Overexpression of RING Domain E3 Ligase ZmXerico1 Confers Drought Tolerance through Regulation of ABA Homeostasis [OPEN]

Norbert Brugière, a,2 Wenjing Zhang, a Qingzhang Xu, a Eric J. Scolaro, a Cheng Lu, a Robel Y. Kahsay, a,1 Rie Kise, a Libby Trecker, a Robert W. Williams, a Salim Hakimi, a Xiping Niu, a Renee Lafitte, a and Jeffrey E. Habben a

aDuPont Pioneer, 7300 NW 62nd Avenue, PO Box 1004, Johnston, Iowa 50131-1004

ORCID IDs: 0000-0002-2681-7559 (N.B.); 0000-0003-1365-8269 (C.L.); 0000-0001-6847-0920 (R.W.); 0000-0002-5762-0652 (R.L.)

Drought stress is one of the main environmental problems encountered by crop growers. Reduction in arable land area and reduced water availability make it paramount to identify and develop strategies to allow crops to be more resilient in water-limiting environments. The plant hormone abscisic acid (ABA) plays an important role in the plants’ response to drought stress through its control of stomatal aperture and water transpiration, and transgenic modulation of ABA levels therefore represents an attractive avenue to improve the drought tolerance of crops. Several steps in the ABA-signaling pathway are controlled by ubiquitination involving really interesting new genes (RING) domain-containing proteins. We characterized the maize (Zea mays) RING protein family and identified two novel RING-H2 genes called ZmXerico1 and ZmXerico2. Expression of ZmXerico genes is induced by drought stress, and we show that overexpression of ZmXerico1 and ZmXerico2 in Arabidopsis and maize confers ABA hypersensitivity and improved water use efficiency, which can lead to enhanced maize yield performance in a controlled drought-stress environment. Overexpression of ZmXerico1 and ZmXerico2 in maize results in increased ABA levels and decreased levels of ABA degradation products diphaseic acid and phaseic acid. We show that ZmXerico1 is localized in the endoplasmic reticulum, where ABA 8’-hydroxylases have been shown to be localized, and that it functions as an E3 ubiquitin ligase. We demonstrate that ZmXerico1 plays a role in the control of ABA homeostasis through regulation of ABA 8’-hydroxylase protein stability, representing a novel control point in the regulation of the ABA pathway.
RESULTS

Maize RING Protein Family

We analyzed the maize RING gene family using publicly available maize genome models. RING-domain-containing proteins were identified using protein families (PFAM) domains as queries with an e-value cutoff threshold approach and classified as RING-H2, RING-HC, RING-C2, and RING-V, which are the main RING domain consensus found in plants (Kosarev et al., 2002; Stone et al., 2005; Lim et al., 2010; Fig. 1; Supplemental Table S1). A total of 442 proteins were identified, which is very similar to the 425 RING proteins reported in rice (Lim et al., 2010) and 462 RING proteins classified in these four categories in Arabidopsis (Stone et al., 2005). The repartition of RING proteins between RING-H2 and RING-HC types is also similar to what was described in Arabidopsis and rice (Stone et al., 2005; Lim et al., 2010), with the RING-H2 class being the largest.

ZmXerico1 and ZmXerico2 have better water use efficiency (WUE) resulting in increased seed number per plant in a controlled drought-stressed environment. We demonstrate that ZmXerico1 can function as an E3 ubiquitin ligase in vitro. Further, we provide evidence that overexpression of ZmXerico1 and ZmXerico2 genes in maize exert a control on ABA homeostasis through regulation of ABA 8′-hydroxylase protein stability and ABA degradation rather than biosynthesis, as previously described by others. This function, likely exerted through the 26S proteasome pathway, represents a novel mode of action for ZmXerico1 and ZmXerico2 gene products in maize and a new control point in the regulation of the ABA pathway.

Figure 1. Classification and representation of consensus domains of maize RING-domain-containing proteins. Graphical representation of multiple sequence alignments of maize RING protein domains for each RING protein type using HMMlogo. The height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each amino acid at that position. Interleaved zinc coordination sites are indicated. The number of proteins for each category of RING domain and percentage of the total number of RING proteins are indicated.
and representing approximately two-thirds of total RING proteins (Fig. 1). RING-HC-, RING-V-, and RING-C2-containing proteins represented 28%, 6%, and less than 1% of the total, respectively.

The large number and diversity of RING-domain-containing proteins suggest that they have been evolving over time to fulfill different functions. For this reason, there is generally a lack of clear phylogenetic relationship between proteins sharing small conserved domains like RING domains and their respective functions. We therefore used a phylogenetic approach with core RING domains of the proteins that were identified. The results show a perfect clustering of RING-H2, RING-HC, RING-C2, and RING-V domains indicative of a common evolutionary pathway of each domain family (Supplemental Fig. S1). RING-C2 clustered close to RING-HC, while RING-V clustered close to the RING-H2 domains clade. It is possible that the less abundant RING-C2 and RING-V domain proteins could have evolved more recently from the RING-HC and RING-H2 domain families, respectively.

Identification of ZmXerico1 and ZmXerico2

A number of RING-H2 proteins have been identified in the literature that can influence drought tolerance when overexpressed in model plant species (Lyzenga and Stone, 2011; Lyzenga and Stone, 2012; Stone, 2014). In particular, transgenic overexpression of a specific set of short Arabidopsis RING-H2 proteins, including Xerico, RHA2A, RING-H2 group A (RHA) 2a, and RHA2b from Arabidopsis (Jensen et al., 1998), as well as OsRHP1 from rice, were shown to confer improved drought tolerance (Ko et al., 2006; Bu et al., 2009; Li et al., 2011; Zeng et al., 2014). We identified two novel genes we called ZmXerico1 and ZmXerico2 that encode small RING-H2 proteins of 158 and 166 aa, respectively. ZmXerico1 and ZmXerico2 are located on different chromosomes (2 and 7, respectively), and their locations are distinct from that of ZmXerico located on chromosome 1 (Supplemental Table S2). The alignment of ZmXerico, ZmXerico1, ZmXerico2, OsRHP1, Arabidopsis Xerico, RHA2a, and RHA2b proteins and their canonical RING-H2 domains is shown in Figure 2A. While ZmXerico1 and ZmXerico2 proteins shared 85% identity, they were more dissimilar to Arabidopsis proteins (less than 30% identity). ZmXerico1 and ZmXerico2 were found to have only 54% and 53% identity to ZmXerico, respectively (Supplemental Table S3). Interestingly, despite the low amino acid identities, ZmXerico, ZmXerico1, and ZmXerico2 RING domains cluster together in the same branch of the maize RING domain phylogenetic tree (Supplemental Fig. S1, red star and insert), which may indicate a similar ancestry and/or function.

