Supporting Information

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SI Materials and Methods

Plant Materials, Experimental Conditions, and Measurements. The *S. viridis* reference accession A10.1 was used in this study unless indicated otherwise. *S. viridis* accessions and sorghum, switchgrass, and teosinte lines were obtained from the the US Department of Agriculture's North Central Regional Plant Introduction Station, Iowa State University. Maize NAM founders were obtained from Torbert Rocheford (Purdue University). Brachypodium seeds were obtained from the laboratory of John Vogel (Department of Energy, Joint Genome Institute).

Deep pots (D25L; Stuewe & Sons) were used for growing plants unless indicated otherwise. Experiments were replicated at least three times. Seeds were germinated in a peat-based soil mixture containing 75% Pro-Mix PGX (Premier Tech) and 25% river sand, imbibed in water to pot capacity. Pot capacity, defined as the amount of water that soil in a pot can hold against the pull of gravity, was estimated to be 230 mL. Plants were grown in a growth chamber (12 h light at 31 °C and 12 h dark at 23 °C with constant relative humidity at 54–55%) and were watered once every third day unless noted otherwise. For WD experiments, seeds were sown in soil imbibed to pot capacity, and no further water was added. To prevent water loss, the bottom of each pot was covered with a plastic bag. To monitor the amount of water loss over the duration of the experiment, pot weight was measured every second day. For WW conditions, the amount of water lost was replenished every third day. Plants were grown in 14-cm-deep pots for the time series analysis of outgrown and arrested crown root formation under WW, WD, and RW conditions. Experimental conditions for teosinte and maize were the same as for *S. viridis*, except that a soil containing 100% Pro-Mix PGX was used, and seeds were germinated at 50% of pot capacity for WD treatments. Soil moisture content measurements (gravimetric) were performed as follows: Soil samples collected from pots were weighed both before (fresh weight) and after drying (dry weight). Soil samples were dried in an oven at 60 °C for 3 days. Soil moisture (%) was calculated using the following formula: soil moisture (%) = 100 × [(fresh weight – dry weight)/fresh weight].

Field experiments were carried out at the Department of Plant Biology, Carnegie Institution for Science between June 19, 2015, and July 29, 2015. Plants were grown in two equal-sized (1 m × 3 m) raised-bed plots filled with field soil. Each plot was further divided into six subplots, each containing ∼25 plants. Both plots were watered up to 14 DAS, and water was withheld from the WD plot for the next 21 d. Soil moisture was measured regularly using a Diviner 2000 probe (Sentek Technologies). Arrested crown roots were observed and quantified using an Olympus SZ61 stereo microscope.

RWC measurements were carried out as described previously (33). Four to five plants were sampled per treatment and genotype. For each plant, the apical-most fully expanded leaf was sampled for the analysis. Leaf tissue was harvested with surgical scissors from the area between the midvein and the edge. Samples were collected in preweighed Eppendorf tubes and immediately processed. All weight measurements were performed using a microbalance. RWC was calculated using the following formula: $RWC\ (\%) = [(W - DW)/(TW - DW)] \times 100$, where W is sample fresh weight, TW is sample turgid weight, and DW is sample dry weight.

Maize *rtcs* Genotyping. Maize *rtcs* plants were genotyped using the Phire Plant Direct PCR kit (F-130, Thermo Fisher Scientific) with the primers listed in Dataset S8. WT samples yielded a 323-bp PCR product with the *rtcs*-ATG-79-fw/*rtcs*-ATG+242-rv primer pair. *rtcs*/*rtcs* samples yielded a 325-bp PCR product with the *rtcs*-ATG-79-fw/*rtcs*-5bpinsert-rv primer pair.

Transcriptomic Analysis. Crown tissue samples were collected from plants grown under either WW or WD conditions to 6 and 9 DAS in deep pots. Three biological replicates consisting of 20 plants per sample were collected at 6 and 9 DAS. The 2-mm region containing the crown section and any attached crown roots was cut and placed into a Covaris TT2 tissue bag. The 2-mm region above the crown was cut and placed into a separate bag. Samples were kept at −70 °C until being prepped. *S. viridis* crowns in tissue bags were frozen in liquid nitrogen and smashed to powder in a Covaris CryoPREP impactor (power setting 4). RNA was extracted using Zymo ZR Plant RNA MiniPrep (R2024). Ribosomal RNA was depleted using Ribozero Plant/Root/Seed (MRZSR116) and strand-specific, barcoded RNAseq libraries were created using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (E7420S; New England BioLabs). Libraries were quantified by qPCR using the Kapa Library Quantification Kit (KK4824; Kapa Biosystems) and pooled in equimolar groups of 12. Pooled libraries were sequenced with HiSeq 2 × 101-bp paired end reads. Reads were mapped to the *S. viridis* v1.1 genome and analyzed via the Tuxedo pipeline (34).

All RNA-seq data were quality controlled via fastqc. Adapters were filtered using cutadapt, low-quality 3′ sequences were filtered using the fastx toolkit (discarding nucleotides <Q35, reads shorter than 50 nt), and read singletons were discarded. Read pairs that survived the foregoing process were aligned against the *S. viridis* v1.1 genome (https://phytozome.jgi.doe.gov/) (35) using BWA-MEM (36).

Alignments of RNA-seq data were grouped by tissue/treatment/duration combinations (e.g., the three biological replicates derived from WW upper root samples at 6 DAS were treated as a group). Replicate groups were compared using the Cuffdiff algorithm (i.e., estimating differential expression via dispersion modeling on a Poisson distribution) (34). All possible pairwise comparisons between replicate groups were conducted, and gene loci exhibiting a change in expression with a false-discovery–corrected P value < 0.05 were identified as differentially expressed. Differentially expressed genes were grouped by their directionality of differential expression and analyzed for overrepresentation of GO terms (37) using the topGO R package (38) from Bioconductor (39). GO terms with a false-discovery–corrected P value <0.05 were deemed overrepresented.

The MapMan ontology enrichment analysis was conducted using *Oryza sativa* annotations (Osa_MSU_v7). Mapping *O. sativa* to *S. viridis* (v1.1) genes was done by finding reciprocal blast hits between the two genomes. Pathway enrichment of genes were measured by calculating P values using Fisher's exact test for each sub-bin.

