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Running Head: Growth of Maize Leaves under Drought Stress

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Research area: Signaling and Response

Drought Induces Distinct Growth Response, Protection and Recovery Mechanisms in the Maize Leaf Growth Zone

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Summary

Drought inhibits cell division and expansion in the maize leaf growth zone by reducing antioxidant levels and increases photosynthetic capacity to allow for enhanced growth upon recovery.

Footnotes

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Abstract

Drought is the most important crop yield-limiting factor and detailed knowledge of its impact on plant growth regulation is crucial. The maize leaf growth zone offers unique possibilities for studying the spatio-temporal regulation of developmental processes by transcriptional analyses and methods that require more material such as metabolite and enzyme activity measurements.

By means of a kinematic analysis we show that drought inhibits maize leaf growth by inhibiting cell division in the meristem and cell expansion in the elongation zone. Through a microarray study, we observed the down-regulation of 32 of the 54 cell cycle genes, providing a basis for the inhibited cell division. We also found evidence for upregulation of the photosynthetic machinery and the antioxidant and redox systems. This was confirmed by increased chlorophyll content in mature cells and increased activity of antioxidant enzymes and metabolite levels across the growth zone, respectively.

We demonstrate the functional significance of the identified transcriptional reprogramming by showing that increasing the antioxidant capacity in the proliferation zone, by overexpression of Arabidopsis *FeSOD*, increases leaf growth rate by stimulating cell division. We also show that the increased photosynthetic capacity leads to enhanced photosynthesis upon re-watering, facilitating the often-observed growth compensation.

Introduction

Drought imposes a major limitation on crop productivity (Boyer, 1982). Currently no less than 75% of world's fresh water supplies are utilized in agriculture and it is more than likely that the expanding world population and unfavourable climate conditions will decrease its availability in the near future (Wallace, 2000). For example, climate change trends towards increasing drought, are predicted to reduce U.S. maize (*Zea mays* L.) yields between 15 and 30% (Lobell et al., 2014). Therefore, increasing crop productivity under conditions of limiting water availability is of major importance. To achieve this, a systems level understanding of how plant growth adapts to drought is a scientific requirement.

Inhibition of leaf growth is one of the earliest responses to limited water availability, leading to reduction of transpiration and water conservation. This response can cost as much as 60% of the potential yield of a maize crop even in absence of visual wilting symptoms (Ribaut et al., 1997).

At the cellular level, division and expansion in the plants' growth zones determines organ and plant level growth responses to drought. The developing maize leaf provides an ideal model system to investigate such a growth zone at various organizational levels. Already in the 1980s and early 1990s, this system has been used to study organ growth by kinematic analysis (Silk and Erickson, 1979). In recent years, it is increasingly being used for studies into the regulation of cell division and expansion (Rymen et al., 2007), and the environmental effects on these processes (Walter et al., 2009), redox regulation (Kravchik and Bernstein, 2013), hormone homeostasis (Nelissen et al., 2012), protein expression and phosphorylation (Riccardi et al., 1998; Bonhomme et al., 2012), and development of the C4 photosynthetic system (Li et al., 2010; Majeran et al., 2010). Several of these studies are currently impossible in the model plant *Arabidopsis thaliana*, due to the small size of its meristematic and elongation zones. The steady-state growth after the leaf emergence and the relatively large size of the leaf growth zone (ca. 1-2 cm for the meristem and ca. 4-6 cm for the elongation zone) are important advantages, allowing sampling for molecular and physiological analyses with sub-zonal resolution.

Next to a reduction of leaf area, plants adapt to drought by avoiding dehydration, due to activation of mechanisms such as stomatal closure and accumulation of osmolytes (proline, soluble sugars), and by increasing drought tolerance through the induction of protective mechanisms against cell damage, such as synthesis of dehydrins and late-embryogenesis abundant (LEA) proteins (Verslues et al., 2006). Drought stress also leads to accumulation of reactive oxygen species (ROS), inducing cells to generate antioxidants and activate redox-regulating enzymes (Cruz de Carvalho, 2008). Although these responses have been studied extensively in mature tissues, little is known about their regulation in the growth zone and their interaction with the growth processes (Considine and Foyer, 2014). Nevertheless, gene expression varies strongly between dividing, expanding, and mature cells (Beemster et al., 2005), and the impact of osmotic and salt stress on each of these processes is distinctly different (Skirycz and Inze, 2010), urging more development-specific studies of the impact of drought.

Here we use the growth zone of the maize leaf to investigate the effect of relatively mild drought that inhibits growth in the absence of other visual signs of stress, and a more severe drought that leads to leaf rolling, but still allows for continued growth.

Results

Leaf Growth

We studied the effect of drought on leaf growth of maize (B73). Control pots were watered daily to maintain a Soil Water Content (SWC) of 54% throughout the experiments. For drought treatments the soil was not watered, until it reached 43% (mild stress, no wilting) and 34% SWC (severe stress, leaves are wilting during the day), respectively (Figure S1), after which it was maintained at that level. We studied the fifth leaf, because it is the first to initiate and develop fully under stress conditions.

The treatments reduced final leaf length by 17 and 40%. This was associated with an even stronger inhibition of leaf elongation rate (*LER*) by 27% and 63% in mild and severe stress conditions, respectively (Table 1), which was partly offset by an increased duration of the leaf growth period (data not shown). Kinematic analysis, based on measurements of *LER*, the cell length profile along the growth zone (Figure S2), and the length of the meristem determined by locating mitotic cells, showed that the decrease in *LER* is primarily due to a strongly reduced cell production in the meristem (P), whereas mature cell length showed only a small reduction that was not statistically significant (L_{mat}; Table 1). The decrease in P, in turn, was caused by a reduction in division rate (and thus a prolonged cell cycle) and a smaller number of dividing cells (Table 1), due to a smaller division zone (Figure S2). Severe drought also inhibited cell expansion rates by 39%, but a tripling of the time in the elongation zone compensated for this, so that mature cell length was not affected (Table 1).

Microarray Analysis

The cell length profile provides a map of the growth zone under each experimental condition, allowing sampling of equivalent meristem, elongation, and mature zones, although their spatial localization has shifted (Figure S2). We used two-color Agilent maize chips (Ma et al., 2008) in a hybridization design, involving three separate loops for the zones, in each of which the three treatments were contrasted (Figure S3). Out of 44,000 probes on the array, 16,850 transcripts were above background levels (Defined as Foreground (FG) > Background (BG) + 2 x Standard Deviation (SD)) in at least one sample. The levels of 5,878 transcripts were significantly affected by drought (Bonferonni corrected $p < 0.05$ and $|\log2FC| > 0.75$), 665 transcripts levels varied