ZmXerico1 GFP Fusion Proteins Are Targeted to the ER in Maize Protoplasts

Analysis of maize Xerico protein sequences using transmembrane domain prediction software (TMHMM) revealed the existence of a putative transmembrane region on ZmXerico1 and 2 as previously reported for ZmXerico and AtXerico (Supplemental Fig. S2A). This analysis found a strong probability for the presence of a transmembrane domain for all three maize proteins and RHA2A as well as a lower probability for AtXerico and RHA2B (Supplemental Fig. S2A; Fig. 2A). These results indicate that ZmXerico1 and 2 could be membrane-anchored proteins. We therefore determined the subcellular localization of ZmXerico1 using a GFP fusion approach and transient expression in maize protoplasts followed by confocal microscopy imaging. We built a construct to express the first 40 aa N-terminal region of the protein up to the end of the putative transmembrane domain of ZmXerico1 fused to GFP. The construct was cotransformed with a control RFP fusion using an N-terminal signal peptide derived from an Arabidopsis vacuolar basic chitinase and the C-terminal amino acid sequence HDEL targeting localization of RFP to the endoplasmic reticulum (ER; Haseloff et al., 1997; Chakrabarty et al., 2007). The 40 aa ZmXerico1:GFP fusion showed fluorescent signal typical of what is observed for GFP ER localization in Arabidopsis (Candat et al., 2014), rice (Zhang et al., 2011), and maize (Lomin et al., 2011), and overlapping with the localization of our control ER-specific RFP fusion (Fig. 2B; Supplemental Fig. S2B). The localization of ZmXerico1 in the ER was further confirmed using a second fusion comprising the first 105 aa containing the N-terminal region of the protein up to the RING-H2 domain and a construct expressing RFP in the cytosol (Supplemental Fig. S2C). Our results indicate that the first 40 aa of the ZmXerico1 protein are sufficient to target the protein to the ER. Because the first 40 aa of ZmXerico1 and ZmXerico2 are identical except for 2 aa and both proteins contain a transmembrane domain, it is likely that both proteins are anchored in the ER membrane, suggesting that they could interact with specific membrane-bound ER proteins.

Expression of ZmXerico, ZmXerico1, and ZmXerico2 in Response to Drought Stress

We examined the native expression pattern of ZmXerico, ZmXerico1, and ZmXerico2 in maize in response to drought stress. B73 seedlings were grown in the greenhouse in pots containing a calcined clay inert substrate (Turface). The advantage of growing plants in this material instead of soil is that they can be rapidly drought stressed by withholding water for distinct periods of time and also that very clean roots can be harvested. In this experiment, well-watered plants were grown until the V3 stage and were drought stressed for 24 and 48 h and then rewatered. After 24 h, plants started showing early signs of wilting indicative of a mild stress, while at 48 h, plants were fully wilted and leaves had a flaccid appearance, 72 h after rewatering plants had fully recovered (data not shown). Total RNA was extracted from leaf and root samples collected at each time
point, and an expression analysis was carried out using specific quantitative reverse transcription (qRT)-PCR assays for each gene (Fig. 3). In absence of stress (0 h), expression levels of ZmXerico and ZmXerico1 were similar in leaf and root tissues but lower than that of ZmXerico2 in both tissues. After 24 h of stress, expression of all three genes was increased in leaves (11.8× for ZmXerico, 6.6× for ZmXerico1, and 1.8× for ZmXerico2) and roots (14× for ZmXerico, 8× for ZmXerico1, and 2.8× for ZmXerico2), and expression levels remained high 48 h after water withdrawal. After rewatering (72 h), expression levels of all genes returned to their respective pre-stress levels. These results indicate that expression of maize Xerico genes quickly increases in response to drought stress, stays high while drought stress persists, but returns to normal levels after stress dissipates (Fig. 3A).

Figure 2. Transmembrane domain of ZmXerico1, ZmXerico2, and related proteins and subcellular localization of ZmXerico1 in the ER. A. Alignment of ZmXerico protein sequences and related protein sequences from Arabidopsis (AtXerico, RAH2a, and RHA2b) and rice (OsRHP1). Putative transmembrane domains identified using TMHMM2.0 are indicated by blue boxes. RING-H2 domains are identified by a red box and position of C and H by asterisks. Non-similar, conserved, similar, identical and weakly similar amino acids are represented by black letters on white background, blue letters on blue background, black letters on green background, red letter on yellow background, or green letters on white background respectively. Arrows indicate aa mutated in ZmXerico1 mutant proteins used in Figure 12. B. The first 40aa of ZmXerico1, including its predicted transmembrane domain, were fused to GFP (ZmXerico1 (40aa):GFP) and the protein fusion was transiently co-expressed in maize protoplasts with an ER-targeted RFP fusion marker (CHIT:RFP:HDEL). Merged confocal microscopy pictures show co-localization of GFP and RFP signals. Bar is 5 microns.

Expression of Maize Xerico Genes Is Diurnally Regulated

We studied diurnal expression and the response of ZmXerico, ZmXerico1, and ZmXerico2 to drought stress in B73 seedlings using RNASeq. Plants were grown in Turface until V6, at which point irrigation was stopped for half of the plants. Plants were sampled every 4 h thereafter for 3 d starting from 2 h after the beginning of the first light period identified as ZT (Zeitgeber) = 0. Expression levels of maize Xerico genes were determined using Illumina next-generation sequencing, and results are presented in Figure 4. A clear diurnal expression pattern was observed for each gene. In leaves, expression of ZmXerico increased progressively after the onset of light to reach maximum expression at the end of the light period under both well-watered and drought-stress conditions. Expression reached its lowest expression at night under both growing
conditions and strongly increased under drought stress after the first day of water withholding (Fig. 4A). In roots, ZmXerico expression was generally lower than in leaves. Expression strongly increased under drought-stress conditions during the light period but rapidly decreased in the dark. ZmXerico1 expression in leaf tissue increased gradually during the day similarly to ZmXerico but peaked 2 h after the beginning of the dark period before gradually reaching its lowest level at the beginning of the day (Fig. 4B). Expression of ZmXerico2 peaked during the night time in leaves and stayed constant through the night before gradually decreasing from the beginning to the end of the light period (Fig. 4C). While all three ZmXerico genes were inducible by drought stress in roots, only expression of ZmXerico and ZmXerico1 significantly increased in shoots under drought-stress conditions. At this developmental stage, ZmXerico2 was not drought inducible in leaves.

Constitutive Overexpression of ZmXerico1 Decreases Drought-Induced Senescence and Improves Drought Stress Recovery in Maize

To learn more about the function of ZmXerico1 and ZmXerico2, we overexpressed these genes in maize under the control of the constitutive maize Ubiquitin promoter (Christensen et al., 1992). We built transformation vectors Ubi::ZmXerico1 and Ubi::ZmXerico2 and used them to create stably transformed maize plants via Agrobacterium sp. mediated transformation as previously described (Zhao et al., 1998). Molecular characterization of Ubi::ZmXerico1 and Ubi::ZmXerico2 transgenic events using semiquantitative RT-PCR and northern blot analyses showed strong and uniform overexpression of ZmXerico1 and ZmXerico2, respectively (Supplemental Fig. S3). Hybrid seed was created and 10 events and control plants grown in drought-stressed or well-watered conditions. No difference in visible canopy phenotypes could be identified between transgenic and control plants grown under well-watered conditions (data not shown). In contrast, under drought stress field conditions, transgenic events overexpressing ZmXerico1 showed a clear delayed drought-induced senescence phenotype (Fig. 5). Transgenic plants demonstrated a much healthier leaf canopy than controls characterized by reduced senescence of lower leaves. This resulted in a significantly higher staygreen score (measured on a 1 to 9 scale based on visible leaf greenness) of transgenics compared to controls in flowering stress conditions (7.3 versus 5.3, Student’s t test; P < 0.001, in 2009) except for non-expressing Event 15 (Supplemental Table S4).

In a complementary experiment, we used a greenhouse assay to study the response of ZmXerico1 overexpressers to an acute drought stress. Hybrid transgenic seeds corresponding to 10 Ubi::ZmXerico1 events and nontransgenic control segregants were planted in pots containing a mixture of Turface and soil to promote rapid drought stress. Plants were grown for 2.5 weeks in well-watered conditions. No visible phenotypical differences between transgenic and control seedlings were observed after this period.

