Starch biosynthesis contributes to the maintenance of photosynthesis and leaf growth under drought stress in maize

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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 *shrunk-2* mutant (*sh2*), defective in ADP-glucose pyrophosphorylase.

Drought altered the abundance of 284 proteins overrepresented for photosynthesis, amino acid, 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 showed a reduced increase of starch levels under drought conditions, leading to soluble sugar starvation at the end of the night and correlating with an inhibition of leaf growth rates. 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 contributes to maintaining leaf growth under drought stress and facilitates enhanced carbon acquisition upon recovery.

Key words: drought, leaf growth, maize, photosynthesis, proteomics, *sh2* mutant, starch

Introduction

One of the first responses of plants to drought is reduced leaf growth, limiting transpirational losses (Ahmad et al., 2016; Avramova et al., 2016). In crops, even mild drought, in absence of visual wilting, significantly reduces yield (Ribaut et al., 1997). Leaf growth responses are mediated by cell division and expansion (Rymen, 2010), regulated by integrated molecular signaling involving 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, photosynthesis and 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). Starch reserves are remobilized to release soluble sugars, which function as osmolites, support growth and serve as signal molecules (Thalmann and Santelia, 2017). In mutants unable to synthesize or degrade starch, growth is impaired (Stitt and Zeeman, 2012).

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) facilitates analyses of 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 environmental stress

85 responses (Avramova et al., 2015a, 2017; Kravchik and Bernstein, 2013; Nelissen et al., 2018;
86 Rymen et al., 2007; Walter et al., 2009).

87
88 The effect of drought on the maize leaf proteome has been studied by gel-based approaches
89 (Benešová et al., 2012; Riccardi et al., 1998; Xin et al., 2018). LC-MS/MS based techniques,
90 which have a higher sensitivity, are increasingly used to study the (phospho)proteome,
91 including responses to drought stress in mature leaves (Zhao et al., 2016) and the growth zone
92 of growing leaves (Vu et al., 2016). Currently, the only large-scale studies comparing the effect
93 of drought in dividing, expanding and mature tissues of maize leaves have been performed at
94 the transcriptome (Avramova et al., 2015a), the phosphoproteome (Bonhomme et al., 2012) and
95 the metabolome (Avramova et al., 2017; Nelissen et al., 2018) levels. In addition to
96 transcriptional regulation, molecular adaptation could be regulated at the post-transcriptional,
97 posttranslational and metabolite levels (Ghatak et al., 2017; Nägele and Weckwerth, 2014).
98 Therefore, integrated studies combining data from different regulatory levels are needed to
99 obtain a mechanistic understanding of the growth response to drought.

100
101 In this study, a mass spectrometry based proteome analysis, complemented with metabolite and
102 biochemical measurements, showed that drought strongly affected starch synthesis in the leaf
103 growth zone. Therefore, we studied the effect of drought on the *shrunk-2* mutant (*sh2*),
104 defective in starch biosynthesis, and found a relationship between starch synthesis and leaf
105 growth during and upon recovery from drought.

Materials and Methods

Plant material

The inbred line B73 (Iowa Stiff Stalk Synthetic), the *shrunk-2* mutant and its wild type (W22), were obtained from MaizeGDB (http://www.maizegdb.org/stock_catalog).

Growth experiments

Maize seedlings were grown in a growth chamber (16h day/8h night, 25°C/18°C day/night [d/n], 300-400 $\mu\text{mol.m}^{-2} \text{s}^{-1}$ Photosynthetically Active Radiation, provided by high pressure sodium lamps). Controls 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, wilting), respectively, where they were maintained (Figure S1). Three days after emergence of the fifth leaf, plants were harvested at the middle of photoperiod (14:00 h, MD), at the end of the day (22:00 h, ED), two hours after beginning of the night (24:00 h, 2hN) and at the end of the night (6:00 h, EN). The first 10 cm from the leaf base of each plant were cut in ten segments of 1 cm, immediately frozen in liquid nitrogen and kept at -80°C for analyses.

Proteome analysis

Protein extraction, digestion, and labeling

Total protein extracts were prepared following 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) according to the manufacturer's protocol (Applied biosystems, Foster City, CA,

USA). Four pools containing equal amounts of labelled peptides from 8 samples were prepared (labeling design in Table S1).

Nano reverse phase liquid chromatography and mass spectrometry

In the first dimension of a 2D-LC fractionation, 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 with mobile phase A (2% acetonitrile and 0.25% formic acid at pH 9 with H₅NO) and mobile phase B (98% acetonitrile, 0.25% formic acid at pH 9 with H₅NO). A linear gradient from 2% B to 60% B in 9.5 min followed by 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 nanoAcquity-UPLC system using an ACQUITY UPLC Peptide BEH C18 nano-ACQUITY 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 an 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). For tandem MS, the five most intense peaks

of a full scan mass spectrum were selected. Collision Induced Dissociation (CID) scans were selected for High energy Collision activated Dissociation (HCD) scans 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.

