Screening for drought tolerance of maize hybrids by multi-scale analysis of root and shoot traits at the seedling stage

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Abstract

We studied the drought response of eight commercial hybrid maize lines with contrasting drought sensitivity together with the reference inbred line B73 using a non-invasive platform for root and shoot phenotyping and a kinematics approach to quantify cell level responses in the leaf. Drought treatments strongly reduced leaf growth parameters including projected leaf area, elongation rate, final length and width of the fourth and fifth leaf. Physiological measurements including water use efficiency, chlorophyll fluorescence and photosynthesis were also significantly affected. By performing a kinematic analysis, we show that leaf growth reduction in response to drought is mainly due to a decrease in cell division rate, whereas a marked reduction in cell expansion rate is compensated by increased duration of cell expansion. Detailed analysis of root growth in rhizotrons under drought conditions revealed a strong reduction in total root length as well as rooting depth and width. This was reflected by corresponding decreases in fresh and dry weight of the root system. We show that phenotypic differences between lines differing in geographic origin (African vs. European) and in drought tolerance under field conditions can already be identified at the seedling stage by measurements of total root length and shoot dry weight of the plants. Moreover, we propose a list of candidate traits that could potentially serve as traits for future screening strategies.

Key words: Kinematics, leaf growth, phenotyping, rhizotron, root growth, water deficiency.

Introduction

Limited water availability is one of the main factors restricting crop production (Boyer, 1982; Tollenaar and Lee, 2002) and it is predicted to become an increasing problem under future climate conditions (Burke *et al.*, 2009; Lobell *et al.*, 2011). Consequently, there is an urgent need to breed more drought-tolerant crops to meet future demands of the

growing world population, for which efficient screening strategies are necessary.

Trait-based selection or ideotype breeding is one such efficient strategy where, by examining the variation between lines with different performance under optimal and drought stress conditions, the interaction between genotype and environment can be elucidated and key phenotypic traits can be chosen as breeding criteria (Lynch, 2007; York *et al.*, 2013; Chimungu *et al.*, 2014).

Efficient phenotyping remains one of the main factors limiting breeding advances (Araus *et al.*, 2012). In this process it is crucial to identify a core set of key parameters to measure plant performance, instead of measuring a large number of data points, which could be highly autocorrelated or not indicative of the target trait (Fiorani and Schurr, 2013). The advance of the non-invasive phenotyping technologies (in particular, 2D and 3D imaging systems) in recent years allowed analyses of dynamic processes such as the acclimation of shoot and root growth and development in response to variations of environmental conditions (Nagel *et al.*, 2009, 2015; Pfeifer *et al.*, 2014). These phenotyping techniques yield crucial information on plant performance to assess genetic predisposition of the different varieties, and to analyze the effect of environmental conditions on the development of a plant phenotype (Nagel *et al.*, 2012).

In addition to whole plant/organ level growth analysis, cell level analyses are of considerable scientific interest as they provide a link between molecular processes and whole plant phenotypes (Nelissen *et al.*, 2013). The maize leaf provides an ideal model system for such studies because different developmental stages can be examined on a single leaf due to its spatial gradient spanning dividing, expanding, and mature tissues (Benhajsalah and Tardieu, 1995; Granier *et al.*, 2000; Rymen *et al.*, 2007; Li *et al.*, 2010). Kinematic analysis (Silk and Erickson, 1979) allows a detailed quantification of cell division and elongation along this gradient, providing a mechanistic insight into the cellular basis of whole organ phenotypes. Moreover, this spatial characterization facilitates effective sampling of equivalent developmental stages for further molecular and physiological studies (Nelissen *et al.*, 2013; Avramova *et al.*, 2015*b*).

Together, imaging-based whole plant phenotyping and kinematics have proven their effectiveness for the analysis of the response to environmental conditions and genetic variations in basic research. This raises the questions: (i) whether using these approaches could help us to better understand the impact of drought on plant growth, (ii) whether these methods allow us to determine differences in drought sensitivity of crops at the stage of early seedlings and (iii) which phenotypic traits could be useful for pre-screening of drought tolerance during the early seedling stage in breeding programs for well-defined environmental scenarios.

To address these questions, we phenotyped eight maize hybrids with different drought sensitivity from Western Europe and from South Africa under well-watered and drought conditions. Because it is well known that hybrids grow significantly better under all conditions compared to inbred lines, we included the reference inbred maize line B73 to increase the expected phenotypic variation in our experiments. We expected the African lines to be more drought-tolerant than European lines and to be able to confirm the tolerance ratings provided by the breeder according to field trials. Moreover, we expected to find parameters from the imaging and kinematic analyses that could potentially serve as traits for future screening strategies at the seedling stage.

Materials and methods

Maize lines

We used nine maize (*Zea mays*) lines as a basis for our studies: the inbred line B73 (Iowa Stiff Stalk Synthetic), four hybrids, derived from Western Europe: PR39D23 (EU1), P7345 (EU2), PR39T83 (EU3), PR39F58 (EU4), and four from South Africa: 33H56 (AF1), 33Y74 (AF2), 3442 (AF3), 31MO9 (AF4). Seeds from the hybrid maize lines were generously provided by DuPont Pioneer. Based on field trait evaluation, four of them were rated as drought tolerant (EU1, EU4, AF1, AF3, see Supplementary Table S1 available at *JXB* online). For more detailed information about the hybrid lines see Supplementary Table S1.

Growth conditions

Two complementary sets of experiments were conducted: (i) rhizotron experiments focusing on imaging-based whole plant level characterization and (ii) growth room experiments focusing on cell-level kinematic analysis.

Growth experiments in rhizotrons

Maize seedlings were germinated on filter paper until primary roots reached a length of ~2 cm (about five days at 22–25°C). At this stage, to eliminate potential seed size effects, seedlings of approximately the same size for all genotypes were selected and transferred into rhizotrons, filled with peat potting medium (Jiffy, The Netherlands) either with 54% Soil Water Content (SWC) as optimal condition or pre-dried to 34% SWC as drought stress treatment, respectively. The experiment encompassed a full factorial design of nine lines, two conditions (optimal and drought stress), and four replicate plants. The rhizotrons were part of the GROWSCREEN-Rhizo setup at Forschungszentrum Jülich GmbH, Germany (Fig. 1), described in Nagel *et al.* (2012). Briefly, the rhizotrons consisted of black polyethylene and one transparent polycarbonate plate, with outer dimensions of 90 × 70 × 5 cm, and were inclined by 43° towards the horizontal plane with the transparent plate facing downwards in order to stay covered throughout the plant's growth period.

Plants were grown in the PhyTec greenhouse of the Institute for Plant Sciences (IBG-2; Forschungszentrum Jülich GmbH, Jülich, Germany), which is fitted with micro-structured glass that has a high transparency for ultraviolet (UV) and photosynthetically active radiation (PAR; for more details see Nagel *et al.*, 2012).

Plants were grown without additional watering until leaf four was fully expanded. Climate conditions in the greenhouse were monitored during the experiment and PAR was around 500 µmol m^{-2} s^{-1} (Supplementary Fig. S1). The open top surface of the rhizotrons was covered with a 2 cm layer of 2.5–4 mm white plastic beads in order to prevent evaporation of water from the soil. We stopped the experiment when leaf 4 reached its final size because, for most genotypes, the deeper roots reached the bottom of the rhizotron at this developmental stage. Therefore, all leaf measurements were performed on the fourth leaf. 2D images of the shoot and the root system of every plant were automatically acquired daily in the GROWSCREEN-Rhizo setup and the resulting image sequences were analyzed using the software GROWSCREEN-Root (Muhlich *et al.*, 2008; Nagel

Fig. 1. GROWSCREEN-Rhizo setup at Forschungszenrum Jülich GmbH, Germany. 72 rhizotrons are aligned in two rows in the greenhouse. The inclination angle of the rhizotrons is 43° with the transparent plate of the rhizotrons facing downwards. Between both rows of rhizotrons, an imaging cabinet is moved automatically along a linear axis. The rhizotrons are drawn in a user-defined order inside the cabinet for image acquisition of roots and shoots (Nagel *et al.*, 2012).

et al., 2012). The software allows extraction of root traits, such as total visible root length, maximum depth, width and root surface coverage, which was calculated by extracting the convex hull area of the whole visible root system, as well as differentiating between the length of the primary, seminal and lateral roots, branched primary and seminal roots, and calculating root elongation, which is the relative difference in the total root length between two time points (the day after transplantation of the pre-germinated seeds into the rhizotrons and the day of the harvesting) per hour.

Color images were used to quantify projected shoot area, which was determined automatically with custom-made algorithms (for details see Nagel *et al.*, 2012; Walter *et al.*, 2007). Shoot growth rate was calculated as the relative difference in shoot area between two time points (the day after transplantation of the pre-germinated seeds into the rhizotrons and the day of the harvesting) per hour. The length of the fourth leaf was measured daily with a ruler from the moment it appeared from the whorl of the older leaves until it reached its final size. Leaf elongation rate (LER) was calculated between two time points during the steady-state growth of the leaf (day 2 and 3). Final leaf width was measured with a ruler at the day of the harvesting.

Rhizotrons were weighed at the beginning and at the end of the experiment to calculate the water use (WU), the total amount of the transpired water during the growth period of the plants.

To correlate visible roots from 2D imaging with total root length and biomass, roots were washed at the final harvest, manually cleaned of clumps of soil and scanned (600 dpi, flatbed scanner, Canon Scan LIDE 60, Canon, Krefeld, Germany). Total root-system length and average root diameter were then determined using the WinRHIZO software (version 2012, Regent Instruments; settings: gray value threshold 30; removal of objects with an area <1 cm^2 and a length-width-ratio <4).

The plants were harvested when the fourth leaf was fully expanded (i.e. the same leaf length was measured on two subsequent days), defining the final leaf length, FLL. Shoot fresh weights were measured at the moment of harvesting. Root and shoot dry weights were determined after samples had been oven-dried at 70°C until constant weight was reached. Shoot water content was calculated as the percentage difference between the fresh and the dry shoot weight by the time of the harvesting.

Photosynthesis and chlorophyll content measurements—Net photosynthetic rate was measured with a portable leaf gas exchange system (LI-6400, LI-COR Inc., Lincoln, NE, USA) on the exposed/mature part of the fourth leaf, at the day when it reached its final size. The CO_2 concentration and temperature in the leaf chamber were maintained at 400 µmol mol^{-1} and 25 ± 0.5°C, respectively. The measurements were conducted at photon flux density of 1500 µmol m^{-2} s^{-1} applied by a red-blue light-emitting diode (LED) light source (LI-6400-02B LED; LI-COR) and at ambient relative humidity.

Chlorophyll content was estimated using a SPAD-502 Chlorophyll Meter (Konica Minolta, Inc.) at three positions along the leaf axis (base, middle and tip) of leaf 4 when it reached its final size. These values were averaged for each plant. To minimize variation in parameter values due to diurnal light intensity fluctuations, all photosynthetic parameters were measured between 10:00 and 12:00 a.m.

Growth room experiment

Seeds were directly sown in peat potting medium (Jiffy, The Netherlands; volume of the pots: 1.5 l, dimensions: 15.1 × 11.9 cm), germinated and grown in a growth room under controlled conditions. Day/night temperatures of 25/18°C were maintained and seedlings were grown at 16 h light (350–400 µmol m^{-2} s^{-1} PAR at leaf level, provided by high pressure sodium lamps)/8 h dark cycles. Air humidity was kept constant at 30%. Pots with plants from different lines/treatments were randomly positioned. Control plants were re-watered daily to maintain SWC at 54% (pots were weighed and the lost water was added). Plants under severe drought treatments were not watered (for ~14 d) allowing the SWC to drop to 34%, which we established earlier as water deficient conditions leading to plant growth reduction (Avramova *et al.*, 2015*a*). Thereafter, the soil moisture content was maintained at this level by daily re-watering. All measurements were performed on the fifth leaf as it emerged from its surrounding leaves at around the time when the drought conditions were fully established in the pots. At least ten plants were used for each line and condition.