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of time. Plants were then drought stressed and were rewatered before they reached the permanent wilting point. Representative pictures of transgenic and control plants after 4 h of recovery are presented in Figure 6. After recovery, all Ubi::ZmXerico1 expressing events displayed faster recovery than controls. Representative pictures of the phenotype of Ubi::ZmXerico transgenic lines (events) compared to control are showed in Figure 6A. While control plants appeared gray and their leaves were rolled and spindly, transgenic plants appeared green and their leaves were turgid. Figure 6B shows that the phenotype of Event 15, a non-expressing transgenic event
Supplemental Fig. S3), was very similar to that of controls, indicating that ectopic overexpression of the transgene is responsible for the phenotype of Ubi::ZmXerico1 expressing events. After 9 h, all transgenic lines, except Event 15, had almost fully recovered from drought stress, while controls and Event 15 still showed signs of wilting. After 24 h, 95% of plants expressing Ubi::ZmXerico1 (n = 117) did not show sign of wilting, while only 55% of control plants (n = 15) did not appear wilted. Similar faster recovery results were observed with Ubi::ZmXerico2 (data not shown). All together, these results indicate that overexpression of ZmXerico1 or ZmXerico2 genes can protect maize plants from drought stress and helps them recover more rapidly from dehydration.

Overexpression of ZmXerico1 and ZmXerico2 Increases Drought Tolerance by Reducing Water Loss in Arabidopsis

To confirm that maize Xerico genes could improve drought stress tolerance in another species, we created Arabidopsis plants overexpressing ZmXerico1 and ZmXerico2 under control of the 35S promoter. We used a standardized drought stress assay to measure the drought tolerance of transgenic plants compared to control segregants and transgenic plants over-expressing a control gene (Allen et al., 2013). Segregating T2 transgenic and control plants were grown in soil for approximately 3 weeks with regular watering before drought stress was applied by stopping water application. Plant status was monitored using a commercial imaging system to quantify plant wilting. Pictures of transgenic and control plants were taken 14 d after drought stress (Fig. 7A). Plants overexpressing ZmXerico1 and ZmXerico2 appeared less wilted and were greener compared to control plants with plants overexpressing the control gene having an identical phenotype to control wild-type plants (Fig. 7A). A drought score for this assay was calculated based on image analysis comparing individual transgenic events and nontransgenic plant features as previously described in detail by Shi et al. (2015) (see also Allen et al., 2010). A drought score of 2 in this assay is typically characteristic of drought-tolerant transgenic plants compared to nontransgenic plants. 35S::ZmXerico1 and 35S::ZmXerico2 had a score of 6.18 and 6.26, respectively, compared to nontransgenic control plants, while transgenic controls had a score of 0.7 compared to nontransgenic plants. In a separate experiment, we grew four independent 35S::ZmXerico1 transgenic Arabidopsis events and corresponding control plant segregants for 20 d in a similar experimental setting described above and then applied a drought stress for 9 d by

Figure 5. Delayed drought-induced senescence of Ubi::ZmXerico1 maize transgenics compared to control plants. Representative pictures of Ubi::ZmXerico1 transgenic and control plants grown under managed drought stress conditions in 2009 and 2011. Transgenic plants displayed reduced leaf rolling and visibly healthier lower canopy leading to statistically significant staygreen phenotypes compared to controls.

Figure 6. Drought stress recovery phenotypes of Ubi::ZmXerico1 and control maize plants. A, Comparison of a representative transgenic Ubi::ZmXerico1 overexpressing event (Event 5) and control null plants 4 h after recovery from water stress showing faster recovery of transgenics. B, Comparison of the recovery of a transgenic non-expressing Ubi::ZmXerico1 event (Event 15) and control null plants 4 h after water stress.
discontinuing watering. We estimated plant water usage by measuring differences in pot weight compared to weight at the last watering over the drought stress period. Results presented in Figure 7B show that pots containing 35S::ZmXerico1 transgenic plants lost less water (mass) over time compared to controls. Similarly, we found that the rate of water loss of detached 35S::ZmXerico2 rosette leaves was significantly slower than control nontransgenic plants (Supplemental Fig. S4; see “Materials and Methods” for details). While no significant difference was observed for the weight of detached leaves of transgenic and control plants at the start of the study, leaves of transgenic 35S::ZmXerico2 transgenic plants significantly conserved more water than control leaves after just 1 h (Student’s t test; P < 0.05). All together, these results indicate that ectopic overexpression of ZmXerico1 and ZmXerico2 genes can improve drought tolerance in both monocot and dicot species, like maize and Arabidopsis, using a mechanism that is related to improved water usage.

Overexpression of ZmXerico1 in Maize Reduces Stomatal Conductance and Improves Water Use Efficiency and Grain Production under a Controlled Drought Stressed Environment

We carried out a greenhouse experiment to examine the possibility that maize Ubi::ZmXerico1 plants recover faster from drought stress because they use less water than control plants. We measured stomatal conductance and photosynthetic rate of V8 transgenic and control plants growing in well-watered conditions in the greenhouse using a LICOR instrument. Figure 8 shows the photosynthetic rate, stomatal conductance, and calculated water use efficiency for three independent Ubi::ZmXerico1 events and control nulls. No significant differences were observed for photosynthetic rate between transgenic and control plants (Student’s t test; P < 0.05; Fig. 8A). However, stomatal conductance of Ubi::ZmXerico1 plants was significantly lower (P < 0.05) than controls (Fig. 8B), resulting in an important net improvement in water use efficiency (Fig. 8C).
results were observed at the R1 stage (Supplemental Fig. S5). To further validate this finding, we evaluated water use of transgenic and control plants by measuring changes in pot weight over 3 d after fully saturating pots with water followed by no further watering. Results presented in Figure 9 for two drought cycles at the V5 to V6 developmental stage (Fig. 9, A and B) for the same three Ubi::ZmXerico1 events of Figure 8 show that transgenic plants used significantly less water (14 mL per plant per day on average) than controls. Ubi::ZmXerico1 and control plants subjected to drought-recovery cycles or grown in well-watered conditions were grown to maturity. Total plant biomass, plant height, and seed weight per plant were measured. Transgenic events subjected to chronic drought stress had increased total aerial plant biomass at maturity compared to controls. In well-watered conditions, two out of the three transgenic events tested had no significant reduction in biomass compared to controls (Student’s t test; P > 0.05; Supplemental Fig. S7), and plant height of transgenic events was not significantly different than controls (P > 0.05; Supplemental Fig. S8). Figure 9C shows that transgenic Ubi::ZmXerico1 plants produced a similar mass of grain as control plants in well-watered conditions. However, all three transgenic events showed an increase in seed weight per plant compared to control plants under drought-stressed conditions (Student’s t test; P > 0.05; Fig. 9C). All together, our results indicate that ZmXerico1-overexpressing plants use water more efficiently than controls and that ectopic overexpression of ZmXerico1 can improve grain production in drought-stressed conditions while maintaining similar production in well-watered conditions.

Arabidopsis and Maize Plants Overexpressing ZmXerico1 Are ABA Hypersensitive

There are several reports documenting the ABA hypersensitivity of transgenic plants overexpressing small RING-H2 proteins (Ko et al., 2006; Bu et al., 2009; Li et al., 2011), and for this reason we decided to study the response of Arabidopsis and maize transgenic plants overexpressing ZmXerico1 to ABA using a germination and a root elongation assay, respectively. In Arabidopsis, both 35S::ZmXerico1 and 35S::ZmXerico2 transgenics showed a markedly reduced germination on Murashige and Skoog media containing increasing concentrations of ABA (Fig. 10A). Germination of Arabidopsis transgenic seeds at 0.6 μM over time was also significantly different than Col-0 controls and never reached 100%, as did the control (Fig. 10B). To characterize ABA sensitivity in maize, we used 4-d-old seedlings to measure the primary root elongation rate of transgenic and control seedlings in presence or absence of 50 μM ABA. ABA treatment significantly lowered the primary root elongation rate of control plants by 36% (Student’s t test; P < 0.05), indicating that our assay can be used to assess ABA sensitivity of maize seedlings (Fig. 10C). Root elongation in transgenic Ubi::ZmXerico1 plants was not affected in the absence of ABA treatment compared to control; however, in the presence of ABA, elongation of transgenic seedling roots was significantly less than controls (2.2-fold), corresponding to only 70% of the length of untreated plants (Fig. 10C). Based on these results, both 35S::ZmXerico1 and 35S::ZmXerico2 Arabidopsis plants and Ubi::ZmXerico1 maize transgenic plants have an ABA

