

# Grasses suppress shoot-borne roots to conserve water during drought

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Many important crops are members of the Poaceae family, which develop root systems characterized by a high degree of root initiation from the belowground basal nodes of the shoot, termed the crown. Although this postembryonic shoot-borne root system represents the major conduit for water uptake, little is known about the effect of water availability on its development. Here we demonstrate that in the model C<sub>4</sub> grass *Setaria viridis*, the crown locally senses water availability and suppresses postemergence crown root growth under a water deficit. This response was observed in field and growth room environments and in all grass species tested. Luminescence-based imaging of root systems grown in soil-like media revealed a shift in root growth from crown-derived to primary root-derived branches, suggesting that primary root-dominated architecture can be induced in *S. viridis* under certain stress conditions. Crown roots of *Zea mays* and *Setaria italica*, domesticated relatives of teosinte and *S. viridis*, respectively, show reduced sensitivity to water deficit, suggesting that this response might have been influenced by human selection. Enhanced water status of maize mutants lacking crown roots suggests that under a water deficit, stronger suppression of crown roots actually may benefit crop productivity.

root development | drought | Poaceae | *Setaria* | *Zea mays*

Drought is the most damaging environmental condition affecting global agricultural productivity, due in large part to the effects of water deficit (WD) ([www.fao.org/home/en/reports.weforum.org/global-risks-2015](http://www.fao.org/home/en/reports.weforum.org/global-risks-2015)). Roots provide the main route through which water is absorbed from the soil environment, and thus represent an important target for breeding efforts aimed at improving drought tolerance in crops (1). In the grass lineage, roots develop from multiple sites aboveground and belowground, substantially distinguishing their development from other eudicot models. The initial root system of species such as maize consists of primary and seminal roots, which originate from the embryonic axis (2, 3) (Table S1). This embryonic root system is important for seedling establishment but is largely ephemeral (2, 4, 5). Later during development, roots borne from the crown initiate and emerge from internal tissues. This important developmental transition represents the beginning of the postembryonic shoot-borne root system (4, 6, 7), which will come to dominate the architecture of the plant below ground (2, 8, 9).

The number of crown roots, their growth rate, and their angle of growth with respect to gravity all vary between different inbred lines of maize (10). The physiological impact of variation in crown root growth has been explored through modeling approaches, which indicates that faster growing roots with a steeper gravity setpoint angle promote access to deep-water resources (11, 12). Greater crown root density comes at a cost, however, with recent studies suggesting that higher density is negatively correlated with the efficiency of nitrogen foraging (13).

To understand how WD affects the balance between embryonic and postembryonic parts of the root system and specifically crown root development, we analyzed root growth through excavation from soil and using the GLO-Roots imaging system (14). These studies used the emerging grass model species *Setaria viridis*, which is a C<sub>4</sub>-grass model for other agronomically important panicoid grasses, such as maize and sorghum (15, 16). Our results define the primary developmental mechanism through which WD affects root system architecture and reveals the physiological relevance of such a response.

## Results

**Suppression of Crown Root Growth Is a Major Response to Water Deficit in *S. viridis*.** The *S. viridis* A10.1 accession was grown in 35.5-cm-deep pots filled with a peat-based soil mixture (*Materials and Methods*). Seeds were planted in soil at full water-holding capacity (pot capacity). For well-watered (WW) conditions, pot weight was maintained near water-holding capacity throughout the experiment. To induce WD, water was withheld after germination, which caused gradual drying of the soil from the top to the bottom of the pot (Fig. S1 A and B). WD treatment led to a reduction in leaf relative water content (RWC); an acceleration in leaf initiation rate,

## Significance

Grasses, whose members constitute key food and bioenergy crops worldwide, use unique developmental programs to establish the root system from the shoot. Shoot-borne crown roots originate near the soil surface and provide the main conduits through which the plant takes up water and nutrients. We show that crown root development is the major target of drought stress signaling. Water deficit-triggered crown root arrest provides an important mechanism to conserve water under drought, and this response is widely conserved across grass species. Substantial phenotypic variation exists in maize for this trait, which may be a useful target in breeding efforts to improve drought tolerance.

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flowering time (date of panicle emergence), and tiller production; and a dramatic reduction in root mass (Fig. 1A and D and Fig. S1C-F).