Both roots and shoots of the plants were harvested at the moment the fifth leaf was fully expanded. Shoot fresh weights were measured at the moment of the harvesting. Dry weights of shoots were determined after oven-drying at 70°C until constant weight was reached. Large aggregates of growing medium were removed from the roots to minimize the

Table 1. *Kinematic analysis of the effect of drought stress on cell division and cell expansion during the steady-state growth of the fifth leaf of eight maize hybrids and the reference inbred line B73*

Data for the hybrids are mean values across the respective line averages ±SD (n=8) and average only for B73. A two-way ANOVA was used as a statistical test and P-values for the two factors, genotype and treatment, as well as the interaction between them, are presented in the table. The ANOVA analysis was conducted twice: including the inbred line B73 (+B73) and excluding it (-B73). Parameters: *LL*, leaf length; *LER*, leaf elongation rate; L_{mat}, mature cell length; *P*, cell production rate; *D*, cell division rate; T_c, cell cycle duration; L_{mer}, length of the meristem; N_{mer}, number of cells in the meristem; T_{div}, time in division zone; R_{el}, cell elongation rate; T_{el}, time in elongation zone; T, treatment; G, genotype.

Parameters	Hybrids			B73			Two-way ANOVA	
	Control	Drought	% change	Control	Drought	% change	*P*-value (+B73) (T/G/T*G)	*P*-value (-B73) (T/G/T*G)
LL (mm)	989±32	522±25	−47	703	401	−43	0.00/0.00/0.00	0.00/0.00/0.00
LER (mm/h)	3.3±0.2	1.3±0.2	−61	3	1.5	−49	0.00/0.57/0.38	0.00/0.49/0.60
l_{mat} (µm)	142±11	115±12	−19	134	117	−13	0.00/0.00/0.05	0.00/0.00/0.08
P (cells/h)	24±2	11±3	−54	22	9	−58	0.00/0.00/0.15	0.00/0.00/0.00
D (cell/cell/h)	0.035±0.008	0.017±0.005	−53	0.029	0.016	−44	0.00/0.00/0.00	0.00/0.00/0.00
T_c (h)	21±5	48±19	+131	26	48	+84	0.00/0.00/0.00	0.00/0.00/0.00
L_{mer} (mm)	15±3	11±2	−27	13	10	−26	0.00/0.00/0.39	0.00/0.00/0.12
N_{mer}	705±160	658±75	−7	829	577	−30	0.04/0.00/0.00	0.36/0.00/0.09
T_{div} (h)	198±56	452±188	+128	253`	437	+73	0.00/0.00/0.00	0.00/0.00/0.00
R_{el} (µm/µm/h)	0.039±0.002	0.016±0.005	−58	0.039	0.02	−45	0.00/0.01/0.02	0.00/0.18/0.04
T_{el} (h)	49±7	126±46	+159	44	131	+195	0.00/0.01/0.00	0.00/0.00/0.00

error in root dry weight, which was determined for each plant after ~20 d of drying in the growth chamber (25°C, 30% air humidity).

Kinematic analysis—A kinematic analysis was performed on the fifth leaf of at least five plants for each genotype/treatment at the third day after its emergence from the whorl of older leaves (during its steady-state growth), following the protocol developed by Fiorani *et al.* (2000) and Rymen *et al.* (2010). This entails determining leaf elongation rate and final leaf length, the cell-length profile along the axis of the leaf, and estimating the size of the leaf basal meristem. The length of the fifth leaf was measured daily with a ruler from the time of its appearance to the time it reached its final length (FLL). LER was calculated during the third day after its appearance as the difference in length (L) divided by the time difference between successive measurements [(L_{d3}–L_{d2})/24 h]. For meristem size measurements, samples were stained with DAPI (4',6-diamidino-2-phenylindole) and analyzed with a fluorescence microscope (AxioScope A1, Axiocam ICm1, Zeiss) at 20× magnification. The length of the meristematic zone of the leaves was estimated by locating the most distal mitosis in the epidermal cells and measuring the distance between the base of the leaf and this position (Fiorani *et al.*, 2000). The length of cells in files adjacent to the stomatal files was measured by light microscopy (Scope A1 Axiocam ICm1, Zeiss), using differential interference contrast (DIC) optics at 40× magnification and the online measurement module in the Axiovision (Rel. 4.8, Zeiss) software. To this end, the growth zone of leaf five was divided into ten segments (1 cm each). Measurements were carried out at four locations in each segment: at the base (0 cm), at one-third (0.3 cm), at two thirds (0.6 cm) and at the top of the segment (1 cm). Around 20 cells were measured at each location. The raw data obtained for individual leaves were smoothed and interpolated to an interval of 1 mm using the kernel smoothing function locpoly of the Kern Smooth package (Wand and Jones, 1995) for the R statistical package (R Foundation for Statistical Computing), which allowed averaging between leaves and comparison between treatments. The calculations of cell division and expansion parameters were based on these data, as described earlier (Rymen *et al.*, 2010).

Statistical analyses

The statistical significance of the phenotypic values was determined for all parameters by means of a two-way ANOVA (Analysis of Variance with factors: genotype, treatment and interaction) using SPSS (Version 20, IBM). To allow for discrimination of the differences between the hybrids and between the hybrids and the B73 inbred line, respectively, the ANOVA was conducted twice, including and excluding B73. Additional three-way ANOVA was conducted for all parameters to discriminate of the differences between the hybrids based on their region of origin and drought sensitivity (excluding B73; Table 2).

To evaluate if the experiments performed under different experimental conditions were comparable, bivariate Pearson correlation analyses were performed in SPSS (Version 20, IBM) between the common parameters, measured in both experiments (FLL, LER, shoot fresh and dry weight, and dry root weight).

Principal Component analysis (PCA) of all measured parameters across the nine maize lines was performed by using XLSTAT (an add-in for Microsoft, Excel).

Results

Shoot growth

We set out to investigate the effect of drought on the growth of early maize seedlings and to determine if differences in growth responses to drought between genotypes are detectable at this stage. To this end, we compared the growth of eight hybrid maize lines with contrasting geographical background (European vs. African) and drought tolerance ratings and the inbred line B73 under optimal and drought conditions. The latter severely inhibits plant growth, but does not cause senescence (Avramova *et al.*, 2015a). The plants were harvested when the fourth leaf became mature, because at that time the roots of most lines reached the bottom of the rhizotrons.

Low water availability had a significant effect on seedling development. The plants were visibly wilting and shoot projected area was significantly reduced from the beginning of our observations under drought stress conditions in all lines, but no significant differences were observed between the genotypes (Fig. 2A).

Table 2. *A three-way ANOVA analysis of all parameters measured during the rhizotron and pot experiments, comparing the effects of region of origin of the eight maize hybrids, drought treatment and differences in drought tolerance between the hybrids (breeder's ranking, Supplementary Table 1) and the interaction between them. Significant differences (P<0.05) are marked in bold.*

Parameters	region	treatment	sensitivity	region*treatment	region*sensitivity	treatment*sensitivity	region*treatment*sensitivity
Parameters measured during the rhizotron experiment							
Final shoot projected area	0.24	**0.00**	**0.04**	0.65	0.10	**0.04**	0.06
Shoot growth rate	0.38	**0.00**	0.13	0.60	0.70	0.74	0.55
Final leaf length	0.09	**0.00**	**0.01**	0.31	0.81	0.18	0.43
Leaf elongation rate	0.49	**0.00**	0.26	0.16	0.54	0.37	**0.02**
Final leaf width	0.31	**0.00**	0.13	0.92	0.73	0.33	0.66
Growth period (d)	0.47	**0.00**	0.57	0.62	0.23	0.49	0.92
Number of leaves at harvest	0.30	**0.00**	0.10	**0.02**	**0.00**	0.19	0.10
Shoot fresh weight	0.24	**0.00**	**0.04**	0.62	**0.01**	**0.03**	**0.01**
Shoot dry weight	0.09	**0.00**	**0.02**	0.43	**0.01**	**0.03**	**0.01**
Shoot water content	0.58	**0.00**	0.85	0.30	0.40	0.94	0.70
Water use	0.68	**0.00**	0.80	0.44	0.17	**0.01**	0.12
Photosynthesis	0.34	**0.00**	0.07	0.89	0.69	**0.04**	0.86
Spad	0.05	**0.00**	0.44	0.16	0.84	0.82	0.92
Root dry weight	**0.02**	**0.00**	**0.04**	0.25	**0.01**	0.14	**0.02**
Shoot-root ratio	0.50	**0.00**	0.49	**0.02**	0.73	0.36	0.88
Total root length	**0.00**	**0.00**	**0.01**	0.10	**0.00**	**0.02**	**0.00**
Total visible root length	**0.01**	**0.00**	0.46	**0.04**	**0.03**	0.15	0.41
Root growth rate	0.77	**0.00**	0.50	0.45	0.36	0.44	0.19
Length of primary root	0.41	**0.00**	0.58	0.81	0.18	0.22	0.42
Length of lateral roots	**0.03**	**0.00**	0.50	0.15	0.05	0.13	0.16
Length of seminal and second order lateral roots	**0.00**	**0.00**	0.54	**0.01**	0.05	0.37	0.81
Average root diameter	**0.02**	**0.00**	0.14	0.47	0.63	**0.02**	0.72
Root system depth	0.79	**0.00**	0.47	0.24	0.94	0.09	0.56
Root system width	0.19	**0.00**	0.19	0.42	0.81	**0.00**	0.69
Root surface coverage	0.16	**0.00**	0.99	0.67	0.35	**0.01**	0.85
Parameters measured during the pot experiment							
Final leaf length	0.08	**0.00**	0.07	0.40	0.34	0.16	0.17
Leaf elongation rate	0.51	**0.00**	0.33	0.79	0.18	0.89	0.51
Meristem length	**0.00**	**0.00**	0.34	0.11	0.21	**0.01**	0.80
Length of the growth zone	**0.00**	**0.01**	0.36	0.05	0.43	0.08	**0.02**
Length of mature cells	**0.00**	**0.00**	**0.00**	0.13	0.12	0.70	0.13
Cell production rate	0.35	**0.00**	**0.04**	0.71	0.27	0.86	0.27
Number of cells in the meristem	**0.00**	0.16	0.47	**0.00**	0.65	0.21	**0.03**
Number of cells in the growth zone	0.98	0.58	0.08	0.29	0.81	0.61	0.31
Number of cells in the elongation zone	**0.00**	0.13	**0.01**	**0.00**	0.57	0.95	0.99
Average cell division rate	**0.00**	**0.00**	**0.01**	**0.00**	0.26	0.64	0.05
Cell cycle duration	**0.01**	**0.00**	0.26	0.34	0.20	0.65	0.10
Time in elongation zone	0.48	**0.00**	0.72	0.08	0.34	0.59	0.31
Time in division zone	**0.00**	**0.00**	0.29	0.38	0.18	0.73	0.07
Length of the cells leaving the meristem	**0.00**	**0.00**	0.60	0.06	0.84	0.10	0.95
Average cell expansion rate	0.35	**0.00**	0.20	**0.00**	0.45	0.47	0.29
Root dry weight	0.49	**0.00**	0.07	0.86	0.56	0.18	0.94
Shoot fresh weight	**0.00**	**0.00**	**0.00**	**0.02**	0.20	**0.00**	0.15
Shoot dry weight	**0.00**	**0.00**	**0.01**	**0.02**	0.06	**0.01**	**0.03**