Proteome data analysis

Proteome discoverer software (version 1.3, Thermo Scientific, San Jose, CA) was used for search against the Uniprot *Zea mays* database using Sequest and Mascot algorithms with precursor mass tolerance set to 10 ppm, fragment mass tolerance to 0.5 Da; trypsin digestion with 2 missed cleavages allowed; methylthio (C) and ITRAQ modifications (N-terminus and lysine residues) defined as fixed modifications and methionine oxidation and phosphorylation (STY) as variable modifications. The results were filtered for confident peptide-to-spectrum matches (PSMs) based on a non-concatenated target-decoy approach (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. The most confident centroid method was used with an integration window of 20 ppm. Reporter ion intensities were corrected for isotope contamination using 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. Data were normalized within each sample pool and expression values of all the peptides matching to the same protein were averaged. All peptides listed with multiple accessions were removed (Bradshaw et al., 2006; Neilson et al., 2011). Hierarchical clustering was performed using MATLAB (version 9.0, MathWorks Inc., Natick, MA, USA). Proteins not identified in all biological replicate samples were excluded from the analysis. Data were log₂-transformed and a one-way ANOVA was performed using the software MeV (Multi Experiment Viewer, Saeed et al., 2003) to determine p-values and False Discovery Rates (FDR). A Quality Threshold

clustering (QT, Heyer et al., 1999) was performed in MeV after normalization using Euclidian distance, cluster diameter 0.4 and minimum cluster population 4. Enrichment studies of the differential protein 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 level 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 first, fifth and tenth centimeter from the leaf base, respectively. First strand cDNA synthesis was performed with superscript II Reverse Transcriptase (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

Activities of Peroxiredoxin (PRX), Glutaredoxin (GRX), Thioredoxin (TRX) and Ferredoxin were determined according to Horling et al., (2003), Lundberg et al., (2001), Wolosiuk et al. (1979) and Smillie, (1962), respectively.

Amino acids and sugars

Cellulose synthase was determined according to Blanton et al., (2000). Amino acids, sugars and activities of 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 Markwell et al. (1986). The contents of chlorophylls *a* and *b* were calculated using the formulas described by Porra et al., (1989) and expressed as $\mu\text{g pigment g}^{-1}$ fresh weight (FW).

Biochemistry

RuBisCo activity was measured with a 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 activities of triosephosphate isomerase and AGPase were measured according to Benítez-Cardoza et al. (2001) and according to Nakamura et al. (1989) in a reaction mixture (5 mM ADPG, 50 mM MgCl_2 , 100 mM Hepes–NaOH), respectively, by monitoring NADH consumption at 340 nm. ATP concentration was measured by using ATP Assay System Bioluminescence Detection Kit (FF2000, 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. 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 using Xylenol Orange (Avramova et al, 2015a).

229

230 **Photosynthesis and stomatal conductance measurements**

231 Net CO₂ assimilation rate (photosynthesis) and stomatal conductance were measured on the
232 exposed/mature part of the fifth leaf using a portable photosynthesis system (LI-COR LI-6400,
233 LI-COR Inc., Lincoln, NE, USA).

234

235 **Sucrose feeding**

236 At the end of photoperiod (at 22:00h) on the day of emergence, the fifth leaf of well-watered
237 plants and plants subjected to mild and severe drought stress was fed through the cut leaf tip
238 with a 0.3 M sucrose solution or demineralized water following Spoehr (1942). Two hours after
239 the start of the treatment and at the end of the night (6:00 h, EN), the first 10 cm from the fifth
240 leaf base were collected for soluble sugar determination.

241

242 **Kinematic Analysis**

243 The kinematic analysis was performed according to Sprangers et al. (2016). The length of the
244 fifth leaf was measured daily with a ruler. Meristem size was determined by locating the most
245 distal mitosis in epidermal cell files on samples harvested 3 days after leaf emergence, using
246 fluorescence microscopy (AxioScope A1, Axiocam ICm1, Zeiss, Germany) at 20×
247 magnification. Cell length was measured using differential interference contrast microscopy at
248 40× magnification and the online measurement module in the Axiovision software (Zeiss,
249 Germany). For this, the leaf growth zone was cut in ten 1 cm segments and cell length of around
250 20 epidermal cells adjacent to the stomatal files were measured at the tip (0 cm), at one-third
251 of the segment (0.3 cm), at two-thirds (0.6 cm) and at the end of the segment (0.99 cm). Raw
252 data 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 R (R Core Team, 2011), before averaging between leaves and comparison between treatments.