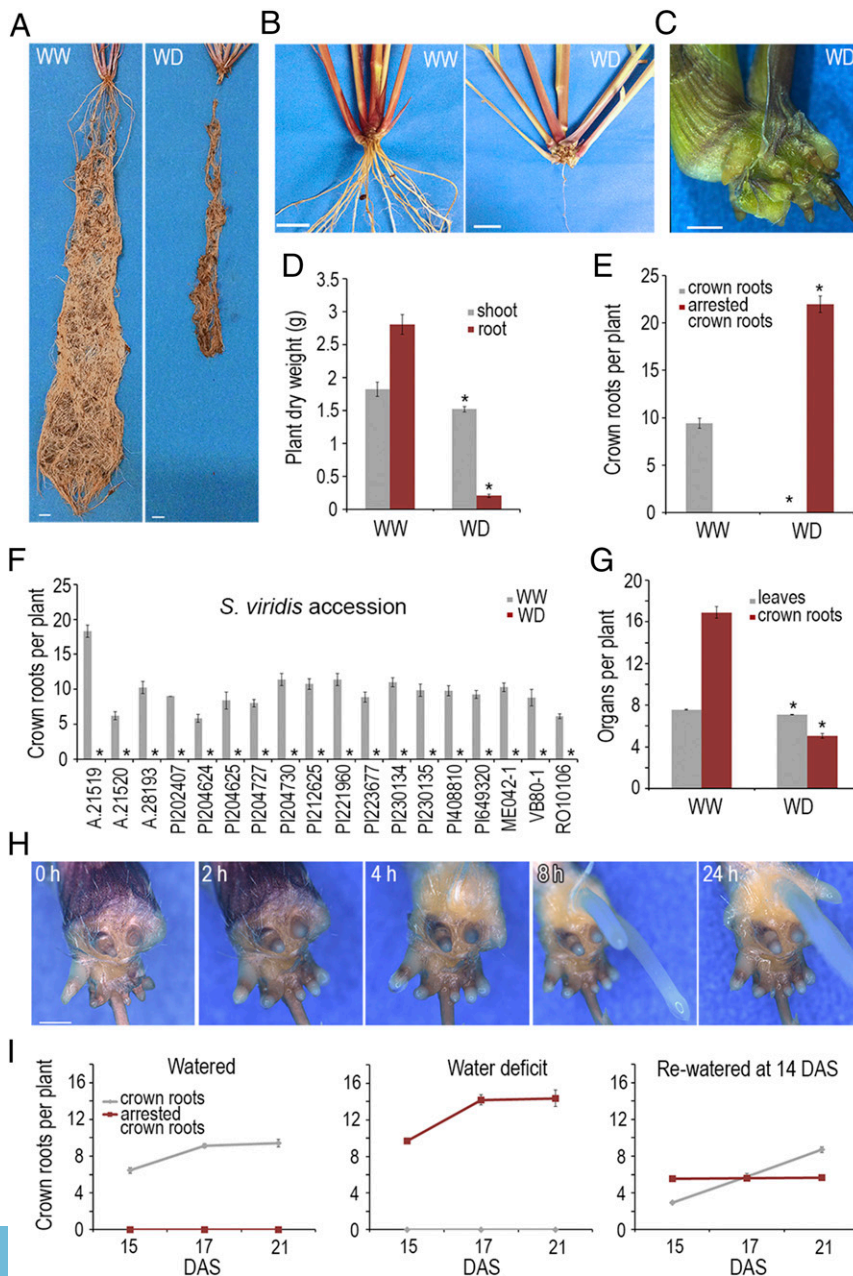
The first crown roots emerged 7 to 9 days after sowing (DAS) under WW conditions, and new roots emerged continuously thereafter (Fig. S1G). Excavation of the root system at 30 DAS revealed that most branches were crown root-derived under WW conditions, while under WD, crown roots were completely absent (Fig. 1B and E and Fig. S1H). Close examination of the crown showed an accumulation of arrested roots, which were absent under WW conditions (Fig. 1B, C, and E). Interestingly, the number of arrested crown roots was greater than outgrown roots under WW conditions, suggesting that WD may promote crown root initiation (Fig. 1E). Similar effects were observed in a survey of WD responses in 18 other *S. viridis* accessions and in plants grown at a lower temperature (Fig. 1F and Figs. S1I-K and S2A). Importantly, the observed suppression of crown root growth also occurred under field conditions simulating WW and WD conditions (Fig. 1G and

Fig. S2B-F). Together, these results show that the postemergence suppression of crown root growth represents a major response to WD in the *S. viridis* root system and is responsible for the dramatic decrease in root system size.