Real-Time qPCR Analysis. Crown tissue samples were collected into Covaris TT2 tissue bags from 30 plants per sample in three biological replicates from WW or WD plants grown in 35.5-cm-deep pots for 9 d after sowing. RW plants were grown in WD conditions for 9 d, then watered and harvested 4 h later. Crown tissue was smashed in a cryoPREP impactor, then extracted with TRIzol (Thermo Fisher Scientific) and purified using the Direct-zol RNA MiniPrep Kit (Zymo Research) to maximize the yield of small RNAs. cDNAs of large transcripts were made using iScript Reverse Transcription Supermix (1708841; Bio-Rad) with 1 μg total RNA as input. qPCR was performed using the Bioline SensiFAST SYBR

No-ROX Kit in a Roche Light Cycler using NV9 (Sevir.6G210400) as a control. cDNA of miRNA was prepared as described previously (40), except using 2 μg of total RNA as input. qPCR was performed as above using 5.8S rRNA as a control. The qPCR primer sequences are listed in Dataset S8.

GLO-Roots Imaging System for *S. viridis*. To visualize *Setaria* root systems in soil using the GLO-Root imaging system, transgenic plants expressing the luciferase gene were generated. A plant codon-optimized luciferase gene, *LUC2o* (14), was cloned into the monocot binary vector pANIC10 (41). Transgenic *S. viridis* plants harboring *ZmUbi1::LUC2o* were regenerated from callus as described previously (42). Transgenic T_2 plants were surface-sterilized and germinated in tissue culture plates as described previously (43). For comparison of growth characteristics of WT and transgenic lines, plants were grown in deep pots filled with Pro-Mix soil. For growth of plants in rhizotrons, seeds were first germinated on agar media and then, at 3 DAS, transferred to rhizotrons and maintained in the growth chamber (12 h light at 31 °C and 12 h dark at 23 °C with constant relative humidity at 54–55%). The WW-treated plants were watered daily using a transfer pipet, but the WD-treated plants received no further watering from 5 DAS onward. Plant imaging and image processing were done as described previously (14). Directionality analysis, which computes the mean direction of roots in the root system, was done as described previously using the GLO-RIA ImageJ plug-in (14). Total root system area was calculated using ImageJ (44).

QTL Analysis. RILs were grown as described above. One plant per RIL per condition was phenotyped. QTL mapping was performed using the R/qtl package (45) on raw experimental values. The analysis was performed on datasets collected within each treatment block and on the numerical difference of values between treatments using 153 RILs and a genetic map containing 1,595 SNP markers. Haley–Knott regression was used to perform a single QTL scan at each marker individually. One thousand permutations were performed to determine significance thresholds for inclusion additive QTL at $\alpha = 0.05$. Automated stepwise model selection was used to determine a final additive QTL model. After refinement of QTL position estimates, phenotypic values were fit to the QTL model using ANOVA to assess the proportion of variance explained and effect size of each locus. All putative protein coding genes (*S. viridis* genome v1.1) found within a 1.5-LOD confidence interval were reported for each QTL.