Figure 8. Physiological analysis and water use efficiency of maize Ubi::ZmXerico1 and control plants grown in well-watered conditions. A, Photosynthetic rate; B, stomatal conductance; and C, WUE of Ubi::ZmXerico1 events and control null plants grown in well-watered conditions in the greenhouse (n = 26). Measurements were obtained from leaves of V8 plants with a LICOR portable photosynthesis system; photosynthetic rate and stomatal conductance were used to calculate WUE. Error bars represent SEM. Asterisks indicate significant statistical differences (Student’s t test; P < 0.05).
hypersensitivity phenotype compared to control plants. Our data therefore suggests that overexpression of ZmXerico1 and ZmXerico2 could affect ABA sensing and signaling and/or ABA levels leading to ABA hypersensitivity.

Ubi::ZmXerico1 and Ubi::ZmXerico2 Transgenic Maize Plants Have Higher ABA Levels and Reduced ABA Catabolite Levels in Leaves and Roots

We decided to assess if the phenotypic differences observed between maize Ubi::ZmXerico1 or Ubi::ZmXerico2 transgenic lines and control plants could be due to differences in ABA levels as reported previously for Arabidopsis plants overexpressing AtXerico (Ko et al., 2006). Control (transgenic nulls) and Ubi::ZmXerico1 or Ubi::ZmXerico2 transgenic plants were planted in two separate field experiments in managed stress environments, where plants were grown in well-watered or drought stressed environments. Stress was applied to drought stressed plots by withholding water at V7 and managing stress intensity through limited irrigation to reach maximum drought stress at flowering time. Leaf samples were collected a week before flowering and ABA-related metabolites measured using a gas chromatography–mass spectrometry approach (Chiwocha et al., 2003). ABA levels in leaves of drought-stressed control plants were increased dramatically compared to leaves of well-watered control plants indicative of the strength of the drought stress applied to experimental plots (Fig. 11, A and B). ABA levels significantly increased in leaves of Ubi::ZmXerico1 and Ubi::ZmXerico2 plants in both well-watered and drought stressed environments (Student’s t test; P < 0.05; Fig. 11, A and B). ABA levels were increased 4.5- and 2.9-fold in leaves of Ubi::ZmXerico1 and Ubi::ZmXerico2 plants under well-watered conditions, and 2.9- and 1.4-fold under drought-stressed conditions compared to control plants, respectively. Similarly, ABA-GE levels were significantly greater in leaves of transgenic plants compared to control plants in both growing conditions (P < 0.05; Fig. 11, A and B). Interestingly, levels of DPA and PA, the most important ABA catabolites derived from 8'-hydroxylation pathway, were significantly lower in leaves of both Ubi::ZmXerico1 and Ubi::ZmXerico2 transgensics in both water regimen conditions compared to their respective nontransgenic controls (Student t test; P < 0.05). Similar differences were observed in roots of transgenic Ubi::ZmXerico1 and control seedlings exposed to water deprivation (Supplemental Fig. S6). Our data suggest that the observed increase in ABA levels in transgenic Ubi::ZmXerico1 and Ubi::ZmXerico2 plants are the results of reduced ABA degradation, and less so to an increase in ABA biosynthesis compared to controls.

ZmXerico1 Functions as an E3 Ubiquitin Ligase

RING-finger-containing proteins, including RING-H2 proteins like SALT- AND DROUGHT-INDUCED RING FINGER1 (SDIR1) (Zhang et al., 2007), RHA2a (Bu et al., 2009), and RHA2b (Li et al., 2011) can often function as E3 ubiquitin ligases. Since ZmXerico1 contains a RING-H2 motif, we determined if it could
function as an E3 ubiquitin ligase. An N-terminal fusion of the maltose binding protein (MBP) and ZmXerico1 was produced in *Escherichia coli* and purified using an amylose column to test its E3 ligase activity. Figure 12 shows detection of the MBP-ZmXerico1 protein in the presence of the different components in the reaction mixture using anti-ZmXerico1 antibodies. In absence of E1, E2, or ubiquitin only one major band was detected corresponding to the Mr of the MBP-ZmXerico1 protein (Fig. 12A). However in the presence of E1, E2, and Ub, several bands of a higher Mr than MBP-ZmXerico1 were detected indicative of sequential addition of Ubiquitin on the protein fusion (Fig. 12A). ZmXerico1 does not have a Lys residue, the usual site of ubiquitination on the E3 substrate, and therefore could only catalyze ubiquitination on Lys residues on the MBP moiety of the fusion protein. Specific amino acids of RING proteins were previously found to be critical for E3-E2 interaction. It was suggested that a mutation in the equivalent of I383 and W408 c-Cbl human RING protein would destabilize the E3-E2 complex and functionally inactivate RING E3s (Deshaies and Joazeiro, 2009). We therefore created a MBP-ZmXerico1 mutant fusion where ZmXerico1 V98 and W126 were mutated to a Q and a R, respectively, and analyzed activity of the mutant fusion. Figure 12B shows the V98Q/W126R mutations result in a complete inactivation of ZmXerico1. Similarly, inactivation of ZmXerico1 zinc coordination sites C96G/C99G/C114G/H119F/C122G resulted in inactivation of E3 ligase activity (Fig. 12C). All together, our results indicate that ZmXerico1 functions as an E3 ubiquitin ligase in vitro capable of ubiquitinating a nearby substrate.

**ZmXerico1 Promotes Destabilization of ZmABA8ox1a and ZmABA8ox3a**

The hydroxylation of ABA by ABA 8'-hydroxylase is the key step in ABA catabolism (Endo et al., 2014). Since reduced ABA catabolism was observed in Ubi::ZmXerico1 transgenic plants, we tested if ZmXerico1 could regulate ZmABA8ox protein stability using a transient expression system in maize protoplasts. Five ABA 8'-hydroxylases, ZmABA8ox1a, ZmABA8ox1b, ZmABA8ox2, ZmABA8ox3a, and ZmABA8ox3b have been identified in maize (Vallabhaneni and Wurtzel, 2010). Among them, ZmABA8ox3a (GRMZM2G065928_T01) showed highest expression in leaves, ZmABA8ox1a (GRMZM2G179147_T02) in roots, and ZmABA8ox2 (GRMZM2G105954_T02) in kernels. Thus, these three major hydroxylases were chosen for stability tests in maize protoplast.

We studied the steady-state level of hemagglutinin (HA) epitope-tagged ZmABA8ox1a and ZmABA8ox3a proteins overexpressed in maize protoplasts together with a functional ZmXerico1 protein or the ZmXerico1 (105aa)-GFP fusion missing ZmXerico1 RING domain. Figure 13A shows that the steady-state levels of ZmABA8ox1a and ZmABA8ox3a were greatly reduced in protoplasts coexpressing the full length ZmXerico1 protein compared to those coexpressing the nonfunctional ZmXerico1 (105aa)-GFP fusion. In contrast, levels of ZmABA8ox2 protein were not affected. The effect of ZmXerico1 overexpression on HA-tagged ZmABA8ox1a and ZmABA8ox3a protein stability was confirmed using protoplasts isolated from Ubi::ZmXerico1 transgenic plants compared to those from the corresponding
transgenic segregants (Supplemental Fig. S9). The effect of ZmXerico1 overexpression on the steady-state level of HA-tagged ZmABA8ox1a and ZmABA8ox3a was further examined by cotransfecting protoplasts with the same amounts of ZmABA8ox1a-HA or ZmABA8ox3a-HA expression plasmids along with increasing amounts of ZmXerico1 expression plasmids. As shown in Figure 13B, the steady-state level of HA-tagged ZmABA8ox1a and ZmABA8ox3a protein gradually decreased as the expression of ZmXerico1 increased, while the protein level of GFP control was not affected by ZmXerico1. Together, our results suggest that ZmXerico1 can destabilize ZmABA8ox1a and ZmABA8ox3a in maize protoplasts.