#### Local Perception of Water Rapidly Induces New Crown Root Development.

Irrespective of the degree or duration of the WD regime, if plants were still alive, they could produce new crown roots on rewatering (Fig. S3A). Newly emerged crown roots appeared between 4 and 8 h after treatment (Fig. 1H). All of the newly formed crown roots emerged de novo after rewatering and not as a result of recovery of growth of arrested crown roots (Fig. 1H and I).

Plants in which water was applied at the bottom of the pot recovered their water status but did not induce crown root growth, likely owing to the lower water content at the crown region under this watering regime (Fig. S3B-I). In contrast, plants for which water was applied directly to the crown region itself rapidly reinitiated



**Fig. 1.** Crown root growth is suppressed in *S. viridis* as a response to WD. (A) Comparison of whole-root systems of *S. viridis* grown under WW and WD conditions (30 DAS). (B) Comparison of crown regions of WW- and WD-treated *S. viridis* plants (25 DAS). (C) Magnified image of WD-treated *S. viridis* crown region showing the presence of arrested crown roots. (D) Comparison of plant dry weight under WW and WD conditions (34 DAS;  $n = 15$ –20 plants per condition). (E) Number of arrested and outgrown crown roots under WW and WD conditions (41 DAS;  $n = 10$ –15 plants per condition). (F) Crown root response to WD treatment in 18 *S. viridis* accessions (40 DAS;  $n = 5$ –10 plants per accession). (G) Number of leaves and crown roots quantified in field-grown plants under WW and WD conditions. Data are an average of results from six subplot replicates ( $n = 120$ –130 plants per treatment). (H) Time-lapse images of the crown region of a WD-treated plant after rewatering. Labels indicate time after rewatering. Image series shows rapid emergence and elongation of new roots from the crown, whereas previously emerged roots remain arrested. (I) Quantification of outgrown and arrested crown root formation in plants grown under WW (Left), WD (Center), or WD followed by rewatering (Rewatered, Right) ( $n = 25$  plants). Plants in the rewatered condition were WD-treated until the 14th DAS and then rewatered. (Scale bars: 1 cm in A and B, 1.5 mm in C and H.) \* $P < 0.05$ , Student's *t* test. Error bars represent SE.

crown root growth. These data suggest that water availability is locally sensed by the crown to regulate crown root growth and may be partly independent of the overall water status of the shoot. Whether such sensing occurs in the entire crown or in initiated crown roots is not clear at present.

**The Crown Region Is Highly Transcriptionally Responsive to WD.** We performed a transcriptomic analysis of the *S. viridis* crown tissue to elucidate the molecular pathways associated with WD responses. Crown tissue samples were collected from plants grown under either WW or WD conditions for 6 and 9 DAS. At 6 DAS, neither sample type had produced any emerged crown roots, whereas at 9 DAS, only WW plants formed outgrowing crown roots (Fig. 2A and Fig. S4A–C). We also sampled a 2-mm region of the stem apical to the crown region for comparison. We found

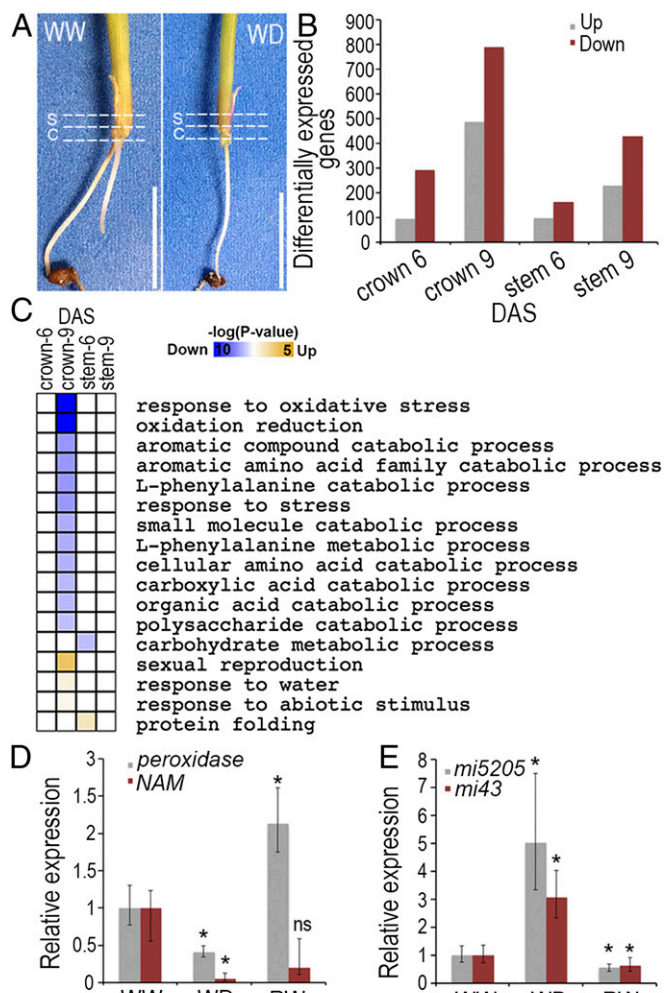
that the crown region at 9 DAS had the most differentially expressed genes between WW and WD conditions (Fig. 2B and Dataset S1), indicating a large relative change in the cellular state of these tissues.