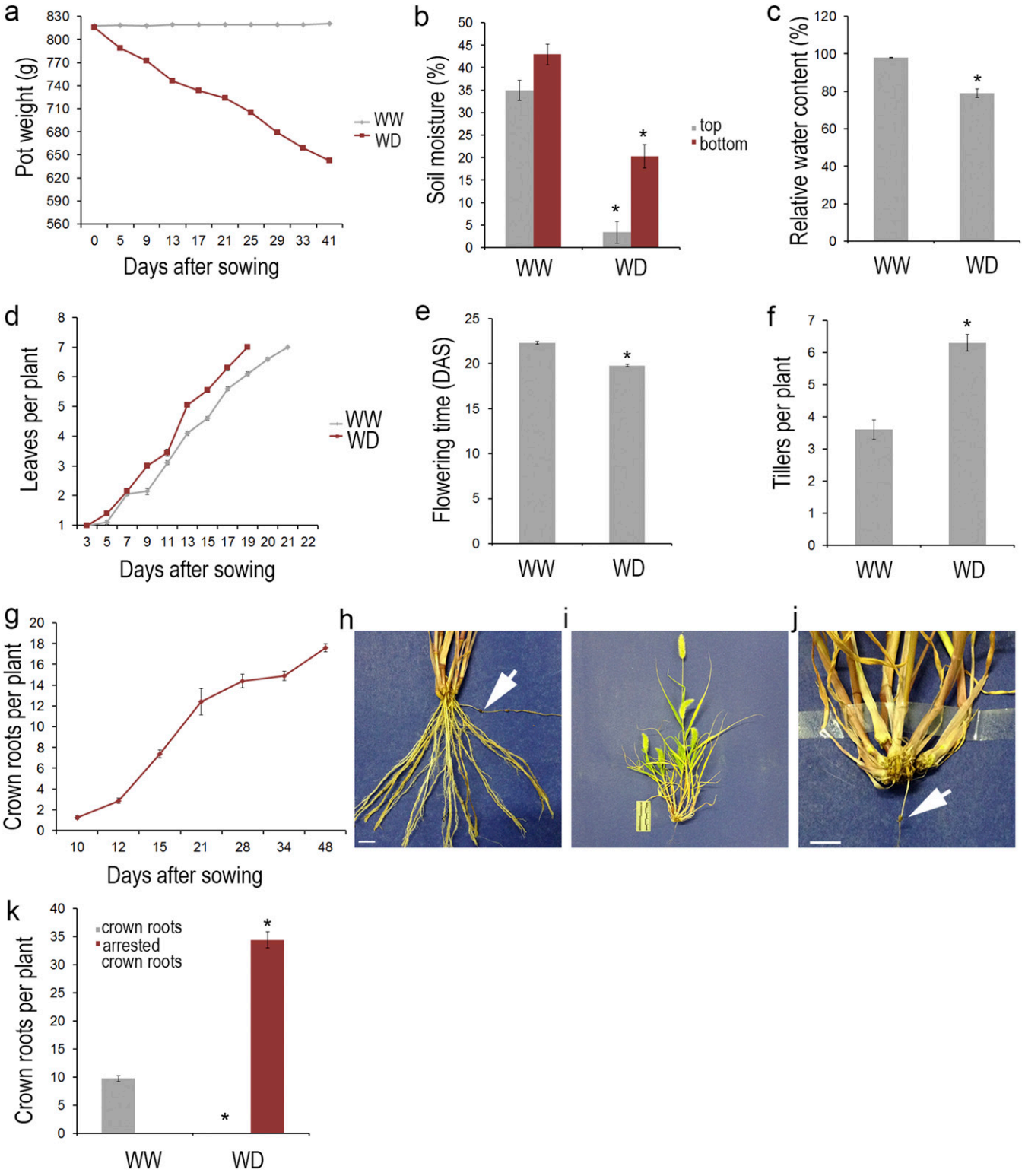

Fig. S1. Crown root growth arrest in *S. viridis* as a WD response. (*A*) Pot moisture content measured through a time series showing the gradual drying of the soil with WD treatment ($n = 10$ pots). Pots with soil weighed on average 586.5 ± 1.3 grams before watering. (*B*) Soil water content of deep pots at 27 DAS subjected to WW or WD conditions ($n = 3$). Top, soil from the top half of the pot; bottom, soil from the bottom half of the pot. (*C*) Relative water content of leaves from WW and WD conditions at 41 DAS ($n = 10$). (*D–F*) Comparison of leaf initiation rate (*D*), flowering time (*E*), and tiller production (*F*) in *S. viridis* grown under WW and WD conditions ($n = 10–15$ plants). (*G*) A time-series analysis of crown root emergence in *S. viridis*. Plants are grown in deep pots under WW conditions. (*H*) *S. viridis* crown region showing primary (arrowhead) and crown roots at 36 DAS. (*I* and *J*) Image of an *S. viridis* plant grown under WD conditions at 22 °C (*I*) and a magnified image of the crown region showing arrested crown roots and the primary root (arrowhead) (*J*) (40 DAS). (*K*) Comparison of arrested vs. outgrown crown roots in *S. viridis* plants grown under WW or WD conditions at 22 °C ($n = 12–14$ plants). *$P < 0.05$, Student's *t* test. (Scale bars: 1 cm.) Error bars represent SE. WW, watered; WD, water deficit.

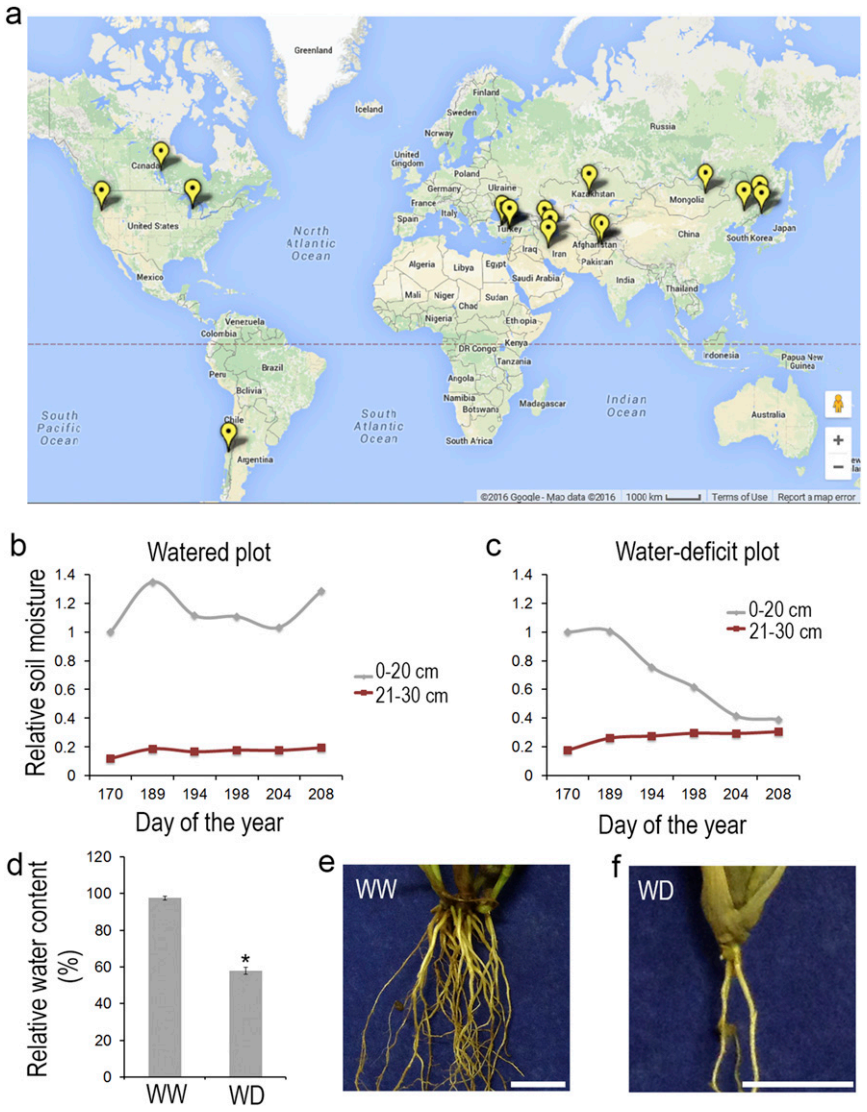

Fig. S2. *S. viridis* accessions and analysis of crown root growth under field conditions. (*A*) A global map showing the approximate location (yellow markers) of the different *S. viridis* accessions used in this study (Ames 21519, China; Ames 21520, Russia; Ames 28193, Kazakhstan; PI202407, Chile; PI204624, Turkey; PI204625, Turkey; PI204727, Turkey; PI204730, Turkey; PI212625, Afghanistan; PI221960, Afghanistan; PI223677, Azerbaijan; PI230134, Iran; PI230135, Iran; PI408810, China; PI649320, Mongolia; ME042-1, Canada; VB80-1, United States; and RO10106, United States). Fig. S2*A* © 2016 Google – Map data © 2016 Google. (*B* and *C*) Relative moisture content in the topsoil measured using a Diviner 2000 probe for WW (*B*) and WD (*C*) field plots. (*D*) RWC (leaf) of field-grown plants ($n = 36$ plants). (*E* and *F*) Images of *S. viridis* plants from the field experiment under WW (*E*) and WD (*F*) conditions. (Scale bars: 1 cm.) Error bars represent SE.

