A group VII ethylene response factor gene, ZmEREB180, coordinates waterlogging tolerance in maize seedlings

Feng Yu, Kun Liang, Tian Fang, Hailiang Zhao, Xuesong Han, Manjun Cai and Fazhan Qiu*

National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China

Summary

Group VII ethylene response factors (ERFVII) play important roles in ethylene signalling and plant responses to flooding. However, natural ERFVII variations in maize (ZmERFVII) that are directly associated with waterlogging tolerance have not been reported. Here, a candidate gene association analysis of the ZmERFVII gene family showed that a waterlogging-responsive gene, ZmEREB180, was tightly associated with waterlogging tolerance. ZmEREB180 expression specifically responded to waterlogging and was up-regulated by ethylene; in addition, its gene product localized to the nucleus. Variations in the 5′-untranslated region (5′-UTR) and mRNA abundance of this gene under waterlogging conditions were significantly associated with survival rate (SR). Ectopic expression of ZmEREB180 in Arabidopsis increased the SR after submergence stress, and overexpression of ZmEREB180 in maize also enhanced the SR after long-term waterlogging stress, apparently through enhanced formation of adventitious roots (ARs) and regulation of antioxidant levels. Transcriptomic assays of the transgenic maize line under normal and waterlogged conditions further provided evidence that ZmEREB180 regulated AR development and reactive oxygen species homeostasis. Our study provides direct evidence that a ZmERFVII gene is involved in waterlogging tolerance. These findings could be applied directly to breed waterlogging-tolerant maize cultivars and improve our understanding of waterlogging stress.

Introduction

Waterlogging is one of the most important abiotic stresses affecting crop growth, development and yield. Flooding events associated with global climate change have occurred increasingly since the start of this century (Bailey-Serres et al., 2012; Hirabayashi et al., 2013). Maize (Zea mays L.) frequently encounters waterlogging stress during its life cycle due to poor drainage and/or long periods of rainfall (Visser et al., 2003). Waterlogged soil results in reduced levels of oxygen in plant tissues and less gas diffusion between cells (Voeseenk and Bailey-Serres, 2013), which restricts mitochondrial respiration and decreases soil pH (Fukao and Bailey-Serres, 2004; Setter et al., 2009). The accumulation of reactive oxygen species (ROS) that cause oxidative damage to plants is also enhanced in conditions of prolonged low-oxygen stress (Shabala, 2011).

To survive and regulate different responses under waterlogging stress, plants modulate numerous morphological, transcriptional and metabolic changes (Bailey-Serres and Colmer, 2014; Bailey-Serres and Voeseenk, 2008; Lee et al., 2011; Nanojo et al., 2011; Nasrai et al., 2009; Zou et al., 2010). Anaerobic pathways, such as ethanolic fermentation and glycolysis coupled with nicotinamide adenine dinucleotide (NAD) regeneration, respond to fulfill adenosine triphosphate (ATP) needs under waterlogging stress, and adaptive traits such as aerenchyma and adventitious root formation are induced to promote gas diffusion (Colmer and Voeseenk, 2009). In addition, endogenous antioxidant enzymes and nonenzymatic molecules are up-regulated to counteract the deleterious effects of ROS (Apel and Hirt, 2004; Mittler et al., 2004).

Research on rice has revealed that two ethylene-mediated opposite signalling pathways are involved in submergence tolerance (Voeseenk and Bailey-Serres, 2015). SUBMERGENCE 1A (SUB1A) confers submergence tolerance in rice (Xu et al., 2006).

Ethylene-induced SUB1A increases the accumulation of gibberellin (GA) signalling repressors to inhibit the transcription of GA-inducible genes, limiting GA-mediated starch breakdown, elongation growth and leaf senescence and enhancing the use of carbohydrate reserves (Fukao and Bailey-Serres, 2008; Fukao et al., 2006, 2012; Hirano et al., 2012). Tolerant genotypes with the SUB1A allele show up-regulation of mRNAs encoding antioxidant enzymes during submergence (Jung et al., 2010; Mustroph et al., 2010). By contrast, the induction of SNORKEL1 (SK1) and SK2 by ethylene in deepwater rice varieties promotes internode elongation by increasing the accumulation of active GA, which also fits within the hormonal hierarchy (Ayano et al., 2014; Hattori et al., 2009; Raskin and Kende, 1984). Similar survival strategies in response to flooding have also been found in other species, such as Rumex species (Bailey-Serres and Voeseenk, 2008; Benschop et al., 2005). Moreover, studies on Arabidopsis have shown that petiole elongation and leaf hyponastic growth are adaptive responses to submergence in partial or complete darkness and likely occur via an ethylene-dependent process (Lee et al., 2011). Recently, the gene determining leaf gas films [LEAF GAS FILM1 (LGF1)] has been shown to enhance underwater photosynthesis while contributing to submergence tolerance in rice via regulating C30 primary alcohol synthesis (Kurokawa et al., 2018). These studies provide the basis for a better understanding of how plants adapt to excess water environments.

The ethylene response factor (ERF) family is a large gene family of plant-specific transcription factors characterized by a single DNA-binding APETALA2 (AP2)/ethylene-responsive element-bind- ing protein domain (Licau et al., 2013; Nakano et al., 2006). A subgroup of this family, the ERFVIs, is characterized by several other motifs in addition to the AP2 domain (Nakano et al., 2006). ERFVIs control flooding responses and low-oxygen tolerance in several plant species (Gibbs et al., 2015). Arabidopsis encodes
five ERFVII s, including two known hypoxia-responsive ERFs (HRE1 and HRE2) that directly affect anaerobic responses (Licausi et al., 2010). The survival rate of the double-knockout mutant hre1hre2 in Arabidopsis is markedly lower in comparison with the wild type, whereas overexpressed HRE1 exhibits improved tolerance to anoxia (0% oxygen), positively regulating core hypoxia-responsive gene expression (Hess et al., 2011; Licausi et al., 2010). Another ERFVII in Arabidopsis, RAP2.2, has a similar function of flooding response (Hinz et al., 2010). RAP2.12 is highly homologous to RAP2.2 and is up-regulated in leaves under hypoxic conditions. The RAP2.12 protein is anchored to membrane-bound ACYL-CoA BINDING PROTEIN1 (ACBP1) under aerobic conditions, but it is released and translocated to the nucleus to activate the expression of hypoxia-responsive genes at the onset of hypoxia (Licausi et al., 2011). Thus, there is strong evidence that ERFVII s in Arabidopsis are directly involved in anoxia/hypoxia signalling and regulate survival ability under stress conditions. Notably, three genes (SUB1A, SK1 and SK2) conferring submergence tolerance in rice also belong to the ERFVII family (Hattori et al., 2009; Xu et al., 2006). Furthermore, the flooding responses of two related Rumex spp. species from contrasting hydrological environments may also be regulated by ERFVII s (van Veen et al., 2014).

