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Molecular and Physiological Analysis of Growth-Limiting Drought Stress in *Brachypodium distachyon* Leaves

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ABSTRACT The drought-tolerant grass *Brachypodium distachyon* is an emerging model species for temperate grasses and cereal crops. To explore the usefulness of this species for drought studies, a reproducible *in vivo* drought assay was developed. Spontaneous soil drying led to a 45% reduction in leaf size, and this was mostly due to a decrease in cell expansion, whereas cell division remained largely unaffected by drought. To investigate the molecular basis of the observed leaf growth reduction, the third *Brachypodium* leaf was dissected in three zones, namely proliferation, expansion, and mature zones, and subjected to transcriptome analysis, based on a whole-genome tiling array. This approach allowed us to highlight that transcriptome profiles of different developmental leaf zones respond differently to drought. Several genes and functional processes involved in drought tolerance were identified. The transcriptome data suggest an increased energy availability in the proliferation zones, along with an up-regulation of sterol synthesis that may influence membrane fluidity. This information may be used to improve the tolerance of temperate cereals to drought, which is undoubtedly one of the major environmental challenges faced by agriculture today and in the near future.

Key words: *Brachypodium*; drought stress; plant growth; leaf.

INTRODUCTION

Drought is one of the most devastating threats to plant productivity and, in our rapidly changing environment, its prevalence is on the increase worldwide (Rosegrant and Cline, 2003; Yang et al., 2010). In both natural populations and cultured fields, the effects of drought—usually coupled with elevated temperatures and soil salinity—can be quite dramatic. A decreased water availability can severely limit growth (Skirycz and Inzé, 2010), and this jeopardizes the organism's primary goal—to survive and sustain growth long enough to ensure the plentiful production of viable seeds within the favorable growth season.

One of the greatest challenges facing us in the nearby future is to feed the growing world population under deteriorating climatic conditions, implying that crop productivity should increase further. Among the most important crops worldwide are temperate C3 cereals such as wheat and barley. Wheat—the basis of bread, pasta, and breakfast cereals, for example—ranks third among the grass crops (after maize and rice) and, in Europe and Russia, for example, it is the undisputed number one cereal crop. Barley (ranking fourth among cereals worldwide) is extremely important as animal fodder, and also for beer brewing (Food and Agriculture Organization of the United Nations, faostat.fao.org; data of the year 2010, last accessed 16 August 2012). Unfortunately, these species are not suitable for a targeted experimental approach to improve drought tolerance and yield: they have large polyploid genomes, and the lack of easy genetic and genomic tools has so far hampered breakthrough

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discoveries in this field. To enable standardized molecular experiments with direct relevance for temperate cereal crops, the wild grass *Brachypodium distachyon*—belonging to the Pooideae—is now being developed as a new model species. It possesses all desirable properties of a model system, and a wide variety of genetic and genomic tools has already been developed (Mur et al., 2011). Its genome is diploid and small (in size, only 2% of the wheat genome), and whole-genome sequencing of the Bd21 accession of *Brachypodium distachyon* (hereafter referred to as *Brachypodium*) has recently been completed (International Brachypodium Initiative, 2010). This grass has a short life cycle (~12 weeks), undemanding growth requirements, and a working transformation system (Garvin et al., 2008; Opanowicz et al., 2008; Vogel and Hill, 2008; Alves et al., 2009).

Phylogenetic surveys have revealed that *Brachypodium* is much more closely related to wheat and barley than rice, sorghum, or maize are. Estimates of divergence times of *Brachypodium* are 29.4 (±4.9) million years ago (MYA) from wheat, 42.1 (±6.9) MYA from rice, and 50.5 (±7.5) MYA from sorghum (International Brachypodium Initiative, 2010). This puts the common ancestor of *Brachypodium* and wheat at an only slightly earlier date than that of *Arabidopsis* and *Brassica* (International Brachypodium Initiative, 2010). Also, synteny appears to be much better between *Brachypodium* and the Triticaceae genomes than between rice, sorghum, and the Triticaceae, thereby facilitating the translatability of data from *Brachypodium* to Triticaceae crops.

The response of plants to drought has been studied extensively in *Arabidopsis*, as well as in other dicots and in a few grass species (Hirayama and Shinozaki, 2010; Lopes et al., 2011). Being a drought-tolerant grass originating in Iraq, *Brachypodium* is a very interesting species to study, as it may possess specific adaptations or tolerance mechanisms, which—once identified and characterized in detail—may be transferred to related grass crops. *Brachypodium* represents a wild, undomesticated grass species, in contrast to cultured crops such as rice, wheat, barley, sorghum, and maize. During domestication of crops, human selection has caused a genetic bottleneck in which many ancestral traits have been lost at random because they were not linked to agronomic traits (Tanksley and McCouch, 1997). When breeding occurred in a favorable climate and/or well-irrigated field conditions, the lack of selective pressure may have led to the random loss of many traits conferring stress tolerance. As a wild grass, *Brachypodium* has never been subjected to the human selection that has instead impoverished the genetic diversity of related temperate cereals during their domestication. For this reason, *Brachypodium* is a very promising system for identifying drought-tolerance mechanisms in grasses that may have been lost during the domestication of temperate cereal crops, for example.

Here, we describe the development of a standardized drought assay in soil for young *Brachypodium* plants. Using this assay, we investigated the effects of drought stress on leaf growth, on both the organ and cellular levels, and on the leaf transcriptome. Leaf growth is the combined result of two developmental mechanisms, namely cell proliferation and cell expansion. In grasses, cell proliferation during leaf development is confined to a narrow zone at the base of the leaf. Distally to this cell proliferation zone is a defined zone in which cells expand until they reach a mature cell size. This study revealed that—in contrast to the situation in *Arabidopsis* (Skirycz et al., 2010) and maize (Tardieu et al., 2000), for example—the *Brachypodium* leaf meristem is nearly unaffected by drought, and drought-induced growth reduction is almost entirely caused by reduced cell expansion. In our molecular analysis, we identified various drought-responsive genes and functional processes, and found that the transcriptomes of different developmental leaf zones respond differently to drought. We conclude that *Brachypodium* is a promising model species for grass research, and that its high level of drought tolerance can be exploited for the identification of stress-tolerance genes with the potential to make grass crops more tolerant to drought.