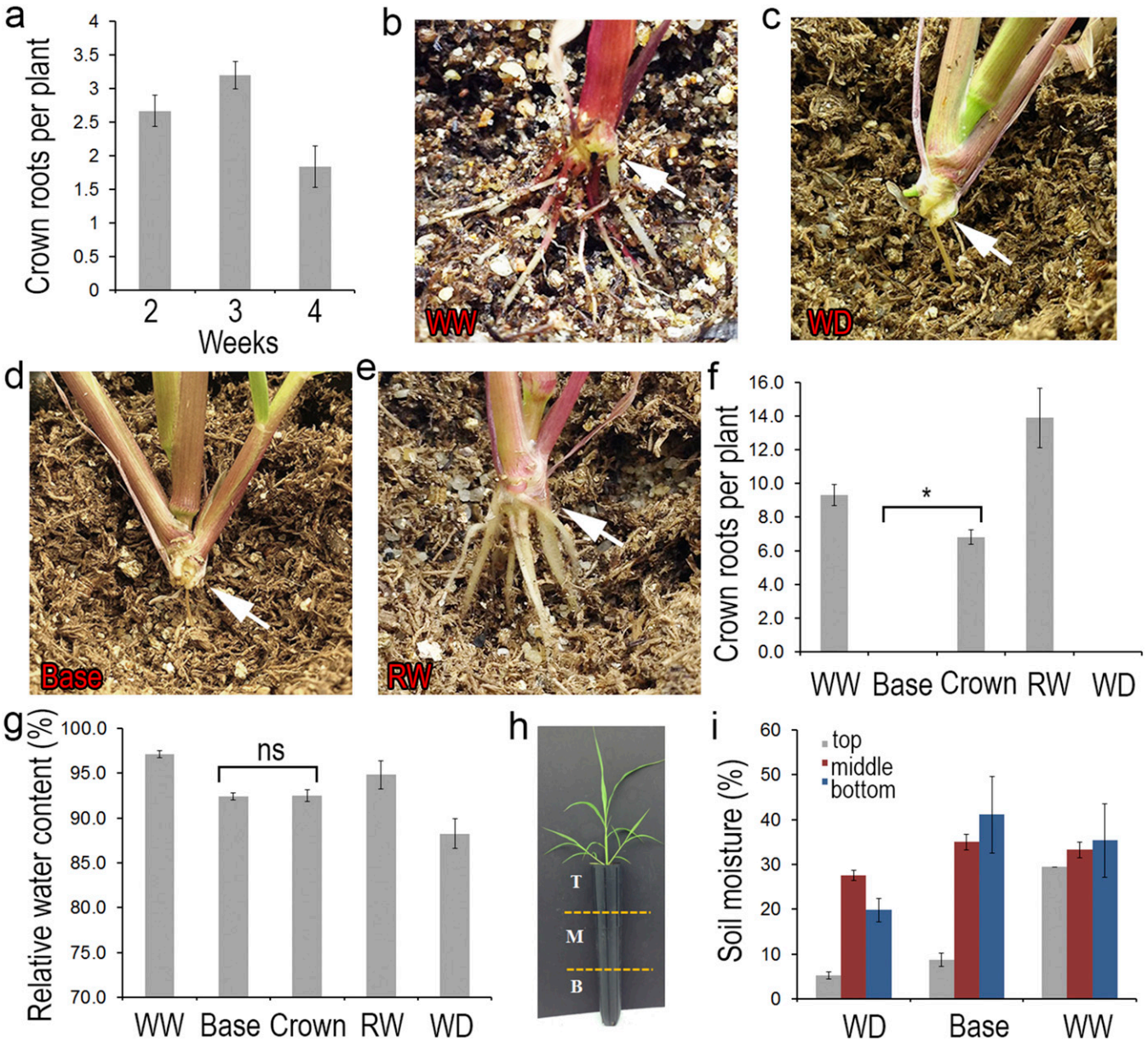

Fig. S3. Local perception of moisture by roots in the crown. (*A*) Number of crown roots formed at 24 h after rewatering. Plants were subjected to varying durations of WD before rewatering ($n = 6–10$ plants). (*B–E*) Images of the crown region. Soil has been removed to visualize the roots. Plants were grown under WW conditions (*B*), WD conditions (*C*), WD conditions for 3 wk then rewatered from the bottom of the pot (base) (*D*), or fully rewatered (brought back to pot capacity) (*E*). The arrowhead indicates the crown region. (*F* and *G*) Number of outgrown crown roots (*F*) and leaf RWC (*G*) in plants grown under WW or WD conditions for 3 wk before being rewatered from the bottom of the pot (base), rewatered at the crown region (10 mL added around the crown region using a pipette), fully rewatered (RW), or continuous WD ($n = 10$ plants). (*H*) Division of regions of the pot for analysis of soil moisture content in *I*. (*I*) Pot soil moisture content under various watering regimes ($n = 3$ samples per section). Error bars represent SE. $^*P < 0.05$, Student's *t* test. ns, not significant.

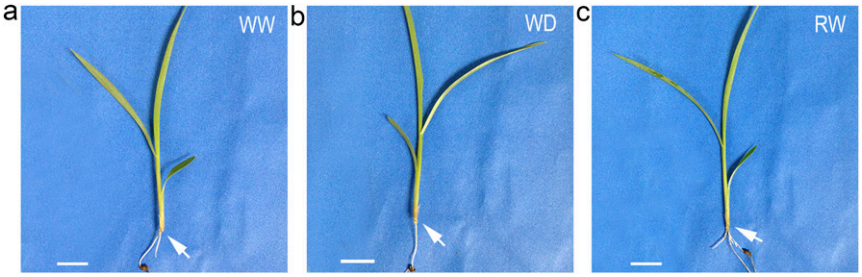

Fig. S4. Developmental stages used for transcriptional analysis of WD responses in crown. Picture of *S. viridis* plants subjected to WW (*A*), WD (9 DAS) (*B*), and RW (10 DAS) (*C*) conditions. (Scale bars: 1 cm.)

