Cheng et al., (2002) and Dekkers et al., (2008) indicated that increased ABA biosynthesis reduces seedling growth. In addition, reduced starch biosynthesis possibly directs the soluble sugars produced by photosynthesis to mitochondria, which increases mitochondrial metabolism including electron transport chain (ETC) activity with potentially harmful consequences such as ROS production (Moller, 2001; Pastore et al., 2007). These observations might explain the increased H_2O_2 levels in the *sh2* mutant. Therefore, despite the overall higher accumulation of sugars in the*sh2* mutant in the mature zone, the mutation leads to stronger inhibition of cell division in the meristem and consequently higher growth impairment in response to drought.

Reduced starch availability and degradation reduces growth during the night.

Even in control conditions, the growth of *sh2* was impaired (Table 1, Figure 8d). Starch accumulates gradually during the photoperiod in the mature zone of the leaf and is remobilized at night by amylase activity providing the energy supply for growth processes in the growth zome (Smith and Stitt, 2007; Stitt and Zeeman, 2012; Thalmann et al., 2016). Consequently, it is not the starch content itself, but the ability to sustain a steady supply of soluble sugar during the night which is crucial for plant growth (Rasse and Tocquin 2006). Starch degradation during the day is induced under stress conditions (Yano et al., 2005, Thalmann et al., 2016). The *sh* 2 mutant had reduced starch levels in the mature leaf tissues (Figures 4c, and 8a) and accumulated higher soluble sugar levels during the day (Figure 5a and 8a), but these concentrations decreased rapidly to very low levels early in the night (Figure 8c). Caspar et al. (1985) linked the rapid declining sugar levels in the early night observed in another starchless mutant to nonproductive respiration and suggested that respiration during the night is proportional to the availability of soluble sugars rather than the energy demand for growth. We showed that the growth impairment during the night is due to the low soluble sugar concentrations in *sh2* and could be fully restored to WT rates by sucrose feeding (Figure 8e and f).

Overall, we showed a strong effect of altered energy and antioxidant metabolism in the maize leaf proteome in response to drought. Subsequent functional analysis of *sh2* allowed us to unravel the central role of starch synthesis in growth regulation and stress defense.

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Conflict of interest

We declare that we have no conflict of interest.

Author contributions

H.A. and V.A. conducted and analysed the experiments and compiled the data; V.A. and H.A. wrote the manuscript; G.T.S.B. conceived the project, developed the ideas and edited the manuscript; G.V., G.B., X.v.O., W.V.d.E, Y.G and H.As. designed and supervised experiments; D.V. contributed to data analysis; all authors read and approved the manuscript.

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Tables

Table 1. Kinematic analysis of the effect of drought on the fifth leaf of maize plants. Cellular growth parameters of wild type (WT) and *sh2* mutant plants grown in well-watered control conditions (C) and severe drought stress conditions (D) were compared. Percentage change in drought stress compared to corresponding controls, as well as between the mutant and the wild type in control conditions are indicated. More detailed analyses including mild drought stress and statistical analysis is provided in Table S5. Parameters are as follows: leaf elongation rate (LER), mature cell length (l_{mat}), cell production rate (P), cell division rate (D), cell cycle duration (Tc), length of the meristem (Lmer), Length of the growth zone (Lgz), length of cells leaving meristem (l_{div}), number of cells in the meristem (Nmer), time in the division zone (Tdiv), cell elongation rate (Rel), and time in the elongation zone (Tel), number of cells in growth zone (Ngz), number of cells in elongation zone (Nel).