RESULTS AND DISCUSSION

A Soil-Based Set-Up for Reproducible Induction of Growth-Limiting Drought Stress in Bd21 Leaves

The grass species *Brachypodium* is an emerging and very promising model species for drought-tolerance studies. In order to provide the community with a valuable tool, we developed and optimized a protocol for reproducibly subjecting young *Brachypodium* plants to growth-limiting, non-lethal drought stress in soil. In contrast to most published drought studies—which were performed in artificial *in vitro* conditions—the data reported here are biologically relevant for agricultural settings. As recently shown by Skirycz et al. (2011), most reports on drought stress in *Arabidopsis* are not physiologically relevant for field conditions. The soil-based approach from the current study is therefore far more reliable, and these findings for *Brachypodium* may have direct relevance for the drought response of cultivated grass crops, such as wheat, barley, and maize.

Seedlings were transferred to soil—after being germinated under controlled conditions, as detailed in the Methods section—and subsequently water was withheld from the pots containing plants that would be subjected to drought stress. At the time of plantlet transfer, all pots contained 2.27 g water per g dry soil, and they were dried down to 1.82 g g^{-1} (control), 0.73 g g^{-1} (moderate drought), or 0.45 g g^{-1} (severe drought). Pot weight was monitored on a daily basis, and water was added only after the target weight had been reached, and just enough to re-adjust the pot weight to the target level. Figure 1 illustrates the synchrony that was achieved at the various stages of the drought stress protocol, which are listed in detail in Supplemental Table 1. Soil drying was highly

reproducible and homogeneous, and the developed protocol ensured that the desired stress level was reached before the third leaf was initiated (Figure 2A). The experimental protocol ensured that the third leaf was growing under persistent stress conditions, as no watering had occurred for several days prior to its emergence. Furthermore, the protocol did not lead to a growth arrest and, in most cases, plants could still initiate the fourth leaf (Figure 1D). This demonstrated that the treatment was not too harsh.

Interestingly, *Arabidopsis* already experiences moderate drought stress at a soil water content of 2 g water per g dry soil, or 30% field capacity (Harb et al., 2010). In our experiments with *Brachypodium*, we worked with a field capacity of about 11% (moderate drought) and 6.7% (severe drought), and control plants were grown at a field capacity of 27%. This clearly illustrates that *Brachypodium* is a relatively drought-resistant species.

Figure 2A illustrates the progression of soil drying in our experimental set-up, which was highly reproducible and predictable. The desired drought level was reached before the third leaf was initiated. Moderate drought and severe drought stress resulted in *Brachypodium* leaves that were 27% and 45%, respectively, smaller at maturity than control leaves. Control leaves were on average 9.3 (±0.2) cm long from base to tip, while, under moderate drought, they reached 6.8 (±0.3) cm, and, under severe