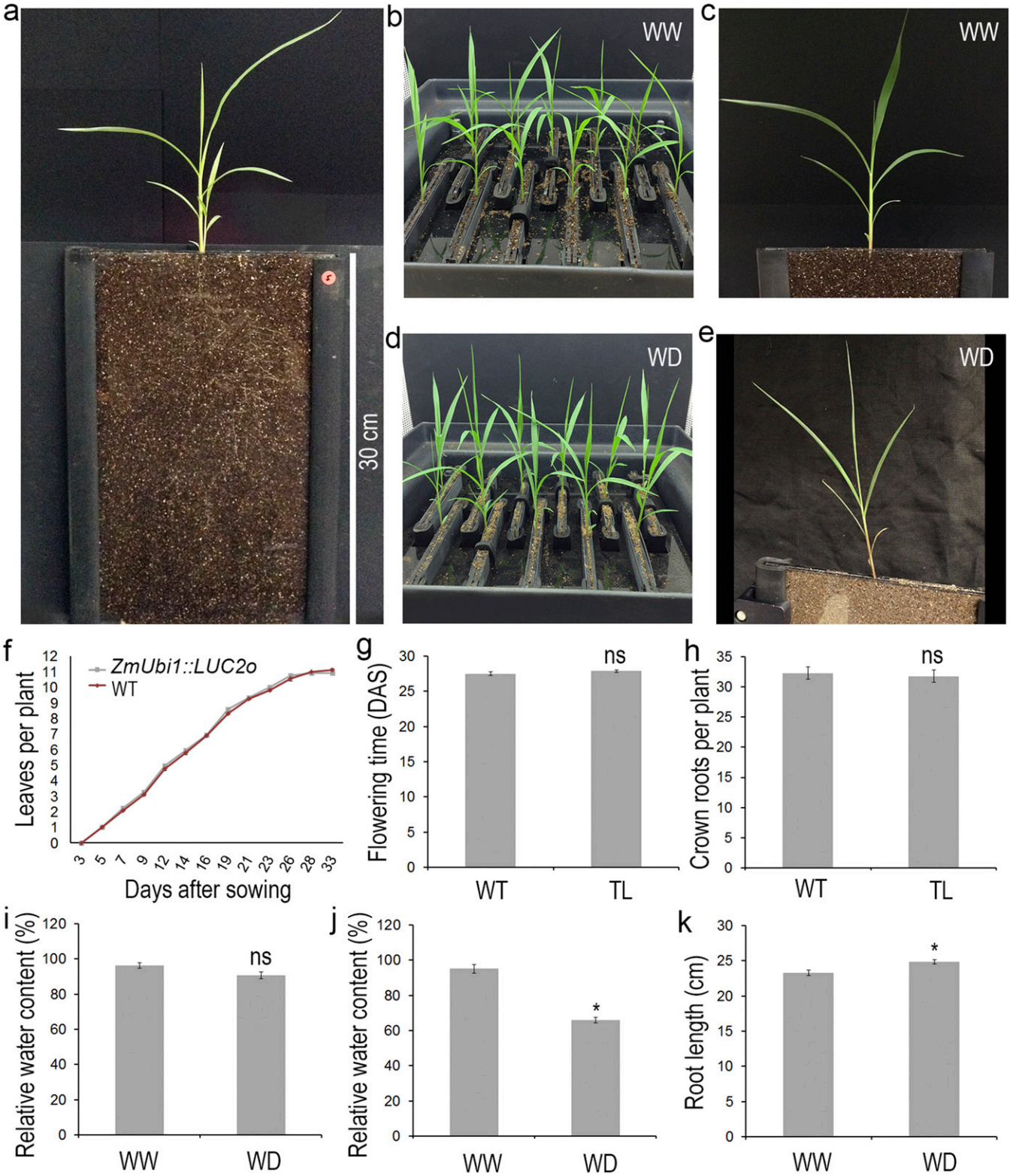

Fig. S5. Growth of *S. viridis* in GLO-Roots rhizotrons. (*A*) Rhizotron with a *S. viridis* plant. The black sheet blocking light exposure of soil was removed to visualize the soil. (*B*) *S. viridis* plants subjected to WW conditions growing in the rhizotron box (11 DAS). (*C*) Shoot of WW plant at 15 DAS. (*D*) Plants subjected to WD conditions growing in the rhizotron box (11 DAS). (*E*) Plant grown under WD at 15 DAS. (*F–H*) Comparison of growth characteristics of leaf initiation rate (*F*), flowering time (*G*), and crown root number (*H*) between the WT and *S. viridis* transgenic line expressing *LUC2o* (*ZmUbi1:: LUC2o*) ($n = 10$ per genotype). (*I* and *J*) Analysis of leaf RWC of plants grown in rhizotrons at 11 DAS (*I*) and 17 DAS (*J*) under WW or WD conditions ($n = 5$). (*K*) Primary root lengths in plants grown in rhizotrons under WW or WD conditions at 11 DAS ($n = 10$). *$P < 0.05$, Student's *t* test. ns, not significant. Error bars represent SE.