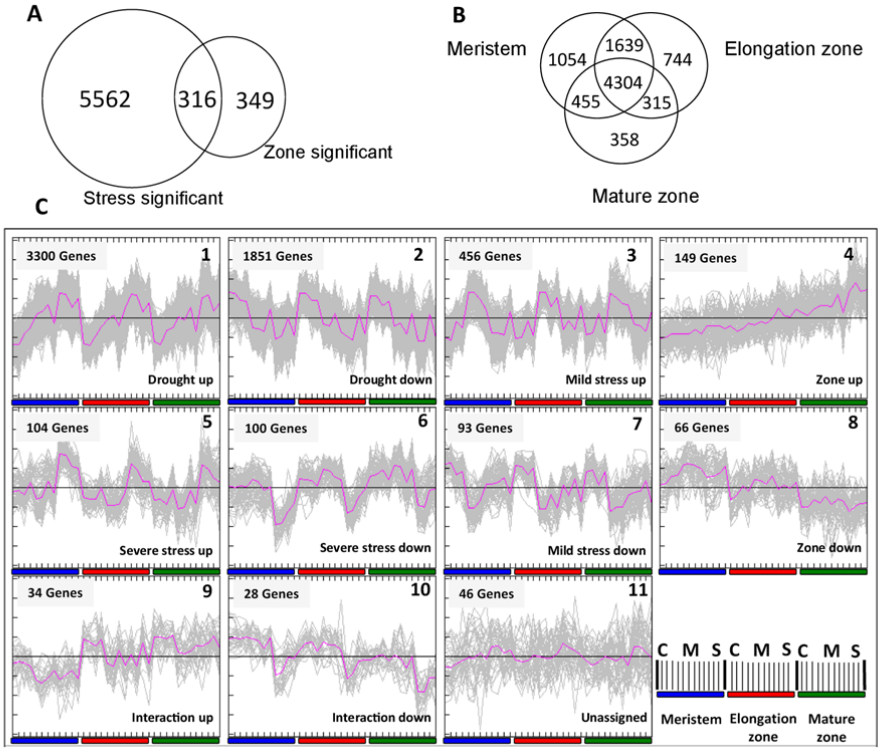

Figure 1. Gene Expression Analysis in the Growth zone in Response to Drought. **(A)** An overview of the 6227 significant (Two-way ANOVA analysis with Bonferroni correction for the stress and a False Discovery Rate (FDR) correction for the zone effect, cutoff: $p < 0.05$ and $|\log2FC|>0.75$) gene transcripts on the microarray; **(B)** An overview of the transcripts, changed significantly in response to drought stress in each developmental zone (meristem, elongation, and mature) along the leaf axis (3 independent one-way ANOVA analyses with FDR correction, cutoff: $p < 0.05$ and $|\log2FC|>0.75$) **(C)** Clustering of gene expression profiles by QT-Clust analysis (Heyer et al., 1999, Pearson correlation measure; cluster diameter=0.5; minimum cluster population=20) of the expression profiles of 6227 significantly modulated genes ($p < 0.05$ and $|\log2FC|> 0.75$). The abscissa, which is enlarged for Cluster 8, denotes three stress treatments (C=control, M=mild and S=severe stress) for each zone (meristem, elongation and mature zone) and 4 biological replicates (each one a pool of 4 plants) for each zone/treatment combination. The ordinate indicates normalized and median-centered expression levels. The colored bar shows the corresponding growth phases based on the kinematic analysis (Figure S2).

between the zones (False Discovery Rate (FDR) < 0.05 and $|\log2FC|> 0.75$), and 316 transcripts showed both responses (Figure 1A). The relatively low number of transcripts showing significantly different levels between the three zones, compared to treatments (despite using a less stringent multiple testing correction), was probably due

to the hybridization design: In contrast to the treatments within each segment, there were no direct comparisons between different zones in the design (Figure S3), limiting the statistical power in their comparison compared to that of the treatment effects. When comparing the effect of stress along the leaf axis, the majority of differentially affected transcripts (4304) were common for the three zones (Figure 1B). Only 358 genes were significantly affected in the mature tissues, compared to 744 in the elongation zone and 1054 in the meristem, indicating that the strongest transcriptional responses occur in the growth zone and particularly in the meristem (Figure 1B). Quality Threshold clustering (Heyer et al., 1999) of the 6,227 differentially expressed transcripts (Figure 1A) resulted in 10 clusters of transcript profile patterns (Figure 1C; Expression values of all genes and their associated cluster are provided in the excel file Supplemental data 2). To identify the major processes represented by the transcription profiles in the different clusters, we performed a gene-enrichment analysis using Pageman (Usadel et al., 2006). The two largest clusters contained 3,300 and 1,851 genes with increasing and decreasing transcript levels in proportion to the severity of the stress conditions, respectively (Figure 1C). It was striking that their opposite expression pattern translated into opposite enrichment and depletion of functional categories (Figure S4). The most prominent (z-score>1.96) transcriptional shifts induced by the drought included the overrepresentation of transcripts related to photosynthesis/light reactions, cellulose synthesis, redox (ascorbate, gluthatione, dismutases, and catalases), oxidases, and the secondary metabolites isoprenoids and flavonoids among upregulated transcripts. Inversely, there was an overrepresentation of lipid metabolism, fermentation, cell wall, amino acid metabolism, RNA regulation, DNA synthesis and repair, protein synthesis, signaling, and cell division and cell cycle transcripts among the downregulated transcripts (Figure S4). The expression patterns indicated that these processes were affected proportionally to the level of stress, starting in mild stress, even before visible signs of wilting occur.

In contrast, there were 545 (cluster 3) and 93 transcripts (cluster 7), whose expression was specifically up- or downregulated under mild stress conditions (Figure 1C). These represented a different set of functional classes. Minor carbohydrate metabolism, ATP synthesis, and ethylene-related transcripts were overrepresented among down regulated transcripts (Figure S4).

Another 104 (cluster 5) and 100 transcripts (cluster 6) were specifically up- or downregulated in response to severe stress (Figure 1C). These were specifically enriched in RNA processing and binding and protein amino acid activation among the upregulated transcripts, and glycolysis, brassinosteroid metabolism, and abiotic stress/heat among the downregulated transcripts (Figure S4). Taken together, these results show that the largest clusters represent pathways that respond proportionally to stress levels, while others are specifically affected by mild and severe stress.
Clusters 4 and 8 contained 149 and 66 transcripts that were gradually increasing and decreasing across the zones with highest levels in the mature zone and in the meristem, respectively (Figure 1C). We found a strong enrichment of major carbohydrate metabolism/sucrose degradation, protein synthesis and posttranscriptional modifications among the transcripts with highest expression in proliferating cells, whereas amino acid metabolism, ethylene metabolism, drought/salt stress, nucleotide metabolism, RNA processing, protein targeting, and development were overrepresented among transcripts that were upregulated in elongating and mature cells (Figure S4). Finally, the smallest clusters, 9 and 10 (34 and 28 transcripts), represented profiles, where the effects of developmental zone and stress were superimposed (Figure 1C). These were enriched in lipid metabolism, protein degradation, and MAP kinase signaling (Figure S4).

In summary, this analysis shows that drought and developmental stage induce severe reprogramming of the maize leaf transcriptome. The largest number of transcripts in our dataset is affected in all zones in proportion to drought levels, with smaller sets of transcripts responding specifically to mild or more severe drought and to developmental differences between the zones.

To understand the significance of these changes, we investigated in more detail the most prominent changes in the largest clusters, the downregulation of DNA duplication and cell cycle gene-expression, the upregulation of the photosynthetic machinery for the light reactions, and changes in redox regulation.

Cell Cycle Regulation

Our kinematic analysis shows that inhibited cell division is the main contributor to the reduced leaf growth in response to drought (Table 1). The cell division cycle is

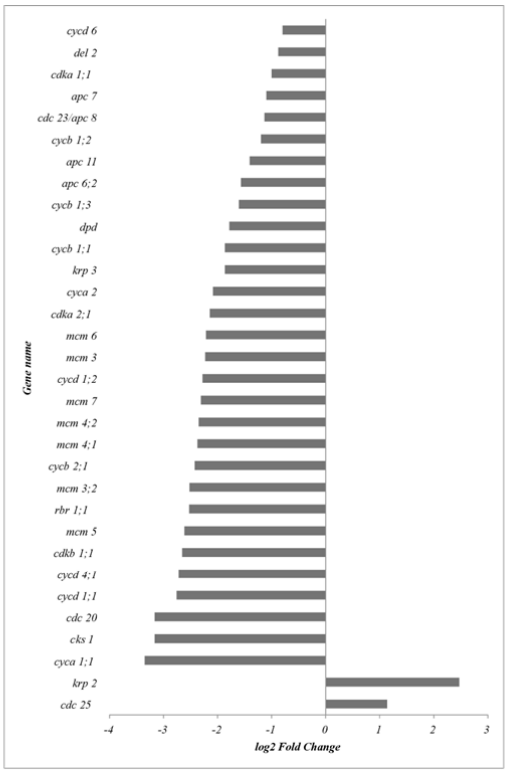

Figure 2. Effect of Drought Stress on Cell Cycle Gene Expression in the Meristem. Presented are the fold changes of the 34 of 57 cell cycle genes present on the array (see Table S2 for the full list of cell cycle genes), which have significantly affected expression levels (|log2 FC|>0.75 and *P* value<0.05, two-way ANOVA with Bonferroni correction) under drought conditions.

transcriptionally regulated (Menges et al., 2005; De Veylder et al., 2007). Therefore we investigated in detail the expression patterns of the maize cell cycle regulators: Cyclin-Dependent Kinases (CDKs), cyclins and their interacting proteins, identified earlier (Rymen et al., 2007, Table S1). Of the 57 cell cycle-related genes on the array, the transcripts of 44 were detected above background levels (FG > BG + 2SD) and 34 of these were significantly affected by drought ($p < 0.05$ and $|Log2FC| > 0.75$), all but two of which were two-fold or more down-regulated (Figure 2). The only two upregulated transcripts were a putative homolog of Cell Division Cycle 25 (*CDC 25*), the function of which in plants is disputed (Boudolf et al., 2006) and a homolog of the inhibitor Kip-Related Protein (*KRP2*) in *Arabidopsis thaliana* (De Veylder et al., 2001). The latter change appears to be offset by an opposite effect on the expression of another member of the KRP-family, *krp3*. Most striking, however, was the suppression of four cyclin-

dependent kinases (*cdka1;1, cdka2;1, cdkb1;1, cdkb2;1*) and ten of their activating cyclins, as well as Retinoblastoma-Related (*rbr1;1*), that acts as a master switch controlling E2 transcription Factor (E2F) transcriptional activation downstream of CDKA for S-phase entry (Sabelli et al., 2013). Related to that, we found 7 minichromosome maintenance (*mcm*) transcripts that control DNA duplication (Chong et al., 1996; Aparicio et al., 1997), which are known targets of E2F transcription factors (Vandepoele et al., 2005), and 4 transcripts of genes encoding subunits of the Anaphase Promoting Complex (APC) that controls the cell cycle at M-phase exit (Eloy et al., 2006). These changes indicate that the reduced cell division activity is a consequence of transcriptional down-regulation of all stages of the cell cycle.