After germination, shoot area increased nearly exponentially. Growth rates (Fig. 2B) differed significantly between the genotypes only when B73 was included in the analysis (P=0.03). No significant differences were detected between the hybrids. The drought treatment significantly reduced shoot growth rate (P<0.01; Fig. 2A, B). However, the

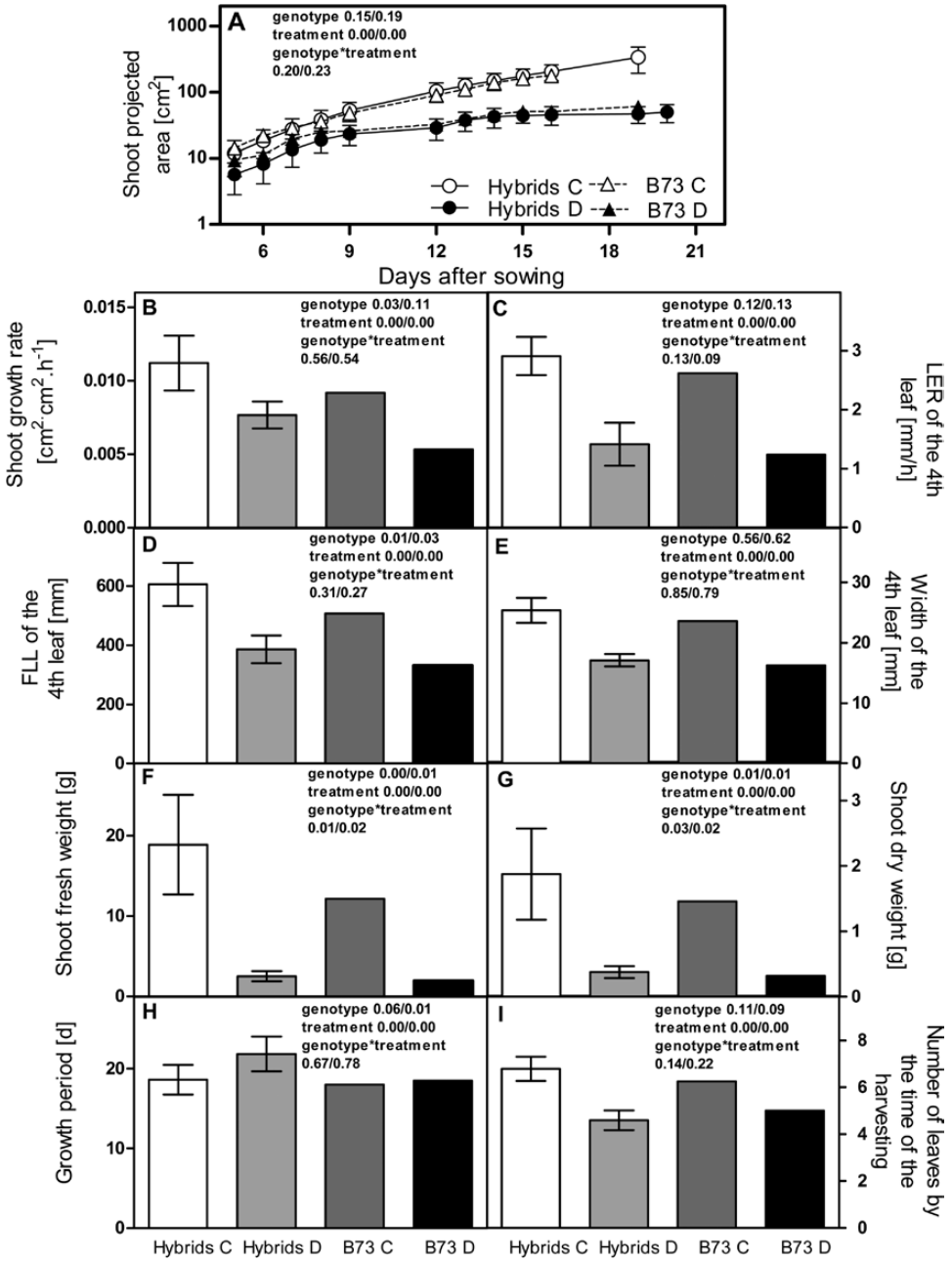

Fig. 2. The effect of drought on shoot-related traits of eight hybrid maize lines and a reference inbred line B73. (A) Shoot projected area, (B) shoot growth rate, (C) leaf elongation rate (LER), (D) final leaf length, (E) width of the fourth leaf, (F) shoot fresh weight, (G) shoot dry weight, (H) growth period, (I) number of leaves at the harvest time point. All parameters in the graph are measured on plants grown in rhizotrons. A two-way ANOVA was used as a statistical test and *P*-values for the two factors, genotype and treatment, as well as the interaction between them, are present in the graph panels. The ANOVA analysis was conducted twice (including B73/excluding B73). The statistical analysis in Panel A relates to the end point. Data for the hybrids are mean values across the respective line averages ±SD (n=8) and mean value only for B73. C, control conditions; D, drought conditions; LER, leaf elongation rate; FLL, final leaf length.

non-significant genotype × treatment interaction showed that differences in drought tolerance between the lines could not be detected based on this parameter.

To assess the effect of the stress at the level of individual organs, we analyzed the growth of the fourth leaf in more detail. Leaf elongation rate (LER) largely reflected the growth response at the whole shoot level and was strongly inhibited by drought (Fig. 2C). As a consequence, the final length of the leaves (FLL) was significantly reduced by the stress. Moreover, FLL was significantly different between the hybrids (P=0.03) and between the hybrids and the inbred line (P<0.01; Fig. 2D). No significant genotype × treatment interaction was found independent of whether or not B73 was included. Leaf width was significantly reduced by the stress (P<0.01), but no significant differences between the genotypes were detected (Fig. 2E). Based on these results we conclude that leaf level macroscopic observations could not discriminate differential responses to drought between the genotypes studied here.

As a result of the reduced leaf growth rates, the accumulated fresh and dry shoot biomass was strongly reduced by drought at

the time of harvesting (P<0.01; Fig. 2F, G). Significant effects of genotype and genotype × treatment were found regardless of the inclusion of B73, demonstrating that significant differences in drought response between the lines were present and could be detected based on biomass measurements (Fig. 2F, G). The effect of drought-induced inhibition of growth on biomass at the final harvest was partly offset by a longer duration for the plants to reach the stage when the fourth leaf reached its final size (Fig. 2H). Drought-stressed plants at that time had fewer visible leaves than controls (Fig. 2I), indicating differential effects on leaf growth and leaf appearance.

Physiological responses

Next, we measured physiological parameters that have previously been shown to respond strongly to drought treatments and may contribute to the observed growth responses (Schurr *et al.*, 2000; Huang *et al.*, 2013). As expected, shoot water content (Fig. 3A) and water use (Fig. 3B) were significantly decreased by the drought stress (P<0.01). However, there were no significant genotype or genotype × treatment interactions for these two parameters (Fig. 3A, B). Under drought-stressed conditions the decrease in SPAD values, which correlates with chlorophyll content (Ling *et al.*, 2011), was modest, but the differences between the hybrids and between the hybrids and the inbred line were significant (Fig. 3C). In contrast, photosynthetic rates were strongly reduced (~60%) by the reduced water availability (Fig. 3D), likely due to reduced stomatal conductance. Photosynthesis rates of B73 were significantly lower than those of the hybrids, particularly under drought conditions. Nevertheless, no significant genotype × treatment effects were found between the hybrids or even between B73 and the hybrids. This indicates that genetically determined differences in the drought response were too small to be detected by using these physiological parameters.

Cellular analysis

In earlier studies, kinematic analysis allowed determination of the cellular parameters underlying stress responses (Bernstein *et al.*, 1993; Sharp *et al.*, 1988; West *et al.*, 2004; Rymen *et al.*, 2007) and genetic variation (Volenec and Nelson, 1981; Fiorani *et al.*, 2000; Beemster *et al.*, 2002) in leaf growth. To determine if these cellular parameters can discriminate differences in sensitivity to drought between contrasting lines, we conducted a separate experiment in a growth room, where seedlings from the same lines were subjected to similar levels of SWC, but in somewhat different experimental conditions (see 'Materials and methods'). The plants were grown in pots instead of rhizotrons and a different drought stress protocol was applied. In the rhizotrons drought stress conditions were established by filling them with pre-dried soil (34% SWC) and no irrigation was applied during the experiment. The drought stress in the growth room experiment, in contrast, started with well-watered (54% SWC) soil in the pots. The soil was then allowed to dry while the plants were growing until 34% SWC was reached, at which level they were maintained until the end of the experiment. Due to differences in drought treatment between the two experiments, we chose to work on a different leaf position. The fifth leaf emerged by the time the 34% SWC was reached in the pots of the drought treatment. It was therefore considered to have developed entirely under the stress conditions, in that way being comparable to the fourth leaf in the rhizotron experiment. Despite these differences in experimental conditions, a similar reduction of 50–60% in LER and 35–45% in FLL across the lines was observed (cf. Fig. 2C and Table 1). Moreover, there was a highly significant (P<0.001) positive correlation between the parameters that were measured in both experiments (Supplementary Table S2). Having established the reproducibility of growth and response to drought between

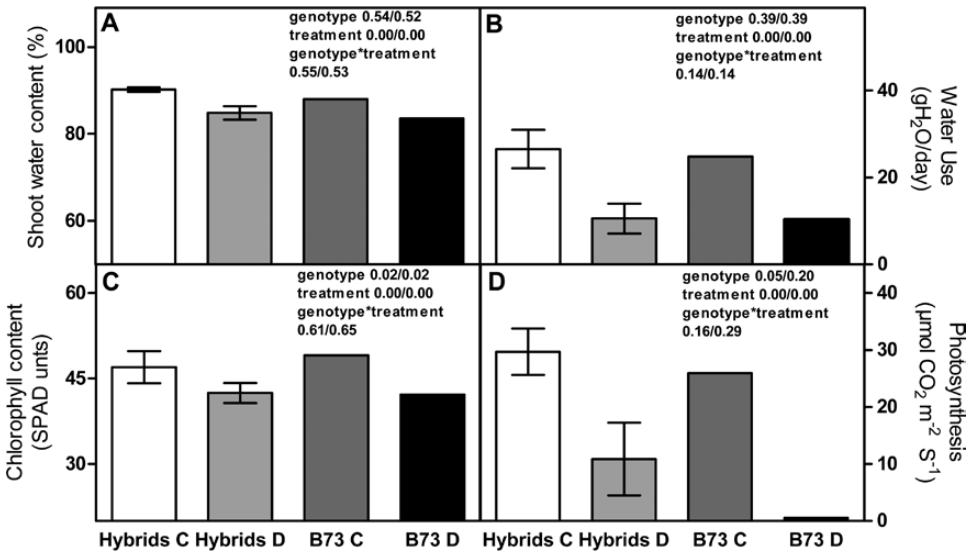

Fig. 3. The effect of drought effect on physiological parameters of eight hybrid maize lines and a reference inbred line B73. (A) Shoot water content, (B) water use (WU), (C) chlorophyll content, (D) photosynthesis. All parameters were measured on plants grown in rhizotrons. A two-way ANOVA was used as a statistical test and P-values for the two factors, genotype and treatment, as well as the interaction between them, are presented in the graph panels. The ANOVA analysis was conducted twice (including B73/excluding B73). Data for the hybrids are mean values across the respective line averages ±SD (n=8) and mean value only for B73. C, control conditions; D, drought conditions.

the experiments, we conducted a kinematic analysis to study the effect of drought stress on growth of leaf five. Similar to leaf four in the rhizotron experiment (Fig. 2C), the final leaf length was reduced by 47% in the growth room experiment (Table 1). Elongation rate (LER) of the fifth leaf was reduced by up to 61%, indicating that an increased duration of the growth of the leaf partly compensated for the reduced growth rates. The inhibition of leaf elongation was mainly due to a lower cell production rate, with a small contribution of a reduced mature cell length (Table 1). The decrease in cell production rate, in turn, was caused by a lower cell division rate, corresponding to increased cell cycle duration, and to a reduction of the number of cells in the meristem, related to a smaller meristem length (Table 1). Drought stress lowered the average cell expansion rate, but this was largely compensated by a doubling of the residence time in the elongation zone (Table 1), which explains the relatively small (but significant) change in the mature cell length.