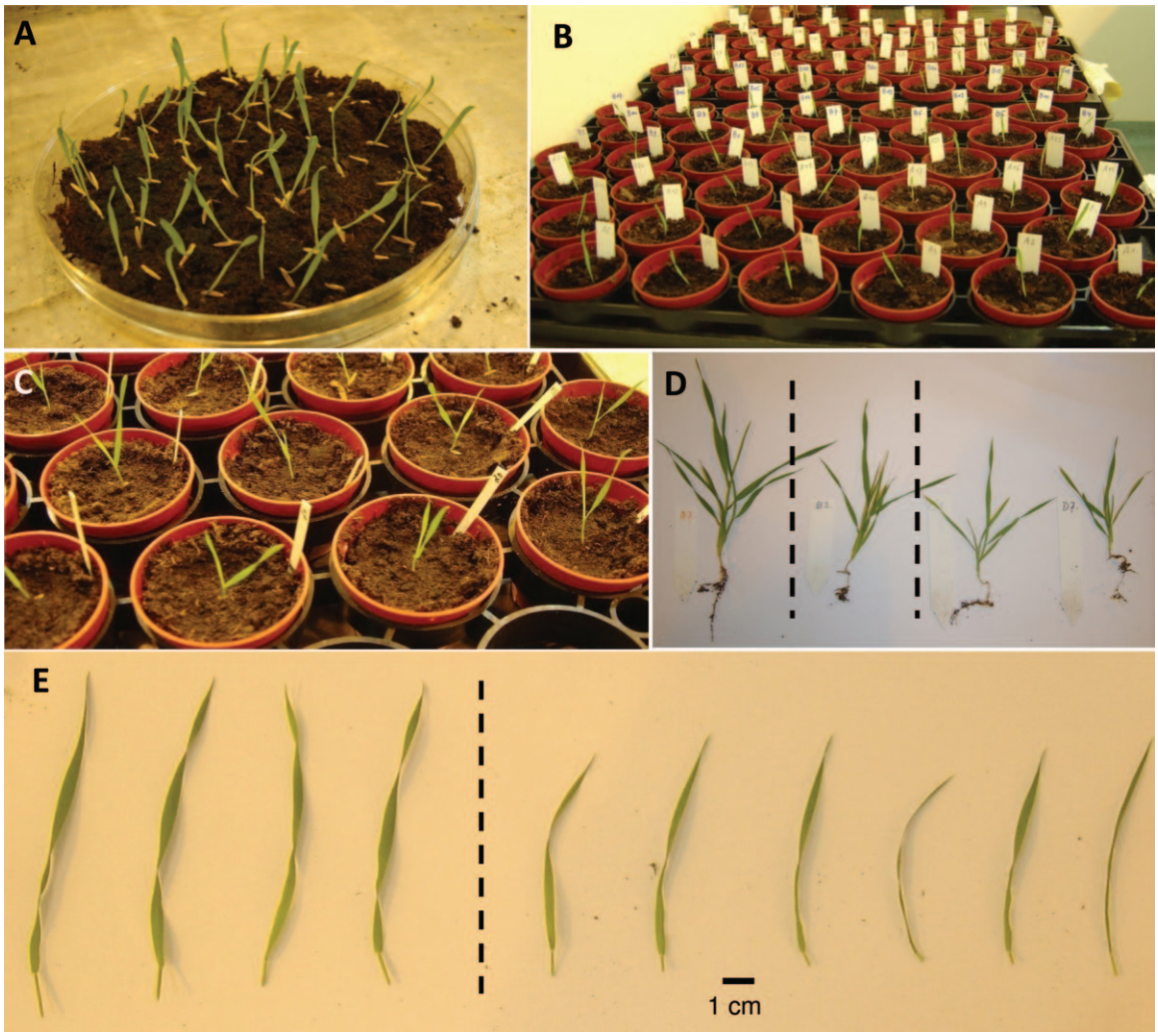

Figure 1. A Reproducible Drought Stress Protocol for *Brachypodium* in Soil.
(A) shows Bd21 seedlings which have germinated synchronously on wet soil in a Petri dish. In **(B)**, all seedlings have just been transferred to individual pots and, in **(C)**, the second leaf has appeared in all plants. In **(D)**, representative Bd21 plants are shown after 20 d of growth in the soil-based system; the plant on the left had been grown under control conditions, the second plant from the left had been grown under moderate drought stress, and the two plants on the right under severe drought stress. Plants from all conditions remained healthy and continued growing, although drought stress greatly slowed down vegetative growth and development. The effect of moderate drought stress on the length of the third leaf is presented in **(E)**: four representative control leaves (left) are compared to six representative leaves from stressed plants (right), harvested 16 d after transfer of the seedlings to soil. Apart from being shorter, their width was clearly also reduced, resulting in a much smaller leaf surface overall.

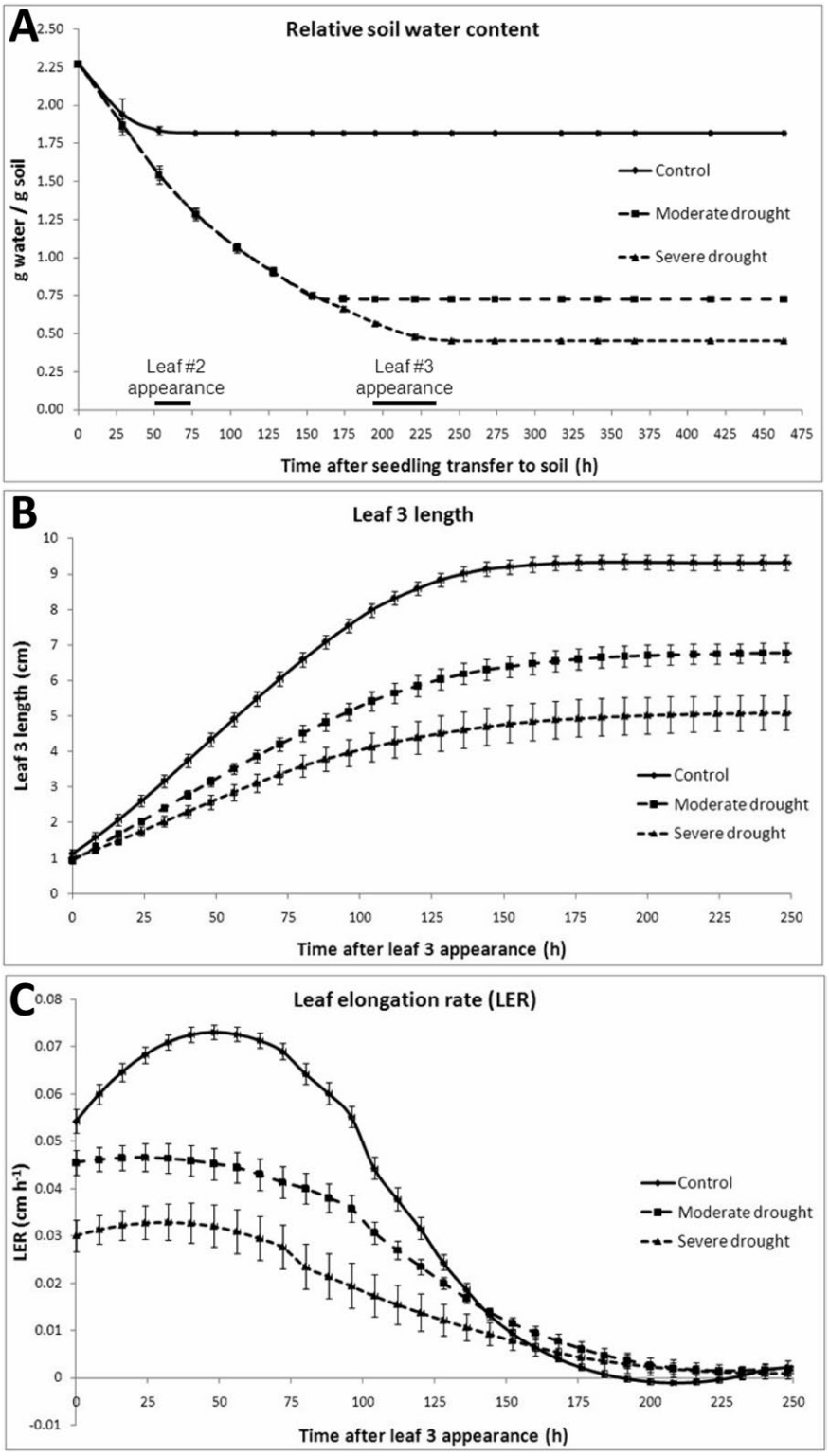

Figure 2. Drought Stress Inhibits *Brachypodium* Leaf Growth.
(A) shows the progression of soil drying in our experimental set-up. The high level of reproducibility and homogeneity is apparent from the very small standard deviations in relative soil water content. Below the *x*-axis, the time span is indicated during which the second and third leaves first became visible. The *x*-axis represents the time (in hours) that was reached since the transfer of Bd21 seedlings to individual, well-watered pots (with 2.27 g water per g dry soil); in **(B)**, the length of the third leaf is shown in relation to the time since its first appearance and, in **(C)**, the corresponding leaf elongation rate (LER) is plotted, in cm per hour. Graphs are from a representative experiment with 25 plants per treatment and six experiments have been conducted, with very comparable results (Supplemental Table 2).

drought, 5.1 (±0.5) cm (Figure 2B). Leaf expansion rates did not exceed 0.5 mm h^{-1}, while control leaves grew at up to 0.85 mm h^{-1} (Figure 2C). Already at their initiation, stressed leaves expanded at a slower rate than control leaves, which indicates that the plants had already been experiencing stress before that time. During the entire leaf growth period of approximately 150 h, the growth rate of control leaves remained much higher than that of stressed leaves. Only between 160 and 230 h after the emergence of the third leaf did the growth rate of stressed leaves exceed that of control leaves, but this was insufficient to compensate for the final size difference, because the absolute growth rate was already insignificantly low at this point. A similar effect was observed in *Arabidopsis* leaves under mild drought stress conditions: low expansion rates were compensated by an extension of the duration of expansion (Aguirrezabal et al., 2006).