Photosynthesis

Transcripts encoding proteins for photosystem I and II, and light reactions were most enriched among upregulated transcripts (and underrepresented in downregulated transcripts; Figure S4). This suggests transcriptional upregulation of the photosynthetic machinery along the whole leaf growth zone under drought conditions. This is unexpected, given the growth inhibition and other studies showing downregulation of the photosynthetic activity (Dwyer et al., 1992; Ashghizadeh and Ehsanzadeh, 2008) and inhibited expression of photosynthesis genes under drought stress (Hayano-Kanashiro et al., 2009).

To validate and understand the upregulation of the photosynthetic apparatus under mild and severe drought stress (Figure 3A and B), we measured biochemical and physiological photosynthetic parameters. Consistent with the increased mRNA levels of photosynthesis-related transcripts (Figure 3A and B) and transcripts encoding enzymes involved in the synthesis of tetrapyrrole, which forms the active core of chlorophyll (Figure S4), chlorophyll content (types a and b) increased up to 5-fold by drought. The mild stress only affected the levels in the mature tissues, whereas in severe stress chlorophyll already started to accumulate in the elongation zone (Figure 3C and E). However, in line with published results, our gas-exchange data indicated that photosynthesis in the mature part of the leaf is progressively inhibited by increasing drought stress levels (Figure 3D). This inhibition was correlated with a strongly reduced stomatal conductance (Figure 3F), indicating that under drought conditions photosynthesis in the mature parts of the leaf is limited by stomatal aperture.

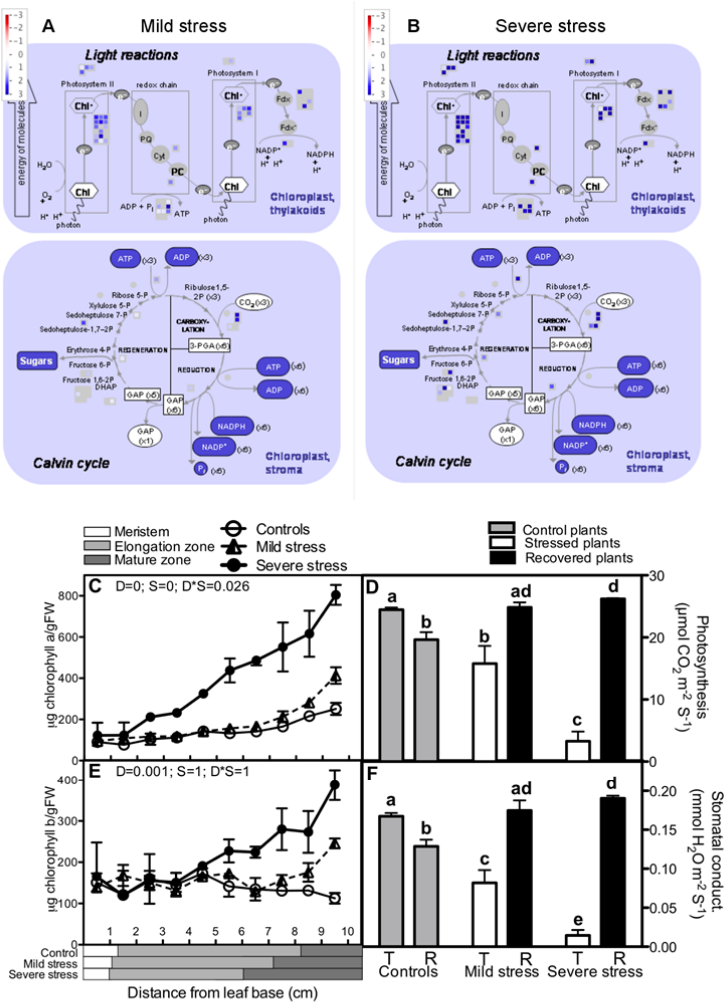

Figure 3. Changes in the Photosynthetic Machinery in the Growth Zone of the Maize Leaf under Mild and Severe Drought Stress.

Transcript abundance of photosynthesis-related genes (log2FC) under mild **(A)** and severe drought stress **(B)**; Chlorophyll a **(C)** and chlorophyll b **(E)** content across the leaf axis in well-watered and stressed plants (a two-way ANOVA was used as a statistical test and *P* values for the two factors, drought (D) and segment (S), as well as the interaction between them, are present in the figure panels; Data are averages +/- SE, n=3), the length of each developmental zone (meristem, elongation, mature) is marked on the x axis of the graph for each treatment (control, mild, and severe stress) according to Figure S2; Rates of photosynthesis **(D)** and stomatal conductance **(F)** before and after recovery of the stressed plants, (T-treatment; R-recovery; Unstressed plants of the same age as the plants that were subjected to stress and allowed to recover are included as a control for ontogenetic differences; Students t-test was used for statistical analysis and significant differences ($p < 0.05$) are marked with different letters; Data are averages +/- SE, n=5).

To reconcile the apparent contradiction of increased photosynthetic capacity with reduced carbon-assimilation rates in drought stressed leaves, we hypothesized that the investment in the photosynthesis machinery facilitates enhanced carbon acquisition upon recovery. To functionally test this, we re-watered drought stressed plants at 3

weeks after sowing (4-5 days after emergence of the fifth leaf). After five days of recovery, we measured the photosynthesis of the fifth leaf, which had developed under stress conditions. As a control, we measured leaves grown under control conditions, but of the same age as the leaves from re-watered plants. In the control plants photosynthesis decreased when the leaves matured (Figure 3D). In contrast, the photosynthetic rates in the leaves that had recovered from the drought were 26% (mild stress, $p = 0.044$) and 33% (severe stress, $p = 0.0005$) higher than controls of the same age and even 1% (mild stress, NS) and 7 % (severe stress, $p = 0.003$) higher than the control leaves that had just emerged. The increased photosynthesis upon recovery was accompanied by enhanced stomatal conductance (Figure 3F). These results demonstrate that leaves developing under drought conditions increase their photosynthetic capacity, to maximize photosynthesis upon recovery when the stomata are allowed to open.

Oxidative Stress

The observed transcriptional changes in redox control are in line with earlier observations that drought induces oxidative stress in mature leaves (Kar, 2011; Cruz de Carvalho, 2008). In such conditions, cellular redox homeostasis is often disturbed, as the consequence of extra ROS generation. A more oxidative environment may result in macromolecule damage (protein oxidation, lipid peroxidation), but also alters regulatory and signaling processes. Redox signaling is essential in a number of processes during plant growth and development as it affects calcium fluxes (Foreman et al., 2003; Mazars et al., 2010) and regulates the activity of redox-sensitive enzymes, containing S-S groups (Klomsiri et al., 2011). In order to evaluate a potential link with the growth response, we characterized changes in redox status and regulation along the growth zone at the molecular level.