Results

Proteome analysis of the maize leaf growth zone

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 5th leaf at three days after emergence. We prepared 4 ITRAQ labeled (Wiese et al., 2007) pools of samples, 3 of these 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 (Table S1). In total, we identified 7636 peptides matching 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 using Mercator (Lohse et al., 2014; Figure S2). In total, 230 proteins were differentially expressed between the zones (FDR < 5%; Table S2) clustering into 7 patterns (Figure 1, Table S2). Two clusters containing the majority of proteins (118 in cluster 1 and 66 in cluster 2), showed opposite patterns. To identify the biological processes represented by these two clusters, we performed an enrichment analysis using Pageman (Usadel et al., 2006; Figure S3). Cluster 1 with gradually increasing levels from the meristem to the mature zone was significantly enriched for the categories “cell”, and “transport”, while “sucrose degradation”, “protein synthesis”, “redox”, and “RNA processing” were underrepresented.

Cluster 2 contained proteins with levels decreasing 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.

Clusters 3 and 4 contained 15 proteins each specifically up- or downregulated in the meristem, respectively. Although the majority of them are uncharacterized, proteins related to ribosomal protein synthesis and lipid metabolism were 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 cluster 5 (7 proteins, among which were RuBisCo large chain, Eukaryotic translation initiation factor and proteasome alpha subunit) and cluster 6 (6 proteins, among which histone H2A, Peroxiredoxin-5, Magnesium-chelatase subunit cell and nuclear transport factor-2), respectively (Table S2). 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 (using a relatively loose FDR of < 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 proteins, 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 by 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 functional 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. Consistent with this, 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 activities, 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 also decreased 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) 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 Saviouré, 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), 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). Consistent with this, 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 this study was that drought stress increased photosynthetic capacity and starch biosynthesis correlating with elevated starch levels in the mature part of the leaf (Figure 3a and b). 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. Elevated 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 contribute to the previously observed induction of the photosynthetic machinery and increased CO₂ assimilation and growth upon re-watering (Avramova et al., 2015a).

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

To evaluate this hypothesis, we studied the response of the *shrunk-2* mutant (*sh2*) to drought and subsequent re-watering. The *Sh2* gene encodes the large subunit of ADP-glucose pyrophosphorylase (AGPase), the rate-limiting enzyme of starch synthesis, which provides the substrate ADP glucose. Consistent with this, 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 and 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 were not affected (Figure 4c). Severe drought stress significantly increased the activity of these

enzymes and led to starch accumulation in the mature zone of WT. In *sh2* however, the induction of starch synthase activity was significantly reduced and induction of AGPase activity was absent. This virtually abolished 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 *sh2* is compromised throughout the growth zone, preventing drought-induced starch accumulation.

According to our hypothesis, the reduced ability of the *sh2* mutant 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. Consistent with this hypothesis, we found reduced starch synthesis and increased soluble sugar accumulation in *sh2* compared WT during the photoperiod, particularly in the mature zone (Figure 5 and S7 a-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 highest in the elongation zone, while the activity of sucrose phosphate synthase gradually increased towards the mature zone (Figure 5 and S7 e and f), suggesting that sucrose synthesis occurred predominantly in photosynthetic tissues, while sucrose degradation was localized in growing tissues. The activities of both enzymes were increased by about 50% throughout the growth zone by drought. Sucrose phosphate synthase activity was elevated in the mature part of the *sh2* leaves, consistent with enhanced export of 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 CO₂-fixing enzymes (PEPC and RuBisCo).

In well-watered conditions, PEPC activity was very similar in WT and *sh2*, but in drought the enzyme activity was increased less 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 are consistent with our hypothesis that the reduced ability of the mutant to accumulate starch under drought stress correlates with impaired induction of CO₂ fixing enzymes and chlorophyll accumulation by drought (Avramova et al., 2015a).

We then tested if this reduced induction of photosynthetic capacity by drought could affect rates of photosynthesis and compromise the recovery upon re-watering. Gas-exchange measurements showed that in well-watered conditions net photosynthesis (A_{net}) was significantly lower in *sh2* than in WT (Figure 6a), whereas stomatal conductance (g_s) was unaffected. As expected, under mild and severe drought, A_{net} and g_s were severely reduced in WT (Figures 6 and S8). However, the reduction in A_{net} was significantly stronger in *sh2* (Figure 6a), while no significant difference between the genotypes was observed in the response of g_s (Figure 6b). In WT leaves, starch concentration increased in response to drought, while in *sh2* this increase was strongly reduced (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 became even higher than under drought. 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. Consistent with this, only a minor non-significant increase in 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 continuously well-watered controls. This compensation was not observed for *sh2*,

where photosynthesis of drought exposed plants barely recovered to the level of the control plants. The response of *sh2* is consistent with our hypothesis that starch synthesis facilitates the induction of the photosynthetic machinery under drought supporting increased CO₂ assimilation and growth upon re-watering.