Maize is sensitive to waterlogging stress. Waterlogging severely affects approximately 18% of the land in south and south-east Asia and annually results in production losses of 25%–30% (Zaidi et al., 2010). Investigations of maize and its tolerant ancestor Zea nicaрагuensis in waterlogged conditions have revealed that enhanced formation of aerenchyma and induction of barriers to radial oxygen loss in adventitious roots contribute to waterlogging tolerance (Abiko et al., 2012; Watanabe et al., 2017). Other research has shown that programmed cell death (PCD) in the root cortex and the formation of aerenchyma and adventitious roots are the most adaptive traits in maize (Thirunavukkarasu et al., 2013; Zhai et al., 2013). Increasing the oxygen available to waterlogged organs is thus vital for maize to adapt to waterlogged conditions, but the regulation mechanisms of these adaptive traits in maize are unclear.

In this study, we identified and characterized 19 ERFVII s of maize (ZmERFVII s). The association between genetic variations of each ZmERFVII gene and waterlogging tolerance, evaluated in terms of survival rate (SR) under long-term stress at the seeding stage, was quantified using a candidate gene association strategy and a diverse population of maize consisting of 368 inbred lines from global germplasm. A key candidate gene, ZmEREB180, was strongly associated with SR under multiple environments. Variations in the 5′ untranslated region (5′-UTR) of ZmEREB180 affected its expression in different varieties and showed significant correlation with SR. Functional analysis showed that ZmEREB180 plays a positive role in maize waterlogging tolerance, seemingly by promoting the formation of adventitious roots and coordinating ROS homeostasis. Our results improve our understanding of the molecular mechanisms in waterlogging responses and accelerate application of marker-assisted breeding for waterlogging tolerance.

**Results**

**Identification and cloning of ZmERFVII genes**

To identify the group VII ethylene response factors in maize (ZmERFVII s), the predicted protein sequences of 212 AP2 and ethylene-responsive element-binding protein (AP2-EREBPs) genes were downloaded from GrassTFDB in GRASSiUS (Burdo et al., 2014). The five Arabidopsis ERFVII genes (Nakano et al., 2006) were used as queries to search against these sequences, and two signatures (alanine at position 13 and aspartic acid at position 18; Magnani et al., 2004) of ERFVII genes were further confirmed manually. Finally, 19 ZmERFVII s were identified. We cloned these genes from the B73 maize inbred line and found that they were identical to the annotated sequences. These genes are located in all chromosomes except chromosomes 8 and 10 (Figure 1, Table S1). Whereas ZmEREB179 had two introns, the other 11 and seven genes had one and zero introns, respectively (Figure S2). Motif analysis revealed numerous conserved motifs in addition to the AP2/ERF domain (Figure S2). The conserved N-terminal motif (CM1) divides ERFVII s into subgroup a and subgroup b depending on whether they contain CM1 (Bailey-Serres et al., 2012). Among the ZmERFVII s, 12 and 7 belonged to subgroup a and subgroup b, respectively. By contrast, none of the 5 ERFVII s in Arabidopsis and only 1 of the 15 ERFVII s in rice belong to subgroup b (Bailey-Serres et al., 2012; Nakano et al., 2006), indicating that ZmERFVII s not only include more family members than in Arabidopsis and rice but also exhibit more sequence variability, implying a greater diversity of functions.

**Association analysis of natural variation in ZmERFVII s with maize waterlogging tolerance**

From previously reported transcriptomic sequencing of a maize panel consisting of 368 inbred lines, high-quality SNP markers with a minor allele frequency of ≥5% (Fu et al., 2013) were used to characterize the DNA polymorphism of 19 ZmERFVII genes. Among these ZmERFVII genes, all were found to be polymorphic; on average, 31.95 SNP markers were identified in each gene, and ZmEREB102 exhibited the most polymorphic markers (Table 1). The waterlogging tolerance of each inbred line was investigated, and the survival rate (SR) of each genotype was determined. The SR of inbred lines exhibited wide phenotypic variations that ranged from 0% to 100%, mainly distributed between 30% and 60% (Figure S3). Combining the genotypic SNP data from 19 ZmERFVII genes with SR phenotypes in three different environments and best linear unbiased prediction (BLUP) data, an association analysis was conducted to quantify significant associations between SNP and traits. Nine genes were significantly associated with SR in at least one environment (P < 0.01), of which ZmEREB179 and ZmEREB180 were identified in multiple environments (Table 1). Only ZmEREB180 was associated with waterlogging tolerance in this panel at P < 0.001 (Table 1).

To identify genetic variations in ZmEREB180, a 3.1-kb genomic sequence containing ZmEREB180 was resequenced in 248 inbred lines randomly selected from the 368 line association panel. In total, 58 single nucleotide polymorphisms (SNPs) and 29 insertion/deletions (InDels) were detected. We again analysed the association of each polymorphism with BLUP data using the compressed mixed linear model (cMLM) and calculated the pairwise linkage disequilibrium (LD) of these polymorphisms (Figure 1). Six variants, InDel-241 (59 bp), InDel-196 (5 bp), SNP-118, SNP-78, InDel-77 (8 bp) and InDel-19 (1 bp) in 5′-UTR, and one variant (InDel214, 3 bp) in the first exon were significantly associated with maize waterlogging tolerance (SR; P < 3.67 × 10−6) and completely mapped within an LD block (Figure 1a,b). These resequenced genotypes were classified into two haplotype (Hap) groups based on the significant variants (Figure 1c). Hap1 had a significantly higher SR than Hap2 (P = 3.58 × 10−6; Figure 1d), and Hap1 was therefore designated as the favourable/tolerant allele, explaining 15.6% of the phenotypic variation. The amplicon of the primer...
To examine whether variants in the 5’-UTR result in altered ZmEREB180 mRNA abundance among different genotypes, we analysed the mRNA abundance of the ZmEREB180 gene among 100 inbred lines under well-watered (control, before waterlogging treatment), short-term (4-h) and long-term (3-day) waterlogging stress using quantitative reverse-transcription PCR (qRT-PCR). ZmEREB180 mRNA abundance was positively correlated with plant SR under both 4-h and 3-day waterlogging stress, but there was no significant correlation under well-watered conditions (Figure 2a). These observations suggest that the increased mRNA abundance in the ZmEREB180 gene is closely associated with the waterlogging tolerance of maize inbred lines investigated in this study under waterlogging stress. Furthermore, the genotypes with Hap1 exhibited significantly higher mRNA abundance of ZmEREB180 than those with Hap2 whether the stress was imposed or not, and the mRNA abundance of ZmEREB180 was up-regulated in both Hap1 and Hap2 to different extents (Figure 2b), indicating that the mRNA abundance of ZmEREB180 is also associated with 5’-UTR variants. Based on these results, we suggest that variations in the 5’-UTR of ZmEREB180 that altered its expression level might be the important causal determinants conferring waterlogging stress tolerance in maize seedlings.