In contrast to most other ROS, hydrogen peroxide levels (H_2O_2) can be quantified. However, existing techniques are sensitive to the reactivity of the H_2O_2 molecule, which possibly creates artifacts (Cheeseman, 2006; Queval et al., 2008). We therefore compared different extraction and detection methods. Using an extraction in 5% TCA, and staining with xylenol orange, we observed a doubling of H_2O_2 content in severe, and a small increase across the growth zone under mild stress conditions (Figure 4A). Extraction in a very distinct environment, phosphate buffer supplemented with catalase inhibitor, and quantification with both xylenol orange and Amplex red hydrogen

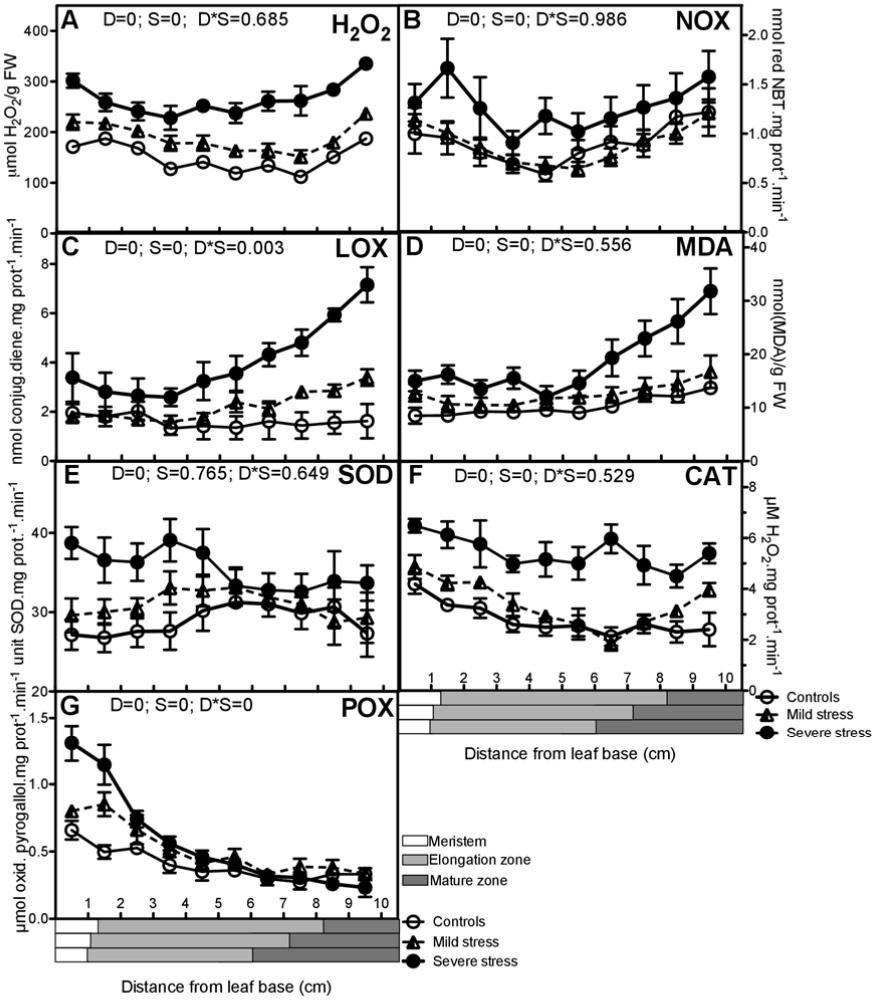

Figure 4. Oxidative Stress Determinants and Main Antioxidant Enzymes. Well-watered control plants are compared to mildly and severely stressed plants. Metabolite concentrations and enzyme activities were determined in each centimeter of the leaf growth zone. **(A)** Hydrogen peroxide contents, **(B)** nicotinamide adenine dinucleotide phosphate-oxidase (NOX) activity, **(C)** Lipoxygenase (LOX) activity, **(D)** malondialdehyde (MDA) contents, **(E)** superoxide dismutase activity (SOD), **(F)** catalase (CAT), and **(G)** peroxidase activity (POX). A two-way ANOVA was used as a statistical test and p values for the two factors, drought (D) and segment (S), as well as the interaction between them, are present on the graph panels. Data are averages +/- SE (n=5). The length of each developmental zone (meristem, elongation, mature) in each treatment (control, mild, and severe stress) is marked on the x axes of the graphs according to Figure S2.

peroxide/peroxidase assay, on independent samples, gave nearly identical results (Figure S5A, B and C). This strongly suggests the independence of these data of extraction conditions and detection assay. Even under carefully controlled conditions, considerable variation is observed in H_2O_2 determinations, possibly related to

extraction efficiency and stability of the molecule (Cheeseman, 2006; Queval et al., 2008). We therefore also performed an in-tissue H_2O_2 staining (3'3-diaminobenzidine, DAB; Thordal-Christensen et al., 1997). In this assay, H_2O_2 is 'captured' inside the cells (Figure S5D). These results also confirmed that increasing drought levels progressively increased H_2O_2 throughout the growth zone. However, the effect is much less pronounced, particularly in the more mature tissues. Differences between DAB, Amplex red and xylenol orange measurements, may be related to limited penetration of DAB, which could be more prominent in mature cells with thicker cell walls. On the other hand, DAB staining is dependent on intracellular peroxidase activity, which also strongly decreases towards the mature-cell zone (Figure 4G). Despite the intrinsic advantages and disadvantages of each of these methods, they consistently indicate increased H_2O_2 levels in the growth zone in response to drought stress.

Increased ROS levels under stress originate primarily from increased photorespiration, altered electron transport in chloroplasts and mitochondria, and increases in Respiratory Burst Oxidase Homologue (RBOH) activity. The latter activity (RBOH) represents membrane-bound NADPH-dependent oxidases (NOX) that catalyze the production of superoxide ($O_2^{\bullet -}$), which is converted to H_2O_2. The levels of *zmrboh a* and *b* transcripts were proportionally increased by mild and severe drought (Table S2). NOX activity levels in mild stress were close to those in the control plants, whereas in the severely stressed plants a dramatic increase occurred throughout the growth zone (Figure 4B). NOX activity appeared to be suppressed in expanding cells in all the conditions, which was not observed in the transcriptome data (Table S2). Together H_2O_2 and NOX activity data clearly indicated increased ROS levels throughout the growth zone in response to drought stress.

One effect of increased ROS levels is the potential increase of levels of oxidation of lipid molecules to lipid hydroperoxides. The activity of lipoxygenase enzymes (LOX) is also a major source of lipid peroxidation (Repetto et al., 2010). Out of six LOX enzymes annotated in our maize microarray data, the expression levels of four transcripts, encoding LOX isoforms (*lox2*, *lox6, lox10* and *lox11*) increased in response to drought stress, while the expression was highest in elongating and mature tissues (significant only for *lox2* and *lox10*; Table S2). During lipid peroxidation, small hydrocarbon fragments such as ketones and malondialdehyde (MDA) are formed. Total LOX activity and MDA content closely followed the LOX transcript profiles in

response to drought, increasing towards the mature part of the leaf (Figure 4C and D), demonstrating that drought induces oxidative damage in all regions of the leaf, with mature tissues showing the strongest response.

Enzymatic Oxidative Stress Defenses

To evaluate the response of the anti-oxidative defense system, we measured the expression and activity of the main antioxidant enzymes along the leaf growth zone. Superoxide dismutase (SOD) is a metalloenzyme associated with copper (Cu), zinc (Zn), manganese (Mn) or iron (Fe) that catalyzes the dismutation $O_2^{\bullet -}$ to H2O2 and O2. In the microarray data, six SOD-encoding transcripts were expressed above background levels. Four of these were 2 to 5 fold upregulated by drought (Table S2). The expression levels of five of these genes were highest in the meristem, compared to the mature parts of the leaf, but these changes were only significant for one homolog of the Arabidopsis *FeSOD* (Table S2). The total SOD enzyme activity correlated with the transcriptional data, showing a progressive induction by drought and highest activity in the meristem (Figure 4E).

Catalases (CAT) and peroxidases (POX) are the primary scavengers of H_2O_2. Their activity protects plants in response to various stress factors (Castillo, 1992; Willekens et al., 1995). Of the three CAT-encoding transcripts, identified in maize, two were up- (*cat1* and *cat2*) and one was downregulated (*cat3*) by drought (Table S2). The activity of the enzyme was highest in the meristem and was enhanced by drought, correlating with the transcript levels (Figure 4F).

Peroxidases (POXs) are localized in the cell wall and vacuoles and use numerous substrates (Carpin et al., 1999). The transcript levels of two plasma membrane-bound POX isoforms (*pmpox1*, *pmpox3-1*) were significantly upregulated in response to severe drought only, whereas those of *pmpox3-2* were significantly upregulated in both stress levels (Table S2). Again, the enzyme activity was consistent with the transcription data, with a higher activity in the meristem and upregulation by drought (Figure 4G).

Together these results show a general correlation between the transcript-level changes of various antioxidant enzymes and their activity. Interestingly, the activity of POX and

CAT, but also SOD under severe drought was highest in the meristem at the base of the leaf, while the oxidative stress (determined by the activity of the enzymes NOX and LOX, and the content of MDA) increased mainly in the mature zone. Our measurements indicate that antioxidant regulation in response to drought differs across the growth zone, the meristem being most actively protected.