Starch contributes to maintaining cell division rates under drought-stress conditions

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. 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). Consistent with this, transcriptome analysis showed that ABA biosynthesis and signaling in the maize leaf growth zone were induced by drought (Avramova et al., 2015a). Consistent with this, drought increased ABA levels throughout the growth zone and this increase was stronger in *sh2* compared to the WT (Figures 7a and S9a).

Drought typically induces oxidative stress in the growth zone, which partly explains the growth inhibition (Avramova et al., 2015a). Consistent with this, drought induced malondialdehyde (MDA), a marker for membrane damage, throughout the growth zone. In *sh2*, this response was enhanced (Figures 7b and S9b), suggesting that the mutant is less able to neutralize oxidative radicals. Indeed, the elevation of H₂O₂ levels induced by drought throughout the growth zone was increased in *sh2* (Figures 7c and S9c). These results show that *sh2* experienced elevated oxidative stress in its growing tissues.

Both ABA and H₂O₂ 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). The *sh2* mutation reduced leaf elongation rate (LER) and final length of the 5th 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 (Tables 1 and 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 *sh2* and WT. The decrease in cell production rate for *sh2* was 53% for plants grown under severe drought, while this reduction was only 31% for WT plants (Table 1). The reduced cell production was due to an 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 *sh2*. The increased number of cells in the meristem of the wild type was due to cells being smaller in the meristem under drought conditions, which compensated for a concurrent reduction of the size of the meristem as a whole. In *sh2* the size of the meristem was reduced to a greater extent, whereas the cell size reduction of meristematic cells was similar to the WT. Taken together these results confirm the hypothesis that *sh2* 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 evaluate this hypothesis, we measured LER, starch and soluble sugar concentrations and amylase activity, responsible for starch degradation, in the leaf growth zone of *sh2* and WT in response to drought throughout the diurnal cycle (Figures 8 and S10). As expected, 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). The *sh2* mutant accumulated significantly less starch by the end of the day compared to WT (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 *sh2* the increase of starch levels under drought conditions was strongly reduced, whereas soluble sugar accumulation was enhanced (Figure 8a and c). Under drought conditions, sugar levels were rapidly depleted in *sh2* during the night (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* (61%) was much higher than in WT (35%; Figure 8d).

We reasoned that if sugar depletion caused hypersensitivity of leaf growth to drought in *sh2*, supplementing the leaves with sugars during the night should, at least partly, restore the mutant phenotype. Therefore, we fed sucrose through the tip of the growing leaf (Spoehr, 1942). Compared to water-fed controls, sucrose feeding increased soluble sugar content in the growth zone during the dark period under both control and drought conditions (Figure 8e). At the end of the night, the difference was larger in the drought-treated leaves (45% increase due to sucrose

feeding) compared to control conditions (16% increase) and in *sh2* (92% increase for the controls and 143% for the drought-stressed plants) compared to the wild type (Figure 8e). Moreover, in contrast to WT and control conditions, the response in *sh2* was already significant after 2 hours under drought conditions. Under control conditions, increasing sugar content in the leaf growth zone correlated with a significant increase in leaf growth of *sh2*, but not WT. During the night, sugar feeding fully restored leaf growth rates of *sh2* to wild type levels (Figure 8f). Moreover, sugar feeding reduced the effect of drought in the wild type and almost doubled leaf elongation rate in *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 synthesis during the day contributes to sustained sugar supply to the growth zone and leaf growth both during the photoperiod and during the night, particularly under drought.

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 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). Nevertheless, we still lack systems level understanding of these responses, requiring 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 standardized drought treatments in the maize leaf growth zone.

In accordance with previous transcriptome data (Li et al., 2010; Avramova et al., 2015a), the categories “protein synthesis”, “RNA processing” and “transport”, were enriched among the differentially expressed proteins in the meristem. In contrast, the proteome of the mature part was dominated by energy generating processes, such as photosynthesis and carbohydrate metabolism, including glycolysis and TCA cycle. Consistent with the protein levels, biochemical activities and metabolite levels showed the induction of light reactions, carbon fixation, 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).