To examine if ZmABA8ox1a and ZmABA8ox3a could be ubiquitination substrates of ZmXerico1 E3 ligase, the physical interaction between ZmABA8ox and ZmXerico1 proteins was studied by coimmunoprecipitation assays using maize protoplasts coexpressing both HA-tagged ZmABA8ox1a and ZmABA8ox3a proteins and a ZmXerico1-FLAG-HA protein. Although a small amount of ZmABA8ox1a and ZmABA8ox3a protein was pulled down by anti-FLAG affinity gel, significantly more ZmABA8ox1a and ZmABA8ox3a proteins were pulled down in protoplasts overexpressing ZmXerico1-FLAG-HA protein (Fig. 14, A and B). This suggests that ZmXerico1 can form a complex with ZmABA8ox1a and ZmABA8ox3a. Taken together, our data shows that ZmXerico1 E3 ubiquitin ligase interacts with ZmABA8ox1a and ZmABA8ox3a, and regulates their protein stability and ABA catabolism.

**DISCUSSION**

**Maize RING Family**

Ubiquitin-mediated regulation of protein stability is a major control point for plant growth and development and has been identified as an important pathway for environmental adaptation (Lyzenga and Stone, 2011, 2012). We conducted an in silico analysis of the publicly available maize gene model sequences (GRMZM) to identify maize RING domain-containing proteins. Comprehensive analysis of the RING protein families of Arabidopsis (Stone et al., 2005) and rice (Lim et al., 2010) has previously been reported, but to our knowledge this is the first genome-wide analysis of this important class of proteins in maize. In Arabidopsis and rice, phylogenetic studies found that RING domains of the same type tended to cluster together with few exceptions (Stone et al., 2005; Lim et al., 2010). Our analysis revealed that RING-type protein domains clustered perfectly together, with RING-H2 and RING-HC forming the main clusters (Supplemental Fig. S1). RING-C2 and RING-V clustered on the fringe of the RING-HC and RING-H2 clusters, respectively. RING-C2 domains may have diverged from a specific ancestral RING-H2 domain, while RING-V could have evolved from an ancestral RING-HC domain during the evolution of maize. Little is known about how plant RING-domain-containing proteins may have evolved during speciation; however, the similarities in total
number of RING proteins among species and their re-
partitions into different RING types indicate that RING-
domain-containing proteins may have originated from a
common ancestor. Recent developments in the study of
the early evolution of the ubiquitin system seem to
substantiate the possibility that eukaryotes acquired
their ubiquitination system from an archeal prokaryote
progenitor (Burroughs et al., 2011). The presence of other
domains on some RING-type proteins would have fur-
ther contributed to the expansion of this family and its
diversification in controlling different aspects of plant
growth and development, including response to abiotic
stress. ZmXerico1 and ZmXerico2 chromosome locations
are highly syntenic, indicating that one of the genes
might have arisen through duplication. Interestingly, we
found that the RING domains of maize ZmXerico,
ZmXerico1, ZmXerico2, and a fourth RING-H2 protein
(GRMZM2G029623) clustered together in the same
subclade of the maize RING protein phylogenetic tree
(Supplemental Fig. S1). While our study revealed that
the overexpression of ZmXerico1 and ZmXerico2 in
transgenic plants result in improved drought tolerance
explained in identifying the drought tolerance provided by ZmXerico1 and ZmXerico2 ectopic
expression (Fig. 7) and will be used to assess other RING-
H2 candidates with similar characteristics.

ZmXerico Genes Expression Pattern

Our study of ZmXerico gene expression in maize
seedlings is in agreement with the results reported
previously by Gao et al. (2012) that native expression of
this gene is stronger in leaf compared to root. Our
finding that ZmXerico expression is strongly induced by
drought stress in maize seedlings (Fig. 3) is also con-
sistent with strong induction of expression by poly-
ethylene glycol (PEG), NaCl, ABA, and cold treatments
(Gao et al., 2012). Similarly, we found that ZmXerico1
and ZmXerico2 gene expression is also induced by
drought stress (Fig. 3), which suggests that maize
Xerico genes could be responsive to endogenous increases in
ABA levels associated with various abiotic stresses. We
also found that expression of maize Xerico genes is di-
urnally regulated. However, in contrast to Gao et al.
(2012), our study shows that ZmXerico is expressed at
higher levels during the light period and at lower levels
during the dark period. Gao et al. (2012) used expres-
sion of an actin gene (GRMZM2G126190, EU969279) to
normalize their qRT-PCR results, but this maize gene is
also strongly diurnally regulated (Hayes et al., 2010),
with low expression during the dark period and high
expression during the light period, which could have

Figure 12. ZmXerico1 E3 ubiquitin
ligase activity. A, Recombinant MBP-
ZmXerico1 fusion protein catalyzes self-
ubiquitination in presence of E1, E2,
and Ubiquitin. B, Mutations in amino
acids critical for E2 and RING-H2 in-
teraction inactivate self-ubiquitination
of MBP-ZmXerico1. C, Mutation in
amino acids critical for the stability of
the RING-H2 domain inactivate self-
ubiquitination of MBP-ZmXerico1.

A - + + + + Ub
   + - + + + E1
   + + - + + E2
   + + + + - MBP-ZmXerico1

B - + + + + Ub
   + - + + + E1
   + + - + + E2
   + + + + - MBP-ZmXerico1
       (V98Q/W126R)

C + + + + + E1
   + - + + + E2
   + + - + + MBP-ZmXerico1
       (C96G/C99G/C114G/H119F/C122G)

MBP-ZmXerico1
       (V98Q/W126R)
resulted in this different interpretation of expression results. *ZmXerico*1 and *ZmXerico*2 have higher expression at night, which is concomitant with stomatal closure (Tallman, 2004; Nováková et al., 2005). Since ectopic expression of *ZmXerico* genes leads to increased ABA levels and reduced stomatal conductance, it is tempting to speculate that maize *Xerico* genes could play a role in stomatal closure through their control on ABA homeostasis. Specific RING-H2 genes could have different roles, i.e. since *ZmXerico* is not expressed at high levels at night it might be more important for the maintenance of high ABA levels in response to drought stress while *ZmXerico*1 and *ZmXerico*2 may, in addition, be involved in dark-induced stomatal closure.

ABA Hypersensitivity

In maize, ABA hypersensitivity of Ubi::*ZmXerico*1 seedlings could be the result of an increase in ABA levels in seeds and/or a consequence of ABA accumulation in elongating roots (Supplemental Fig. S6). Similarly, the ABA hypersensitivity of 35S::*ZmXerico*1 and 2 overexpressers could be the result of increased ABA levels in seeds and/or roots of germinating seedlings. This is in agreement with the finding that a mutation in Arabidopsis *AtXerico* results in ABA hyposensitivity in a green cotyledon germination assay and a reduction in seed ABA levels (Zentella et al., 2007). Similarly, rha2a and rha2b single and double mutants have been shown to be hyposensitive to ABA; however, ABA levels in mutants have not yet been reported (Bu et al., 2009; Li et al., 2011). A complete examination of ABA metabolites of 35S::RHA2a and 35S::RHA2b overexpressers as well as *AtXerico*, *rha2a*, and *rha2b* single, double, and triple mutants in Arabidopsis would be valuable to better understand the roles and functional redundancy of these RING-H2 proteins in ABA signaling.