We found significant genotype and/or genotype × treatment effects for leaf length, cell production rate, cell division rate, cell cycle duration, residence time in both division and elongation zones, and cell expansion rate, even in the absence of B73. Overall these results suggest differences between the studied genotypes and in their response to drought could be detected based on these cellular parameters.

Root growth and architecture

The development of an extensive root system is closely related to drought tolerance (Yu *et al.*, 2008, 2013; Comas *et al.*, 2013). The rhizotron experiment facilitated the quantification of root growth dynamics and the relationship between shoot and root development under water-deficient conditions. As is typical for maize, the lateral and seminal roots represented the major part of the root system during early seedling development in all lines studied (Fig. 4A). B73 and the hybrid maize lines exhibited clear differences in root growth dynamics. During the first days of its growth B73 had a larger fraction of lateral roots branched from primary roots and less seminal roots than the hybrids in control as well as in drought conditions. However, at the last time point (day 15) the root systems were very similar (Fig. 4A, Supplementary Fig. S2). Drought strongly reduced the length of the root system (Fig. 4A, B) and its growth rate (Fig. 4C), with the strongest impact on the development of the seminal and second order lateral roots for all genotypes (Fig. 4A, Supplementary Fig. S2). This root fraction was also significantly different between the hybrids (Fig. 4A). Even though the growth rate of B73 was lower than the average of the hybrids, specifically under control conditions, no significant genotype or genotype × treatment effects could be detected (Fig. 4C).

At the final harvest, the image-based root growth analysis in the rhizotrons was complemented with the more classical approach of scanning root systems after washing. Although only 15–20% of the roots were visible on the transparent plate of rhizotrons, the results of the 2D imaging showed the same pattern as the scans (Fig. 4B). However, significant differences between the genotypes were detected only in terms of the total, but not in terms of the visible root length. Moreover, the genotype × treatment interaction was significant for the total root length even when B73 was excluded from the ANOVA analysis (Fig. 4B). Similar differences in growth and drought response were detected between the hybrids in terms of root dry weight (Fig. 4D). In all lines, a slight increase in average root diameter was observed in the drought conditions (Fig. 4E). This could be a consequence of the reduced fraction of lateral roots branched from primary and seminal roots (Fig. 4A), which are generally thinner than primary and seminal roots.

As a consequence of reduced root growth rates, drought reduced the extent of the root system in all lines, affecting root system depth, width and root surface coverage (Fig. 4F, G). Next to having a shorter root system than the hybrids at the final harvest in both control and stress conditions, the root system of B73 was more compact, as it was less deep and wide, and consequently a smaller soil area was covered by the root system (Fig. 4F, G).

Drought inhibited growth of the shoot more than that of the roots, as evidenced by a significantly decreased shoot/root ratio under drought conditions in the hybrid lines and B73 (Fig. 4H). There was no significant difference between the genotypes.

We found significant genotype and/or genotype × treatment interaction effects, indicative of different growth and drought sensitivity between the hybrids in terms of total root length, root dry weight, root diameter and root system width. For shoot-root ratio a significant interaction was found only when B73 was included, suggesting that the inbred line was less able to reallocate resources from shoot to root growth under drought conditions (Fig. 4H). Root system width and root surface coverage also had a significant genotype × environment interaction, indicating differences in response to drought between the genotypes.

Correlations between measured traits

In order to assess correlations between parameters and performance, we performed a Principal Component Analysis (PCA) based on all the parameters measured across the two experiments (Fig. 5). PC1, accounting for 58% of the data variation, separates the treatments (control and drought stress conditions). PC2, which accounts for 13% of the data variation, separates the lines. As expected, the biggest differences were observed between the inbred line B73 and the commercial hybrids, both under control and stress conditions. Curiously, despite their different origin and different drought sensitivity, the growth of the commercial lines was similar under stress conditions, while African and European lines were separated under control conditions (Fig. 5A). There was no grouping of the lines, based on their drought sensitivity rating (Supplementary Table S1). Interestingly, most of the shoot and root-related parameters primarily contributed to the treatment grouping (PC1, Fig. 5B), whereas the kinematic parameters mainly contributed to the grouping of the lines (African and European; PC2, Fig. 5B).

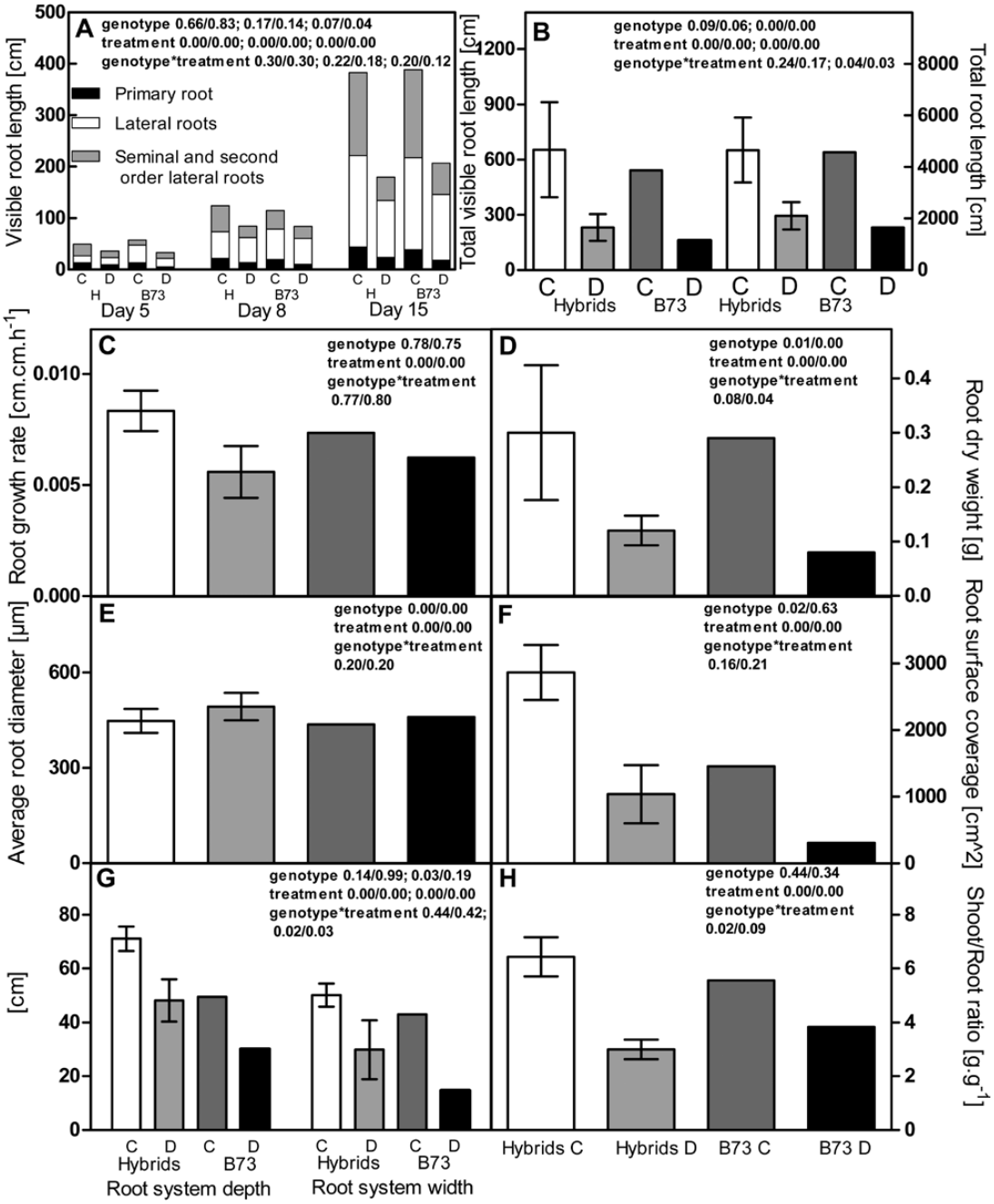

Fig. 4. The effect of drought effect on root growth and architectural traits of eight hybrid maize lines and a reference inbred line B73. (A) Visible root length, (B) total root length, (C) root growth rate, (D) root dry weight, (E) average root diameter, (F) root surface coverage, (G) root system depth and root system width, (H) shoot/root ratio. All parameters were measured on plants grown in rhizotrons. Visible root length was obtained by analyzing rhizotron images and total root length was measured by scanning the washed roots. A two-way ANOVA was used as a statistical test and *P*-values for the two factors, genotype and treatment, as well as the interaction between them, are present in the graph panels. The ANOVA analysis was conducted twice (including B73/excluding B73). In panel A the three statistical datasets correspond to primary root, lateral roots, and seminal and second order lateral; in panel B to total visible root length and total root length; and in panel H to root system depth and root system width. Data for the hybrids are mean values across the respective line averages ±SD (*n*=8) and mean value only for B73. C, control conditions; D, drought conditions.