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 drought response. 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 under well-watered conditions. We found that *Sh2* expression was significantly higher in expanding tissues than in the mature part of the leaf and induced by drought stress, particularly in the meristem (Figure S11). 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 CO₂ acquisition upon re-watering (Avramova et. al., 2015a). While net CO₂ assimilation (A_{net}) is reduced due to stomatal closure, presumably driven by increased ABA levels, an induction of the two photosystems, chlorophyll levels, and CO₂ fixation capacity by upregulation of RuBisCo and PEPC occurs in the mature cells under drought stress conditions. Increased ATP synthase levels facilitate increased conversion of light energy into ATP. Additionally, increased thioredoxin activity stimulates the Calvin cycle (Buchanan et al., 2002), which leads to higher biosynthetic rates 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 CO₂ uptake decreases due to stomatal closure, starch in the mature part of the leaf is degraded to sucrose and transported to the meristem. Accordingly, sucrose transporter genes were upregulated under drought (Figure S12), which could contribute 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, hexoses localized in the vacuole for osmoregulation are 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). Consistent with this, our transcriptome analysis showed induced expression of *Krp2* and downregulation of *Cdk* and *Cycb* in the meristem (Avramova et al., 2015a). ABA induces ROS production by plasma membrane-associated NADPH oxidases (Kwak et al., 2003) and mitochondria (He et al., 2012). ABA 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, preventing starch accumulation in the mature part of the leaves (Figure 9, red arrows) and increasing the accumulation of soluble sugars. The ratio of soluble sugars to starch increases significantly in the mature part of the leaf in response to drought (Figure S13), possibly inducing feedback inhibition of photosynthetic genes (Figure 9) as demonstrated for glucose and sucrose (Carvalho et al., 2010; Paul and Foyer, 2001; Sheen, 1993). Soluble sugars, including glucose and sucrose repress promoter activity of several maize photosynthetic genes e.g., RuBisCo, chlorophyll-binding protein and PEPC (Sheen, 1990). The reduced ability of *sh2* to accumulate starch possibly results in accumulation of triose-6-phosphate (Figure 9), which inhibits RuBisCo and ATP synthase activities (Paul and Foyer, 2001; Yang et al., 2016) further reducing photosynthetic capacity. Consistent with this, A_{net} in the mutant is much lower than WT when stomatal conductance is restored upon re-watering (Figure 5). In meristematic cells, the *sh2* mutation increased sensitivity to drought by increasing ABA levels and ROS accumulation, but had no effect on hexose-induced osmoprotection (Figure 9).

This could be explained by reduced starch biosynthesis directing soluble sugars produced by photosynthesis to mitochondria, increasing mitochondrial metabolism, including electron transport chain (ETC) activity, and ROS production (Moller, 2001; Pastore et al., 2007), consistent with the increased H₂O₂ 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 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 zone (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). The *sh2* mutant had reduced starch levels in the mature leaf tissues (Figures 4c, and 8a) and accumulated soluble sugars during the day (Figure 5a and 8c), but their concentrations decreased rapidly to very low levels early in the night (Figure 8c). We showed that the growth impairment during the night is due to the low soluble sugar concentrations in *sh2* as we could restore its growth to WT rates by sucrose feeding (Figure 8e and f).

Overall, we found a strong effect of altered energy and antioxidant metabolism in the maize leaf proteome in response to drought. Subsequent functional analysis of *sh2* demonstrate the contribution 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.E. and V.A. conducted and analysed the experiments, compiled the data and 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.A. 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	<i>sh2</i> C	<i>sh2</i> 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%)

l_{mat} (μm)	174.9±1.8	159.4±5.6 (-9%)	125.1±2.8 (-28%)	116.9±2 (-6%)
l_{div} (μm)	71.6±0.79	39.5±0.9 (-45%)	55.6±1.72 (-22%)	32±0.4 (-42%)
Rel (μm μm⁻¹ h⁻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 different 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 (meristem, elongation and mature zones) along the leaf axis are based on Avramova et al (2015a) and indicated in the bottom row in green. Syn, synthase; TPI, Triosephosphate isomerase

Figure 4. The effect of drought on starch metabolism and photosynthetic enzyme activities and pigment levels in the *shrunk2* (*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-bisphosphate carboxylase/oxygenase (RuBisCo) activity; (f) Chlorophyll a concentration; (g) Chlorophyll b concentration. Data are means $\pm$ 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 (meristem, elongation and mature zones) along the leaf axis are based on the kinematic analysis in Table 1 and indicated with bars in the bottom row for both wild type (WT) and *sh2* mutant in control (C) and drought treatments (D).

Figure 5. The effect of drought on the sugar metabolism of the *shrunk2* (*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 $\pm$ 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 (meristem, elongation and mature zones) along the leaf axis are based on the kinematic analysis in Table 1 and indicated with bars in the bottom row for both wild type (WT) and *sh2* mutant in control (C) and drought treatments (D).

Figure 6. The effect of drought on photosynthesis (A_{net}) and stomatal conductance (g_s) in the *shrunk2* (*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 $\pm$ SE ($n = 5$).