P35S:AtFeSOD Overexpressing Maize Has Increased ROS Tolerance and Improved Growth Rates under Control and Drought Stress Conditions

The increased activity of the antioxidant enzymes in the meristem, where inhibition of cell division is at the basis of the growth response, leads to the question if enhanced antioxidant production in this zone could reduce the growth inhibition imposed by drought. Expression of transgenic *Arabidopsis thaliana FeSOD* in chloroplasts enhanced oxidative stress tolerance in tobacco plants, by protection of the plasma membranes and Photosystem II (VanCamp et al., 1996; Van Breusegem et al., 1999). To test the effect of elevated *FeSOD* chloroplast-targeted expression in maize Van Breusegem et al. (1999), produced transgenic maize lines overexpressing *Arabidopsis thaliana FeSOD* under a 35S promotor, inducing enhanced tolerance towards methyl viologen (Paraquat) and improved growth under control and cold stress conditions. Because we found highest SOD levels in the meristem and increased levels in response to drought, we hypothesized that the improved growth of this line would also occur under drought conditions and opposite to the effect of drought (Table 1), would be due improved cell division. To prove this we first validated that the overexpression of the Arabidopsis gene in this line resulted in increased SOD activity and improved redox state in the growth zone of maize leaves. Indeed, the activity of the enzyme was significantly higher throughout the growth zone in the *FeSOD*-overexpressing line than in the wild type in control and drought conditions (Figure 5A). This increased activity led to lower levels of ROS, as evidenced by a significant reduction in the levels of the MDA in the transgenic plants, showing reduced lipid peroxidation (Figure 5B).

To test the effect of the transgene on growth, we subjected the AtFeSOD overexpressing plants and the wild type (H99) to a kinematic analysis. We observed a 17% increase in LER of the transgenic plants under control conditions confirming the previous observations (Van Breusegem et al., 1999), but obtained also 30% faster growth under mild and 9% under severe drought, respectively. Consistent with our hypothesis, these enhanced growth rates were due to increased cell production rates in

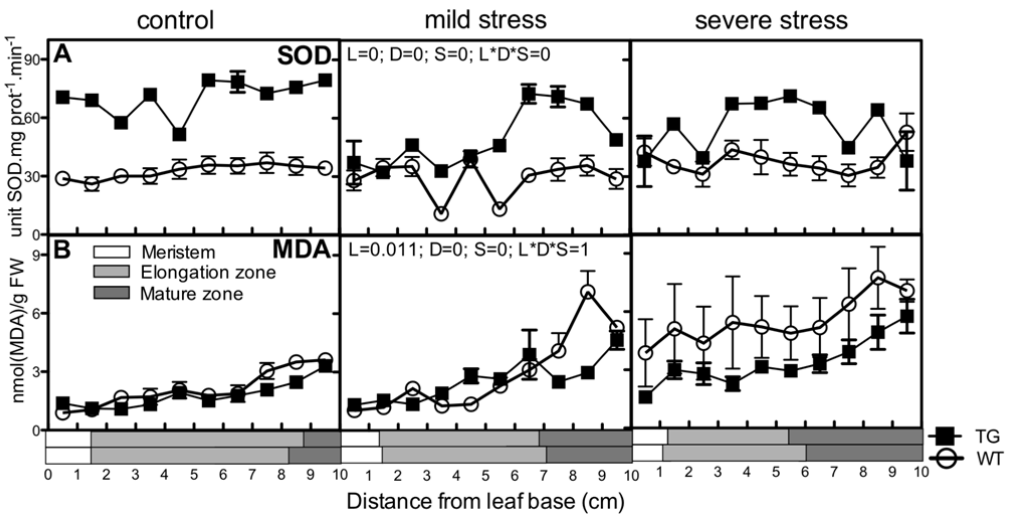

Figure 5. SOD Activity and MDA Levels in the Leaf Growth Zone of P35S:ATFeSOD Line and its Wild Type.

Biochemical determination of SOD activity **(A)** and MDA **(B)** levels was done in the growing zone of maize leaves from control (well watered) plants and plants, exposed to mild and severe water stress, comparing wild type (WT) and 35S-AtFESOD (TG) lines. A three-way ANOVA was used as a statistical test and p values for the three factors, line (L), drought (D), and segment (S), as well as the interaction between them, are present on the middle graph for each band. Data are averages +/- SE (n=3). The length of each developmental zone (meristem, elongation, mature) is marked on the x axes of the graphs for each line (WT and TG).

444 the transgenic line, whereas no significant differences were observed between the cell
445 lengths and cell elongation rates (Table 2).
446
447

Discussion

Drought is one of the major factors limiting plant growth in ecological and agricultural contexts. Although its effects have been studied extensively, most molecular and physiological studies have focused on mature leaves of plants exposed to severe stress treatments that lead to clear signs of wilting. Our results show that well before such signs appear, growth is already inhibited up to 30%. These more mild stress levels are important determinants of crop yields in modern agricultural conditions (Skirycz et al., 2011).

Our studies confirm major differences in gene expression and physiology (Beemster et al., 2005; Li et al., 2010) between mature and growing tissues. This implies that to understand the effect of drought on growth, growing tissues need to be analyzed separately. So far such studies have been performed more extensively in maize roots and differences in regulation of growth-related processes between the apical and the basal region of the primary root tip have been reported (Yamaguchi and Sharp, 2010). Similar to our results (Figure 1C), specific changes in gene expression and protein composition in these zones were described between well-watered and drought-stressed plants (Zhu et al., 2007; Spollen et al., 2008).

Kinematic analysis shows that a significant decrease in cell division rates in the meristem is the main cause of the growth reduction (Table 1). The microarray data obtained from the meristem show downregulation of the entire cell cycle machinery (Figure 2) and upregulation of an inhibitor of cell cycle progression (*krp*2, De Veylder et al., 2001). It has previously been demonstrated that CDKA kinase activity correlates with reduced cell division rates in maize leaves subjected to drought conditions (Granier et al., 2000), but the regulatory mechanism was not determined. Previously, we found that in response to cold nights, cell cycle transcript levels in the maize leaf meristem correlated with inhibited cell division rates (Rymen et al., 2007). Generally, our results are consistent with other studies of cell cycle regulation and abiotic stress: a decrease of transcript levels of A- and B-type cyclins occurred in response to cold, drought, and salinity stress (West et al., 2004; Rymen et al., 2007; Kakumanu et al., 2012). Studies in Arabidopsis have shown that altered expression of A-and B-type cyclins indeed results in altered cell proliferation (Doerner et al., 1996; Vanneste et al.,

2011). In addition to these core cell cycle regulators, several subunits of the anaphase-promoting complex, cyclosome (APC/C) were also downregulated (Table S1). This complex promotes the transition from anaphase to metaphase by destruction of B-type cyclins and increased levels of its subunit APC10 enhanced leaf growth (Eloy et al., 2011). Together, our results indicate a broad effect on multiple control points of the cell cycle rather than a single key regulator that is responsible for the reduced cell division activity.

The upregulation of genes involved in the photosynthetic machinery (Figure 3A and B) in the microarray study was surprising, particularly given the reduced rates of photosynthesis in our (Figure 3D) and other published studies (Dwyer et al., 1992; Ashghizadeh and Ehsanzadeh, 2008; Hayano-Kanashiro et al., 2009). In other studies either little effect (Chaves et al., 2009) or downregulation (Kilian et al., 2007; Hayano-Kanashiro et al., 2009; Humbert et al., 2013) of photosynthesis-related transcripts was observed in response to drought and other abiotic stresses. However, most of these results have been obtained by sampling mature leaves and therefore may relate to the maintenance of the fully developed photosynthetic system during stress conditions. Our results indicate that leaves developing in drought conditions, in contrast, increase their photosynthetic capacity possibly to compensate for the smaller size of their leaves. This potential can be used upon recovery from the drought, when the stomata open. To our knowledge these changes have not been reported at a transcriptional level. They represent an important finding and may explain at least to some extend the often observed phenomenon that stressed plants upon recovery grow faster than unstressed control plants, so that the effect of the stress on plant size reduces (Hayano-Kanashiro et al., 2009; Xu et al., 2009).