**ZmEREB180 overexpression confers seedling waterlogging tolerance**

Considering that ZmEREB180 expression is positively correlated with waterlogging tolerance of maize seedlings, we performed transgenic assays overexpressing the coding sequence of ZmEREB180 (from the B73 genotype) in both *Arabidopsis* and maize. For *Arabidopsis*, two independent lines (OE5 and OE8) were used to evaluate the phenotypes after submergence treatment. The transgenic Arabidopsis displayed significantly enhanced submergence tolerance in comparison with the wild type (WT), and there were no remarkable morphological changes under normal conditions in transgenic seedlings (Figure S4). After recovery, the fresh weight of aboveground seedlings in transgenic plants was significantly higher than in WT (Figure S4). Moreover, four anaerobic metabolism genes (*AtADH1*, *AtPDC1*, *AtSUS1* and *AtSUS4*; Licau et al., 2010) were significantly up-regulated in the transgenic plants compared with WT under submerged conditions (4 h), but not significantly altered under normal conditions (0 h; Figure S4), indicating that expression of ZmEREB180 in Arabidopsis enhanced the effect of submerging treatment. The physiological assays of OE5, OE8 and WT demonstrated that MDA content, indicating the degree of oxidative damage, was not significantly different between transgenic lines and WT under normal conditions (Figure S5). However, WT exhibited significantly higher MDA contents after 1 and 2 days of submergence stress. The H$_2$O$_2$ content was significantly
lower in transgenic lines than in WT whether the stress occurred or not (Figure S5), whereas the activity of POD in transgenic lines was significantly higher than in WT after 2 days stress.

We also examined the waterlogging tolerance of transgenic maize transformed by \textit{ZmUbi:ZmEREB180} under waterlogging stress. Two independent transgenic lines (OE115 and OE240) were analysed in the T\textsubscript{2} generation. The transgenic lines overexpressed \textit{ZmEREB180} by fourfold to sevenfold compared to the transformation receptor (C01) and that waterlogging stress enhanced the expression until 3 days in transgenic lines but not in C01 (Figure 3a). We subsequently investigated dynamic growth under normal and waterlogged conditions in parallel over 6 days. There were no significant differences in seedling height under normal conditions (Figure S6a), but seedling height in the transgenic lines was significantly higher than in C01 from the fourth day (Figure S6b), and SPAD values of the 1st leaf of transgenic lines were significantly higher than in C01 from the third day under waterlogging conditions (Figure S6d). In addition, there were no significant changes in transgenic lines before waterlogging treatment, measured in terms of shoot fresh weight, root fresh weight and root length (Figure S6c,f,g). However, measured traits increased in these lines after 6 days of waterlogging stress, and significant alterations were observed in transgenic lines compared to C01. Moreover, there was significantly less leaf injury in transgenic lines compared to C01 (Figure S6e). These results suggest that overexpression of \textit{ZmEREB180} does not significantly influence the growth of maize seedlings under normal conditions, but transgenic plants strongly maintain growth ability under waterlogged conditions.

We further observed enhanced tolerance under long-term waterlogging stress in transgenic lines, which generated at least two complete green leaves (Figure 3b,c). The transgenic lines had significantly higher shoot fresh weight and length of the longest root compared with C01 (Figure 3d,e). Furthermore, the transgenic lines had well-developed ARs compared to C01 (Figure S7), measured in terms of number of ARs, maximum AR length and average AR length (Figure 3f–h). Nevertheless, there is no obvious difference of the seedling characterizations that parallelly measured between transgenic lines and C01 under normal condition (Figure 3). Since oxidative stress, which affects plant development, was imposed on roots under waterlogged conditions, five physiological parameters that characterize oxidation state were also measured in OE115, OE240 and C01 seedling roots. The MDA content was not significantly different between transgenic maize lines and C01 under normal conditions, but C01 exhibited significantly higher MDA after 1 and 3 days of waterlogging stress (Figure 4). The content of H\textsubscript{2}O\textsubscript{2} was significantly higher in C01 than in transgenic lines after 3 days stress (Figure 4), and inhibition capacity of hydroxyl free radical, representing the capacity of scavenging hydroxyl free radical, was significantly lower in transgenic lines than in WT whether the stress occurred or not (Figure S5), whereas the activity of POD in transgenic lines was significantly higher than in WT after 2 days stress.

We also examined the waterlogging tolerance of transgenic maize transformed by \textit{ZmUbi:ZmEREB180} under waterlogging stress. Two independent transgenic lines (OE115 and OE240) were analysed in the T\textsubscript{2} generation. The transgenic lines overexpressed \textit{ZmEREB180} by fourfold to sevenfold compared to the transformation receptor (C01) and that waterlogging stress enhanced the expression until 3 days in transgenic lines but not in C01 (Figure 3a). We subsequently investigated dynamic growth under normal and waterlogged conditions in parallel over 6 days. There were no significant differences in seedling height under normal conditions (Figure S6a), but seedling height in the transgenic lines was significantly higher than in C01 from the fourth day (Figure S6b), and SPAD values of the 1st leaf of transgenic lines were significantly higher than in C01 from the third day under waterlogging conditions (Figure S6d). In addition, there were no significant changes in transgenic lines before waterlogging treatment, measured in terms of shoot fresh weight, root fresh weight and root length (Figure S6c,f,g). However, measured traits increased in these lines after 6 days of waterlogging stress, and significant alterations were observed in transgenic lines compared to C01. Moreover, there was significantly less leaf injury in transgenic lines compared to C01 (Figure S6e). These results suggest that overexpression of \textit{ZmEREB180} does not significantly influence the growth of maize seedlings under normal conditions, but transgenic plants strongly maintain growth ability under waterlogged conditions.

We further observed enhanced tolerance under long-term waterlogging stress in transgenic lines, which generated at least two complete green leaves (Figure 3b,c). The transgenic lines had significantly higher shoot fresh weight and length of the longest root compared with C01 (Figure 3d,e). Furthermore, the transgenic lines had well-developed ARs compared to C01 (Figure S7), measured in terms of number of ARs, maximum AR length and average AR length (Figure 3f–h). Nevertheless, there is no obvious difference of the seedling characterizations that parallelly measured between transgenic lines and C01 under normal condition (Figure 3). Since oxidative stress, which affects plant development, was imposed on roots under waterlogged conditions, five physiological parameters that characterize oxidation state were also measured in OE115, OE240 and C01 seedling roots. The MDA content was not significantly different between transgenic maize lines and C01 under normal conditions, but C01 exhibited significantly higher MDA after 1 and 3 days of waterlogging stress (Figure 4). The content of H\textsubscript{2}O\textsubscript{2} was significantly higher in C01 than in transgenic lines after 3 days stress (Figure 4), and inhibition capacity of hydroxyl free radical, representing the capacity of scavenging hydroxyl free radical, was significantly...
higher in transgenic lines than in C01 after 1 and 3 days of stress. The POD activity and GSH contents were significantly higher in transgenic lines than in C01 whether the stress was imposed or not. These results suggest that overexpressing ZmEREB180 could increase antioxidants to alleviate oxidative damage and indicate that increased expression of ZmEREB180 may promote AR development and enhance antioxidant ability as mechanisms for adapting to waterlogging stress.

Multidimensional responses of nucleus-localized ZmEREB180 to waterlogging

Given that ZmEREB180 is an ethylene (ET) response factor gene and its orthologs in Arabidopsis (HRE1 and HRE2) specifically respond to hypoxia (Licau et al., 2010), we analysed the mRNA abundance of ZmEREB180 under different stress and hormone treatments in B73 seedling roots. ZmEREB180 specifically responded to waterlogging, while the NaCl and chilling treatments did not affect mRNA abundance (Figure 5a). ZmEREB180 was markedly induced by ET and GA and NAA treatments but not by ABA (Figure 5b). Moreover, the GFP fluorescence of ZmEREB180-GFP expressed in maize protoplasts colocalized with the Nucleus-Tracker Red-labelled nuclei (Figure 5c), indicating that ZmEREB180 targets to the nucleus. These results collectively demonstrate that ZmEREB180 is a nucleus-localized ethylene response factor and specifically responds to waterlogging.