Figure 7. The effect of drought on stress parameters in *shrunk2* (*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) Absciscic acid (ABA) concentration (in control conditions *sh2* and WT overlap due to very low values); (b)

Malondialdehyde (MDA) concentration; (c) Hydrogen peroxide (H₂O₂) concentration. Data are means $\pm$ 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 (meristem, elongation and mature zones) along the leaf axis are based on the kinematic analysis in Table 1 and indicated with bars in the bottom row for both wild type (WT) and *sh2* mutant in control (C) and drought treatments (D).

Figure 8. The effect of drought on starch metabolism in the *shrunk2* (*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 $\pm$ 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 $\pm$ SE (n=6). In a separate experiment, plants were fed with 0.3 M sucrose and compared to water-fed control plants 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 $\pm$ 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 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 CO₂ 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.

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Supporting Information

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

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Figure S1. Comparison between well-watered maize plants to plants developed in mild and severe drought stress conditions. Maize seedlings were grown in a growth chamber (16h day/8h night, 25°C/18°C day/night [d/n], 300-400 $\mu\text{E}\cdot\text{m}^{-2}\text{ s}^{-1}$ 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. The picture was taken at the time of harvesting: three days after emergence of the fifth leaf.

Table S1. Experimental design of ITRAQ proteome analysis. A total of four pools of samples were used for mass spectrometry analysis. Each pool consisted of eight samples (each sample comprising four individual plants), labeled with the 8 ITRAQ reagents (113-121, marked with different colors in the table). Pools 1, 2, and 3 compared the drought stress effect in each developmental zone of the maize leaf, and pool 4 compared the 3 developmental zones (meristem, elongation, and mature zone) in the control conditions.

Pool 1 Meristem								
Sample	Control	Control	Control	Mild stress	Mild stress	Severe stress	Severe stress	Severe stress
ITRAQ label	113	114	115	116	117	118	119	121
Pool 2 Elongation zone								
Sample	Control	Control	Control	Mild stress	Mild stress	Severe stress	Severe stress	Severe stress
ITRAQ label	118	119	121	113	114	115	116	117
Pool 3 Mature zone								
Sample	Control	Control	Control	Mild stress	Mild stress	Severe stress	Severe stress	Severe stress
ITRAQ label	115	116	117	118	119	121	113	114
Pool 4 Control condition								
Sample	Meristem	Meristem	Meristem	Elongation zone	Elongation zone	Elongation zone	Mature zone	Mature zone
ITRAQ label	113	114	115	116	117	118	119	121

Figure S2. Functional categories of proteins identified in the growth zone of the maize leaf. A BLAST search of 1194 identified proteins in the growth zone of the maize leaf under control conditions (Pool 4; Table S1), using the Mercator tool (Lohse *et al.*, 2014) resulted in a Mapman mapping (Thimm *et al.*, 2004), where the proteins were assigned to one of the 35 Mapman functional category bins.

Figure S3. Enrichment analysis of protein levels along the maize leaf growth zone.

Pageman (Usadel *et al.*, 2006) software was employed to perform a gene enrichment analysis of the first two clusters from Fig. 1, containing the majority of proteins with differentially changed levels (FDR<0.05) along the maize leaf growth zone. Red: overrepresented functional categories; blue: underrepresented functional categories

Figure S4. Hierarchical clustering of the total of 24 proteome samples of different developmental zones along the leaf axis from plants, subjected to well-watered, mild, and severe drought stress conditions. The clustering of the samples (horizontal supporting tree, main clusters labeled in red and blue) and the proteins (vertical supporting tree) was performed using MATLAB (version 9.0, MathWorks Inc., Natick, MA, USA). Data were normalized. The number of the ITRAQ-labeled pool to which each sample belongs is marked in brackets, corresponding to the pool number from Table S1 (1-Meristem, 2-Elongation zone, 3-Mature zone). Green: downregulation, red: upregulation, black: no change.

Figure S5. Enrichment analysis of the differentially affected proteins in response to drought stress (D) in the meristem (Me), elongation zone (EZ), and the mature zone (MZ) of the maize leaf. The data represents proteins with significantly affected levels (FDR < 0.1) along the leaf axis, resulting from three one-way ANOVA analyses, each performed in one of the three developmental zones, comparing the effects of drought stress. Pageman (Usadel et. al., 2006) software was employed to perform the gene enrichment analysis. Red: overrepresented functional categories; blue: underrepresented functional categories

Table S4. *P* values for Figures 4, 5, 7 and 8. A three-factor ANOVA was used as a statistical test, and *P* values for the three factors, genotype (G), treatment (T), and segment (S) for Figures 4,5,7 and genotype (G), treatment (T), and timepoint (TP) for Figure 8, respectively, as well as the interactions between the factors are presented. PEPC, Phosphoenolpyruvate carboxylase; RuBisCo, Ribulose-1,5-bisphosphate carboxylase/oxygenase; AGPase, ADP-glucose pyrophosphorylase; SPS, Sucrose-phosphate synthase; ABA, Absciscic acid; MDA, Malondialdehyde