The observed increase in chlorophyll levels in the stressed plants could be also linked to the redox status in the leaf. We showed a significant increase in the activity of redox enzymes in the growth zone of the maize leaf. It has been shown that there is a direct link between ROS levels and photosynthetic activity during leaf development, which could additionally influence plant growth and leaf aging (Chen and Gallie, 2006).

The growth reduction under drought stress could also be explained by changes in the redox status of the stressed plants. Our measurements show a significant increase in

515 H_2O_2 levels especially in severe stress conditions. Besides their oxidative effect, H_2O_2
516 and other ROS are demonstrated to play a role in growth-related processes as signaling
517 molecules. In mammalian cells, it is well established that ROS can act as positive
518 growth regulators depending on their concentration and pulse duration (Sauer et al.,
519 2001; Menon and Goswami, 2007). In plants, ROS are involved in the regulation of
520 several processes (for review see Considine and Foyer, 2014), including both cell
521 division and cell elongation. For example, low concentrations of ROS are needed to
522 induce cell proliferation (Fehér et al., 2008) and cell differentiation (Tsukagoshi et al.,
523 2010). H_2O_2 is shown to block cell cycle progression (Reichheld et al., 1999; Kovtun et
524 al., 2000) and is needed for cell elongation (Rodriguez et al., 2002). ROS also play a
525 role in cell wall stiffening and thus may inhibit cell expansion (Hohl et al., 1995;
526 Schopfer, 1996). Our measurements show that H_2O_2 in maize leaves progressively
527 increases with the severity of the drought treatments, suggesting risk of oxidative
528 damage in the meristem, elongation and maturation zones. Therefore, the observed
529 inhibition of both cell division and cell expansion in response to drought can possibly
530 be linked to the negative impact of elevated H_2O_2 in the corresponding leaf zones
531 directly on the regulation of these two processes.
532
533 Our analysis of redox regulation shows that different antioxidant systems dominate in
534 specific parts of the growth zone during drought stress. POX is mainly active in the
535 meristem and in the very beginning of the elongation zone, whereas SOD and CAT
536 activity slightly decrease over the growth zone, with highest activity in the meristem.
537 SOD activity is most strongly upregulated in this zone. The meristem therefore appears
538 to be the part of the leaf, with highest antioxidant enzymatic activity (Figure 4E, F, and
539 G). Similarly, salinity stress induced higher transcription of genes involved in
540 antioxidant protection in young compared to old cells across the maize leaf (Kravchik
541 and Bernstein, 2013). Possibly as a consequence, the mature zone shows higher levels
542 of lipid peroxidation during drought stress (Figure 4E). High MDA in turn can also
543 affect cellular processes such as gene expression and activate defense responses (Weber
544 et al., 2004).
545
546 The increases in lipid peroxidation also correlates with increased LOX activity in the
547 more mature leaf segments (Figure 4C). This could possibly point to increased stress-
548 related jasmonate production, as jasmonate biosynthesis involves the synthesis of

549 oxylipins, through lipid oxidation. Particularly LOX 6, whose transcript levels were
550 significantly induced in our conditions (Table S2), is responsible for stress-induced
551 jasmonate accumulation in roots (Grebner et al., 2013). Jasmonates are involved in
552 stomatal closure (Suhita et al., 2004) during drought stress and negatively regulate cell
553 cycle progression, keeping the cells in the G1 stage (Noir et al., 2013). Our microarray
554 data showed significant changes in the transcripts of three other key-regulatory
555 enzymes of jasmonate-biosynthesis (12-OXO-PHYTODIENOIC ACID REDUCTASE
556 5 and 6, and ALLENE OXIDE CYCLASE 1; Table S2). The transcription pattern of
557 12-OXO-PHYTODIENOIC ACID REDUCTASE 5 followed the one of LOX. Isoform
558 6 of the same reductase was downregulated in the stress conditions, but to a much
559 smaller extend compared to the upregulation of isoform 5's transcription, suggesting
560 that the latter plays a more important role during water stress. The transcription of the
561 enzyme ALLENE OXIDE CYCLASE 1, shown to be linked to ROS regulation during
562 salinity stress (Hazman et al., 2015), was only induced in the elongation zone of
563 severely stressed plants.
564
565 The existence of a transgenic line, overexpressing an Arabidopsis gene for FeSOD
566 (Van Breusegem et al., 1999), allowed a direct investigation of the link between
567 antioxidant activity in the meristem and the inhibitory effect of drought on cell division
568 rates. Several papers have already demonstrated enhanced performance of plants with
569 increased antioxidant levels under stress conditions (Mckersie et al., 1993; VanCamp et
570 al., 1996; Van Breusegem et al., 1999), but the cellular basis for this was never
571 determined. We demonstrated that the better growth of the *FeSOD*-overexpressing line
572 was due to increased cell production rates. In the Arabidopsis root tip, a correlation
573 between levels of glutathione (a non-enzymatic antioxidant metabolite) and cell cycle
574 regulation was established (Vernoux et al., 2000). Our results are consistent with this,
575 and to our knowledge show for the first time that increased enzymatic antioxidant
576 levels in the leaf meristem, can positively regulate cell division and thereby improve
577 growth. We demonstrate that combining molecular genetic insights from Arabidopsis
578 with studies of the maize growth zone not only validates results in a crop species, but
579 also increases knowledge of plant growth regulation in general.
580
581

Methods

Maize Lines

All measurements were performed using the inbred line B73 (Iowa Stiff Stalk Synthetic). A transgenic line, overexpressing the *FeSOD* gene from *Arabidopsis thaliana* under control of the CaMV 35S promoter (P35S:ATFeSOD) and its wild type, a backcross of Pa91xH99 to the H99 parent were used to test the impact of increased antioxidant capacity on plant growth. Seeds from the transgenic and wild type lines were obtained from the corresponding author (Van Breusegem et al., 1999).

Growth Experiment

Maize seedlings were grown in a growth chamber under controlled conditions (16h day/8h night, 25°C/18°C day/night [d/n], 300-400 $\mu E.m^{-2}\ s^{-1}$Photosynthetically Active Radiation, provided by high pressure sodium lamps). For control plants the 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. Three days after emergence of the fifth leaf, randomly chosen plants were harvested and the growth zone (the first 10 cm from the leaf basis) of leaf five of each plant was cut in ten segments of 1 cm and the samples were stored at -80°C for further measurements (total RNA extraction, pigment and antioxidant quantification, enzyme activities). The remaining plants were used to determine the length of the fifth leaf until it reached maturity. Five independent reproducible drought experiments were conducted, each one of them on a batch of at least 20 plants for each condition.

Kinematic Analysis

The kinematic analysis was done according an established protocol (Rymen, 2010). It entails leaf-elongation rates and final leaf length measurements, measurements of the cell-length profile along the axis of the leaf, 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 until the moment it reached its final leaf size. Leaf elongation rate (LER) was calculated during the first three days of leaf growth as the difference in length divided by the time difference between successive measurements (Day3-Day2/24 h). For meristem measurements, samples harvested 3

615 days after leaf emergence were analyzed by fluorescence microscopy (AxioScope A1,
616 Axiocam ICm1, Zeiss) at 20x magnification. The size of the meristematic zone of the
617 leaves was estimated by locating the most distal mitosis in the cell files. Cell length
618 was measured by light microscopy (Scope A1 Axiocam ICm1, *Zeiss*), using
619 Differential Interference Contrast (DIC) at 40x magnification and the online
620 measurement module in the Axiovision (version, Zeiss) software. Measurement was
621 carried out at four locations on each segment, at the tip (0 cm), at third of the segment
622 (0.3cm), at two thirds (0.6cm) and at the end of the segment (0.99cm). Around 20 cells
623 were measured at each location. The raw data obtained for individual leaves were
624 smoothed and interpolated at an interval of 50 mm using the kernel smoothing function
625 locpoly of the KernSmooth (Wand and Jones, 1995) package for the R statistical
626 package (R Foundation for Statistical Computing), which allowed averaging between
627 leaves and comparison between treatments. The calculations of all of the other
628 parameters (Tables 1 and 2) were done, based on these data as described earlier
629 (Fiorani et al., 2000).
630
631 **RNA Extraction, labeling and Hybridization**
632 Total RNA was extracted from each zone (meristem, elongation and mature zone) of
633 the fifth leaf at the third day after its appearance of control plants, and plants subjected
634 to mild and severe drought stress. Four biological replicates (each one a pool of four
635 plants) were used for each zone/treatment combination. The total RNA was extracted
636 using the TRIZOL reagent (Invitrogen) and purified using the RNeasy Plant Mini Kit
637 (Qiagen, Netherlands). Probe concentrations and purity were determined, using a
638 NanoDrop ND-1000 UV-VIS Spectrophotometer (Thermo Scientific, USA), and the
639 quality was assessed, by using a gel cartridge on a QIAxcel platform (Qiagen, Hilden,
640 Germany). Samples were labeled using the Quick Amp Labeling Kit (Two Color,
641 Agilent). The labeled samples were purified (by RNeasy Mini Kit), and cRNA yield
642 and relative amount of incorporated labeled dCTPs were determined on a NanoDrop
643 ND-1000 UV-VIS Spectrophotometer (Thermo Scientific, USA). The microarray
644 analysis was conducted using the Agilent 44K maize chips (Ma et al., 2008). Three
645 separate hybridization loops (Figure S3), each consisting of 6 arrays, were used on five
646 4-pack formatted microarray slides. Labeling, hybridization, and washing were
647 performed as described in the Agilent Two-Color Microarray-Based Gene Expression
648 Analysis Protocol.