	WT C	**WT D**	*sh2* **C**	*sh2* **D**
LER (mm h^{-1})	3.3±0.03	2±0.04 **(-38%)**	2.5±0.04 **(-25%)**	1.1±0.04 **(-57%)**
Lmer (mm)	21.2±0.3	15.7±0.2 **(-25%)**	15.5±0.2 **(-27%)**	9±0.4 **(-42%)**
Lgz (mm)	74±0.03	54±0.11 **(-26%)**	74±0.14 **(0%)**	60±0.2 **(-19%)**
$\lambda_{\mu\alpha\tau}$ ($\mu\mu$)	174.9±1.8	159.4±5.6 **(-9%)**	125.1±2.8 **(-28%)**	116.9±2 **(-6%)**
$\lambda_{\delta\iota}$ ($\mu\mu$)	71.6±0.79	39.5±0.9 **(-45%)**	55.6±1.72 **(-22%)**	32±0.4 **(-42%)**
$P\varepsilon\lambda$ ($\mu\mu$ $\mu\mu^{-1}$ η^{-1})	0.04±0.04	0.052±0.1 **(31%)**	0.041±0.06 **(2.5%)**	0.053±0.1 **(31%)**
Tel (h)	22.7±0.4	46.3±1.5 **(104%)**	26±1.5 **(15%)**	60.7±4.9 **(134%)**
P (cells h^{-1})	18.9±0.3	13±0.4 **(-31%)**	20.2±0.58 **(7%)**	9.4±0.4 **(-53%)**
D (cell $cell^{-1}$ h^{-1})	0.031±0.01	0.018±0.02 **(-41%)**	0.025±0 **(-19%)**	0.013±0.0 **(-50%)**
Nmer	603.5±10.8	721.3±37 **(19%)**	848.4±35 **(41%)**	810.4±69 **(-4%)**
Tdiv (h)	205.8±5.8	363.8±14 **(76%)**	293.8±18 **(43%)**	573.9±43 **(95%)**
Tc (h)	22.3±0.6	38.3±1.2 **(72%)**	30±1.76 **(35%)**	59.2±3.8 **(97%)**
Ngz	1015±10.9	1321.8±59 **(30%)**	1372.6±44 **(35%)**	1344.6±75 **(-2%)**
Nel	411.5±4.1	600.5±25 **(45.9%)**	524.1±26.9 **(27%)**	534.3±25.7 **(2%)**

Figure Legends

Figure 1. Clustering of protein levels along the maize leaf growth zone.

A QT-Clust analysis (Heyer *et al* ., 1999) was used to cluster a total number of 230 proteins with significantly differential levels (FDR < 0.05, one-way ANOVA), between the three developmental zones (meristem, elongation zone, and mature zone). Euclidian distance, cluster diameter = 0.4, and minimum cluster population = 4 were used in MeV (Saeed *et al* ., 2003) to generate the clusters.

Figure 2. Global overview of protein expression across developmental zones of the maize leaf and the effect of drought stress.

The total number of identified proteins (a), and the differentially expressed proteins in each developmental zone (meristem, elongation, mature zone) in response to drought stress (b). The data in (b) results from three one-way ANOVA analyses (FDR corrected P -values < 0.1), each performed in one of the three developmental zones, comparing the strongest contrast between stress (mild or severe) and control conditions drought stress.

Figure 3. The effect of drought stress on metabolite and biochemical activities in the growth zone of the maize leaf.

Enzyme activities and metabolite concentration patterns related to photosynthesis (a), amino acid and sugar metabolism (b) and redox regulation (c) were compared in response to mild and severe drought stress in each developmental zone of the growing maize leaf (meristem, elongation zone, and mature zone). Metabolite and biochemistry measurements were performed in each centimeter (from 1 to 10) of the growth zone of the 5th leaf of plants, grown in well-watered conditions (C1–C10) and plants, subjected to mild (M1–M10) and severe (S1–S10) drought. Data are means of at least 3 biological replications (each consisting of three pooled plants). Data are hierarchically clustered and mean-centered, which effectively removed the differences in concentration and activity values between the different metabolites and enzymes to highlight the magnitude of differences across the growth zones and in response to the treatments. The lengths of the different zones along the leaf axis are based on Avramova et al (2015a) and indicated in the bottom row. Syn, synthase; TPI, Triosephosphate isomerase

Figure 4. The effect of drought on starch metabolism and photosynthetic enzyme activities and pigment levels in the *shrunken2* (*sh2*) maize mutant and its wild type (W22).

Well-watered control plants (C) were compared to severely drought (D) stressed plants (more detailed analysis including mild drought stress is presented in Figure S6). Measurements were done in each 1 cm of the leaf growth zone. (a) Activity of the enzyme ADP-glucose pyrophosphorylase (AGPase); (b) Starch synthase activity; (c) Starch concentration; (d) Phosphoenolpyruvate carboxylase (PEPC) activity; (e) Ribulose-1,5-bisphospate carboxylase/oxygenase (RuBisCo) activity; (f) Chlorophyll a concentration; (g) Chlorophyll b concentration. Data are means ± SE (n=3). FW, Fresh weight; 3-PGA, 3-phosphoglycerate. A three-factor ANOVA was used as a statistical test, and P -values are present in Table S4. The lengths of the different zones along the leaf axis are based on the kinematic analysis in Table 1 and indicated in the bottom row.