Screening potential of phenotypic traits

By analyzing the effect of drought on shoot and root growth of the eight commercial hybrids and the B73 inbred line, we identified whole plant and cellular parameters that identify differences in growth and drought sensitivity between the lines, as evidenced by significant genotype and genotype × environment interactions. This allowed us to address the question whether differences in growth between hybrids from different regions of origin and between hybrids with contrasting drought tolerance in the field (based on the breeder's ranking; Table S1) can be identified at the early seedling stage and which parameter is a good trait to detect those differences. To this end, we performed a three-way ANOVA on all parameters for both experiments separately (pot and rhizotron, respectively). Consistent with the dominance of

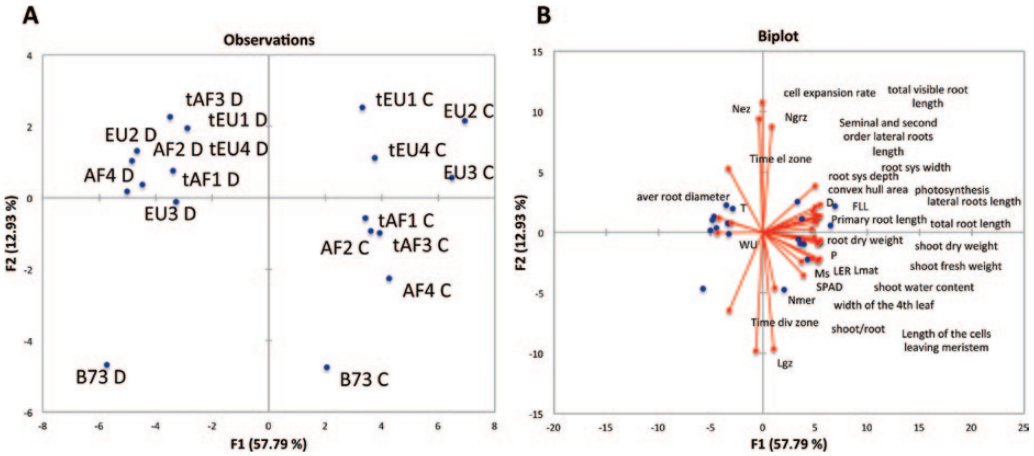

Fig. 5. Principal component analysis of the nine lines under control and drought conditions (A) Grouped according to all measured traits, (B) in rhizotrons and pot experiments. EU, European hybrids; AF, African hybrids; t, drought tolerant line.

the effect in the PCA (Fig. 5), most of the measured parameters were responsive to the drought treatment (Table 2). Consistent with the PCA grouping of the lines according to their region of origin in the control conditions (Fig. 5), a relatively high number of parameters were significant for the difference between the South African and Western European hybrids (Table 2). However, for the majority of parameters there was no significant region × treatment interaction effect (Table 2), which explains the lack of region-related grouping of the lines in the stress conditions (Fig. 5). Nevertheless, the African and the European lines responded differently to the drought conditions (region × treatment) in terms of their number of leaves at the time of the harvesting, shoot-root ratio, total visible root length, length of seminal and second order lateral roots, number of cells in the meristem and in the elongation zone (Table 2).

In relation to the sensitivity of the lines, we found a significant difference for final shoot projected area, final leaf length, shoot and root biomass, total root length, mature cell length, cell production rate, and number of cells in the elongation zone (Table 2). This indicates that the tolerant lines in this panel could be identified irrespective of whether they are grown under control or drought stress conditions. On the other hand, the different response to drought (sensitivity × treatment) between these two groups of hybrids was significant for final shoot projected area, shoot biomass, WU, photosynthesis, total root length, root diameter, root system width, surface coverage, and leaf meristem length. This indicates that these parameters respond differently in drought tolerant lines. Overall, this means that we have identified parameters at the early seedling stage that can discriminate between lines with established differences in drought tolerance.

Such parameters also allow us to test our hypothesis that: (i) drought-tolerant lines would respond less to drought and (ii) African lines would be more drought tolerant than European lines. To evaluate this, we looked in more detail at total root length in the rhizotron experiment and shoot dry weight in the pot experiment, because these parameters appeared most informative given that they had the highest number of significant factors across the two experiments (six out of seven; Table 2).

Indeed, the drought-tolerant European and African hybrids appear to have different strategies in coping with limited water availability: the total root length of the tolerant European hybrids was similar to the African lines under control conditions, whereas the other European lines had much longer roots. However, the reduction in root length in response to the drought stress was the lowest (47%) for the tolerant European, compared to all other lines (Fig. 6A), indicating that in that respect they are the least sensitive to drought.

On the other hand, the drought tolerant African hybrids had smaller shoots than the other African and European hybrids and experienced significantly less shoot growth reduction during water stress (Fig. 6B). Therefore, we conclude that, albeit based on different parameters, tolerant hybrids from both continents were proportionally less affected by drought.

Additionally, shoot dry weight of the tolerant African lines was lower under optimal conditions than that of the European lines and the other African lines, but they all became very similar under drought conditions (Fig. 6B). Although the reduction was the lowest in the tolerant African lines, their response did not differ significantly from the European lines. Therefore, we could not confirm our second hypothesis that the impact of drought on the African lines is overall smaller than on the European lines. Instead our results show that the hybrids with different origins had different strategies to cope with the drought. Tolerant European lines had small root systems that were relatively insensitive to drought, whereas tolerant African lines had small shoot dry weight that was relatively insensitive.

Discussion

Drought is the environmental factor that imposes the strongest limitations on crop yield (Boyer, 1982). Therefore, development of drought-tolerant crop varieties is a primary concern for breeders and agronomists. To achieve this, effective screening is of crucial importance. Here we used image-based analysis of root and shoot growth and detailed kinematic analyses of leaves to characterize in detail the response of young maize seedlings to drought. Our findings contribute to a deeper understanding of this response and

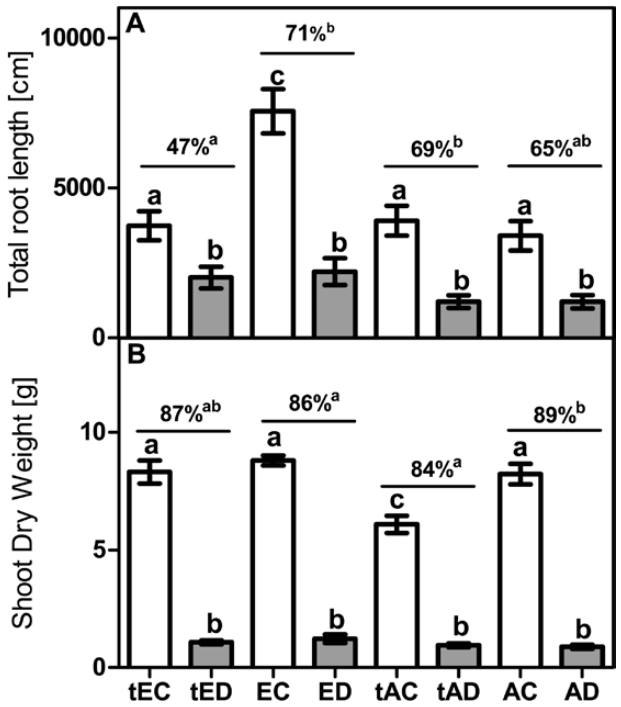

Fig. 6. Phenotypic traits discriminating between differences in response to drought between groups of hybrids with different region of origin and different drought sensitivity. Total root length (A) and shoot dry weight (B) of Western European and South African hybrids, differing in their drought tolerance. Data are mean values across the respective groups of hybrids ±SE (n=8) and Student's t-test was used as a statistical test and significant differences between the groups are marked with different letters above the bars on the graph. Percentage values above the bars show the average reduction in response to the treatment. Student's t-test was used as a statistical test on all pairs of control and drought values and significant differences are marked next to the percentage values. E, Western European hybrids; A, South African hybrids; t, tolerant lines; C, control conditions; D, drought conditions.

which traits characterize a drought-tolerant maize plant. The main aim of this study was to evaluate whether detailed phenotyping at the young seedling stage offers possibilities to screen for drought tolerance. The results clearly demonstrate that the methods yield a detailed insight in which parameters (i) contribute to the effect of drought on growth and (ii) contribute to differences in growth between genotypes of young maize seedlings. Most importantly, (iii) we could identify significant genotype × treatment interactions, indicating differences in drought tolerance between hybrids. This allowed us to identify significant differences between lines from Africa and Europe and between lines that have contrasting drought tolerance in field conditions. Therefore, early seedling stage traits could potentially be used in drought-tolerance screens.

Main effects of drought on seedling growth

Whole plant

Consistent with other studies, shoot and root growth were inhibited by drought and because shoot growth is more sensitive than root growth, shoot-root ratio is typically reduced (Li *et al.*, 2014; Naveed *et al.*, 2014). This means that under drought stress, plants allocate more resources to root than to shoot growth in order to enhance water acquisition and limit evaporation (Sharp and Davies, 1979; Palta and Gregory, 1997; Lynch and Ho, 2005). However, our results show that both having a smaller shoot (African lines), and smaller root system (European lines) can be related to drought tolerance (Fig. 6A, B).

Based on genotype effects in ANOVAs, the most significant differences in shoot related parameters between genotypes were found for final leaf length, fresh and dry biomass, and growth period (Table 2, Figs 2, 3). Differences in the response to drought stress between the hybrids were detected only in terms of fresh and dry biomass (significant treatment × genotype interaction; Fig. 2F, G). In terms of roots, drought stress mainly reduced the growth of the seminal root system, including branched lateral roots, preventing the plant to form a dense root network. This is consistent with observations in *Arabidopsis thaliana*, where mutants that grow better under drought conditions have more tertiary roots than wild-type plants (Karaba *et al.*, 2007). Our results show significant treatment × genotype interaction in terms of total root length, root dry weight and root system width, implicating them as parameters that can discriminate differences in drought response between the studied lines (Fig. 4B, D, G).

Cell level

To understand the impact of drought on shoot growth at the cellular level we applied a kinematic analysis. Our and earlier findings (Tardieu *et al.*, 2000) point at the importance of reduced cell division as the main cause of the reduced leaf growth. Similar effects on cell division were also observed in other abiotic stress conditions as chilling temperatures (Rymen *et al.*, 2007), N-fertilization (Volenec and Nelson, 1983), salt stress (West *et al.*, 2004), drought stress (Sacks *et al.*, 1997), soil compaction (Beemster *et al.*, 1996), and light and temperature stress (Granier and Tardieu, 2000). Growth reduction caused by abiotic factors is often due to a combination of both reduced cell production and mature cell size (Beemster *et al.*, 1996; Sacks *et al.*, 1997; Granier and Tardieu, 2000; West *et al.*, 2004). In our experimental setup, the impact of the stress on mature cell size was relatively small. This was due to an increase in the time cells spend in the elongation zone of the leaf, which compensated for the reduced cell expansion rates. Similar to our observations, in many cases abiotic stresses limit the overall cell division activity by reducing meristem size (Volenec and Nelson, 1983; Beemster *et al.*, 1996; West *et al.*, 2004; Rymen *et al.*, 2007). Recently, it was shown that a local peak of gibberellic acid determines the location of the meristem boundary (Nelissen *et al.*, 2013), opening the possibility that this mechanism is also involved in growth responses to abiotic stresses.

Many kinematic parameters showed significant differences in drought response between the lines (significant genotype × treatment interaction; Table 1) among the hybrids. However, the different response between the groups of hybrids with different origin and drought sensitivity could only be measured by shoot dry weight and total root length. This means that either the differences between the maize lines were too small and the number of replicates was too limited to detect underlying cellular mechanisms, or that complex compensation

mechanisms between different cellular parameters exist. A well-known example of such compensation mechanisms is the compensation between cell division and cell expansion (Beemster *et al.*, 2003; Horiguchi and Tsukaya, 2011). Based on statistical modeling, Tisne *et al.* (2008) found that causal/regulatory relationships between leaf size and cell number and size operated in both directions (from the cellular to the whole leaf, but also from the whole leaf and to cellular level parameters), implying a complex interaction between whole leaf growth and cellular processes. Such interactions obviously disturb the direct link between the individual parameters and plant level growth, making these parameters less suitable for screening purposes.

Next to their ability to detect parameters underlying growth differences between maize genotypes and even identify genotype × treatment interactions, these detailed analyses are extremely valuable for experimental purposes. An important aspect of our study is that detailed whole plant and cellular measurements are combined, so that the observed dynamics of phenotype development can be understood in terms of spatio-temporal changes at the cellular level. This provides a more solid basis for investigating the molecular mechanisms behind the development of the observed phenotype by allowing specific sampling of those parts of the plant where the response occurs and at the time it is occurring (Granier *et al.*, 2000; Nelissen *et al.*, 2013; Avramova *et al.*, 2015*a*). The phenotypic data provided in this study represent the first step in establishing gene-to-phenotype associations that can be assessed in a high-throughput approach under more strictly controlled and reproducible than field conditions. Moreover, multiple parameters may be independent and could be selected in parallel.