Drought Stress Mainly Affects Final Cell Size, Not Cell Number

To investigate whether the leaf size reduction in *Brachypodium* was due to a stress effect on cell division, cell expansion, or both, we microscopically examined fully grown leaves. We found that the total cell number was not significantly lower in stressed leaves, and that the smaller leaf length was almost entirely accounted for by a reduced mature cell size. The total cell number from base to tip was 389.7 (±26.5) in control leaves, and 366.8 (±12.8) and 368.0 (±17.2) in leaves grown under moderate and severe drought stress, respectively (Figure 3A), and the calculated average mature cell size was 207.9 (±11.6) μm, 160.4 (±6.0) μm, and 135.0 (±7.0) μm, respectively (Figure 3B). Hence, drought stress induced a reduction of the cell number per row only by about 6%, while the average cell size decreased by 23% (at moderate drought) and by 35% (at severe drought). In conclusion, the 27% leaf size reduction under moderate drought stress was almost entirely the result of a reduced cell expansion, and the same holds true for the 45% leaf size reduction under severe drought stress.

The fact that, in *Brachypodium* leaves, cell expansion is affected by drought, while cell proliferation is not, is in sharp contrast to prior observations made in other plant species such as *Arabidopsis*, barley, maize, rice, and wheat. Both growth processes were consistently reported to be equally affected by drought stress, thereby not only resulting in a smaller average cell size, but also in a smaller number of cells per leaf (Lu and Neumann, 1998; Schuppler et al., 1998; Tardieu et al., 2000; Skirycz et al., 2010). In *Arabidopsis*, for example, drought reduces the cell number by up to 40% (Skirycz et al., 2010) but, in *Brachypodium*, it has virtually no effect on the final cell number in a leaf (Figure 3A). The leaf size reduction in response to drought stress in Bd21 is thus nearly entirely caused by an effect on cell expansion, namely a reduced final cell size. This observation suggests that *Brachypodium* possesses mechanisms to protect its dividing cells against the negative impact of drought stress.

Measurement of individual cell lengths in function of the relative cell position within a growing leaf (harvested 3 d after appearance, at a developmental stage when the proliferation, expansion, and mature zones were all present within the leaf) demonstrated that the increase in cell size within the cell expansion zone had a quite similar slope in both cases (Figure 3C). These observations therefore suggested that not the speed, but rather the duration of cell expansion was reduced under drought stress (Figure 3C), and that drought stress prompts leaves to stop expanding after a shorter period of expansion. The developmental switch from expansion to maturity thus appears to occur earlier under drought stress. This indicates that the smaller leaf size under drought stress is mostly caused by physical constraints (i.e. a lack of sufficient turgor pressure in the leaf cells due to a highly reduced water availability), rather than by a deliberate physiological growth arrest instructed by the plant.

The Molecular Response to Drought Strongly Depends on the Developmental Stage

To identify the molecular changes underlying the growth reduction that we observed in *Brachypodium* leaves under drought stress, we performed Affymetrix tiling array analysis on RNA samples from proliferating, expanding, and mature zones of both control and stressed leaves (Supplemental Table 3). Three biological replicates of each developmental zone were sampled in control and severe drought stress conditions, two biological replicates in the mild drought stress condition. As previously reported for *Arabidopsis* leaves (Skirycz et al., 2010), the drought response is highly dependent on the developmental stage. Also in *Brachypodium*, there is only a very limited overlap between—on the one hand—genes of which the expression is modulated in the proliferation zone and—on the other hand—genes that are differentially expressed in the cell expansion and mature zones. Apart from developmental differences in the transcriptome, we identified numerous transcripts that were drought-responsive in specific developmental zones (Figure 4 and Supplemental Table 3). Overall, the effect of severe drought on gene expression was most pronounced in the mature leaf zone, where we detected the significant up- or down-regulation of 457 transcripts. In the expansion zone, 300 transcripts were affected by drought, compared to 121 in the proliferation zone (Supplemental Table 4). Only 20 transcripts responded generally to drought in all three leaf zones. Remarkably, the effect of moderate drought stress was far smaller on the transcript level, especially in the growth zone of the leaf: only 16 transcripts were affected in the proliferation zone, 15 in the elongation zone, and 204 in the mature zone.

Of the genes that were differentially regulated by severe drought stress, 94 were also affected by moderate drought. Severe drought affected an additional 656 genes, whereas