*ZmXerico*1 Functions as an E3 Ubiquitin Ligase, and Its Overexpression Affects ABA 8'-Hydroxylases Stability

The ubiquitin-proteasome system has been implicated in the control of the ABA response at different points of the ABA pathway (Yu et al., 2016). A yeast two-hybrid screen identified that *AtXerico* can interact with UBC8, an E2 ubiquitin conjugating enzyme, which strongly suggests that it may function as E3 ubiquitin ligase (Ko et al., 2006). We conducted a yeast two-hybrid screen using *ZmXerico*1 as bait and identified that it can also interact with specific E2-conjugating enzymes (data not shown). Moreover, we tested the activity of *ZmXerico*1 as an E3 ubiquitin enzyme and found that it is active in vitro (Fig. 12). Ko et al. (2006) hypothesized that overexpression of *AtXerico* in Arabidopsis increased leaf ABA levels by regulating expression of nine-cis-epoxycarotenoid dioxygenase 3 (NCED3), an ABA biosynthetic gene. Such regulation could for example be exerted through a mechanism that would reduce the stability of specific repressors of NCED3 expression, considered a control point in ABA biosynthesis. However, several reports indicate that

![Figure 13. *ZmXerico*1 destabilizes *ZmABA8ox1a* and *ZmABA8ox3a* proteins. A, The steady-state level of *ZmABA8ox1a*, *ZmABA8ox2*, and *ZmABA8ox3a* proteins in maize protoplasts coexpressed with either *ZmXerico*1-FLAG-HA or *ZmXerico*1 (105 aa)-GFP. B, The steady-state level of *ZmABA8ox1a* and *ZmABA8ox3a* protein in maize protoplast with increasing *ZmXerico*1 expression. GFP was used as a control.](https://www.plantphysiol.org/content/175/3/1363/F13.large.jpg)
ZmABA8ox proteins for degradation through the 26S proteasome pathway. Whether ZmXerico1 interacts directly or indirectly with ZmABA8ox proteins remains to be determined. The mechanism we have discovered represents a new control point for regulation of ABA homeostasis. Specific ZmXerico genes could be implicated in different abiotic and biotic responses such as drought stress and stomatal closure at night or in response to pathogen attacks. In Arabidopsis, other small RING-H2 proteins like RHA2a and RHA2b have been implicated as positive regulators of ABA signaling (Bu et al., 2009; Li et al., 2011). Whether these proteins function through regulation of ABA homeostasis or another mode of action is unknown, but multiple RING-H2 proteins involved in ABA signaling indicates that they could have redundant roles in Arabidopsis.

Maize Drought Tolerance Improvement via Overexpression of ZmXerico1 and ZmXerico2

Because of its central role in the drought stress response, the ABA pathway has been an important target for the improvement of crop performance in drought stress conditions (Du and Xiong, 2014). Manipulating ABA metabolism, signaling, and the regulation of the pathway provides an opportunity to not only enhance the plants’ response to drought stress by increasing its sensitivity and amplifying its magnitude but also modulate the plant’s water economy. By preventing excessive transpiration and maintaining close to normal photosynthesis under well-watered conditions, plants can decrease water removal from the soil so that soil water is conserved and available during periods of stress (Gholipoor et al., 2013; Messina et al., 2015). We show that such a trait (Fig. 8) is beneficial for Ubi::ZmXerico1 drought-stressed plants grown in the greenhouse and that it can lead to increased grain weight compared to control nontransgenic plants (Fig. 9). The balance between CO2 uptake and transpiration is an important determinant of water use efficiency and plant productivity in drought-stressed conditions (Lawson and Blatt, 2014). The benefits of a limited transpiration trait on maize productivity can vary with environment types (Messina et al., 2015), and it could be necessary to optimize a transgenic trait using maize ZmXerico1, ZmXerico2, or related RING-H2 genes to achieve improved drought stress tolerance in a variety of target environments (Tardieu, 2012).

MATERIAL AND METHODS

Plant Material, Transformation and Growing Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 (Col-0) was used for Agrobacterium tumefaciens transformation using a dipping method (Clough and Bent, 1998). After transformation, glufosinate-resistant T2 plants and control plants were sown on Scotts Metro-Mix 360 soil. Flats were conformed with eight square pots each. Each of the square pots was filled to the top with soil. Each pot (or cell) was sown to produce nine seedlings in a 3x3 array. Within a flat, four pots consist of glufosinate-resistant plants and four pots consist of control plants. The soil was watered to saturation, and plants were grown under standard conditions (i.e. 16 h light, 8 h dark cycle; 22°C; ~60% relative humidity). Plants were grown in well-watered conditions for approximately 3 weeks, at which time water was withheld and drought stress was monitored.
and analyzed using a Leica Imaging system and Leica TCS-SP5 IV CCD image analysis software. Drought scores were calculated as previously described (Allen et al., 2010; Shi et al., 2015). A drought score greater than 2 indicates drought-tolerant plants. Pictures of representative plants were taken 14 d after water withdrawal.

Agrobacterium strain LBA4404 was used to transform maize (Zea mays) embryos from a proprietary inbred (Zhao et al., 1998). After regeneration and characterization of transgenics, T1 transgenic plants were used for seed increase and top crossed with a proprietary inbred to create hybrid seeds that were used in all experiments. Greenhouse experiments with maize were conducted between October and December 2011 in Johnston, IA. Plants were grown in 6" pots (water use experiment) or 9" pots (LICOR measurements) filled with Fafard 3B soil mix and Turface (1:1 mix in volume). Day-night temperature was around 28°C-20°C. Supplemental lights were on at 6:00 a.m. and off at 7 p.m. and provided 500 μmol m⁻² s⁻¹ of light intensity. Two seeds were placed into each pot according to a randomized scheme. Pots were thinned to one uniform plant per pot at V2. Field experiments with maize hybrids of Ubi::ZmXerico1 were grown in managed stress environments in well-watered (fully irrigated) or drought-stressed conditions (including a non-expressing event, Event 15) and null segregants were grown in a randomized nested design. Stayscores were measured for each entry from four field replicates using a 1 to 9 scale, 9 indicating a plot with >90% green biomass, 7 indicating a plot with >70%–80% green biomass, 5 indicating a plot with 40%–60% green biomass, 3 indicating a plot with 20%–30% green biomass, and 1 indicating a plot with <10% green biomass. Each replicate value corresponds to the average score of approximately 50 plants in a two-row plot.

Construction of Vectors for Plant Transformation

The pBC Yellow Gateway vector was used as a destination vector to clone the ZmXerico1 or ZmXerico2 coding sequences between the 35S promoter of the cauliflower mosaic virus and the phaseolin terminator as previously described (de la Luz Gutiérrez-Nava et al., 2008). Resulting vectors were transformed in Agrobacterium tumefaciens GV3101 (Helliens et al., 2000).

The Ubi::ZmXerico1 and Ubi::ZmXerico2 constructs were created by fusing the ubiquitin promoter (Christensen et al., 1992) to the coding sequence of ZmXerico1 and ZmXerico2 cDNAs. The gene fusion was terminated with the poly(A) addition site from the potato (Solanum tuberosum) proteinase inhibitor (PinII) termination sequence (An et al., 1989; Unger et al., 1993). This construct uses a maize optimized phosphohistidinorcin acetylsyrphase gene as a selectable marker and a cassette using the promoter of lipid transfer protein 2 (LTP2) from barley fused to the 104 bp sequence of Dioscorea sp. red fluorescent protein (DsRed [ALTII]) as marker to identify transgenic seeds (Wu et al., 2016). Resulting vectors were transformed in Agrobacterium strain LBA4404.