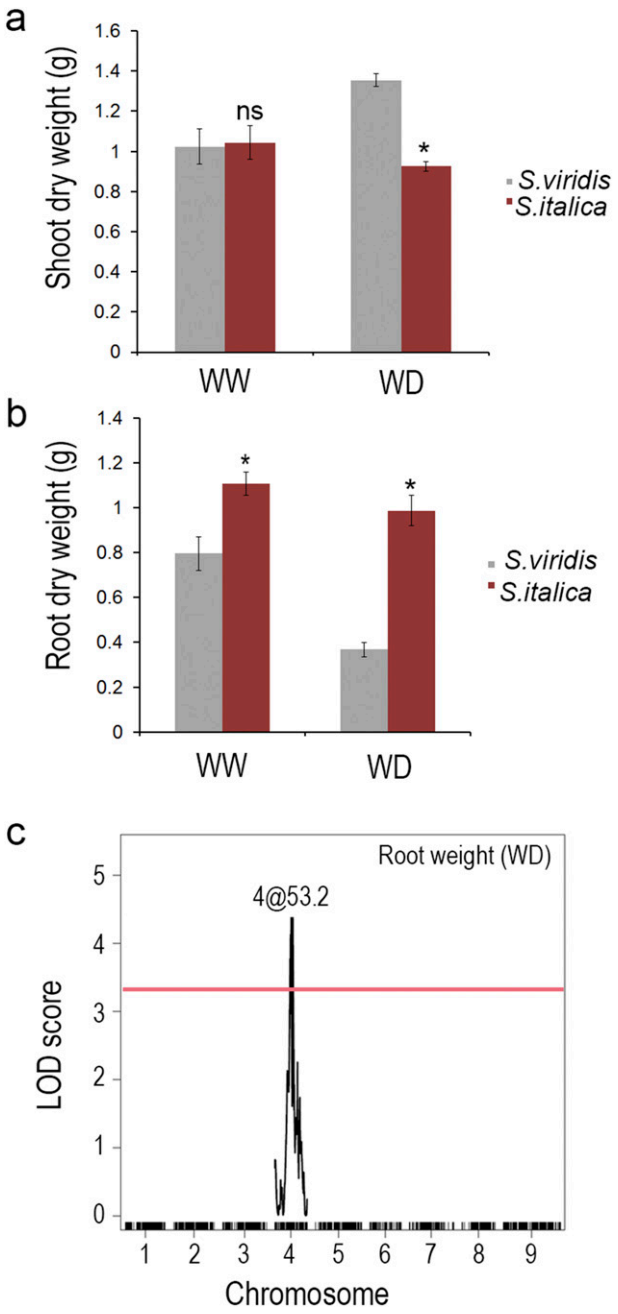

Fig. S6. Variation in shoot and root biomass under WD conditions between *S. viridis* and *S. italica*. (*A* and *B*) Comparison of shoot (*A*) and root (*B*) system dry weight between *S. viridis* and *S. italica* grown under WW and WD conditions (30 DAS; $n = 5$ plants for WW, 15 plants for WD). (*C*) LOD score plot showing the QTL region involved in regulation of the root biomass under WD. The scanone 95% threshold is indicated for reference. The number above the peak represents the chromosomal location and cM position. Error bars represent SE. $*P < 0.05$, Student's *t* test. ns, not significant.