To investigate the regulatory network involving ZmEREB180, transcriptomes of the transgenic line OE240 and C01 plants under normal and waterlogged conditions were compared. A total of 1423 and 730 genes (adjust \( P < 0.01 \), fold change \( >2 \) or \(<0.5 \) ) were up-regulated and down-regulated in OE240 compared to C01 under normal conditions, respectively (Figure S8a, Tables S4 and S5). Gene Ontology (GO) analysis revealed that the up-regulated list was mainly enriched for response to stress, including fungus, wounding, chitin, hypoxia and hormone stimuli such as ethylene, auxin, jasmonic acids and hormone-mediated signalling (Figure S8b). The most enriched category was regulation of transcription, which supports the conclusion that ZmEREB180 is a transcription factor. At the level of molecular function, protein serine/threonine kinase activity, transcription factor activity and calcium/haem binding were the most enriched categories. These results indicate that ZmEREB180 is involved in a stress-related pathway. Under waterlogging stress, 662 and 431 genes were twofold up-regulated and down-regulated in OE240 relative to C01, respectively (Figure 6a, Tables S6 and S7). GO analysis also showed that up-regulated genes were most enriched for stimulus-related pathways, including response to fungus, external stimuli, chitin, hormone stimuli and starvation (Figure 6b). Auxin and ET response, two endogenous hormone stimulus pathways, were enriched among the up-regulated gene list; these are the two most important modulators of AR formation (Bellini et al., 2014). Programmed cell death (PCD) of the epidermal layers adjacent to AR primordia is a key phase before AR emergence (Atkinson et al., 2014). Among the 662 up-regulated genes, many genes involved in PCD were identified.
including those related to cell wall hydrolysis (three xyloglucan endotransglucosylase), cell wall loosening (six expansions) and Ca²⁺ signalling related (six calmodulin-related genes). Moreover, eight glutathione transferase genes that are critical for H₂O₂ detoxification (Dixon et al., 2010) were significantly up-regulated in OE240 under waterlogging stress, which was reflected in the enrichment in glutathione transferase activity at the GO molecular function level (Figure 6b). The increased expression of these genes was further validated by qRT–PCR analysis (Figure 6c, Table S2). These genes may be directly or indirectly involved in ZmEREB180-mediated signalling to coordinate development and defence under waterlogging stress.

**Discussion**

There are 98 members of the plant-specific ERF transcription factor family in maize (Liu et al., 2013) and over 100 members in rice and Arabidopsis, all of which share an AP2 DNA-binding domain (Nakano et al., 2006). ERFVII, a subgroup of the ERF family characterized by two conserved amino acid signatures within the AP2 domain (Magnani et al., 2004), are involved in a wide range of plant growth, development and stress responses (Gibbs et al., 2015). Fifteen rice ERFVII have been identified, with only SUB1C (OsERF73) classified as a subgroup b ERFVII (ERFVIIb); members of this subgroup lack the conserved N-terminal region...
found in the ERFVIIa subgroup (Singh et al., 2010; Bailey-Serres et al., 2012). Arabidopsis encodes five ERFVIs that belong to ERFVIIa (Nakano et al., 2006). In this study, 19 ZmERFVII genes were identified in the maize genome (Figure S1), a number higher than that found in rice and Arabidopsis. We consider maize to contain fewer than the 20 ZmERFVIs reported by Liu et al. (2013) because ZmEREB118 did not share the conserved residue at position 18 of the AP2 domain and was therefore removed. Seven of the 19 ZmERFVIs belonged to subgroup b (Figure S2), in marked contrast to rice and Arabidopsis (Nakano et al., 2006; Singh et al., 2010), indicating possible functional divergence in maize since amino acid variations often directly affect protein function. However, most of the ZmEREBVIs share the conserved N-terminal motif, suggesting that similar functions to ERFVIs in rice and Arabidopsis may also exist.

Investigations of Arabidopsis and rice revealed that ERFVII family members play a vital role in response to flooding. Four out of five ERFVIs in Arabidopsis (HRE1, HRE2, RAP2.12 and RAP2.2) are involved in anoxia/hypoxia stress (Licausi et al., 2010; Hinze et al., 2010; Hess et al., 2011; Licausi et al., 2011) and three ERFVII members in rice (SUB1A, SK1 and SK2) confer submergence tolerance (Hattori et al., 2009; Xu et al., 2006). Given that homologous genes that evolved from the same ancestor can have a similar function, we hypothesized that ERFVIs in maize may also respond to waterlogging, which is frequently encountered in the maize life cycle. Candidate gene association was thus conducted to identify associations between variations in ZmERFVII genes and SR phenotype in our association panel (Table 1). Some possible signals were identified, implying that these ZmERFVIs are potentially involved in regulating waterlogging tolerance. ZmEREB180 was associated with waterlogging tolerance in multiple environments and as such is a key candidate for importance in waterlogging tolerance (Table 1). ZmEREB180 has a conserved N-terminal motif (Figure S2), and ectopic expression ZmEREB180 in Arabidopsis increased survival after submergence treatment (Figure S4), indicating that ZmEREB180 functions similarly to Arabidopsis ERFVIs such as HRE1 (Licausi et al., 2010) and RAP2.2 (Hess et al., 2011). Overexpression of ZmEREB180 in maize further confirmed that transgenic lines exhibited increased survival ability under long-term waterlogging stress (Figure 3). These results collectively demonstrate that ZmEREB180 confers waterlogging tolerance at the maize seedling stage.

Molecular and physiological characterization of the key candidate genes involved in plant waterlogging tolerance is needed to understand the adaptation of plants to excess water conditions. However, very few loci associated with waterlogging stress have hitherto been cloned and used in breeding to enhance the waterlogging tolerance of maize; instead, most related research has involved mapping to wider genetic intervals along chromosomes (Mano and Omori, 2013; Mano et al., 2005; Qiu et al., 2007; Watanabe et al., 2017; Zaidi et al., 2015). Almost all of these studies were conducted using morphological- and biomass-related traits, such as formation of aerenchyma and adventitious roots, root length, seedling height, fresh and dry weight of seedlings, and formation of radial oxygen loss barriers that indirectly reflect adaptive ability to waterlogging stress. In this study, the SR phenotype of inbred line seedlings was evaluated under long-term stress (Figure S3), showing substantial differences in response to waterlogging. Recently, candidate gene association analysis combining the SR phenotype of drought tolerance in maize seedlings with natural variations in certain genes has successfully been conducted to identify favourable alleles of ZmDERB2.7, ZmNAC111, ZmPP2C-A10 and ZmVPP1 for enhancing maize drought tolerance (Liu et al., 2013; Mao et al., 2015; Wang et al., 2016; Xiang et al., 2017). The same method was used in this study, and a favourable allele (Hap1) of ZmEREB180 was detected.
demonstrating that this method is also suitable for dissecting the genetic basis of waterlogging tolerance traits. Hap1 of ZmEREB180 is considered to be favourable because (i) waterlogging-tolerant maize inbred lines tend to exhibit Hap1, while waterlogging-sensitive lines tend to exhibit Hap2 (Figure 1d); (ii) variants of Hap1 and Hap2 in ZmEREB180 are highly associated with gene expression; that is, lines with Hap1 showed higher expression of ZmEREB180 during waterlogging stress and tended to be waterlogging-tolerant, whereas lines with Hap2 showed lower expression of ZmEREB180 and tended to be waterlogging-sensitive (Figure 2). Moreover, ZmEREB180 is located within a genomic interval previously identified as associated with root length under waterlogging conditions (Osman et al., 2013). Therefore, InDel59 could be applied in