Transient Expression in Protoplasts

ZmXerico1:GFP fusions were built using a PCR strategy. A section of the 5’ end coding sequence of ZmXerico1 corresponding to the first 40 or 105 aa of ZmXerico1 was first PCR amplified with a forward primer corresponding to the 5’ end of ZmXerico1 coding sequence and a reverse primer containing a sequence corresponding to a protein linker (aa sequence GGGGSGGGGSGGGGSGGG). Similarly, the GFP coding sequence was PCR amplified using a forward primer corresponding to the 5’ end of the GFP coding sequence (CDS) with a 5’ end extension corresponding to the protein linker. Twenty-five nanograms of each PCR product was then used in a new PCR using the ZmXerico1 forward primer and the GFP reverse primer to obtain the fusion, which was cloned in pENTRY/D, sequenced, and recombined in a pBBSK+ vector between the 35S promoter and the phaseolin terminator. Maize protoplasts were prepared as previously described (Sheen, 1991) and transformed using a PEG protocol (Cao et al., 2014). Protoplasts were visualized using a Nikon Y-FL microscope with appropriate RFP and GFP filters and in bright field. The overlay image was generated using Spot advanced software. The ER-targeted TagRFP fusion contains an N-terminal signal peptide derived from an Arabidopsis vacuolar basic chitinase and the C-terminal amino acid sequence HDEL (Hasekoff et al., 1997; Hasekoff and Siemering, 1998; Chakraborty et al., 2007). The CDS was assembled by PCR using extension primers, cloned in pENTRY/D (Invitrogen), sequence verified, and inserted between the 35S promoter and the phaseolin terminator in a pBBSK+ vector background as described above.

ZmXerico1:FLAG-HA and ZmABA8ox2-HA plasmids were built using PCR of cDNA plasmids containing the corresponding CDS with the following primers: for ZmXerico1:FLAG-HA, 5’-CACCATGGGATCCCTGCAGCATG-3’ and 5’-AAGGT-3; for ZmABA8ox2-HA, 5’-CACCAGGTTGATTCTGAGTTTCTCAGG-3’. For ZmABA8ox2-HA, 5’-CACCAGGTTGATTCTGAGTTTCTCAGG-3’. For ZmABA8ox2-HA, 5’-CACCAGGTTGATTCTGAGTTTCTCAGG-3’. For ZmABA8ox2-HA, 5’-CACCAGGTTGATTCTGAGTTTCTCAGG-3’.

Photosynthesis, Stomatal Conductance, Drought Stress Recovery, and Water Use Measurements

To measure water loss, Arabidopsis 35S::ZmXerico1 transgenic and control plants corresponding to four independent transgenic events were grown for 20 d in well-watered conditions using the experimental design outlined above. The weight of each pot containing nine plants each was recorded after days 1, 2, 6, 7, 8, 9, and 10 after water withdrawal, and relative water loss between transgenic and controls was calculated by subtracting measured pot weights from original pot weights at last watering. For water loss measurement of 35S::ZmXerico2 (Supplemental Fig. S4), young rosette leaves from segregating transgenic and control plants at the same developmental stage were excised (time zero) and weighed at different times (n = 11 and 23 for control and transgenic plants, respectively).

To evaluate drought stress recovery, 10 Ubi::ZmXerico1 events and control plants were grown in the greenhouse in September 2009. Uniform seedlings were grown to V1, mixed soil of 60% sand and 40% controlled under fully irrigated condition until V3. Water was then withheld for 4 d until plants appeared fully wilted and were rewatered to assay recovery. The percentage of plants fully recovered for transgenic and control seedlings (12–15 seedlings per entry) was measured 24 h after rewatering. For measurements of maize water use, three events and three wild-type control entries with 9 to 12 entries each were grown in well-watered conditions in 6" pots until V5. One night before withholding water, pots were watered thoroughly until soil was saturated. Pot weight was taken at 8:00 a.m. on day 1 and day 3 of treatment (Fig. 9A). Water use was calculated by (pot weightday1 – pot weightday3)/3 (g water plant⁻¹ day⁻¹). Plants in the drought treatment were wilted within 4 d of treatment. When soil moisture was about 30% of soil capacity, drought-stressed plants were fully rewatered (saturated soil) again to start another drought cycle. Drought treatment cycle was repeated a second time 5 d after normal watering (Fig. 9B).

For photosynthesis measurements, three transgenic events and a wild-type entry with 26 plants per entry were grown in the greenhouse in well-watered conditions in a randomized complete block pattern. Li6600XT Portable Photosynthesis System (LICOR) was used to measure gas exchange. Reference CO₂ was set at 40 μmol·mol⁻¹, flow rate was 500 μmol·s⁻¹, and internal light intensity was 1,800 μmol·m⁻²·s⁻¹. Humidity was controlled around 40% during measurements. At V8, the sunlit middle portion of upmost fully expanded leaf was measured between 10:00 a.m. to 2:00 p.m. on a cloudless day. At R1, the ear leaf was used to take measurements. Standard survey procedure was followed based on the User Manual provided by LICOR Biosciences.

Yield performance was measured from transgenic events, and control plants grown in well-watered conditions (n = 15) or subjected to five drought cycles of 4 to 5 d per cycle (n = 6). When almost all drought-stressed plants showed signs of wilting, pots were filled to capacity before starting next cycle. Seed was harvested and weight of seed per plant was used as a measure of yield performance.

Bioinformatics Analysis

PFAM domains RINGv, zf-RING-like, zf-RING_2, zf-RING_3, zf-RING_4, zf-RING_5, zf-C3HC4, zf-C3HC4_2, zf-C3HC4_3, and zf-C3HC4_4 were used to retrieve RING domain proteins from publically available CRMZM models (AGP_v3) 29 with an e-value threshold cutoff using trusted cutoff and gathering threshold approaches. The obtained protein sequences were classified as RING-V, RING-H2, RING-HC, and RING-C2 based on determination of their actual RING domain architectures (CCCHHCCH, CCCHHCC, CCCCCHCC, and CCCCCCCCCC, respectively). RING domain sequences were obtained and used to generate HMM-logos models using Skyline software with default parameters (Wheeler et al., 2014). A neighbor-joining phylogenetic tree was produced using a CLUSTALW alignment of RING domains using MEGA6 software with default parameters and 1,000 bootstrap trees (Tamura et al., 2013).
Protein identities were calculated with Vector NTI Advance 10 (ThermoFisher Scientific) using the BLOSUM62 matrix. An alignment of proteins was produced using AlignX from the VNTI suite using the same matrix. Trans-membrane predictions were calculated using TMHMM2.0 (Krogh et al., 2001).