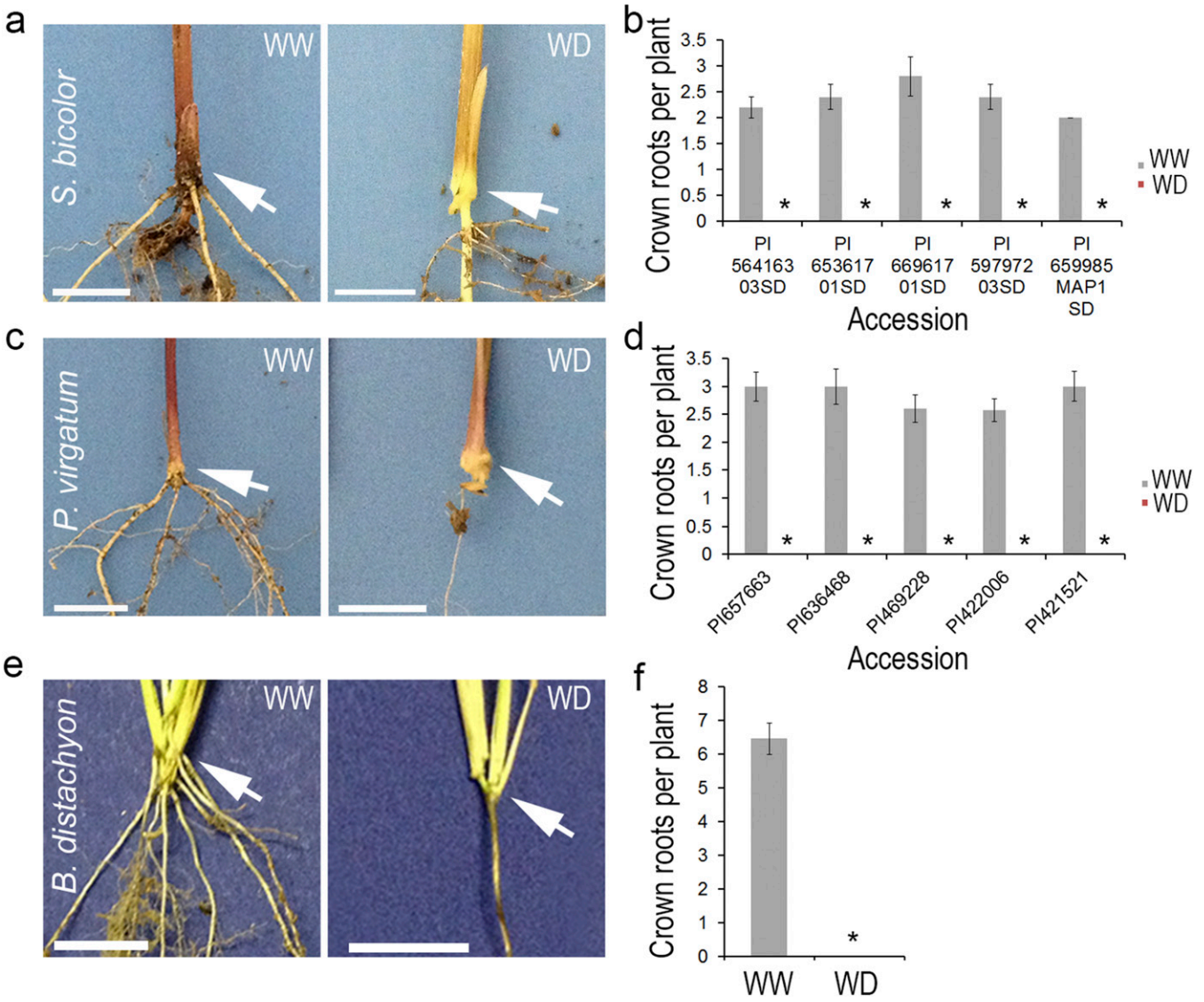

Fig. S7. Inhibition of postemergence crown root growth is a conserved WD response in the Poaceae. (*A*) Crown region of sorghum (*S. bicolor*) plants subjected to WW or WD treatments. (*B*) Number of arrested vs. outgrown crown roots in WW- and WD-treated plants (21 DAS; $n = 5$ plants per accession per condition). (*C*) Crown region of switchgrass (*P. virgatum*) plants subjected to WW or WD treatment. (*D*) Number of arrested vs. outgrown crown roots in WW and WD-treated plants (21 DAS; $n = 5$–8 plants per accession per condition). (*E*) Crown region of Brachypodium (*B. distachyon*) plants subjected to WW or WD treatment. (*F*) Number of arrested vs. outgrown crown roots in WW- or WD-treated plants (38 DAS; $n = 10$ plants per condition). (Scale bars: 1 cm.) Error bars represent SE.

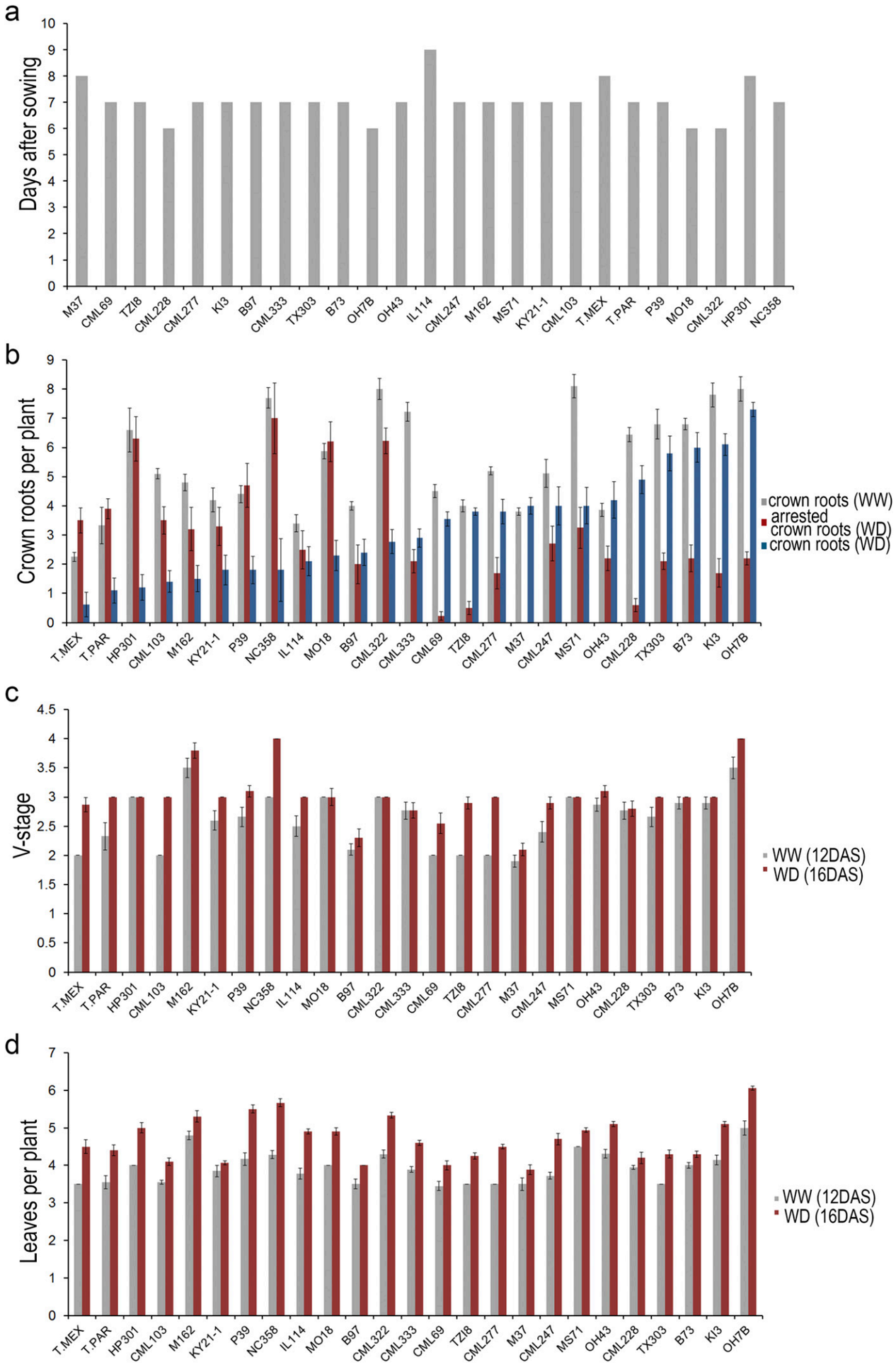

Fig. S8. Variation in crown root development under WD in maize inbred lines. (*A*) Survey of variation in crown root emergence among maize NAM founders. Data indicate DAS at which 50% or more plants exhibit emerged crown roots (n = 10 plants). (*B*) Extent of crown root growth in different maize NAM founders (n = 10 plants). Data collected at 12 DAS for WW and 16 DAS for WD conditions. (*C* and *D*) Comparison of V-stage (*C*) and leaf number (*D*) of plants grown under WW and WD conditions at 12 and 16 DAS. Error bars represent SE.