649

650 **Microarray Analysis**

651 Microarrays were scanned using a Genepix Personal 4100A confocal scanner (Axon
652 Instruments), at a resolution of 5 μm and excitation wavelengths of 635 nm and 532
653 nm. All spots were identified and quantified by GenePix Pro 6.0 software (Axon
654 Instruments). The R packages arrayQualityMetrics and arrayQualitywere used to
655 perform a quality control: Spot filtering was done for each array, and spots that did not
656 pass the criteria of FG >BG + 2SD (FG = foreground, BG = background, SD =
657 standard deviation on the local backgrounds of the entire array (Sclep et al., 2007),
658 were excluded from the analysis. A variance stabilization (Huber et al., 2002), which is
659 a between-array normalization was used, following the function
660 normalizeBetweenArrays (x, method="vsn",lts.quantile=0.5), contained in the package
661 LIMMA (Smyth, 2005). Statistical analysis for differences between the stress
662 conditions and the developmental zones was conducted, by using a two-way ANOVA
663 on the software MeV (Multi Experiment Viewer, Saeed et al., 2003). After a stringent
664 cutoff (Bonferroni multiple testing correction for the stress effect and false discovery
665 rate (FDR) correction for the zone effect and a cutoff of P>0.05 and |log2Fold Change|
666 >0.75), differentially expressed genes were visualized and clustered using Quality
667 Threshold clustering (Pearson correlation measure; Cluster diameter=0,5; minimum
668 cluster population= 20) in MeV. For the comparison of the stress effect in each
669 developmental zone 3 separate one-way ANOVA analyses (for meristem, elongation,
670 and mature zone) were done and FDR was used as a multiple testing correction. Only
671 significant values (FDR < 0,05 and |log2Fold Change| > 0.75) were taken into account.
672 Data from 4 different databases (Ware et al., 2002; Thimm et al., 2004; Coetzer et al.,
673 2011; Van Bel et al., 2012) were combined in order to functionally annotate the
674 differentially expressed genes in the analysis. Gene enrichment studies were carried out
675 by PageMan (Usadel et al., 2006). MapMan (Thimm et al., 2004) was employed to
676 show the differences in gene expression in different cellular and metabolic processes.
677

678 **Photosynthesis Measurements**

679 Net photosynthesis rate and stomatal resistance were measured on the exposed/mature
680 part of the fifth leaf, using a portable photosynthesis system (LI-6400, LI-COR Inc.,
681 Lincoln, NE, USA). The CO_2 concentration and temperature in leaf chamber were
682 respectively kept at 400 μmol/mol^{-1} and 25 ± 0.5°C. The measurements were

683 conducted at photon flux density (1500 μmol m^{-2} s^{-1}) by a red-blue light-emitting
684 diode (LED) light source (LI-6400-02B LED; LI-COR) and at ambient relative
685 humidity. All parameters were measured at noon inside the growth room.
686 Measurements were done once during the stress treatment and four times after
687 recovery, using five plants for each treatment.
688
689 **Biochemical Measurements**
690 Photosynthetic pigments
691 Photosynthetic pigments were extracted and determined according to the method
692 described by (Markwell et al., 1986). The contents of chlorophyll *a* and b were
693 calculated using the formulas, described previously (Porra et al., 1989)and expressed as
694 μg pigment/g fresh weight.
695
696 Determination of H_2O_2 and Malondialdehyde (MDA)
697 For H2O2 determination four independent assays were used. 1. 100 mg of the samples
698 were homogenized in 1 ml of 5% TCA (Velikova et al., 2000), by using a
699 MagNALyser (Roche, Vilvoorde, Belgium). Homogenates were centrifuged (14000
700 rpm, 30 min) and xylenol orange dye reagent (Bellincampi et al., 2000) was added to
701 supernatant. After 45 minutes incubation, the Fe^{3+}-xylenol orange complex was
702 measured at 595 nm; 2. Extraction in 50 mM phosphate buffer (pH=6.5) containing the
703 catalase inhibitor (hydroxylamine; 1 mM) followed by quantification with xylenol
704 orange reagent; 3. Extraction in the phosphate buffer with hydroxylamine as above,
705 followed by a quantification with the Amplex red hydrogen peroxide/peroxidase assay
706 (Molecular Probes, Shin and Schachtman, 2004). 4. Localization along the leaf axis
707 using 3,3'-diaminobenzidine (DAB) staining (Thordal-Christensen et al., 1997).
708
709 MDA was extracted in 2 ml 80% ethanol and measured by using a thiobarbituric acid-
710 malondialdehyde (TBA-MDA) assay (Hodges et al., 1999). The quantity of MDA
711 (μM) was calculated by the formula: ([6.45 x (A532-A600)-0.56 x A440]/0.478).
712
713 Enzyme Extraction and enzyme Activity Assays
714 Around 100 mg frozen leaf tissue was homogenized in 1 mL of K -Phosphate buffer
715 (0.05 M pH 7.0), containing 2% (w/v) polyvinyl pyrrolidone, EDTA (0.4 mM), PMSF
716 (0.2mM) and ascorbic acid (1 mM). Peroxidase (POX) activity was measured by

717 monitoring the production of purpurogallin at 430 nm (Kumar and Khan, 1982).
718 Catalase (CAT) activity was calculated out of the decrease in H_2O_2 concentration,
719 measured at 240 nm (Aebi, 1984). Measuring the inhibition of NBT reduction at 550
720 nm was used to assay superoxide dismutase (SOD) activity to (Dhindsa et al.,
721 1981). The activity of lipoxygenase (LOX) was assayed according to Axelrod et al.
722 (1981), by monitoring the production of conjugate diene at 234nm. NADPH oxidase
723 (NOX) was assayed according to Sarath et al. (2007), where NADPH-dependent
724 superoxide generation was measured by the reduction rate of NBT into monoformazan
725 at 530 nm.
726

727 Soluble Protein Content
728 Soluble protein was determined according to Lowry method (Lowry et al., 1951)

729 **Statistical Analysis**

730 For all of the biochemical measurements a two-way ANOVA (Analysis of Variance)
731 was performed (factor 1: the segment of the growth zone and factor 2: the stress
732 treatment) using the statistical package SPSS (Version 20, IBM). Data are presented as
733 means of three biological replicates ± standard error (SE). Effects were considered
734 significant at $p < 0.05$.
735

736 **Accession numbers:**
737 Raw microarray data have been deposited with NCBI's Gene Expression Omnibus
738 (GEO, http://www.ncbi.nlm.nih.gov/geo) and are accessible through the GEO series
739 accession number GSE55592.
740

741 **Supplemental Material**
742

743 The following materials are available in the online version of this article:
744 **Supplemental Data 1, containing:**
745 **Figure S1** Soil Water Content During the Growth Experiment.
746 **Figure S2** The Effect of Drought on the Cell Length Profile.
747 **Figure S3** Hybridization Design.
748 **Figure S4** Gene Enrichment Analysis.

Figure S5 Comparison Between Different Methods of Determination of H2O2 Concentration Across the Growth Zone of Maize Leaves, Subjected to Mild and Severe Drought Stress.

Table S1 An Overview of Core Cell Cycle Genes in Maize.

Table S2 An Overview of Expression Levels of Genes Coding Different Isoforms of the key Redox Enzymes in Response to Mild and Severe Drought Stress in the Different Positions of the Leaf Growth Zone.

Supplemental Data 2 A table with microarray data, including the expression values of the genes, statistical analysis and presence in clusters illustrated in Figure 1C.