Potential of (pre-)screening of early seedlings for drought tolerance

Our results clearly demonstrate that phenotyping young seedlings under controlled conditions is a useful approach to identify candidate drought-tolerant genotypes, and can reduce, at least in part, the laborious and time-consuming selection under field conditions.

The eight commercial hybrid lines with contrasting drought tolerance ratings based on field trials and the reference line B73 showed significant differences in their growth at the seedling stage under drought conditions in both experiments. The lines differed in terms of their origin (four Western European and four South African) and according to our PCA analysis those differences were already visible at the seedling stage, as in control conditions the lines were clearly grouped together based on their origin. The different origin and drought sensitivity of the hybrids were detectable in terms of the total root length and the shoot dry weight of the plants. Despite the differences between the hybrids being relatively small and the limited sample sizes (i.e. two tolerant European, two sensitive European, two tolerant African, two sensitive African), we could demonstrate that hybrids classified as drought tolerant by the breeder showed less growth reduction than the other hybrids. However, we could not confirm our hypothesis that the African lines were more tolerant than European. Instead, the hybrids from different origins appeared to have different strategies to cope with drought stress. Curiously, both European and African tolerant lines grew less in terms of root length and shoot mass respectively under control conditions. Because under drought conditions these values became similar, the reduction was less for the tolerant lines. It is tempting to conclude that next to being less sensitive to the same reduction in soil water potential, tolerant lines have a more restricted growth, which under field conditions conserves water, so that under drought more water remains available, partly alleviating the stress. From this perspective drought tolerance would entail both somewhat restricted growth under control conditions as well as reduced response to drought conditions. However, as we specifically tested the plants under equal soil water contents, we cannot verify this based on the current experiments.

In contrast to the more complex differences between the hybrids, the well-known differences between hybrids and inbred lines (Meeks *et al.*, 2013) are much clearer and could be related to growth period, shoot growth rate, photosynthesis, root system width and root surface coverage.

An important finding of this study is that shoot and root biomass, which have already successfully been implemented in existing high-throughput screening platforms, appear to provide the most powerful traits to screen drought-tolerant genotypes prior to more laborious field trials, corresponds well with similar findings for cotton (Riaz *et al.*, 2013).

More detailed phenotyping can add new candidate traits such as shoot projected area, total root length, average root diameter, root system width and root surface coverage (image analyses), as well as final leaf length, cell production rate, cell division rate, length of mature cells, meristem length and number of cells in the elongation zone (kinematic analysis). Moreover, detailed phenotyping analyses provide insight into the cellular mechanisms underlying the observed whole plant growth differences, serving as an important bridge between molecular changes and whole plant growth responses.

Supplementary data

Fig. S1. Environmental conditions in the greenhouse, monitored during the experiment.

Fig. S2. Typical color-coded images showing root development along the transparent side of rhizotrons.

Table S1. Description of eight commercial maize hybrid lines with contrasting drought sensitivity.

Table S2. Pearson correlation between the parameters measured in the glasshouse (GH) and in the growth chamber (GR) experiments.

Table S3. A four-way ANOVA between the shoot fresh and dry weight, measured during both glasshouse and growth chamber experiments.

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References

Araus JL, Serret MD, Edmeades G. 2012. Phenotyping maize for adaptation to drought. Frontiers in Physiology **3,** http://dx.doi.org/10.3389/fphys.2012.00305.

Avramova V, AbdElgawad H, Zhang Z, *et al.* 2015*a*. Drought induces distinct growth response, protection and recovery mechanisms in the maize leaf growth zone. Plant Physiology **169,** 1382–1396.

Avramova V, Sprangers K, Beemster GTS. 2015*b*. The maize leaf: another perspective on growth regulation. Trends in Plant Science , in press.

Beemster GT, De Vusser K, De Tavernier E, De Bock K, Inze D. 2002. Variation in growth rate between Arabidopsis ecotypes is correlated with cell division and A-type cyclin-dependent kinase activity. Plant Physiology **129,** 854–864.

Beemster GTS, Fiorani F, Inzé D. 2003. Cell cycle: the key to plant growth control? Trends in Plant Science **8,** 154–158.

Beemster GTS, Masle J, Williamson RE, Farquhar GD. 1996. Effects of soil resistance to root penetration on leaf expansion in wheat (*Triticum aestivum* L): Kinematic analysis of leaf elongation. Journal of Experimental Botany **47,** 1663–1678.

Benhajsalah H, Tardieu F. 1995. Temperature affects expansion rate of maize leaves without change in spatial-distribution of cell length – analysis of the coordination between cell-division and cell expansion. Plant Physiology **109,** 861–870.

Bernstein N, Silk WK, Lauchli A. 1993. Growth and development of sorghum leaves under conditions of nacl stress – spatial and temporal aspects of leaf growth-inhibition. Planta **191,** 433–439.

Boyer JS. 1982. Plant productivity and environment. Science **218,** 443–448.

Burke MB, Lobell DB, Guarino L. 2009. Shifts in African crop climates by 2050, and the implications for crop improvement and genetic resources conservation. Global Environmental Change-Human and Policy Dimensions **19,** 317–325.

Chimungu JG, Brown K, Lynch J. 2014. Large root cortical cell size improves drought tolerance in maize (*Zea mays* L.). Plant Physiology .

Comas LH, Becker SR, Cruz VV, Byrne PF, Dierig DA. 2013. Root traits contributing to plant productivity under drought. Frontiers in Plant Science **4**.

Fiorani F, Beemster GTS, Bultynck L, Lambers H. 2000. Can meristematic activity determine variation in leaf size and elongation rate among four *Poa* species? A kinematic study. Plant Physiology **124,** 845–855.

Fiorani F, Schurr U. 2013. Future scenarios for plant phenotyping. Annual Review of Plant Biology **64,** 267–291.

Granier C, Inze D, Tardieu F. 2000. Spatial distribution of cell division rate can be deduced from that of p34(cdc2) kinase activity in maize leaves grown at contrasting temperatures and soil water conditions. Plant Physiology **124,** 1393–1402.

Granier C, Tardieu F. 2000. Sunflower leaf growth under changing environmental conditions. Ocl-Oleagineux Corps Gras Lipides **7,** 219–228.

Horiguchi G, Tsukaya H. 2011. Organ size regulation in plants: insights from compensation. Frontiers in Plant Science **2,** 24.

Huang CY, Kuchel H, Edwards J, Hall S, Parent B, Eckermann P, Herdina, Hartley DM, Langridge P, McKay AC. 2013. A DNA-based method for studying root responses to drought in field-grown wheat genotypes. Scientific Reports **3**.

Karaba A, Dixit S, Greco R, Aharoni A, Trijatmiko KR, Marsch-Martinez N, Krishnan A, Nataraja KN, Udayakumar M, Pereira A. 2007. Improvement of water use efficiency in rice by expression of HARDY, an Arabidopsis drought and salt tolerance gene. Proceedings of the National Academy of Sciences, USA **104,** 15270–15275.

Li PH, Ponnala L, Gandotra N, *et al.* 2010. The developmental dynamics of the maize leaf transcriptome. Nature Genetics **42,** 1060-U1051.

Li X, Mu C, Lin J. 2014. The germination and seedlings growth response of wheat and corn to drought and low temperature in spring of Northeast China Journal of Animal & Plant Sciences **21,** 3212–3222.

Ling QH, Huang WH, Jarvis P. 2011. Use of a SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. Photosynthesis Research **107,** 209–214.

Lobell DB, Banziger M, Magorokosho C, Vivek B. 2011. Nonlinear heat effects on African maize as evidenced by historical yield trials. Nature Climate Change **1,** 42–45.

Lynch JP. 2007. Rhizoeconomics: the roots of shoot growth limitations. Hortscience **42,** 1107–1109.

Lynch JP, Ho MD. 2005. Rhizoeconomics: carbon costs of phosphorus acquisition. Plant and Soil **269,** 45–56.

Meeks M, Murray SC, Hague S, Hays D. 2013. Measuring maize seedling drought response in search of tolerant germplasm. Agronomy **3,** 135–147.

Muhlich M, Truhn D, Nagel K, Walter A, Scharr H, Aach T. 2008. Measuring plant root growth. Pattern Recognition **5096,** 497–506.

Nagel KA, Bonnett D, Furbank R, Walter A, Schurr U, Watt M. 2015. Simultaneous effects of leaf irradiance and soil moisture on growth and root system architecture of novel wheat genotypes: implications for phenotyping. Journal of Experimental Botany **66,** 5441–5452.

Nagel KA, Kastenholz B, Jahnke S, *et al.* 2009. Temperature responses of roots: impact on growth, root system architecture and implications for phenotyping. Functional Plant Biology **36,** 947–959.

Nagel KA, Putz A, Gilmer F, *et al.* 2012. GROWSCREEN-Rhizo is a novel phenotyping robot enabling simultaneous measurements of root and shoot growth for plants grown in soil-filled rhizotrons. Functional Plant Biology **39,** 891–904.

Naveed M, Mitter B, Reichenauer TG, Wieczorek K, Sessitsch A. 2014. Increased drought stress resilience of maize through endophytic colonization by Burkholderia phytofirmans PsJN and Enterobacter sp FD17. Environmental and Experimental Botany **97,** 30–39.

Nelissen H, Rymen B, Coppens F, Dhondt S, Fiorani F, Beemster G. 2013. Kinematic analysis of cell division in leaves of mono- and dicotyledonous species: a basis for understanding growth and developing refined molecular sampling strategies. Methods in Molecular Biology **959,** 247–264.

Palta JA, Gregory PJ. 1997. Drought affects the fluxes of carbon to roots and soil in C-13 pulse-labelled plants of wheat. Soil Biology & Biochemistry **29,** 1395–1403.

Pfeifer J, Faget M, Walter A, Blossfeld S, Fiorani F, Schurr U, Nagel KA. 2014. Spring barley shows dynamic compensatory root and shoot growth responses when exposed to localised soil compaction and fertilisation. Functional Plant Biology **41,** 581–597.

Riaz M, Farooq J, Sakhawat G, Mahmood A, Sadiq MA, Yaseen M. 2013. Genotypic variability for root/shoot parameters under water stress in some advanced lines of cotton (*Gossypium hirsutum* L.). Genetics and Molecular Research **12,** 552–561.

Rymen B, Coppens F., Dhondt S., Fiorani F., Beemster GTS. 2010. Kinematic analysis of cell division and expansion. Methods in Molecular Biology **655,** 203–227.

Rymen B, Fiorani F, Kartal F, Vandepoele K, Inze D, Beemster GTS. 2007. Cold nights impair leaf growth and cell cycle progression in maize through transcriptional changes of cell cycle genes. Plant Physiology **143,** 1429–1438.

Sacks MM, Silk WK, Burman P. 1997. Effect of water stress on cortical cell division rates within the apical meristem of primary roots of maize. Plant Physiology **114,** 519–527.

Schurr U, Heckenberger U, Herdel K, Walter A, Feil R. 2000. Leaf development in Ricinus communis during drought stress: dynamics of

growth processes, of cellular structure and of sink-source transition. Journal of Experimental Botany **51,** 1515–1529.

Sharp RE, Davies WJ. 1979. Solute regulation and growth by roots and shoots of water-stressed maize plants. Planta **147,** 43–49.

Sharp RE, Silk WK, Hsiao TC. 1988. Growth of the maize primary root at low water potentials .1. spatial-distribution of expansive growth. Plant Physiology **87,** 50–57.