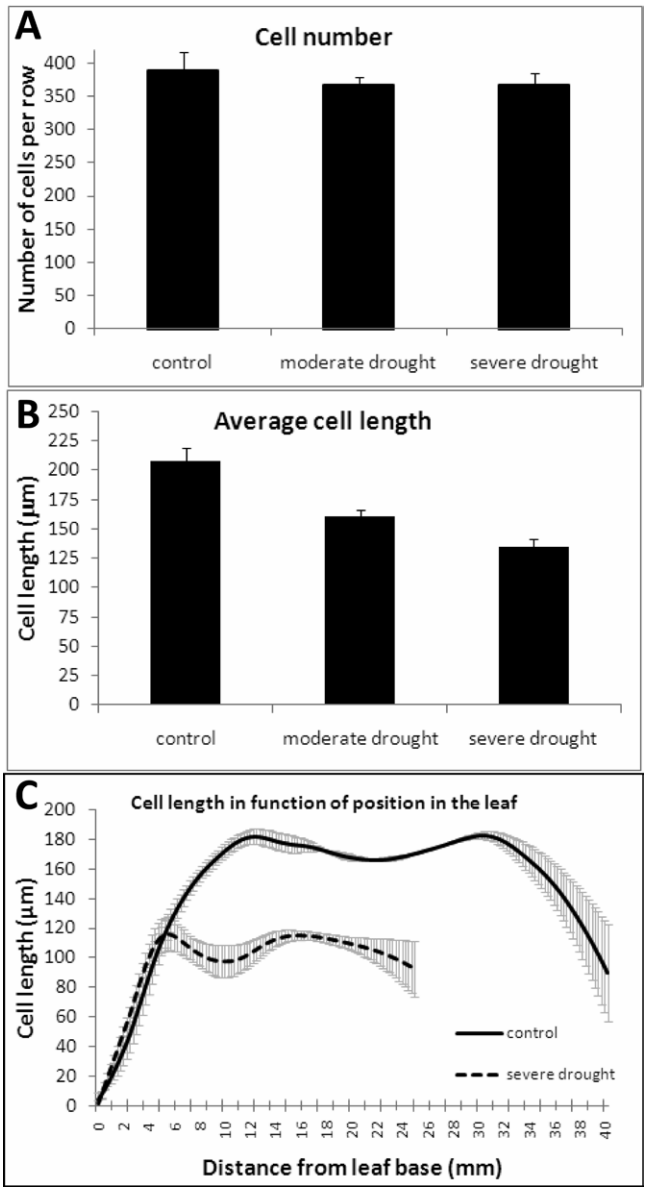

Figure 3. Drought Stress Mainly Affects Final Cell Size, Not Cell Number in *Brachypodium*.

(A) illustrates that the average cell number in the third leaf (counted in a single cell file from leaf base to leaf tip) is not significantly different under control and drought conditions, whereas average cell length **(B)** is greatly reduced by drought stress. In **(C)**, the average cell length is plotted as a function of the cell's position in the leaf, whereby the *x*-axis indicates the distance from the leaf base. This graph indicates that leaf cell expansion occurs at the same rate under control and drought stress conditions, but that cell expansion in stressed leaves is arrested after a shorter period of time. This shorter expansion period accounts almost entirely for the smaller mature size of drought-stressed leaves. Measurements in (A) and (B) are taken from six leaves per treatment, from a representative experiment, and from three leaves per treatment in (C).

134 genes were only affected by moderate drought. Of the 16 genes that were affected by moderate drought in the proliferation zone, 13 were also affected by severe drought. In the expansion zone, moderate drought only affected 15 genes, and these were all also affected by severe drought. In the mature leaf zone, the difference between moderate and severe drought was most pronounced: only 73 genes were affected by both conditions, while 677 genes were only affected by severe drought and 131 genes only by moderate drought.

To corroborate our tiling array results, we first investigated the transcript levels of eight selected, differentially expressed genes by quantitative RT–PCR, using RNA from leaf samples from an independent biological experiment. We used three validated reference genes to standardize gene expression across the samples (as described in the Methods section). The results—shown in Supplemental Figure 1—confirmed the observations for these genes in our tiling array analysis.

In the leaf's proliferation zone, drought stress had no effect on the expression level of genes related to cell division, which is in agreement with our observation that cell division remains unaffected in drought-stressed *Brachypodium* plants (Supplemental Table 5).

We observed that drought stress has a strong effect on various genes from the lipid and sterol biosynthesis pathways. In the proliferation zone, a lipoxygenase gene (Bradi1g09270) was strongly up-regulated, and the most drought-responsive gene in our entire data set was a C4-methylsterol oxidase (Bradi4g23800), which plays an important role in the synthesis of phytosterols. In the expansion zone, two other genes involved in sterol biosynthesis were up-regulated: sterol-4-alpha-carboxylate 3-dehydrogenase (Bradi3g18240) and estradiol 17-beta-dehydrogenase (Bradi1g43160). These observations at the transcriptome level suggest the overall up-regulation of sterol synthesis in response to drought. Physiologically, an increase in sterols likely influences membrane fluidity (Schaller, 2003), which is a classic protective response in times of decreased water availability. Another lipid-related gene that was induced by drought stress in the expansion zone was a wax synthase (Bradi2g17080). This gene plays a very important role during drought, as it produces the complex lipid polymers of which the cuticle is composed, and which greatly restrict the loss of water through the plant's surface (Kunst and Samuels, 2009; Buschhaus and Jetter, 2011). In addition, three aquaporin genes were down-regulated (Bradi3g30540, Bradi5g18170, and Bradi1g75290).

Apart from the classical molecular responses to drought, such as the up-regulation of wax synthase and of the anti-oxidant metabolism, we also observed the up-regulation of trehalose synthesis: a trehalose-6-phosphate synthase (Bradi1g69420) and (in the mature zone) a trehalose synthase (Bradi2g19710) gene. The accumulation of trehalose is thought of as a protective strategy against the effects of severe drought, which is used by various desiccation-tolerant plants, fungi, and invertebrates (Garg et al., 2002). However, in yeast, trehalose is neither necessary nor sufficient for desiccation tolerance (Ratnakumar and Tunnacliffe, 2006). Trehalose has previously also been reported in a desiccation-tolerant