Parameters Figures 4,5,7	G	T	S	G*T	G*S	T*S	G*T*S
Chlorophyll a	0.000	0.000	0.004	0.000	0.000	0.000	0.000
Chlorophyll b	0.000	0.001	0.000	0.001	0.000	0.000	0.000
PEPC	0.000	0.000	0.000	0.000	0.120	0.000	0.420
RuBisCo activity	0.000	0.000	0.006	0.000	0.530	0.210	0.950
AGPase	0.000	0.002	0.000	0.000	0.220	0.420	0.920
Starch synthase	0.000	0.000	0.000	0.000	0.510	0.210	0.900
Invertase	0.310	0.000	0.000	0.000	0.630	0.840	0.500
SPS	0.000	0.003	0.002	0.000	0.120	0.000	0.160
Soluble sugars	0.221	0.000	0.000	0.000	0.600	0.820	0.600
Starch	0.000	0.000	0.000	0.000	0.000	0.800	0.920
Glucose	0.043	0.000	0.001	0.000	0.049	0.630	0.330
Sucrose	0.050	0.001	0.000	0.003	0.200	0.370	0.320
Fructose	0.000	0.000	0.000	0.000	0.042	0.850	0.720
ABA	0.051	0.000	0.040	0.000	0.700	0.000	0.000
MDA	0.000	0.000	0.000	0.000	0.300	0.200	0.700
H ₂ O ₂	0.000	0.000	0.000	0.000	0.000	0.200	0.100
Parameters Figure 8	G	T	TP	G*T	G*TP	T*TP	
Soluble sugars	0.000	0.000	0.010	0.000	0.036	0.067	
Starch	0.000	0.000	0.000	0.021	0.043	0.050	
Amylase	0.000	0.021	0.000	0.000	0.020	0.013

Figure S6. 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 with mildly (M) and severely (S) stressed plants. 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-bisphosphate carboxylase/oxygenase (RuBisCo) activity; (f) Chlorophyll a concentration; (g) Chlorophyll b concentration. Data are means $\pm$ 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 (meristem, elongation and mature zones) along the leaf axis are based on the kinematic analysis in Table 1 and indicated with bars in the bottom row for both wild type (WT) and *sh2* mutant in control (C), mild (M) and severe (S) drought.

Figure S7. The effect of drought on the sugar metabolism of the *shrunk2* (*sh2*) maize mutant and its wild type (W22). Well-watered control plants (C) were compared with mildly (M) and severely (S) stressed plants. 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 $\pm$ 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 (meristem, elongation and mature zones) along the leaf axis are based on the kinematic analysis in Table 1 and indicated with bars in the bottom row for both wild type (WT) and *sh2* mutant in control (C), mild (M) and severe (S) drought.

Figure S8. The effect of drought on photosynthesis (A_{net}) and stomatal conductance (g_s) in the *shrunk2* (*sh2*) maize mutant and its wild type (W22). Rates of net photosynthesis (a), stomatal conductance (b) and starch concentration in the leaves (c) of young seedlings, grown in mild (M) and severe (S) drought 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. 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 $\pm$ SE ($n = 5$).

Figure S9. The effect of drought on stress parameters in *shrunken2* (*sh2*) maize mutant and its wild type (W22). Well-watered control plants (C) were compared with mildly (M) and severely (S) stressed plants. Measurements were done in each 1 cm of the leaf growth zone. (a) Absciscic acid (ABA) concentration (in control conditions *sh2* and WT overlap due to very low values); (b) Malondialdehyde (MDA) concentration; (c) Hydrogen peroxide (H_2O_2) concentration. Data are means $\pm$ 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 (meristem, elongation and mature zones) along the leaf axis are based on the kinematic analysis in Table 1 and indicated with bars in the bottom row for both wild type (WT) and *sh2* mutant in control (C), mild (M) and severe (S) drought.

Figure S10. The effect of drought on starch metabolism in the *shrunken2* (*sh2*) maize mutant and its wild type (W22). Well-watered control (C), mildly (M) and severely (S) drought 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 $\pm$ SE (n=3). A three-factor ANOVA was used as a statistical test, and *P* values are present in Table S2. (d) Leaf elongation rate (LER) was measured during the day and during the night. Data are means $\pm$ SE (n=6). In a separate experiment, plants were fed with 0.3 M sucrose and compared to water-fed control plants 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 $\pm$ 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. FW, Fresh weight; HS, hydrolyzed starch.