Silk WK, Erickson RO. 1979. Kinematics of plant growth. Journal of Theoretical Biology **76,** 481–501.

Tardieu F, Reymond M, Hamard P, Granier C, Muller B. 2000. Spatial distributions of expansion rate, cell division rate and cell size in maize leaves: a synthesis of the effects of soil water status, evaporative demand and temperature. Journal of Experimental Botany **51,** 1505–1514.

Tisne S, Reymond M, Vile D, Fabre J, Dauzat M, Koornneef M, Granier C. 2008. Combined genetic and modeling approaches reveal that epidermal cell area and number in leaves are controlled by leaf and plant developmental processes in Arabidopsis. Plant Physiology **148,** 1117–1127.

Tollenaar M, Lee EA. 2002. Yield potential, yield stability and stress tolerance in maize. Field Crops Research **75,** 161–169.

Volenec JJ, Nelson CJ. 1981. Cell-dynamics in leaf meristems of contrasting tall fescue genotypes. Crop Science **21,** 381–385.

Volenec JJ, Nelson CJ. 1983. Responses of tall fescue leaf meristems to n-fertilization and harvest frequency. Crop Science **23,** 720–724.

Walter A, Scharr H, Gilmer F, *et al.* 2007. Dynamics of seedling growth acclimation towards altered light conditions can be quantified via GROWSCREEN: a setup and procedure designed for rapid optical phenotyping of different plant species. New Phytologist **174,** 447–455.

Wand MP, Jones MC. 1995. Kernel Smoothing . Chapman & Hall/CRC.

West G, Inze D, Beemster GTS. 2004. Cell cycle modulation in the response of the primary root of Arabidopsis to salt stress. Plant Physiology **135,** 1050–1058.

York LM, Nord EA, Lynch JP. 2013. Integration of root phenes for soil resource acquisition. Frontiers in Plant Science **4,** http://dx.doi.org/10.3389/fpls.2013.00355.

Yu H, Chen X, Hong YY, Wang Y, Xu P, Ke SD, Liu HY, Zhu JK, Oliver DJ, Xiang CB. 2008. Activated expression of an Arabidopsis HD-START protein confers drought tolerance with improved root system and reduced stomatal density. Plant Cell **20,** 1134–1151.

Yu LH, Chen X, Wang Z, Wang SM, Wang YP, Zhu QS, Li SG, Xiang CB. 2013. Arabidopsis Enhanced Drought Tolerance1/HOMEODOMAIN GLABROUS11 confers drought tolerance in transgenic rice without yield penalty. Plant Physiology **162,** 1378–1391.

Supplementary data

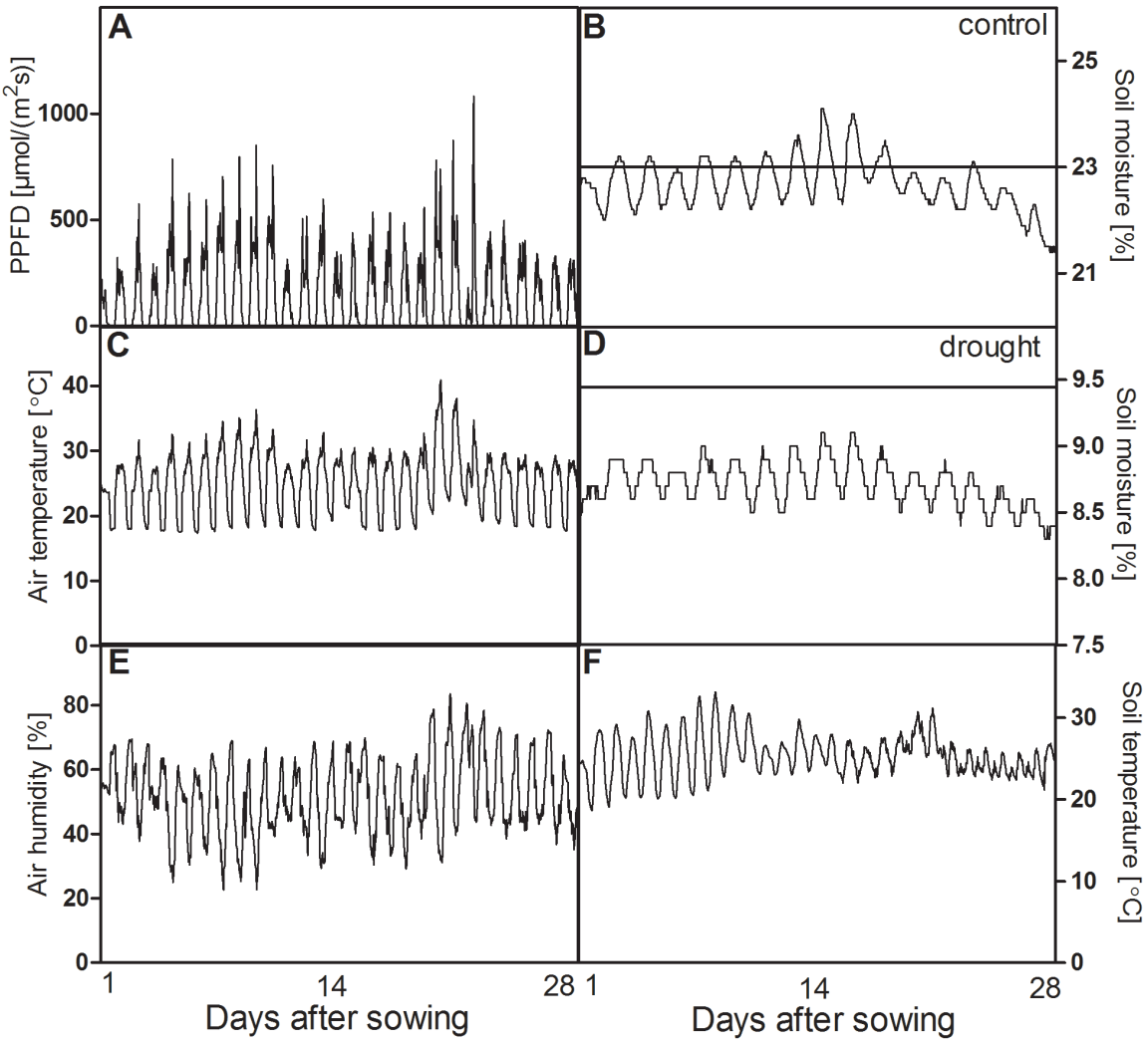


Figure S1. Environmental conditions in the green house, monitored during the experiment. Climate conditions in the green house were monitored by using four sensors (one in each of the four compartments of the GROWSCREEN-Rhizo setup) to monitor Photosynthetic Photon Flux Density (PPFD;A), air temperature (C) and air humidity (E). Another four sensors were situated inside the soil of four rhizoboxes (two in each condition) to monitor soil moisture (B and D) and soil temperature (F). Data ware recorded every half an hour for 28 days.

Table S1. Description of eight commercial maize hybrid lines with contrasting drought sensitivity.

A.

Line	Drought tolerance	Description	Country where available	More information
EU 1 (PR39D23)	Tolerant	Early/Medium early silage and medium early grain	Poland	http://public.pioneer.com/portal/site/Public/template.CMI/guid.E3F796D6-022E-45D4-C2E4-E8BCBD214372/?attributes=region
			The Netherlands	http://public.pioneer.com/portal/site/Public/template.CMI/guid.19A4806A-DEAE-8A46-296A-0732F57CC4E8/?attributes=region
EU 2 (P7345)		Very early maturity with high dry matter yields (Maturity class 9, Less favorable sites)	Germany	http://public.pioneer.com/CMRoot/international%5Cpublic%5CGerman%5CGermany%5CMais%5CVersuchsergebnisse%5CSM_Gebiet22_24980_Wallsbuell_2011.pdf
EU 3 (PR39T83)		Late maturity (Maturity class 4) good starch content, high silage dry matter	UK and Ireland	http://public.pioneer.com/portal/site/Public/template.MAXIMIZE/corn/productoverview/?javax.portlet.tpst=f83e6ef89acd1be6d58aef46310093a0_ws_MX&javax.portlet.prp_f83e6ef89acd1be6d58aef46310093a0=viewID%3Dnonassociated_content_display_view&beanID=895776695&viewID=nonassociated_content_display_view&javax.portlet.begCacheTok=com.vignette.cachetoken&javax.portlet.endCacheTok=com.vignette.cachetoken&guid=93D0BBE2-6A8C-10A5-2A94-A1A6FC04B550
EU 4 (PR39F58)	Tolerant	Medium early maturity; High starch and sugar content	The Netherlands	http://public.pioneer.com/portal/site/Public/template.MAXIMIZE/corn/productoverview/?javax.portlet.tpst=f83e6ef89acd1be6d58aef46310093a0_ws_MX&javax.portlet.prp_f83e6ef89acd1be6d58aef46310093a0=viewID%3Dnonassociated_content_display_view&beanID=895776695&viewID=nonassociated_content_display_view&javax.portlet.begCacheTok=com.vignette.cachetoken&javax.portlet.endCacheTok=com.vignette.cachetoken&guid=FBBD7E78-CEA0-1303-F1DD-4C551E9110A5
			Poland	http://public.pioneer.com/portal/site/Public/template.MAXIMIZE/corn/productoverview/?javax.portlet.tpst=f83e6ef89acd1be6d58aef46310093a0_ws_MX&javax.portlet.prp_f83e6ef89acd1be6d58aef46310093a0=viewID%3Dnonassociated_content_display_view&beanID=895776695&viewID=nonassociated_content_display_view&javax.portlet.begCacheTok=com.vignette.cachetoken&javax.portlet.endCacheTok=com.vignette.cachetoken&guid=32A73A7A-59CF-2910-C757-D12F97D5E004

B.

Line	Days to Physiological maturity	Yield Potential	Prolificacy	Productive Tillers	Standibility	Drought tolerance	Dry Down	Silage yield	Silage quality
AF1 (33H56)	120 (full season maturity)	8	7	5	9	9	9		
AF2 (33Y74)	122 (full season maturity)	9	4	3	9	6	9		
AF3 (3442)	130 (full season maturity)	7	8	5	8	9	7	9	8
AF4 (31MO9)	125 (full season maturity)	9	6	6	6	6	6	8	9
Information provided from Pioneer catalogue									

Table S2. Pearson correlation between the parameters measured in the glasshouse (GH) and in the growth chamber (GR) experiments.

	FLL GR	LER GR	FLL GH	LER GH	Shoot Fresh Weight GH	Shoot Dry Weight GH	Root Dry Weight GH	Root Weight GR	Shoot Fresh Weight GR	Shoot Dry Weight GR
Final Leaf Length GR	1									
Leaf Elongation Rate GR	**0.946**	1								
Final Leaf Length GH	**0.905**	**0.861**	1							
Leaf Elongation Rate GH	**0.911**	**0.911**	**0.940**	1						
Shoot Fresh Weight GH	**0.896**	**0.853**	**0.942**	**0.907**	1					
Shoot Dry Weight GH	**0.850**	**0.816**	**0.933**	**0.891**	**0.993**	1				
Root Dry Weight GH	**0.752**	**0.695**	**0.888**	**0.826**	**0.941**	**0.967**	1			
Root Weight GR	**0.915**	**0.880**	**0.845**	**0.823**	**0.872**	**0.836**	**0.753**	1		
Shoot Fresh Weight GR	**0.980**	**0.967**	**0.916**	**0.915**	**0.909**	**0.874**	**0.785**	**0.918**	1	
Shoot Dry Weight GR	**0.974**	**0.956**	**0.911**	**0.898**	**0.891**	**0.855**	**0.763**	**0.935**	**0.994**	1
Correlation is significant at the 0.01 level (2-tailed).										