Acknowledgments

We would like to thank Prof. Frank Van Breusegem, who kindly provided the seeds of the FeSOD overexpressing maize line.

Figure Legends

Figure 1. Gene Expression Analysis in the Growth zone in Response to Drought. **(A)** An overview of the 6227 significant (Two-way ANOVA analysis with Bonferroni correction for the stress and a False Discovery Rate (FDR) correction for the zone effect, cutoff: $p < 0.05$ and $|\log2FC|>0.75$) gene transcripts on the microarray; **(B)** An overview of the transcripts, changed significantly in response to drought stress in each developmental zone (meristem, elongation, and mature) along the leaf axis (3 independent one-way ANOVA analyses with FDR correction, cutoff: $p < 0.05$ and $|\log2FC|>0.75$) **(C)** Clustering of gene expression profiles by QT-Clust analysis (Heyer et al., 1999, Pearson correlation measure; cluster diameter=0.5; minimum cluster population=20) of the expression profiles of 6227 significantly modulated genes ($p < 0.05$ and $|\log2FC|> 0.75$). The abscissa, which is enlarged for Cluster 8, denotes three stress treatments (C=control, M=mild and S=severe stress) for each zone (meristem, elongation and mature zone) and 4 biological replicates (each one a pool of 4 plants) for each zone/treatment combination. The ordinate indicates normalized and median-centered expression levels. The colored bar shows the corresponding growth phases based on the kinematic analysis (Figure S2).

Figure 2. Effect of Drought Stress on Cell Cycle Gene Expression in the Meristem. Presented are the fold changes of the 34 of 57 cell cycle genes present on the array (see Table S2 for the full list of cell cycle genes), which have significantly affected expression levels (|log2 FC|>0.75 and $p < 0.05$, two-way ANOVA with Bonferroni correction) under drought conditions.

Figure 3. Changes in the Photosynthetic Machinery in the Growth Zone of the Maize Leaf under Mild and Severe Drought Stress.
Transcript abundance of photosynthesis-related genes (log2FC) under mild **(A)** and severe drought stress **(B)**; Chlorophyll a **(C)** and chlorophyll b **(E)** content across the leaf axis in well-watered and stressed plants (a two-way ANOVA was used as a statistical test and p values for the two factors, drought (D) and segment (S), as well as the interaction between them, are present in the figure panels; Data are averages +/-SE, n=3), the length of each developmental zone (meristem, elongation, mature) is marked on the x axis of the graph for each treatment (control, mild, and severe stress) according to Figure S2; Rates of photosynthesis **(D)** and stomatal conductance **(F)** before and after recovery of the stressed plants, (T-treatment; R-recovery; Unstressed plants of the same age as the plants that were subjected to stress and allowed to recover are included as a control for ontogenetic differences; Students t-test was used for statistical analysis and significant differences ($p < 0.05$) are marked with different letters; Data are averages +/- SE, n=5).

Figure 4. Oxidative Stress Determinants and Main Antioxidant Enzymes.
Well-watered control plants are compared to mildly and severely stressed plants. Metabolite concentrations and enzyme activities were determined in each centimeter of the leaf growth zone. **(A)** Hydrogen peroxide contents, **(B)** nicotinamide adenine dinucleotide phosphate-oxidase (NOX) activity, **(C)** Lipoxygenase (LOX) activity, **(D)** malondialdehyde (MDA) contents, **(E)** superoxide dismutase activity (SOD), **(F)** catalase (CAT), and **(G)** peroxidase activity (POX). A two-way ANOVA was used as a statistical test and p values for the two factors, drought (D) and segment (S), as well as the interaction between them, are present on the graph panels. Data are averages +/- SE (n=5). The length of each developmental zone (meristem, elongation, mature) in

each treatment (control, mild, and severe stress) is marked on the x axes of the graphs according to Figure S2.

Figure 5. SOD Activity and MDA Levels in the Leaf Growth Zone of P35S:ATFeSOD Line and its Wild Type.

Biochemical determination of SOD activity **(A)** and MDA **(B)** levels was done in the growing zone of maize leaves from control (well watered) plants and plants, exposed to mild and severe water stress, comparing wild type (WT) and 35S-AtFESOD (TG) lines. A three-way ANOVA was used as a statistical test and p values for the three factors, line (L), drought (D), and segment (S), as well as the interaction between them, are present on the middle graph for each band. Data are averages +/- SE (n=3). The length of each developmental zone (meristem, elongation, mature) is marked on the x axes of the graphs for each line (WT and TG).

828 # Tables

829

830 **Table 1.** Kinematic Analysis of the Effect of Drought on Cell Division and Expansion
831 in the Growing Maize Leaf.
832 Results are averages of five independent experiments ±SE. Statistical significance is
833 based on Student's t-test and p values > 0.05 are marked as NS (Not Significant).
834 Parameters: Leaf Length (*LL, measured on the fifth leaf at the time of the harvesting,*
835 *Fig.S1*), Leaf Elongation Rate (*LER, calculated during the first 3 days after leaf*
836 *appearance*), Mature cell length (L_{mat}), Cell production rate (P), Cell division rate (D),
837 Cell cycle duration (T_c), Length of the meristem (L_{mer}), Number of cells in the meristem
838 (N_{mer}), Time in division zone (T_{div}), Cell elongation rate (R_{el}), Time in elongation zone
839 (T_{el})

Growth parameters	**control**	**mild stress**	**severe stress**	**% change in mild /severe stress**
LL (mm)	727±15	603±16	436±23	-17/-40
LER (mm/h)	3.0±0.1	2.2±0.1	1.1±0.2	-27/-63
L_{mat} (μm)	134±6	126±7	117±8	NS/NS
P (cells/h)	22±2	17±1	9±1	-24/-58
D (cell/cell/h)	0.029±0.004	0.026±0.003	0.016±0.002	NS/-44
T_c (h)	26±2	28±3	48±7	NS/+84
L_{mer} (mm)	13±1	10±1	10±1	NS/-26
N_{mer}	867±58	685±41	591±17	-21/-32
T_{div} (h)	253±25	267±26	437±67	NS/+73
R_{el} (μm/μm/h)	0.040±0.002	0.032±0.003	0.024±0.006	NS/-39
T_{el} (h)	44±3	60±8	131±30	NS/+195

840

Table 2. Kinematic Analysis, Describing the effect of Mild and Severe Drought Stress on Cell Division and cell Expansion Parameters During the Steady-State Growth of the Fifth Leaf of Wild Type and Transgenic Plants, Overexpressing an Arabidopsis Gene, Encoding a FeSOD Enzyme.

A two-way ANOVA statistical analysis was used to determine statistically significant differences between the three treatments and the two maize lines and p values > 0.05 are marked as NS (Not Significant) change. Data are averages +/- SE (n=4). Parameters: Leaf Length (*LL, measured on the fifth leaf at the time of the harvesting, Fig.S1*), Leaf Elongation Rate (*LER, calculated during the first 3 days after leaf appearance)*), Mature cell length (L_{mat}), Cell production rate (P), Cell division rate (D), Cell cycle duration (T_c), Length of the meristem (L_{mer}), Number of cells in the meristem (N_{mer}), Time in division zone (T_{div}), Cell elongation rate (R_{el}), Time in elongation zone (T_{el})

Parameter	WT control	35S-FeSOD control	WT mild stress	35S-FeSOD mild stress	WT severe stress	35S-AtFeSOD severe stress	Treatment	Lines
LL (mm)	802±21	894±8	662±51	755±43	402±73	439±54	S	S
LER (mm/h)	2.5±0.1	3.0±0.1	1.7±0.3	2.3±0.2	1.0±0.2	1.1±0.4	S	S
L_{mat} (µm)	135±12	112±11	126±10	116±4	103±4	105±4	NS	NS
P (cells/h)	19±2	28±2	13±2	20±3	10±2	11±4	S	S
D (cell/cell/h)	0.025±0.003	0.033±0.003	0.016±0.002	0.023±0.004	0.013395±0.004	0.015±0.005	S	NS
T_c (h)	29±3	22±2	46±7	33±5	62±19	76±32	S	NS
L_{mer} (mm)	20±1	20±0	15±0	14±1	11±0	13±1	S	NS
N_{mer}	810±109	865±122	799±63	883±46	782±99	757±100	NS	NS
R_{el} (µm/µm/h)	0.031±0.002	0.035±0.002	0.021±0.005	0.030±0.003	0.014283±0.001	0.017±0.006	S	NS
T_{el} (h)	52±6	42±5	81±19	64±8	102±4	133±41	S	NS

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