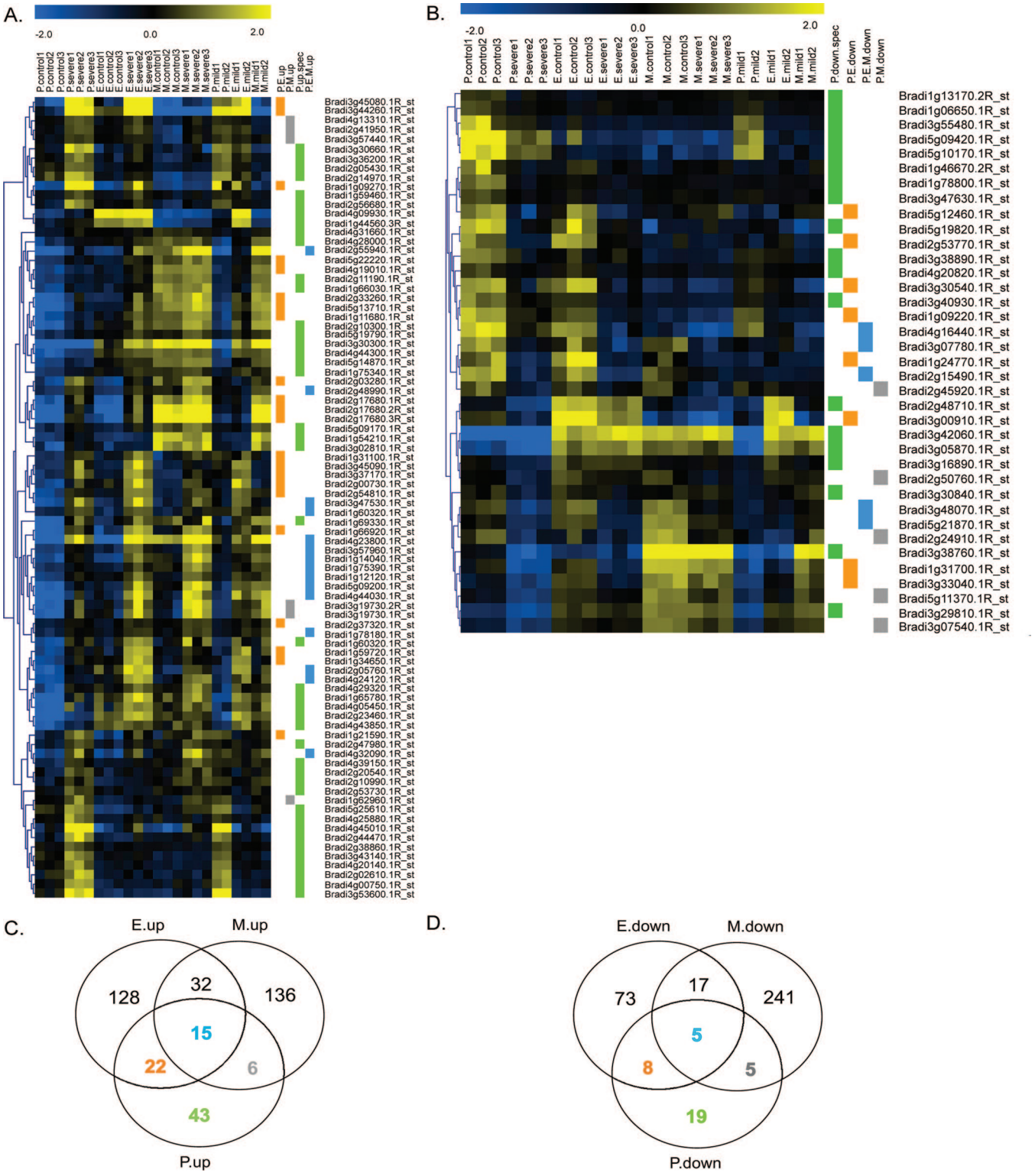

Figure 4. Molecular Response to Drought Stress in the Developmental Zones of *Brachypodium* Leaves.
The expression profile of the up- **(A)** and down-regulated **(B)** genes of severe drought response in the proliferation zone are displayed using heatmap. Log2 transformed, mean-centered expression values are shown. Rectangles next to the heatmap indicate the presence of each of the genes in the different Venn diagram sections. The Venn diagrams show the number of up- **(C)** and down-regulated **(D)** genes in the proliferation (P), expansion (E), and mature (M) zones of the third leaf of Bd21, when comparing control and severe drought stress conditions. The molecular response to drought stress is highly dependent on the developmental stage, as there is little overlap between the genes whose transcript level is affected by drought stress in all three leaf zones.

grass species from Mexico, *Sporobolus atrovirens* (Iturriaga et al., 2000). Since transgenic overexpression of a trehalose-6-phosphate synthase gene resulted in an improved abiotic stress tolerance in rice, in increased trehalose and proline concentrations, and in an altered expression of stress-related genes (Li et al., 2011), the natural drought tolerance of Bd21 may—at least in part—be related to its ability to up-regulate trehalose synthesis in adverse conditions.

Intriguingly, in addition to acting as an osmoprotectant, trehalose also influences the biosynthesis of storage carbohydrates (Iordachescu and Imai, 2008; Smeekens et al., 2010). In our experiments, we observed the up-regulation of various genes that are functionally involved in starch and sucrose metabolism. These include two sucrose synthase genes (Bradi1g62960 and Bradi1g60320), two glucose-1-phosphate adenylyltransferase genes (Bradi2g14970 in the proliferation zone and Bradi4g27570 in the expansion zone), and a starch synthase gene (Bradi2g41590) in the expansion zone.

Furthermore, we detected the strong up-regulation of cystathionine gamma-synthase (CGS, catalyzing the first step in methionine biosynthesis), of methylthioribose kinase (Bradi5g25610, involved in an alternative pathway for methionine synthesis), and of asparagine synthase (Bradi4g45010) in drought-stressed Bd21 leaves, most markedly in the proliferation zone. In *Brachypodium* leaves, the increased expression of methionine and asparagine biosynthesis genes was accompanied by the down-regulation of genes encoding an amino acid transporter (Bradi2g24910) and a peptide transporter (Bradi3g33040). Recently, levels of amino acids were shown to increase in leaf tissues of three different wheat cultivars exposed to drought stress (Bowne et al., 2012). On the other hand, the up-regulation of genes involved in amino acid biosynthesis is in apparent contrast to the recent report by Aranjuelo et al. (2011), which described that drought stress reduces the content of asparagine and other amino acids in alfalfa leaves.

We generally observed the up-regulation of various pathways that reflect a high energy content (such as starch, sucrose, and amino acid synthesis pathways), which indicates that the energy availability increases in the proliferation zone of drought-stressed *Brachypodium* leaves. Although this tendency has also been observed in *Arabidopsis* (Skirycz et al., 2010), drought stress has a strong negative effect on cell division in *Arabidopsis*, but not in *Brachypodium*. In *Arabidopsis*, drought stress appears to affect both developmental transitions, from proliferation to expansion and from expansion to maturity, while, in *Brachypodium*, only an effect on the latter transition is seen.

In the expansion zone, we also found that the expression of various genes related to cell wall formation and expansion was elevated by drought, such as a pectinesterase inhibitor (Bradi3g45080), a 1,3-β-glucan synthase (Bradi1g76620), and a xyloglucan galactosyltransferase (Bradi1g75450) gene.