Genes that were highly down-regulated in the crown region at 9 DAS are significantly associated with the oxidative-stress response ( $P = 4.7\text{e-}20$ ) and oxidation-reduction reactions ( $P = 1.2\text{e-}18$ ) and include several putative peroxidases (Fig. 2C and Datasets S1 and S2). Peroxidase activity promotes root growth, and their repression under WD may prevent continued growth of crown roots (17, 18). Sexual reproduction is the most enriched Gene Ontology (GO) enrichment term for up-regulated genes, which is consistent with the accelerated flowering exhibited by *S. viridis* under WD conditions (Fig. S1E). MapMan pathway analysis showed significant enrichment for genes associated with degradation of branched-chain amino acids in WD-repressed genes of the crown (Dataset S3). This alternative respiration pathway has recently been shown to affect drought tolerance in *Arabidopsis* (19).

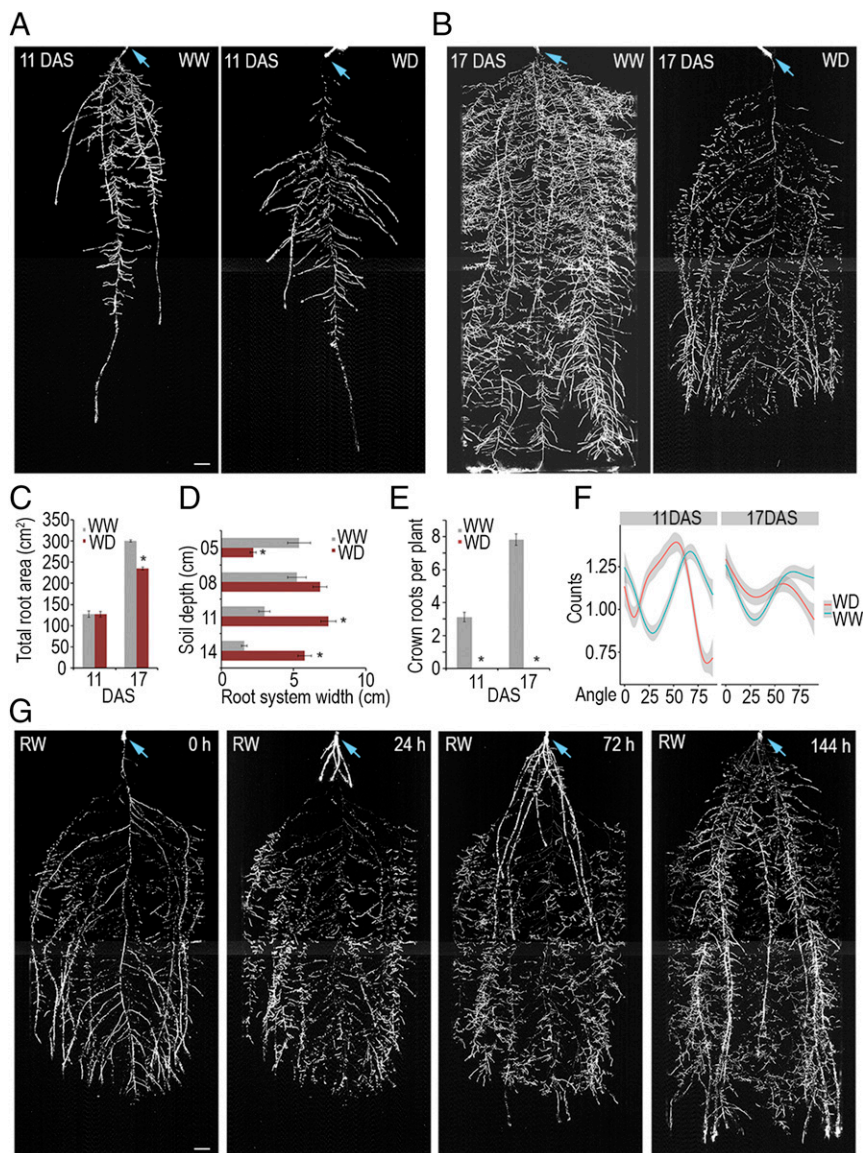
We found that 5% of the differentially expressed genes in the crown region are predicted targets of miRNAs, including *Sevir.5G028500*, a class III peroxidase that is the most down-regulated gene under WD, and *Sevir.7G133100*, a *NO APICAL MERISTEM (NAM)* gene (refs. 20 and 21 and Dataset S4). *NAM* family genes are plant-specific transcriptional regulators that control growth and development (22, 23). We analyzed levels of the miRNAs, *mi5205b*, targeting the peroxidase gene, and *miR43*, targeting the *NAM* gene, and found that both were significantly induced under WD (Fig. 2D and E). At 4 h after rewatering, the miRNA levels were down-regulated and the peroxidase gene expression was up-regulated; however, *NAM* gene expression showed no significant response (Fig. 2D and E). These data show coordinated regulation of miRNAs and their predicted targets, identifying a potential regulatory cascade affecting growth in the crown region; however, further functional studies are warranted to directly test this hypothesis.