Table S3. A four-way ANOVA between the shoot fresh and dry weight, measured during both glasshouse and growth chamber experiments. P < 0.05 is considered as a significant difference.

ANOVA	p-value	
	Shoot fresh weight	Shoot dry weight
Region (African and European maize lines)	**0.027692**	**0.000744**
Treatment (control and severe drought stress)	**6.62E-15**	**9.28E-16**
Sensitivity of the lines (sensitive and tolerant)	**0.020014**	**0.003381**
Experiment (glasshouse and growth chamber)	**9.05E-12**	**3.25E-14**
region * treatment	0.125375	**0.012121**
region * sensitivity	0.571272	0.780206
region * experiment	0.346207	**0.045294**
treatment * sensitivity	**0.03383**	**0.007932**
treatment * experiment	**1.91E-10**	**9.75E-13**
sensitivity * experiment	0.806895	0.395916
region * treatment * sensitivity	0.589201	0.64253
region * treatment * experiment	0.349706	0.079456
region * sensitivity * experiment	**0.035836**	**0.004906**
treatment * sensitivity * experiment	0.884939	0.534271
region * treatment * sensitivity * experiment	**031982**	**0.002923**

EU1 control

EU1 drought

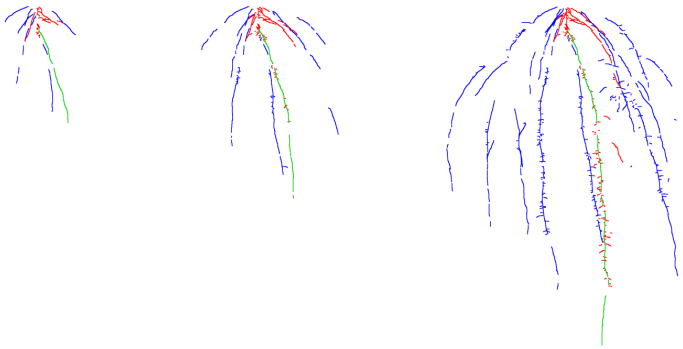
primary root

lateral roots

seminal and second order lateral roots

Day 5 Day 8 Day 15

EU2 control



EU2 drought

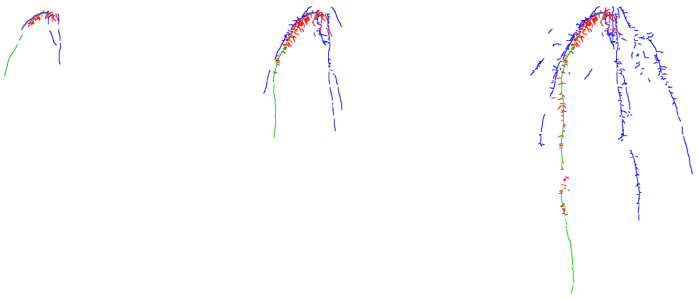
primary root

lateral roots

seminal and second order lateral roots

Day 5 Day 8 Day 15

EU3 control



EU3 drought

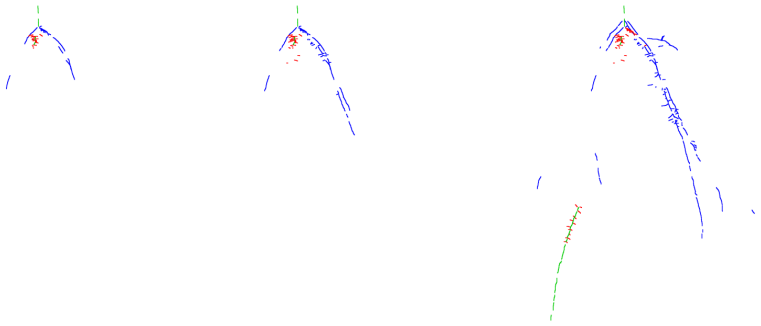
primary root

lateral roots

seminal and second order lateral roots

Day 5 Day 8 Day 15

EU4 control



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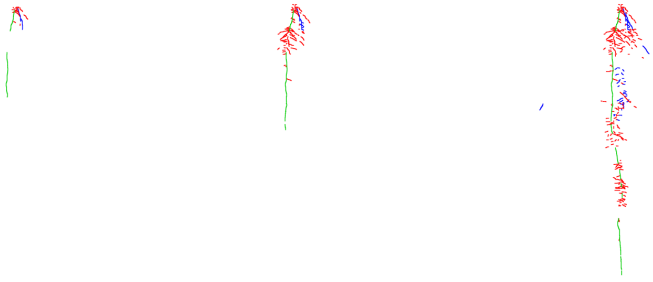


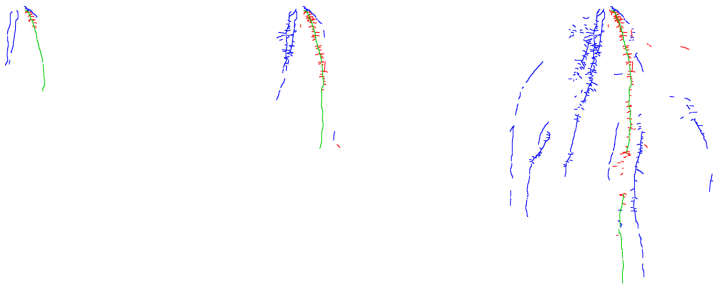
primary root

lateral roots

seminal and second order lateral roots

Day 5 Day 8 Day 15

AF1 control



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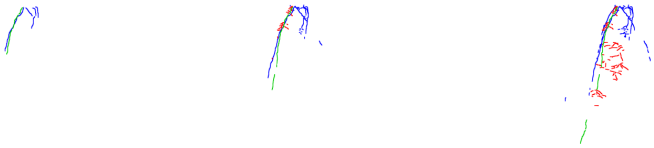


primary root

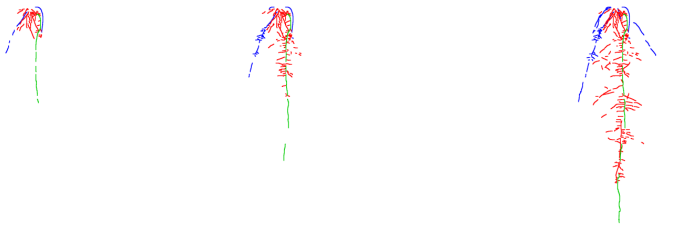
lateral roots

seminal and second order lateral roots

Day 5 Day 8 Day 15

AF2 control

AF2 drought



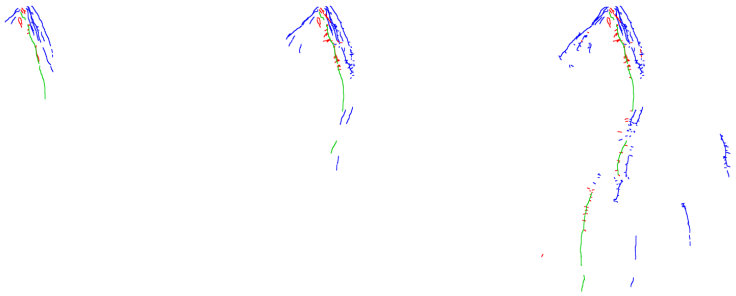
primary root

lateral roots

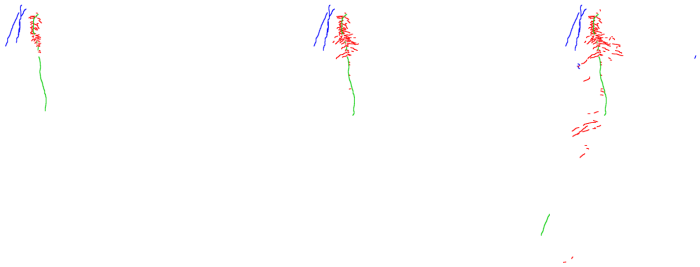
seminal and second order lateral roots

Day 5 Day 8 Day 15

AF3 control



AF3 drought



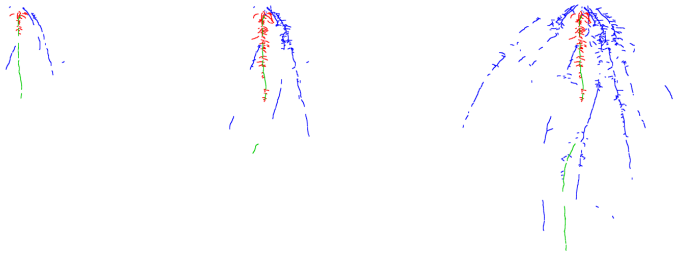
primary root

lateral roots

seminal and second order lateral roots

Day 5 Day 8 Day 15

AF4 control



Af4 drought

primary root

lateral roots

seminal and second order lateral roots

Day 5 Day 8 Day 15

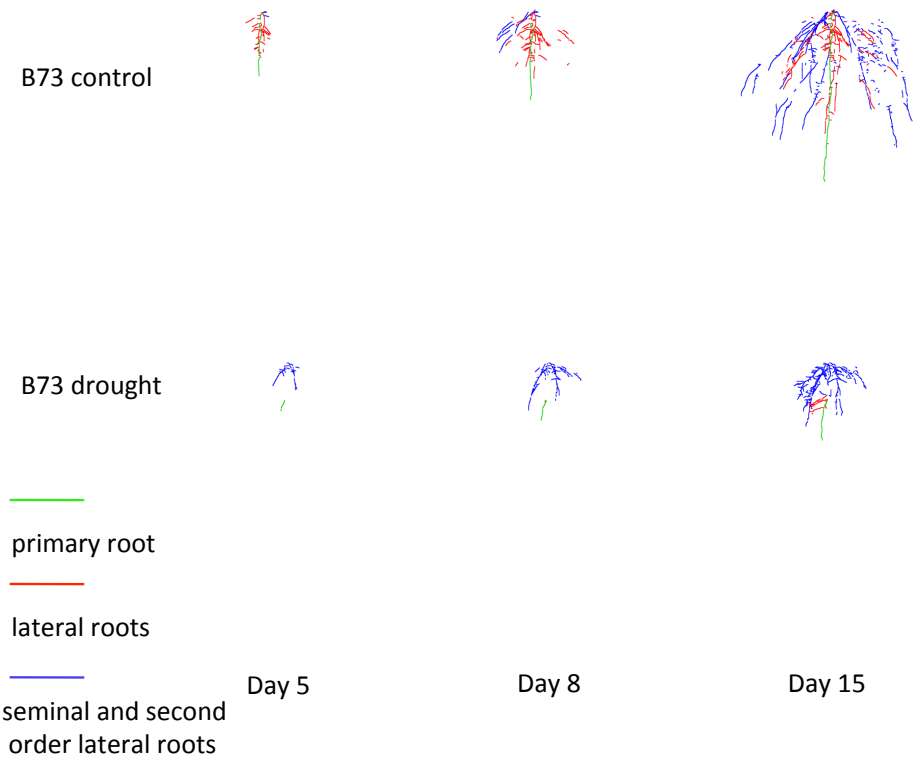


Figure S2. Typical color-coded images showing root development along the transparent side of rhizotrons. The development of root systems eight commercial hybrid maize lines (EU1, EU2, EU3, EU4, AF1, AF2, AF3, and AF4) and a reference inbred line B73 are compared in control and drought conditions at three time points during their growth development (day 5, 8, and 15).