Another interesting observation was the effect of drought stress on cytokinin and gibberellic acid (GA) metabolism. While our study revealed only minor effects on hormone synthesis genes, it did indicate an elaborate regulation at the level of hormone turnover and signaling. Our data suggest that cytokinin degradation in the apoplast may be affected by drought, through the concerted regulation of various genes. A cytokinin dehydrogenase gene (Bradi2g60460) was specifically up-regulated in the expansion zone, along with a laccase gene (Bradi1g66720). Both enzymes have been proposed to function together in the apoplast to degrade cytokinins (Galuszka et al., 2005). Interestingly, two other cytokinin dehydrogenase genes (Bradi2g51530 and Bradi3g29130) were down-regulated, while, in the mature zone, a cytokinin-*O*-glucosyltransferase gene (Bradi5g16100) was up-regulated. The latter enzyme reversibly converts cytokinins to *ortho*-glucosyl derivatives, thereby making them resistant to degradation by cytokinin oxidases (Martin et al., 1999). This observation suggests that cytokinin concentrations in the apoplast may be differentially affected in the expansion and mature zones of the drought-stressed Bd21 leaf.

Cytokinin activity promotes cell expansion through cell wall loosening (Thomas et al., 1981), and hence the tight apoplastic control of cytokinin levels may be the key mechanism through which cell expansion is controlled in *Brachypodium* leaves. This could also explain why the suppression of cell expansion is reversible for a long period of time, almost until full maturity of the leaf (data not shown): no major changes in gene expression are required, as the regulation may occur at the level of cytokinin dehydrogenase, laccase, and cytokinin-*O*-glucosyltransferase activities, whose transcript levels are elevated in the expansion zone of drought-stressed Bd21 leaves.

Based on transcript profiling, GA synthesis appears to be drought-induced in the growing parts of the leaf: the gibberellin-2-beta-dioxygenase (GA2ox, Bradi2g50280, involved in gibberellin catabolism) was down-regulated in the proliferation and expansion zones, accompanied by a modest up-regulation of GA20ox (Bradi1g14580, involved in GA synthesis) transcript levels throughout the leaf. Direct measurement of GA levels and GA metabolites needs to confirm the biological significance of these observed changes in gene expression. Because GA is also known to promote cell expansion by cell wall loosening (Behringer et al., 1990), an increased cell expansion would be expected as a result of this molecular response. However, we observed that the stressed leaves remained much smaller than control leaves, and that they stopped expanding earlier than control leaves. In contrast to our findings in *Brachypodium*, environmental stress has previously been shown to activate the expression of the GA catabolic enzymes—GA-2-oxidases (GA2ox)—that reduce GA levels and in turn stabilize DELLA proteins and repress growth (Achard et al., 2008; Magome et al., 2008). However, we also observed a complex differential response for putatively different isoforms of the GA receptor GID1L2: Bradi1g56860 and Bradi4g32300 were down-regulated in response to drought, while Bradi4g32310 was up-regulated. This suggests that the

lack of a GA effect on cell expansion was the result of a more downstream regulation, acting not on the degradation of GA, but on GID1L2 signaling. Taken together, these observations indicate that drought stress causes concerted and specific changes in GA levels and in the sensitivity of growing leaf tissues to GA.

In conclusion, our molecular analysis provided valuable indications about the elusive mechanisms with which *Brachypodium* protects its growing leaf tissues against the negative impact of drought stress. The results and tools provided by this study can serve as a starting point for a better understanding of drought-tolerance mechanisms in grasses, which can ultimately lead to the durable improvement of crop species. Due to its robustness and reproducibility, our drought stress protocol can be a useful tool for the research community. Although, in this study, it was mainly used to study the physiological and molecular effects of drought stress on leaf growth and development in the model accession Bd21, this highly standardized assay can also be used to compare the relative drought tolerance of different *Brachypodium* accessions, mutants, or transgenic lines, for example. Pilot studies have revealed a great amount of natural variation for agriculturally relevant traits (Opanowicz et al., 2008) and Luo et al. (2011) reported that the phenotypic variation in drought tolerance is also quite large between various accessions of *Brachypodium*. A systematic comparative approach may thus lead to the identification of the genetic factors that underlie this natural variation. The tiling array data sets—along with the standardized protocols—can hereby serve as a reference, which will greatly facilitate the identification of potentially relevant transcripts in the leaf proliferation and expansion zones of other *Brachypodium* accessions with a higher or lower tolerance to drought, for example.

METHODS

Standardized Growth Protocol for Bd21 in Soil

After harvest, *Brachypodium distachyon* Bd21 seeds were air-dried and stored at room temperature for 2 weeks. Then they were transferred to 4°C for at least 2 weeks, before being used for experiments. After manual removal of palea and lemma, the seeds were placed loosely on wet soil. We used peat pellets (Jiffy-7, 44-mm diameter; Jiffy Products), which were first soaked in distilled water until saturated; then the soil was taken out of the Jiffy and spread evenly on a large Petri dish (145/20 mm), on top of which the seeds were placed. Sealed with micropore tape, the plates were incubated at 4°C for 2 d, in darkness. Subsequently, the plates were transferred to a growth room, with 16 h of light (120 μmol $m^{-2} s^{-1}$ photosynthetically active radiation), at 24°C and 55% relative humidity, where the plants remained for the duration of the experiments. After 3 d, when all seeds had germinated synchronously, the lid was removed to let plantlets accustom to the ambient atmosphere in the growth chamber.

The next day (when the first leaf was about 3 cm in length), individual plantlets were carefully transferred to small plastic pots (5.5-cm diameter, 5 cm high), which contained 11 g soil (potting compost; Beroepspotgrond, Saniflor), to which 25 ml water had been added. The soil had first been air-dried, and adding water to the dry soil a day before plantlet transfer ensured that soil and water could properly mix. Just before plantlet transfer, the pot weight was adjusted to the desired weight with water. At the time of plantlet transfer, all pots contained 2.27 g water per g dry soil.

After the transfer of the seedlings, water was withheld from all pots. Pots containing control plants were dried down to 1.82 g g^{-1}, while pots containing plants that would be subjected to drought stress were dried down further, down to 0.73 g g^{-1} (moderate drought) or 0.45 g g^{-1} (severe drought). Pot weight was monitored on a daily basis, and water was added only after the target weight had been reached, and just enough to readjust the pot weight to the target level and to compensate for evaporation. Care was taken not to deposit the water in the direct vicinity of a plant, but rather on the outer edges of the pots, and watering always occurred about 8 h after the beginning of the light period, so as not to interfere with the time of most active plant growth (during the early morning hours).