**WD Inhibits the Transition to a Crown-Root Dominated Root System in *S. viridis*.** To explore acclimatization to water-limited growing conditions at a whole root system level, we used the newly developed luminescence-based imaging system GLO-Roots (Growth and Luminescence Observatory for Roots), which enables imaging of root systems in sheets of peat-based soil (14). Growth of plants in soil-filled rhizotrons enabled implementation of a similar WD regime as used in our pot-based experiments (Fig. S5). We generated transgenic *S. viridis* plants that constitutively expressed the *LUCIFERASE2* codon-optimized transgene (*ZmUbi1::LUC2o*) and identified lines that had no measurable growth differences compared with the wild type (WT) A10.1 progenitor background (Fig. S5 F–H). Root systems were imaged at two developmental phases: an early stage, at 11 DAS, when the primary and associated lateral roots form a major portion of the root system and a later stage, at 17 DAS, when crown root-derived branches predominate (Fig. 3A and B). At 11 DAS, WD-treated root systems grew to encompass a similar area of the soil as WW plants; however, the proliferation of roots occurred deeper in the soil profile (Fig. 3 C–F and Fig. S5 I and K).

At 17 DAS, a significant difference in the size of the root systems was apparent (Fig. 3 C, E, and F and Fig. S5J). The origin of roots was also markedly different, where WD-treated roots showed proliferation of primary root-derived branches, whereas WW root systems were dominated by crown root-derived branches (Fig. 3 B and E). Rewatering of WD-treated plants revealed a remarkable transformation of these root systems, with crown root growth filling the soil volume within 6 d after watering (Fig. 3G). These data indicate that crown root development is heavily dependent on the availability of water, and that the primary root-derived root system expands to complement the suppression of shoot-borne roots. Thus, the transition from a primary root-dominated system to a crown root-dominated system typical of



**Fig. 2.** The crown region is highly transcriptionally responsive to WD. (A) Images of WW- and WD-treated *S. viridis* plants at 9 DAS. Dashed lines indicate the sampled regions (C, crown; S, stem) for RNAseq analysis. (B) Numbers of differentially expressed genes from pairwise comparisons of WW and WD in the two regions and time points. (C) Heatmap showing the GO category enrichment in the *S. viridis* RNAseq analysis. Blue indicates significant enrichment of GO category in down-regulated genes; orange indicates significant enrichment in up-regulated genes ( $P < 0.05$ , corrected  $P$  value). (D) qRT-PCR showing the relative expression levels of peroxidase (*Sevir.5G028500*) and *NO APICAL MERISTEM (Sevir.7G133100)* genes under WW, WD, and RW conditions. (E) qRT-PCR showing the relative expression levels of *mi5205b* and *miR43* under WW, WD, and RW conditions. (Scale bars: 1 cm.) \* $P < 0.05$ , Student's  $t$  test. Error bars represent SE. ns, not significant.