RNA Extraction, qRT-PCR, and Northern Blots

Total RNA extraction and northern blots (Supplemental Fig. S3) were performed as previously described (Brugière et al., 2008). cDNA synthesis for RT-PCR analysis was obtained using reverse transcription of total RNA with SuperScript cDNA Synthesis Kit (ThermoFisher Scientific). The semiquantitative RT-PCR of Supplemental Fig. S3 was carried out using a forward primer in ZmXerico1 (5'-TGGTGTCTCGAGAACGAG-3') and a reverse primer in the PinII terminator (5'-CACATAACACACAACTTTTGAGCCCA-3'). qRT-PCR analysis of ZmXerico1, ZmXerico2, and ZmXerico2 expression was performed using gene specific primers designed in the 3' UTR of each gene (ZmXerico1, 5'-GGGACAGAGGAGGACGAAAGG-3'; ZmXerico1, 5'-GTGTTGAGCATCAGTATGAG-3' and 5'-GCGACGGAACACGAAAGA-3'; ZmXerico2, 5'-AGAGCTTAGGACGCTGAGATA-3' and 5'-GCCGCGGAAACAAAGA-3'). Expression levels were normalized to the expression of a set of validated reference genes previously described by Manoli et al. (2012), including GRMZM2G102471 (5'-TCACCTCACCAGGATTA-3' and 5'-GACAGATGTCGAGGAA-3'), GRMZM2G053577 (5'-GCCACCTGAGACATAAT-3' and 5'-GCAGATCTGATACCTTCTTCA-3') and GRMZM2G166694 (5'-GAGGGCATGTTGCTACATT-3' and 5'-CCGAAATGCTCTGCTTAC-3'). Total RNA was prepared using TRIzol (ThermoFisher Scientific). One microgram of total RNA was used for cDNA synthesis using QiaGen QuantiTect Reverse Transcription kit (Venio, PL). Twenty-five nanograms (~4 μL) was used in a 20-μL reaction containing 0.5 μM of primers with Bio-Rad SoFast Evagreen master mix (Bio-Rad) cycled with a Bio-Rad CFX 96 thermocycler according to the manufacturer’s instructions. Primers were designed using the PrimerQuest design tool from Integrated DNA Technologies with parameters that were optimal for use with the SoFast Evagreen master mix. Each sample for each target was run in triplicate and analyzed using the Bio-Rad CFX Manager Software with all three reference genes used in the normalization analysis.

Diurnal Regulation through NextGen RNASeq

Plants were grown in Turface in growth chambers until V6 and subjected to water stress by withholding water at zt = 0 (60±a.m.). Root and shoot tissue was sampled every 2 h in four replicates consisting of two plants. Total RNAs was isolated from frozen tissue by using the QIAshredder MiniBEST Tissues Kit (Qiagen) and purified using RNaseeasy columns, and then eluted in RNase-free water. Sequencing libraries were prepared using Illumina’s TruSeq stranded kit and quality checked using Bioanalyzer DNA 7500 chip. Ten nanomolar pools made up of 12 samples with unique indices were generated. Sample pools were hybridized and clustered on a single run flowcell as per Illumina’s Rapid Cluster kit and loaded on an Illumina HiSeq. Sequence alignment was performed using Cufflinks, and gene expression was estimated using Kallisto. Genes were filtered to keep only those with an expression value > 10 in at least 10 samples.

Ubiquitination Assay

The maize homolog of UBE11 from wheat (GI214896, ZP_00207716) was cloned from B73 cDNA (GI214896, ZP_00207716). The generated reads per kilobase of transcript per million mapped reads (RPKM) data matrix was visualized and analyzed in GeneData Analyst software. The number of bases that fall into the exonic regions of each gene is summed to obtain gene length counts. The normalized values are calculated as reads per kilobase of transcript per ten million mapped reads (RPKM). The normalized values for genes and exons are counted as follows: Exons/genes Reads Per Kilobase of transcript per Million mapped reads (RPKM) = (10 × C)/N/L, with RPKM = RPKM*10 = reads per kilobase of exon model per ten million mapped reads, C = the number of reads that aligned to the feature, N = total number of mapped reads in the experiment, and L = the length of the feature in bp.

Plant Protein Extraction, Communoprecipitation Assay, and Immunoblotting

Maize protoplasts were transfected with corresponding plasmids, incubated for 16 h, and harvested. Total protein was extracted with 2× Laemmli sample buffer and subjected to immunoblotting. For communoprecipitation assays, total protein extracts were prepared in lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM Ethylenediaminetetraacetic acid (EDTA)), 0.5% Triton X-100, 200 μM MG132, 5 μM phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail (Roche). Samples were subjected to immunoblotting analysis using antibodies against different epitopes, and then incubated for 1 h at room temperature. After washing, the membranes were incubated with a secondary antibody (anti-mouse, anti-rabbit) and detected using an appropriate chemiluminescent reagent. All experiments were performed in triplicate. For detection of the UB11 protein, a specific antibody was used (described in more detail in the manuscript). For detection of the Ub-11 protein, a specific antibody was used (described in more detail in the manuscript). For detection of the Ub-11 protein, a specific antibody was used (described in more detail in the manuscript). For detection of the Ub-11 protein, a specific antibody was used (described in more detail in the manuscript). For detection of the Ub-11 protein, a specific antibody was used (described in more detail in the manuscript). For detection of the Ub-11 protein, a specific antibody was used (described in more detail in the manuscript). For detection of the Ub-11 protein, a specific antibody was used (described in more detail in the manuscript).

ABA Sensitivity Assays and ABA Metabolite Analysis

Germination of Arabidopsis seeds was measured on 1/2 Murashige and Skoog media containing different concentrations of (+)-ABA (Sigma Aldrich).
All tested seeds were collected from plants grown in the same conditions side by side. Around 100 sterilized seeds for each line were plated on medium supplemented with ABA. Seeds were stratified at 4°C for 96 h. Plates were then placed in growth chambers set at 16 h of light at 22°C temperature and 50% relative humidity. Germination was scored as the emergence of radicle over a period of 3 d. Each experiment has been repeated at least three times.

Transgene-positive maize kernels for Ubi::ZmXerico1 event 7, and segregating nontransgenic kernels were germinated on paper rolls wetted with tap water and grown for 4 d. The lengths of the primary root of 15 transgenic and control germinated seedlings of similar length were measured, and seedlings were transferred on a roll wetted with a 50 μM (+)-ABA solution or a roll wetted with water plus a corresponding amount of MeOH used to prepare the ABA stock solution as control treatment. Root lengths were measured after 72 h at 23°C in the lab in daylight conditions and used to calculate root elongation per day.

Measurements of ABA-related metabolites were performed at National Research Council Canada-Plant Biotechnology Institute according to the methodology described by Chiwocha et al. (2003).

**Accession Numbers**

Sequence data from this article can be found in the MaizeGDB database under GRMZM2G018070 (ZmXerico1), and GRMZM2G093349 (ZmXerico2).

**Supplemental Data**

The following supplemental materials are available.

Supplemental Figure S1. Phylogenetic tree representing maize RING-domain-containing protein family.

Supplemental Figure S2. Transmembrane region of ZmXerico and related short RING-H2 proteins predicted using TMHMM and ER subcellular localization of ZmXerico-GFP fusions.

Supplemental Figure S3. Ectopic overexpression of ZmXerico1 and ZmXerico2 in leaves of Ubi::ZmXerico1 and Ubi::ZmXerico2 T0 maize transgenic events compared to control.

Supplemental Figure S4. Reduced water loss of detached leaves of 35S::ZmXerico2 Arabidopsis transgensics compared to controls.

Supplemental Figure S5. Water use efficiency of maize Ubi::ZmXerico1 transgensics compared to controls at R1 developmental stage.

Supplemental Figure S6. ABA, ABA-GE, DPA, and PA levels in roots of maize Ubi::ZmXerico1 transgenic and control seedlings.

Supplemental Figure S7. Effect of ZmXerico1 overexpression on maize plant biomass.

Supplemental Figure S8. Effect of ZmXerico1 overexpression on plant height.

Supplemental Figure S9. Stability of epitope-tagged ZmABA8ox1a and ZmABA8ox3a proteins overexpressed in maize protoplasts isolated from transgenic Ubi::ZmXerico1 events and null segregant plants.

Supplemental Table S1. List of identified nonredundant RING-containing GRMZM proteins, their size, RING domain sequences, and types.

Supplemental Table S2. Chromosome locations of ZmXerico, ZmXerico1, and ZmXerico2 genes in the maize genome.

Supplemental Table S3. Percentage identity between ZmXerico1 and ZmXerico2 with related proteins.

Supplemental Table S4. Statistical analysis of staygreen scores of controls and Ubi::ZmXerico1 plants.

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