Two to three days later, the second leaf emerged in a highly reproductive manner and, another 3 d later, the third leaf appeared. In our hands, nearly all plants grew synchronously, and the third leaf of plants within the same experiment always appeared within a 24-h time window.

Leaf Growth Measurements

The growth kinetics of the third leaf were measured on a daily basis. The distance between the leaf tip and soil level was measured for at least 15 similarly treated plants, every day at about 8 h after the beginning of the light period. When the third leaf had reached maturity, all collected data points were interpolated using a custom-made excel macro.

For microscopic examination, leaves were first incubated overnight at room temperature in 100% ethanol. Subsequently, the leaves were transferred to lactic acid (De Veylder et al., 2002; Rymen et al., 2010). The total number of epidermal cells was counted along one cell row adjacent to a vasculature bundle, on the abaxial side. In the Bd21 epidermis, two regular cells are nearly always separated by a much smaller hair cell (Supplemental Figure 2); for the sake of consistency, a regular cell was therefore always taken together with its neighboring hair cell as one unit, and they were counted together as a single cell.

To calculate the average mature cell length, the total length of the leaf (from base to tip) was divided by the total number of cells along its length. Due to high variability in mature cell size, which was especially pronounced towards the leaf tip (as illustrated in Figure 3C), direct cell size measurements proved to be not very useful.

Bd21 Leaf Sampling for Molecular Analysis

Plants were harvested at a fixed time point in the afternoon, about 24 h after the emergence of the third leaf. The growing third leaf—between 1.5 and 2 cm in size at that point—was carefully removed from the leaf sheath of the older two leaves, without damaging the fragile meristem at its base. Samples were immediately stored in RNA-*Later* solution (Ambion, Austin, Texas). After overnight incubation at 4°C, the leaves were dissected into three distinct developmental zones with a sharp razor blade. Based on microscopic observations, we defined the *proliferation zone* as the first 2 mm from the leaf base, the *expansion zone* as the next 4 mm, and the *mature zone* as the remaining distal part of the leaf. The leaf zones—pooled from at least 10 individual plants—were immediately frozen at –80°C, and stored until RNA isolation could be performed. This sampling approach was taken in four independent experiments, resulting in three independent biological replicates that were subsequently used for tiling array hybridization, and a fourth biological replicate that was used for confirmatory qPCR analysis.

Real-Time qPCR

Total RNA was isolated using the RNeasy protocol (Qiagen, Hilden, Germany) and RNA quality, purity, and quantity were verified using a Nanodrop (Thermo Fisher, Waltham, MA, USA). cDNA was prepared from 1 μg RNA, using the iScript™cDNA Synthesis Kit (Bio-Rad, Roche Diagnostics) and subsequently used in a quantitative RT–PCR (qPCR) reaction. qPCR was performed in a LightCycler 480 (Roche Diagnostics) on 384-well plates with LightCycler 480 SYBR Green I Master (Roche) according to the manufacturer's recommendations. Primers were designed using the Primer 3 (Rozen and Skaletsky, 1999) website (Supplemental Table 6). Melting curves were analyzed to check primer specificity.

As reference genes for standardization, we used an actin gene (Bradi4g41850), an EF1α gene (Bradi1g06860), and a ubiquitin gene (Bradi1g32860), which we had previously identified as suitable housekeeping genes (Hong et al., 2008; Coussens et al., 2012). Primer sequences can be found in Supplemental Table 6. The quantification of the expression level of each gene was normalized to the average of these housekeeping genes. ΔCt values were used for the statistical analysis and the relative expression level was expressed as $2^{-\Delta Ct}$. Ct refers to the number of cycles at which SYBR Green fluorescence reaches an arbitrary value during the exponential phase of the cDNA amplification.

Tiling Array Hybridization

RNA concentration and purity were determined spectrophotometrically using a Nanodrop ND-1000 (Nanodrop Technologies) and RNA integrity was assessed using a Bioanalyzer 2100 (Agilent). Using the Ambion WT Expression Kit, per sample, an amount of 100 ng of total RNA spiked with bacterial poly-A RNA positive controls (Affymetrix) was converted to double-stranded cDNA in a reverse transcription reaction. Next, the sample was converted and amplified to antisense cRNA in an *in vitro* transcription reaction which was subsequently converted to single-stranded sense cDNA. Finally, samples were fragmented and labeled with biotin in a terminal labeling reaction according to the Affymetrix WT Terminal Labeling Kit. A mixture of fragmented biotinylated cDNA and hybridization controls (Affymetrix) was hybridized on a custom Affymetrix Brachypodium Tiling array, followed by staining and washing in a GeneChip® fluidics station 450 (Affymetrix) according to the manufacturer's procedures. To assess the raw probe signal intensities, chips were scanned using a GeneChip® scanner 3000 (Affymetrix).

Tiling Array Analysis

Expression data were processed with Robust Multichip Average (RMA; background correction, normalization, and summarization) as implemented in Affymetrix Power Tools (apt-probeset-summarize) (Irizarry et al., 2003a, 2003b; Gentleman et al., 2004). The BioConductor package Limma was used to identify differentially expressed genes (Smyth, 2004). Pairwise comparisons between conditions were tested with moderated *t* statistics and the eBayes method as implemented in Limma. *P*-values were corrected for multiple testing (for each contrast separately with topTable; Hochberg and Benjamini, 1990). Genes are considered to be differentially expressed when the corrected *p*-value is smaller than 0.05.

Apart from the gene descriptions provided by the International Brachypodium Initiative, functional annotations were also retrieved using the PLAZA comparative genomics platform (Van Bel et al., 2012). Based on orthologous rice genes identified using the Integrative Orthology Viewer (considering phylogenetic trees, OrthoMCL families, and Best-Hits-and-Inparalogs families from PLAZA 2.5), InterPro protein domain and Gene Ontology information was used to study the functional role of the differentially expressed *Brachypodium* genes.

Accession Numbers

Microarray data have been deposited in the NCBI Gene Expression Omnibus, and the GEO series accession number is GSE38247.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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