---

Figure 6 Transcriptomic analysis of the ZmEREB180-overexpressing maize line under waterlogging stress. (a) Hierarchical clustering of differentially expressed genes in OE240 relative to C01 plants. (b) Significantly enriched GO in terms of up-regulated genes in OE240. (c) qRT–PCR verification of up-regulated expression genes in OE240 under waterlogging stress. C01 is a transgenic receptor, and OE240 is an independent transgenic line.
breeding programmes to screen tolerant maize varieties to improve maize waterlogging tolerance.

As a simple gaseous phytohormone, ethylene plays a key role in plant responses to biotic and abiotic stresses (van Loon et al., 2006; Morgan and Drew, 1997) and is a major regulator of several flooding-adapted plant traits (Sasidharan and Voesenek, 2015). SUB1A and SK1/SK2 confer opposite submergence-adapted responses in rice and are induced by ethylene (Hattori et al., 2009; Xu et al., 2006), and RAP2.2 in Arabidopsis regulates the ability of plants to resist hypoxia stress via an ethylene-controlled signal transduction pathway (Hinz et al., 2010). In this study, ZmEREB180 was also ethylene-induced and specifically responded to waterlogging stress (Figure 5), and overexpressing ZmEREB180 enhanced the formation of adventitious roots under waterlogged conditions (Figure 3). Adventitious roots facilitate gas transport and water and nutrient uptake during flooding to ensure plant survival (Sauter, 2013; Steffens and Rasmussen, 2016), which is a key response of many species such as rice (Loribiecke and Sauter, 1999), maize (Zhai et al., 2013), Rumex spp. (Visser et al., 1996), tamarack (Larix laricina; Calvo-Polanco et al., 2012) and Eucalyptus spp. (Argus et al., 2015). Ethylene is the major hormone that induces adventitious root growth in rice (Loribiecke and Sauter, 1999) and tomato (Kim et al., 2008; Negi et al., 2010; Vidoz et al., 2010). Ethylene-mediated development of adventitious roots in rice requires auxin signalling (Pacurar et al., 2014). These two hormones synergistically promote the growth of adventitious root primordia, and subsequent epidermal PCD above root primordial and adventitious root emergence are also regulated through ethylene (Atkinson et al., 2014; Steffens and Rasmussen, 2016). Transcriptome analysis on root samples of the ZmEREB180-over-expressing transgenic line OE240 and maize inbred line C01 showed that the gene categories of auxin and ethylene signalling and genes involved in PCD were markedly enriched in OE240 in comparison with C01 under waterlogged conditions (Figure 6, Table S5), and they are critical for the formation of adventitious roots. This suggests that ZmEREB180 regulates the formation of adventitious roots to enhance waterlogging tolerance.

Reactive oxygen species (ROS) are generated under flooding conditions, with the amount of ROS determined via either increased production of ROS or changes in antioxidant levels, which directly damage cells and cause lipid peroxidation (Bourain et al., 2013; Steffens, 2014). ROS signals have also been implicated in mediating ethylene-induced adventitious root growth through altering epidermal cell fate (Steffens et al., 2012). The genes that encode GST and POD were specifically enriched and up-regulated in the transgenic line OE240 (Figure S6, Table S5), and the activity of POD and the content of GSH were significantly higher in OE240 than C01 whether waterlogging stress occurred or not (Figure 4). Primary roots were relatively more intact in transgenic lines than in C01 (Figure 3), which agrees with the lower MDA and H₂O₂ concentration and higher inhibition capacity of hydroxyl free radical, GSH content and the activity of POD in transgenic lines after waterlogging stress (Figure 4). Moreover, overexpressing ZmEREB180 in Arabidopsis also demonstrated that transgenic lines had an enhanced recovery ability (Figure S4) and lower MDA and H₂O₂ content (Figure S5) after submergence treatment. These results indicate that ZmEREB180 coordinates ROS homeostasis to mitigate oxidative damage in plants. Based on these results, we proposed a model explaining how ZmEREB180 enhances survival ability in waterlogging stress, although further direct molecular evidence will be needed. In our model, increased ethylene levels, stimulated by the onset of waterlogging, activate ZmEREB180 expression. ZmEREB180, as an activator for up-regulating the mRNA abundance of genes encoding endogenous hormones, subsequently promotes root primordial initiation through auxin and ethylene signalling and epidermal cell death through PCD. ZmEREB180 also enhances the mRNA level of genes encoding antioxidants to regulate ROS status via changing antioxidant levels to sustain cell homeostasis, but whether ZmEREB180 in maize is directly involved in epidermal PCD is unclear. Our results thus provide clues for understanding the molecular mechanisms underlying waterlogging stress responses and open the door for genetic improvements in breeding programmes.

**Experimental procedures**

**Plant growth and waterlogging treatment**

To investigate the survival rate of inbred lines in an association panel, seeds were planted in a temperature-controlled greenhouse (~28 °C/22 °C day/night cycle) with 60% average humidity at Huazhong Agricultural University, Wuhan, China. A commercial growing mix (SunGro Horticulture, Agawam, MA) was used for the substrate, and 10 uniform seedslings of each line were planted in a plastic pot (20 cm in diameter and 30 cm deep) containing adequate substrate and sterile deionized water. Waterlogging protocols were similar to those described previously (Yu et al., 2018) and were applied at the second-leaf stage by maintaining a 2- to 3-cm water layer above the substrate. Three potted plant experiments were conducted using a randomized complete-block design with three replicates. Root samples for gene expression analyses were collected directly before waterlogging treatment (control), after 4 h (short-term) of stress and after 3 days (long-term) of stress.

**Evaluating waterlogging tolerance in the maize association panel**

A maize association panel consisting of 368 inbred lines (Li et al., 2013) was planted, and survival rates (SRs) were recorded, calculated as the ratio of the number of surviving plants to the total number of plants in each plot. The time point for recording the SR was determined by the performance of inbred line B73. When the SR of B73 decreased to 50%, the SR of the other inbred lines was assessed. The average value of three replicates per genotype was calculated to represent the SR phenotype, and the average SR value from three different environments was estimated using the BLUP via linear mixed models, in which genotype and environment were set as random effects.