Figure S11. Expression of the *Sh2* maize gene in the meristem, the elongation zone and the mature zone of the growing leaf 5 in the inbred line W22, measured in plants grown in well-watered control (C) and drought stress conditions (D). Expression data is shown relative to the meristem control samples. Data are means $\pm$ SE ($n=3$). Two-way ANOVA and Tukey's test were used as statistical analyses and significant differences ($P < 0.05$) are marked with different letters above the bars.

Figure S12. Effect of drought stress on gene expression of sucrose transporters. Data is from Avramova et al., 2015. Presented are fold change values of the 5 genes assigned as sucrose transporters present on the microarray that show significantly affected expression levels ($P < 0.05$, two-way ANOVA) under drought conditions.

Figure S13. The effect of drought on the ratio between soluble sugars and starch in the leaf growth zone of the *shrunk2* (*sh2*) maize mutant and its wild type (W22). Well-watered control plants were compared to drought stressed plants. Measurements of starch and soluble sugars are done in each cm of the 10 cm growth zone of the maize leaf. Data are means $\pm$ SE (n=3).

Table S5. 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), mild (M) and severe (S) drought stress conditions were compared. A two-factor ANOVA was performed to statistically test the differences between the treatments (T) and the genotypes (G) and the *P*-values are provided in the table. Percentage change in mild or severe stress compared to corresponding controls, as well as between the mutant and the wild type in control conditions, are indicated. 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 M	WT S	<i>sh2</i> C	<i>sh2</i> M	<i>sh2</i> S	G	S	G*S
LER (mm h⁻¹)	3.3±0.03	2.4±0.05 (-26%)	2±0.04 (-38%)	2.5±0.04 (-25%)	1.8±0.09 (-28%)	1.1±0.04 (-57%)	0.00	0.00	0.05
Lmer (mm)	21.2±0.3	19.9±0.34 (-6%)	15.7±0.2 (-25%)	15.5±0.2 (-27%)	13.3±0.38 (-14%)	9±0.4 (-42%)	0.00	0.00	0.74
Lgz (mm)	74±0.03	63±0.22 (-15%)	54±0.11 (-26%)	74±0.14 (0%)	65±0.15 (-13%)	60±0.2 (-19%)	0.29	0.00	0.83
l_{mat} (μm)	174.9±1.8	155±1.5 (-11%)	159.4±5.6 (-9%)	125.1±2.8 (-28%)	118±2.1(-6%)	116.9±2 (-6%)	0.00	0.06	0.86
l_{div} (μm)	71.6±0.79	50.4±1.6 (-30%)	39.5±0.9 (-45%)	55.6±1.72 (-22%)	44.8±4 (-20%)	32±0.4 (-42%)	0.05	0.00	0.79
Rel (μm μm⁻¹ h⁻¹)	0.04±0.04	0.05±0.06 (25%)	0.052±0.1 (31%)	0.041±0.06 (2.5%)	0.046±0.1(14%)	0.053±0.1 (31%)	0.80	0.00	0.32
Tel (h)	22.7±0.4	46.9±2.1(106%)	46.3±1.5 (104%)	26±1.5 (-15%)	37.9±3.2 (46%)	60.7±4.9 (134%)	0.49	0.00	0.24
P (cells h⁻¹)	18.9±0.3	15.7±0.3 (-16%)	13±0.4 (-31%)	20.2±0.58 (7%)	15.4±0.9 (-24%)	9.4±0.4 (-53%)	0.20	0.00	0.28
D (cell cell⁻¹ h⁻¹)	0.031±0.01	0.024±0.02 (-23%)	0.018±0.02 (-41%)	0.025±0 (-19%)	0.016±0.0 (-36%)	0.013±0.0 (-50%)	0.00	0.00	0.79
Nmer	603.5±10.8	650.7±13.8 (8%)	721.3±37 (19%)	848.4±35 (-41%)	997.3±71 (17%)	810.4±69 (-4%)	0.03	0.71	0.54
Tdiv (h)	205.8±5.8	268.2±3.7 (30%)	363.8±14 (76%)	293.8±18 (-43%)	453.8±22.8 (54%)	573.9±43 (95%)	0.00	0.01	0.64
Tc (h)	22.3±0.6	28.7±0.3 (30%)	38.3±1.2 (72%)	30±1.76 (35%)	45.6±2.1 (51%)	59.2±3.8 (97%)	0.00	0.00	0.53
Ngz	1015±10.9	1377.7±14 (35%)	1321.8±59 (30%)	1372.6±44 (35%)	1527.3±54 (11%)	1344.6±75 (-2%)	0.09	0.17	0.38
Nel	411.5±4.1	727±21.6 (76.7)	600.5±25 (45.9%)	524.1±26.9 (27%)	530±19 (1%)	534.3±25.7 (2%)	0.30	0.04	0.03

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