Figure 5. The effect of drough on the sugar metabolism of the*shrunken2* (*sh2*) maize mutant and its wild type (W22).Well-watered control plants (C) were compared to severely drought (D) stressed plants. More detailed analysis including mild drought stress is presented in Figure S7. Measurements were done in each 1 cm of the leaf growth zone. (a) Soluble sugar concentration; (b) Sucrose concentration; (c) Glucose concentration; (d) Fructose concentration; (e) Invertase activity; (f) Sucrose phosphate synthase activity; Data are means ± SE (n=3). FW, Fresh weight. A three-factor ANOVA was used as a statistical test, and P -values are present in Table S4. The lengths of the different zones along the leaf axis are based on the kinematic analysis in Table 1 and indicated in the bottom row.

Figure 6. The effect of drought on photosynthesis ($\mathbf{A_{net}}$) and stomatal conductance ($\mathbf{g_s}$) in the *shrunken2* (*sh2*) maize mutant and its wild type (W22).The rates of net photosynthesis (a) and stomatal conductance (b) of young seedlings, grown in severe drought (D) conditions before, one day after and one week after re-watering (RW). Unstressed plants of the same age as the plants that were subjected to stress and allowed to recover were included as a control (C) for ontogenetic differences. More detailed analysis including mild drought stress is presented in Figure S8. A three-factor ANOVA and Tukey test were used for statistical analysis, and significant differences ($P < 0.05$) are marked with different letters. Data are averages ± SE ($n = 5$).

Figure 7. The effect of drought on stress parameters in*shrunken2* (*sh2*) maize mutant and its wild type (W22).Well-watered control plants (C) were compared to severely drought (D) stressed plants. More detailed analysis including mild drought stress is presented in Figure S9. Measurements were done in each 1 cm of the leaf growth zone. (a) Abscisic acid (ABA) concentration; (b) Malondialdehyde (MDA) concentration; (c) Hydrogen peroxide (H_2O_2) concentration. Data are means ± SE (n=3). FW, Fresh weight. A three-factor ANOVA was used as a statistical test, and P -values are present in Table S4. The lengths of the different zones along the leaf axis are based on the kinematic analysis in Table 1 and indicated in the bottom row.

Figure 8. The effect of drought on starch metabolism in the*shrunken2* (*sh2*) maize mutant and

its wild type (W22). Well-watered control (C) and severely drought (D) stressed plants were compared in terms of (a) Starch concentration, (b) Amylase activity and (c) Soluble sugar concentration at four different timepoints: Midday (MD), End of the Day (ED), 2 hours into the night (2hN) and End of the Night (EN); Data are means ± SE (n=3). A three-factor ANOVA was used as a statistical test, and P -values are present in Table S4. (d) Leaf elongation rate (LER) was measured during the day and during the night. Data are means ± SE (n=6). Part of the plants in the experiment were fed with 0.3 M sucrose during the night and soluble sugar concentration (e) and LER (f) were measured at two time points: 2hN (two hours after feeding) and EN. Data are means ± SE (n=5). A three-factor ANOVA and Tukey's test were used as a statistical analyses and significant differences ($P < 0.05$) are marked with different letters above the bars. More detailed analysis including mild drought stress is presented in Figure S10. FW, Fresh weight; HS, hydrolyzed starch.

Figure 9. Model of the metabolic regulation of the drought response and the effect of the *sh2* mutation, encoding the starch biosynthesis limiting enzyme ADP-glucose pyrophosphorylase (AGPase), on the drought response in meristematic and mature cells of maize leaves.Up or down regulated gene expression, protein levels, enzyme activities, or metabolite concentrations are indicated by a black (drought stress effect) or a red (effect of *sh2* mutation on drought response) arrow. Blue arrows indicate activation or inhibition and purple arrows indicate transport. Interrupted arrows indicate indirect regulation, while uninterrupted arrows indicate direct regulation. Interrupted red lines indicate hypothesis, based on literature. Red question mark indicates that the assumption is made based on the literature due to lack of own data. FBI, feedback inhibition; A_{net}, net photosynthesis (net CO_2 assimilation); PEPC, phosphoenolpyruvate carboxylase; ABA, abscisic acid; Fd, ferredoxin; FDR, ferredoxin reductase; Thx, thioredoxin; PS, photosystem; ATP SYN, ATP synthase; Tri-6-P, triose-6-phosphate; SPS, sucrose phosphate synthase; SSY, starch synthase; SUC, sucrose; INVs, invertases; ROS, reactive oxygen species; CDK, cyclin-dependent kinase.