**Association analysis of ZmERFVII genes**

Association analysis of ZmERFVII genes was performed using the SR phenotypes from three experiments and BLUP data. Among 525 105 high-quality SNPs with a minor allele frequency (MAF) of ≥5% in the association panel (Fu et al. 2013), 607 SNPs were located within the gene region of all 19 ZmERFVII. A cMLM was estimated in TASSEL v3.0 (Bradbury et al., 2007) to quantify significant associations as functions of population structure (Q matrix) and familial kinship (K matrix; Yu et al., 2005; Zhang et al., 2010). Two thresholds (P < 0.01; P < 0.001) were used for determination of significant trait–SNP associations.
ZmEREB180 gene sequence and association with waterlogging tolerance

For the purpose of ZmEREB180 resequencing, three pairs of primers (Table S2) were synthesized to amplify the promoter (1.1 kb), 5'- and 3'-UTR (untranslated region), and all introns and exons of the gene in 248 randomly selected inbred lines from the association panel, using the B73 genome sequence as a reference (B73 RefGen v4, Jiao et al., 2017). All amplified sequences were aligned using MEGA v5 (Tamura et al., 2011). Nucleotide polymorphisms including SNPs and InDels were identified, and variants with MAF ≥5% were used for association analysis. Variants significantly associated with SR according to BLUP data were calculated again using the cMLM.

ZmEREB180 gene expression analysis

For analysis of ZmEREB180 mRNA abundance in 100 maize inbred lines, root samples were separately collected from seedlings grown under normal (0 h), short-term stress (4 h) and long-term stress (3 days) treatments, and roots from five seedlings were pooled for RNA extraction. Total RNA from 300 samples was isolated using TRIzol reagent (Invitrogen, Gaithersburg, MD) and treated with RNase-free DNase (Invitrogen). The purified RNA was used to synthesize single-stranded cDNA using recombinant M-MLV reverse transcriptase (Invitrogen). Quantitative reverse-transcription PCR (qRT–PCR) was performed using gene-specific primers (Table S2) with 2x iTaq™ Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). ZmActin1 (GRMZM2G126010) was employed as the internal control to normalize the expression data. Relative expression levels were calculated according to the 2^−ΔΔCt (cycle threshold) method (Livak and Schmittgen 1991). PCR involved an initial denaturation step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 58 °C for 10 s and 72 °C for 20 s.

To analyse the expression regulation of ZmEREB180 under stress and hormone treatment, waterlogging, sodium chloride (NaCl, 200 mM), chilling (10 °C), high temperature (37 °C) and drought stress were imposed on the second-leaf stage B73 seedlings, and root samples were collected after 4 h of stress. For hormone treatment, the second-leaf stage B73 seedlings were cultured in water with the following treatments: ethylene (ET, 100 µM), abscisic acid (ABA, 100 µM), gibberellin (GA, 100 µM) and 1-naphthaleneacetic acid (NAA, a synthetic plant hormone in the auxin family, 100 mM), abscisic acid (ABA, 100 µM) and 1-naphthaleneacetic acid (NAA, a synthetic plant hormone in the auxin family, 100 µM) and cytokinin (KIN, 100 µM).

For analysis of subcellular localization of ZmEREB180, the full-length coding sequence of ZmEREB180 in B73 seedlings was amplified and inserted into the pM999-GFP vector to generate a GFP fusion construct (ZmEREB180-GFP). The ZmEREB180-GFP construct was placed under the cauliflower mosaic virus 35S promoter for constitutive expression. Maize mesophyll protoplasts were isolated from etiolated leaves, and the fusion construct was introduced into protoplasts with polyethylene glycol (PEG)/calcium-mediated transformation (Ren et al., 2017; Yoo et al., 2007). Nucleus-Tracker Red was used for Nuclear labelling.

For transient expression assays in maize protoplasts, two different types of promoter (with 5'-UTR) sequence of inbred lines B73 and 835B that have the same sequence of promoter were cloned into vector pGreen0800 II (Zhang et al., 2016) to compare the differential expression of 5'-UTR, which was inserted in front of the mini35S promoter to drive the expression of the LUC reporter gene. The expression of the LUC reporter gene under mini35S promoter (Vector, Zhang et al., 2016) was set as background activity. These constructs were transformed into maize protoplasts, and Firefly and Renilla luciferase activities were quantified with a dual-luciferase reporter assay according to the manufacturer’s instructions (Promega, Madison, USA).

Arabidopsis submergence tolerance assays

The ZmEREB180 coding sequence in B73 inbred lines was amplified and inserted into pCAMBIA1300 under the CaMV 35S promoter using Smal restriction sites. The recombinant plasmid was then transformed into Agrobacterium tumefaciens strain GV3101. Arabidopsis thaliana ecotype Col-0 was transformed by Agrobacterium-mediated transformation, and independent T2 transgenic lines were obtained using PCR and kanamycin-based selection. Expression of ZmEREB180 in transgenic plants was determined by qRT–PCR, with AtSg08290 used as an internal control for normalization (Czechowski et al., 2005; Hinz et al., 2010). Two independent overexpression lines, OE5 and OE8, were selected based on the level of transgene expression and subjected to further analyses. For the submergence tolerance assays, 3-week-old plants were submerged under 13/11 day/night at 22 °C. Rosettes were submerged 5 cm below the water surface. Fresh weights of seedlings growing above the substrate were measured, and samples were photographed after 10 days of treatment following recovery for 7 days. Whole seedlings growing under 0 (normal), 4 and 8 h of stress were collected for qRT–PCR, and at least five seedlings were combined for RNA extraction. The expression of four anaerobic metabolism-related genes (AtADH1, AtPDC1, AtSUS1 and AtSUS4) was quantified in overexpression plants and Col-0 (wild type, WT). Empirical data were obtained from three independent experiments. Gene-specific primers used for qRT–PCR analysis are listed in Table S2.

Generation and analysis of transgenic maize

The coding region of ZmEREB180 was amplified from B73 and inserted into the pCAMBIA1300 vector using the Vazyme recombination kit under the control of the Zmubi1 promoter. The constructed plasmid was then transformed into Ag. tumefaciens EHA105. The EHA105 strain with a recombinant plasmid was then used to deliver Zmubi1:ZmEREB180 into the C01 maize inbred line. The China National Seed Group carried out the genetic transformation. Positive transgenic plants were determined in each generation by PCR analysis. Expression of ZmEREB180 in transgenic plants was determined by qRT–PCR. Two independent T2 lines, OE115 and OE240, were selected for further analyses. Using a commercial growing mix as substrate (SunGro Horticulture), OE115, OE240 and C01 plants were planted in a light room under 13-h light/11-h dark, 25 °C day/22 °C night conditions. Waterlogging treatment was applied to plants at the second-leaf stage. Phenotypes, namely, seedling height, root length, leaf injury (LI) and soil and plant analyser development (SPAD) values, of first leaves were measured before treatment (0 h) and 2, 4 and 6 days after the initiation of stress. After ∼15 days, the SR of each line was recorded, as well as the longest root length, shoot fresh weight, number of adventitious roots (ARs), length of the longest AR and average AR length. Statistical analysis was based on data obtained from at least eight seedlings for each plant line with three independent experiments.
Physiological assay

For analysis of physiological characteristics, root samples from no fewer than six maize seedlings for OE115, OE240 and C01 were collected at 0 h, 1 and 3 days after waterlogging stress at the second-leaf stage, and no fewer than 10 seedlings for OE5, OE8 and WT of 3-week-old Arabidopsis plants were collected at 0 h, 1 and 2 days after submergence. Weighed samples were manually ground to a fine powder in liquid nitrogen and then homogenized in fourfold w/v 0.9% normal saline under ice-cold conditions to form a 20% tissue homogenate. The homogenates were centrifuged (2500 g, 10 min, 4 °C), and the supernatants were used for further analysis. Malondialdehyde (MDA), peroxidase (POD) and glutathione (GSH) measurements proceeded according to previous descriptions (Yu et al. 2015). Briefly, GSH concentrations were obtained using thiobis-(2-nitrobenzoic acid) for colour development and monitoring wavelengths at 420 nm, and the concentrations were expressed as micromoles per gram of protein; MDA measurements were based on the thiobarbituric acid (TBA) assay kits, respectively (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Three replicates were performed for each assay.