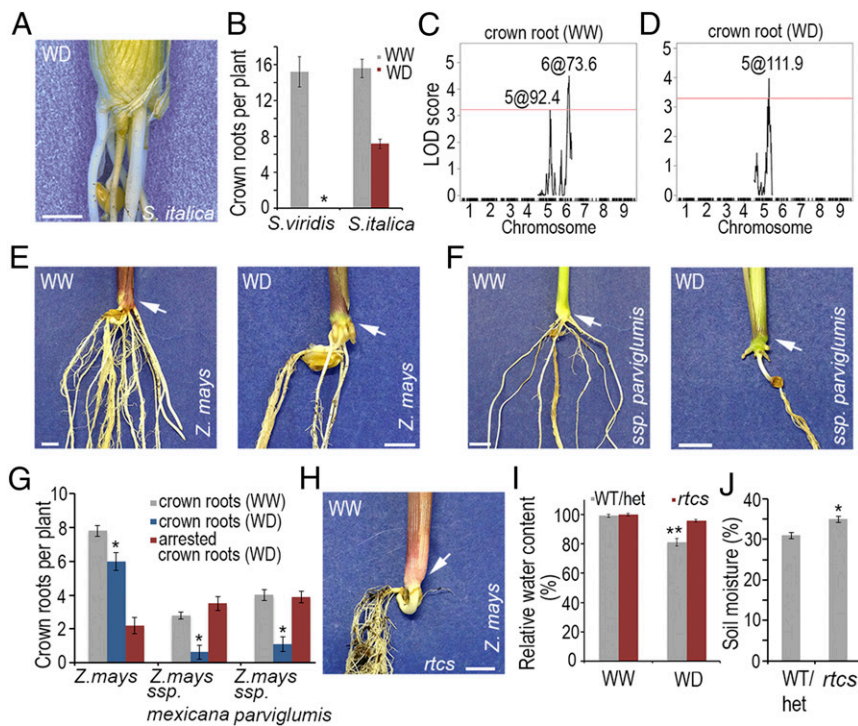
**Fig. 3.** Transition to a crown-root dominated root system is suppressed in response to WD in *S. viridis*. (A and B) Luminescence-based images of *S. viridis* root systems at 11 DAS (A) and 17 DAS (B) subjected to WW and WD conditions. The blue arrowhead indicates the crown region. (C) Quantification of total root system area using ImageJ at 11 and 17 DAS ( $n = 9-10$  plants). (D) Root system width in WW- and WD-treated *S. viridis* plants measured at varying depths of the rhizotron where lateral roots are present (11 DAS;  $n = 10$ ). (E) Number of crown roots observed in plants grown in rhizotrons at 11 and 17 DAS ( $n = 8-11$  plants). (F) Analysis of root system directionality in *S. viridis* root systems at 11 and 17 DAS ( $n = 8-11$  plants). Gray regions indicate the 95% confidence interval. (G) Time-lapse series showing the rapid induction of crown root growth on rewatering. Plants were rewatered at 17 DAS. (Scale bars: 1 cm.) \* $P < 0.05$ , Student's *t* test. Error bars represent SE.

grasses actually may be an environment-dependent transition and an adaptation that allows grasses to rapidly increase root growth in response to recent precipitation events.

**Variation in Crown Root Growth Under WD Conditions Explained by a Small Number of Quantitative Trait Loci.** Unlike *S. viridis*, cultivated foxtail millet, *Setaria italica*, maintained an ability to produce a small number of crown roots under WD conditions (Fig. 4A and B). This difference in crown root number under WD between *S. italica* and *S. viridis* is not likely to have an allometric basis, because *S. italica* has a lower shoot dry weight at this stage (Fig. S6A). *S. italica* is domesticated from its wild ancestor *S. viridis*, and the two species are intercrossable (24). We performed a quantitative trait loci (QTL) analysis using a recombinant inbred line (RIL) population from a cross between *S. viridis* and *S. italica*. We phenotyped a panel of 153 RIL lines under WW and WD conditions for crown root number and total root system dry weight. Overall, 86.5% of the variation in crown root number is explained by treatment. Two QTLs explaining 7.8% and 11.2% of crown root number variation under WW conditions were identified on chromosomes 5 and 6 (*CR-WW5* and *CR-WW6*) (Fig. 4C and Dataset S5). We found no significant QTLs under WD using total crown root number as a

continuous trait; however, when treating the data as a binary trait (presence or absence of crown root), we identified a QTL on chromosome 5 (*CR-WD5*) (Fig. 4D). Because of the 10% overlap between *CR-WW5* and *CR-WD5* confidence intervals, whether *CR-WD5* is WD-specific is unclear (Dataset S5). Of the 380 genes in the confidence interval of *CR-WD5*, 28 are differentially expressed in the crown region under WD in our *S. viridis* RNAseq dataset, thus identifying potential candidates for further study (Dataset S6).