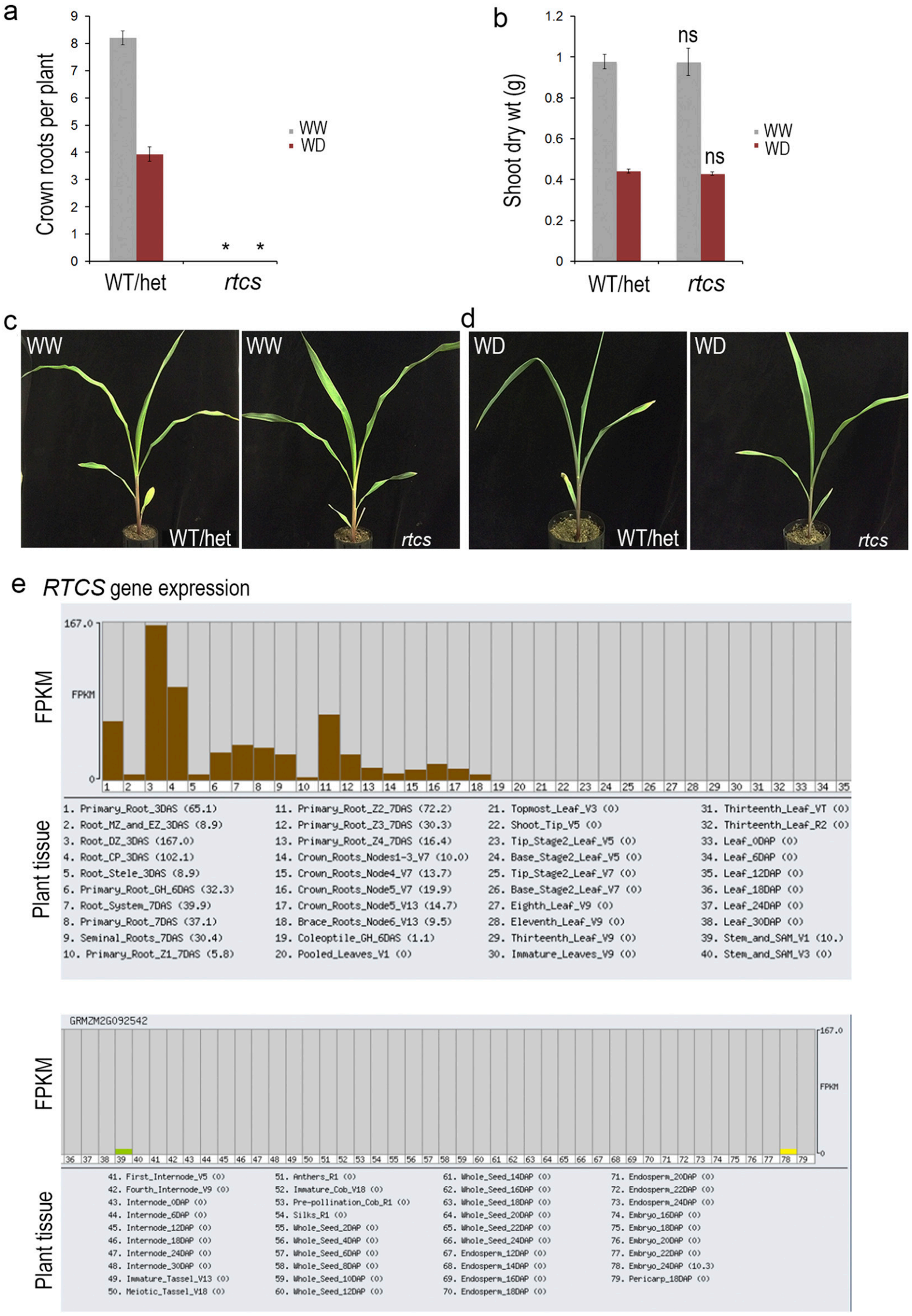

Fig. S9. Analysis of maize *rtcs* plants subjected to WW and WD conditions. (*A*) Crown root number between WT/het and *rtcs*/*rtcs* maize plants (17 DAS; $n = 15$ plants). (*B*) Shoot dry weight of WT/het and *rtcs*/*rtcs* maize plants after 17 DAS ($n = 15$ plants). (*C*) WT/het and *rtcs*/*rtcs* mutant plants at 17 DAS grown under WW conditions. (*D*) Unlike the *rtcs*/*rtcs* mutant, WD-treated WT/het plants showed wilting by 17 DAS. (*E*) Graph showing the expression pattern of the *rtcs* gene in different tissues of the maize plant (15). $*P < 0.05$, Student's *t* test. het, heterozygous for the *rtcs* mutation; ns, not significant. Error bars represent SE. *rtcs*/+, *Rtcs*/*rtcs*.

Table S1. Glossary of commonly used terms describing root system features

Term used	Synonymous terms	Description	References
Crown		Basal nodes of the shoot in grasses	plantscience.psu.edu/research/centers/turf/extension/factsheets/cool-season
Embryonic root system		Comprises roots that originate from the embryo (primary and seminal roots)	2, 7, 46, 47
Postembryonic root system		Comprises roots that emerge after germination (lateral and shoot-borne roots)	2, 7, 46, 47
Primary root	Taproot, radicle	First root of a plant; emerges from the basal pole of the seed	2, 7, 31, 46, 47
Seminal roots		Roots originating from the scutellar node of the embryo (embryonic in origin)	2, 7, 31, 46, 47
Lateral roots		Roots formed from the pericycle and endodermis of other roots	2, 7, 46, 47
Shoot-borne roots	Nodal roots	Roots formed from the nodes (underground and aboveground) of the shoot	2, 7, 46, 47
Crown roots		Shoot-borne roots formed from underground nodes (crown) of the shoot	2, 7, 46, 47

Dataset S1. Differentially expressed genes from the *S. viridis* RNAseq analysis

Dataset S1

Dataset S2. List of GO category enrichment from the *S. viridis* RNAseq analysis

Dataset S2

Dataset S3. MapMan ontology enrichment analysis of the *S. viridis* RNAseq data

Dataset S3

Dataset S4. Predicted miRNA targets differentially expressed under WD in *S. viridis*

Dataset S4

Dataset S5. Summary of the QTL analysis

Dataset S5

Dataset S6. Genes in the *CR-WD5* QTL interval showing differential expression in the *S. viridis* RNAseq analysis

Dataset S6

Dataset S7. Genes in the *RW-WD4* QTL interval showing differential expression in the *S. viridis* RNAseq analysis

Dataset S7

Dataset S8. Primers used in this study

Dataset S8