RNA-seq analysis of transgenic maize

For maize RNA-seq analysis, roots from five-two-leaf stage seedlings were collected from transgenic and C01 plants before and after 4 h of waterlogging stress. The samples from OE115 and OE240 were pooled for total RNA isolation with three biological replicates. The 150-bp paired-end Illumina sequencing was conducted at the National Key Laboratory of Crop Genetic Improvement (Huazhong Agricultural University, Wuhan, China) using HiSeq2500 (Illumina Inc., San Diego, CA). An average of six gigabases of raw data were generated for each sample. A bioinformatic analysis was carried out as previously described (Du et al., 2017). High-quality clean reads were aligned to the maize reference genome (B73 RefGen_v4, Jiao et al., 2017). GO enrichment analysis was performed using agrigo v2.0 (Tian et al., 2017). All RNA samples for transcriptome sequencing were also used to validate the mRNA abundance of the differential expression genes (Table S3).

Acknowledgements

This research was supported by the National Natural Science Foundation of China (Project 31801369 and 31571675) and supported by the Fundamental Research Funds for the Central Universities (Program No. 2662015PY102).

Conflict of interest

The authors declare that they have no conflict of interest.

References

Abiko, T., Kotula, L., Shiono, K., Malik, A.I., Colmer, T.D. and Nakazono, M. (2012) Enhanced formation of aerenchyma and induction of a barrier to radial oxygen loss in adventitious roots of Zea nicaraguensis contribute to its waterlogging tolerance as compared with maize (Zea mays ssp. mays). Plant Cell Environ. 35, 1618–1630.

Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373–399.

Argus, R.E., Colmer, T.D. and Grierson, P.F. (2015) Early physiological flood tolerance is followed by slow post-flooding root recovery in the dryland riparian tree Eucalyptus camaldulensis subsp. refugens. Plant Cell Environ. 38, 1189–1199.

Atkinson, J.A., Rasmussen, A., Traini, R., Voß, U., Sturrock, C., Mooney, S.J., Wells, D.M. et al. (2014) Branching out in roots: uncovering form, function, and regulation. Plant Physiol. 166, 538–550.

Ayano, M., Kani, T., Kojima, M., Sakakibara, H., Kitaoka, T., Kuroha, T., Angeles-Shim, R.B. et al. (2014) Gibberellin biosynthesis and signal transduction is essential for internode elongation in deepwater rice. Plant Cell Environ. 37, 2313–2324.

Bailey-Serres, J. and Colmer, T.D. (2014) Plant tolerance of flooding stress–recent advances. Plant Cell Environ. 37, 2211–2215.

Bailey-Serres, J. and Voosen, J.A. (2008) Flooding stress: acclimations and genetic diversity. Annu. Rev. Plant Biol. 59, 313–339.

B CAMERA, J., Fukao, T., Gibbs, D.J., Holdsworth, M.J., Lee, S.C., Licausi, F., Peñata, P. et al. (2012) Making sense of low oxygen sensing. Trends Plant Sci. 17, 129–138.

Bellini, C., Pacurar, D.I. and Perrone, I. (2014) Adventitious roots and lateral roots: similarities and differences. Annu. Rev. Plant Biol. 65, 639–666.

Benschop, J.J., Jackson, M.B., Gühl, K., Vreeburg, R.B., Croker, S.J., Peeters, A.J. and Voosen, L.A. (2005) Contrasting interactions between ethylene and abscisic acid in Rumex species differing in submergence tolerance. Plant J. 44, 756–768.

Bouranis, D.L., Chorianopoulos, S.N., Sylaniotis, V.F., Protonotarios, V.E., Bouranis, D.L., Chorianopoulos, S.N., Siyiannis, V.F., Protonotarios, V.E. and Sylaniotis, V.F. (2014) The Maize TF ome-developing a transcription factor open reading frame collection for functional genomics. Plant J. 80, 356–366.

Calvo-Polanco, M., Sejrørans, J. and Zwiazek, J.J. (2012) Role of adventitious roots in water relations of tamarack (Larix laricina) seedlings exposed to flooding. BMC Plant Biol. 12, 99.

Colmer, T. and Voosen, L. (2009) Flooding tolerance: suites of plant traits in variable environments. Funct. Plant Biol. 36, 665–681.

Czechowske, T., Stitt, M., Altmann, T., Udvardi, M.K. and Scheible, W.R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 139, 5–17.

Dixon, D.P., Skipsey, M. and Edwards, R. (2010) Roles for glutathione transferases in plant secondary metabolism. Phytochemistry, 71, 338–350.

Du, Y., Uu, L., Li, M., Fang, S., Shen, X., Chu, J. and Zhang, Z. (2017) UNBRAVENCH3 regulates branching by modulating cytokinin biosynthesis and signaling in maize and rice. New Phytol. 214, 721–733.

Fukao, T. and Bailey-Serres, J. (2004) Plant responses to hypoxia is survival a balancing act? Trends Plant Sci. 9, 449–456.

Fukao, T. and Bailey-Serres, J. (2008) Submergence tolerance conferred by SuB1A is mediated by SRL1 and SRL1 restriction of gibberellin responses in rice. Proc. Natl. Acad. Sci. USA, 105, 16814–16819.

Fukao, T., Xu, K., Ronald, P.C. and Bailey-Serres, J. (2006) A variable cluster of ethylene response factor-like genes regulates metabolic and developmental acclimation responses to submergence in rice. Plant Cell, 18, 2021–2034.

Fukao, T., Yeung, E. and Bailey-Serres, J. (2012) The submergence tolerance gene SUB1A delays leaf senescence under prolonged darkness through hormonal regulation in rice. Plant Physiol. 160, 1795–1807.

Fu, J., Cheng, Y., Linghu, J., Yang, X., Kang, L., Zhang, Z., Zhang, J. et al. (2013) RNA sequencing reveals the complex regulatory network in the maize kernel. Nat Commun 4, 2832.

Gibbs, D.J., Conde, J.V., Berckhan, S., Prasad, G., Mendiondo, G.M. and Holdsworth, M.J. (2015) Group VII ethylene response factors coordinate...
oxygen and nitric oxide signal transduction and stress responses in plants. Plant Physiol. 169, 23–31.

Hattoni, Y., Nagai, K., Furukawa, S., Song, X.J., Kawano, R., Sakakibara, H., Wu, J. et al. (2009) The ethylene response factors SNORKEL1 and SNORKEL2 allow rice to adapt to deep water. Nature, 460, 1026–1030.