Total root system biomass in *S. italica* is significantly higher than that of *S. viridis* (Fig. S6B). Some 79% of the variation in root weight observed in the RILs is explained by the interaction between the genotype and the treatment, suggesting that the genotypes respond differently to the WD condition. Our analysis revealed one QTL on chromosome 4 (*RW-WD4*) explaining 12.4% of the total root system biomass variation under the WD condition, whereas no significant QTL was identified under the WW condition (Fig. S6C and Dataset S5). Of the 1,665 genes in this interval, 63 are differentially expressed in our RNAseq dataset, including four putative peroxidases that are strongly down-regulated under the WD condition (Dataset S7). These data indicate that domestication of *S. italica* may have involved changes at specific loci that contribute to total root system mass and crown root responses to WD.



**Fig. 4.** Inhibition of postemergence crown root growth is a conserved WD response in the Poaceae that conserves water. (A) Image of WD-treated *S. italica* crown region showing arrested crown root growth (21 DAS). (B) Comparison of arrested vs. outgrown crown roots in *S. viridis* and *S. italica* plants grown under WW and WD conditions (30 DAS;  $n = 15$  plants). (C and D) Logarithm of odds (LOD) score plots showing the QTL regions affecting crown root growth under WW (C) and WD (D) conditions. The scanone 95% threshold is shown (red line) for reference. The number above the peak represents the chromosomal location and cM position. (E) Crown region of maize (B73) plant grown under WW (Left) and WD (Right) conditions (16 DAS). (F) Crown region of *Z. mays* ssp. *parviglumis* plants grown under WW (Left) and WD (Right) conditions (16 DAS). (G) Comparison of crown root development between *Z. mays* ssp. *parviglumis* and *Z. mays* ssp. *mexicana* (16 DAS;  $n = 10$  plants per condition). The asterisk indicates significant difference in the percentage of arrested crown roots by Student's *t* test. (H) Crown region of the *rts* mutant lacking crown roots. The arrowhead indicates the crown region. (I) Relative leaf water content of WT or *Rtcs/rts*, and *rts/rts* mutant plants (17 DAS;  $n = 15$  for *rtcs/rts* and  $n = 45$  WT/*Rtcs/rts* plants per condition). \*\*Significant genotype  $\times$  treatment interaction ( $P < 0.05$ ), two-way ANOVA. (J) Soil moisture content of deep pots with WT or *Rtcs/rts* and *rtcs/rts* maize plants subjected to WW or WD conditions ( $n = 15$ ). \* $P < 0.05$ , Student's *t* test. (Scale bars: 1.5 mm in A, 1 cm in E, F, and H.) Error bars represent SE. *rtcs*+, *Rtcs/rts*.

**Inhibition of Postemergence Crown Root Growth Is a Conserved WD Response in the Poaceae.** We analyzed crown root growth in four additional species under WD conditions to test the conservation of the responses observed in *S. viridis* (Materials and Methods). Sorghum (*Sorghum bicolor*), switchgrass (*Panicum virgatum*), and *Brachypodium distachyon* each showed strong suppression of crown root growth in response to WD, similar to *S. viridis* (Fig. S7). Five different accessions of sorghum and switchgrass species were analyzed, and all showed similar responses (Fig. S7 A–D). In contrast, the *Zea mays* (maize) inbred B73 maintained an ability to form some outgrown crown roots under WD. This response differed from the wild relatives of maize, teosinte (*Zea mays* ssp. *mexicana* and *Z. mays* ssp. *parviglumis*), which showed near-complete suppression of crown root growth (Fig. 4 E–G).

To explore whether the response of the B73 inbred was representative of maize, we surveyed the response of the nested association mapping (NAM) founder inbred lines, which represent a large portion of the genetic diversity of maize (25). Plants were phenotyped at 12 DAS for WW conditions and 16 DAS for WD, to ensure similar developmental stages were compared. Under WW conditions, the timing of crown root emergence showed limited variation among inbreds [coefficient of variation (CV) = 0.10], whereas the number of outgrown crown roots showed greater variability (CV = 0.28) (Fig. S8 A and B). WD resulted in an increase in phenotypic variation in crown root traits among inbreds. The number of arrested (CV = 0.69) and outgrown crown roots (CV = 0.49) varied substantially across inbred lines. NAM founders, such as M37 and CML69, showed a near-complete resistance to WD-triggered crown root growth arrest, whereas HP301 and NC358 exhibited near-complete arrest of crown root growth comparable to that of teosinte subspecies (Fig. S8B). In contrast to crown root traits, developmental stage of the shoot (V-stage: WW, CV = 0.18; WD, CV = 0.15) and the number of leaves produced showed lower variation across inbreds (WW, CV = 0.11; WD, CV = 0.13) (Fig. S8 C and D). These data show that crown root development remains a highly variable trait in maize inbred lines relative to other developmental traits, particularly under WD stress. Taken together, our results show that inhibition of postemergence

crown root growth is a conserved WD response in the Poaceae, and that significant genetic variation exists for this response in maize.

**Suppression of Crown Root Development Under WD Conditions Preserves Shoot Water Status.** To understand the physiological significance of changes in crown root development during WD, we used the maize *rootless concerning crown and seminal roots* (*rtcs*) mutant, which completely abolishes crown root growth while having no significant effect on primary and lateral root growth (26, 27) (Fig. 4H and Fig. S9A). A segregating population of *rtcs* mutants was germinated and grown under either WW or WD conditions for 17 DAS. RWC measurements demonstrated that homozygous *rtcs* mutant plants maintained shoot water status better than WT and *Rtcs/rts* plants (Fig. 4I and Fig. S9 C and D). Soil moisture content was greater for *rtcs* mutant plants compared with WT and *Rtcs/rts* plants (Fig. 4J). Importantly, shoot biomass was not significantly different between genotypes (Fig. S9B), consistent with the root-specific expression of the *Rtcs* gene (Fig. S9E). Taken together, these data suggest that *rtcs* mutants conserve more water under WD conditions, likely owing to reduced water uptake by a smaller root system. We hypothesize that for wild species such as *S. viridis*, suppression of crown root growth under WD may have a similar effect as the *rtcs* mutant and prevent overdraw of soil water resources (28), an adaptive strategy known as water banking. The suppression of crown root growth itself also may conserve water, because root tissues require water for cell expansion (29).

## Discussion

Here we demonstrate the importance of shoot-borne roots in enabling members of the grass family to acclimate to changing water availability in their environment. We find that a key site for sensing water availability is the crown region, and demonstrate that withholding water can dramatically inhibit the postemergence development of crown roots and maintain the root system in its initial primary root-dominated state. We hypothesize that such severe reductions in shoot-borne root growth are crucial to prevent overdraw of water from the soil and water loss through crown root growth.

Crown roots are necessary to provide mechanical support to the shoot and prevent lodging (7), as well as a level of redundancy required to minimize the damaging effects of biotic stress (30). Based on the results presented here, we can also infer that they provide a strong benefit to the plant in terms of water uptake, given that their development is rapidly activated after the crown locally senses an increase in moisture. The multiaxial nature of the grass root system may enable rapid capture of water from recent precipitation events and provide redundant routes by which water and nutrients can be transported to the shoot. Unlike in eudicots, secondary growth is absent in grasses, which otherwise would increase the vascular capacity of the shoot-root nexus (31).

Perhaps most intriguing from an agricultural standpoint is the difference in WD sensitivity of crown roots in wild and domesticated species of *Setaria* and maize. The large variation in crown root sensitivity to WD in inbreds of maize suggests that breeders might have inadvertently selected for different response strategies based on the water availability dynamics of the field conditions where selection occurred. In this regard, it is interesting to note that sorghum, which completely abolishes crown root growth under WD, is known to exhibit significant drought tolerance for a crop species (32). Perhaps more targeted breeding for varieties with enhanced WD responses of crown roots may

benefit maize productivity when resources are scarce or highly variable.

## Materials and Methods

Plant materials and methods for physiological and genetic analysis, plant growth conditions, transgene construction, plant transformation, and the GLO-Roots methodology are described in *SI Materials and Methods*. Table S1 provides a list of terms used in this work to describe root types, brief definitions of the terms, and associated references. Datasets S1–S8 provide processed RNAseq gene expression values, lists of differentially expressed genes and significantly enriched GO category and MapMan ontology terms, predicted miRNA targeted genes, summarized QTL analysis results, genes within QTL intervals, and a list of primer sequences used in this study.

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