Hess, N., Klode, M., Anders, M. and Sauter, M. (2011) The hypoxia responsive transcription factor genes ERF71/HRE2 and ERF73/HRE1 of Arabidopsis are differentially regulated by ethylene. Physiol. Plant. 143, 41–49.

Hirabayashi, Y., Mahendran, R., Koirala, S., Konoshima, L., Yamazaki, D., Jiao, Y., Peluso, P., Shi, J., Liang, T., Stitzer, M.C., Wang, B., Campbell, M.S., Magnani, E., Sjolander, K. and Hake, S. (2004) From endonucleases to transcription factor genes of the submergence tolerance regulator Sub1A mediates stress-responsive expression of AP2/ERF transcription factors. Plant Physiol. 152, 1674–1692.

Kim, H.J., Lynch, J.P. and Brown, K.M. (2008) Ethylene insensitivity impedes a subset of responses to phosphorus deficiency in tomato and petunia. Plant Cell Environ. 31, 1744–1755.

Kurokawa, Y., Nagai, K., Huan, P.D., Shimazaki, K., Qu, H., Mori, Y., Toda, Y., Hirabayashi, Y., Mahendran, R., Koirala, S., Konoshima, L., Yamazaki, D., Watanabe, S., Kim, H. et al. (2013) Global flood risk under climate change. Nat. Clim. Change, 3, 816–821.

Lorbiecke, R. and Sauter, M. (1999) Adventitious root growth and cell-cycle pathway for protein destabilization. Plant Cell, 11, 225–327.

Mao, H., Wang, H., Liu, S., Li, Z., Yang, X., Yan, J., Li, J. et al. (2015) A transposable element in a NAC gene is associated with drought tolerance in maize seedlings. Nat. Commun. 6, 8326.

Mittler, R., Vanderauwera, S., Gollery, M. and Van Breusegem, F. (2004) Reactive oxygen gene network of plants. Trends Plant Sci. 9, 490–498.

Morgan, P.W. and Drew, M.C. (1997) Ethylene and plant responses to stress. Physiol. Plant. 100, 620–630.

Mustroph, A., Lee, S.C., Ososumi, T., Zanetti, M.E., Yang, H., Ma, K., Yaghoubi-Mashi, A. et al. (2010) Cross-kingdom comparison of transcriptomic adjustments to low-oxygen stress highlights conserved and plant-specific responses. Plant Physiol. 152, 1484–1500.

Nanjo, Y., Koiwai, K., Yazeu, H., Yamauchi-Shinozaki, K., Shinozaki, K. and Komatsu, S. (2010) Transcriptional responses to flooding stress in roots including hypocotyl of soybean seedlings. Plant Mol. Biol. 77, 129–144.

Natsui, R., Howell, K.A., Carroll, A., Ivanova, A., Millar, A.H. and Whelan, J. (2009) Defining core metabolic and transcriptional responses to oxygen availability in rice embryos and young seedlings. Plant Physiol. 151, 306–322.

Osman, K.A., Fang, B., Wang, Y., Chen, J., Yu, F., Li, L., Han, X. et al. (2013) Dynamic QTL analysis and candidate gene mapping for waterlogging tolerance at maize seedling stage. Plos ONE, 8, e79305.

Pacurar, D.I., Perrone, I. and Bellini, C. (2014) Auxin is a central player in the hormone cross-talks that control adventitious rooting. Plant Physiol. 151, 83–96.

Qiu, F., Zheng, Y., Zhang, Z. and Xu, S. (2007) Mapping of QTL associated with waterlogging tolerance during the seedling stage in maize. Ann. Bot. 99, 1067–1081.

Raskin, I. and Kende, H. (1984) Role of gibberellin in the growth response of submerged deep water rice. Plant Physiol. 76, 947–950.

Shabala, S. (2011) Physiological and cellular aspects of phytotoxicity tolerance in plants: the role of membrane transporters and implications for crop breeding for waterlogging tolerance. New Phytol. 190, 457–471.

Shiozaki, K., Mori, Y., Nakamura, T., Suzuki, K., Fujimura, T. and Shinozaki, H. (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol. 140, 411–432.

Septiningsih, E.M., Morgan, P.W., Sticher, L., Jetter, R., Geigenberger, P. and Perata, P. (2010) Molecular characterization of the submergence tolerance in breakdown of the Arabidopsis thaliana ecotype Columbia. New Phytol. 190, 457–471.

Liu, S., Wang, X., Wang, H., Xin, H., Yang, X., Yan, J., Li, J. et al. (2013) Genome-wide analysis of ZmDRED genes and their association with natural variation in drought tolerance at seedling stage of Zea mays L. PLoS Genet. 9, e1003790.

van Loon, L.C., Geraerts, B.P. and Linthorst, H.J. (2006) Ethylene as a modulator of disease resistance in plants. Trends Plant Sci. 11, 184–191.

Lorbecke, R. and Sauter, M. (1999) Adventitious root growth and cell-cycle induction in deepwater rice. Plant Physiol. 119, 21–30.

Magnani, E., Sjolander, K. and Hake, S. (2004) From endonucleases to transcription factors: evolution of the AP2 DNA binding domain in plants. Plant Cell, 16, 2265–2277.

Mano, Y. and Omori, F. (2013) Flooding tolerance in interspecific introgression lines containing chromosome segments from teosinte (Zea miliarum) in maize (Zea mays subsp. mays). Ann. Bot. 112, 1125–1139.

Mano, Y., Muraki, M., Fujimori, M., Takamizo, T. and Kindiger, B. (2005) Identification of QTL controlling adventitious root formation during flooding conditions in teosinte (Zea mays ssp. huehuetenangensis) seedlings. Euphytica, 142, 33–42.

ZmEREB180 regulates maize waterlogging response 2297
Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., Xu, W. et al. (2017) agrigo v2.0: a GO analysis toolkit for the agricultural community, 2017 update. *Nucleic Acids Res.* 45, W122–W129.

van Veen, H., Akman, M., Jamar, D.C., Vreugdenhil, D., Kooker, M., van Tienderen, P., Voesenek, L.A. et al. (2014) Group VII ethylene response factor diversification and regulation in four species from flood-prone environments. *Plant Cell Environ.* 37, 2421–2432.

Vidoz, M.L., Loret, E., Munsal, A., Alpi, A. and Perata, P. (2010) Hormonal interplay during adventitious root formation in flooded tomato plants. *Plant J.* 63, 551–562.

Visser, E., Cohen, J.D., Barendse, G., Blom, C. and Voesenek, L. (1996) An ethylene-mediated increase in sensitivity to auxin induces adventitious root formation in flooded Rumex palustris Sm. *Plant Physiol.* 112, 1687–1692.

Visser, E., Voesenek, L., Vartapetian, B. and Jackson, M. (2003) Flooding and plant growth. *Ann. Bot.* 91, 107–109.

Voesenek, L.A. and Bailey-Serres, J. (2013) Flooding tolerance: QTL, sensing and survival strategies. *Curr. Opin. Plant Biol.* 16, 647–653.

Voesenek, L.A. and Bailey-Serres, J. (2015) Flood adaptive traits and processes: an overview. *New Phytol.* 206, 57–73.

Wang, X., Wang, H., Liu, S., Ferjani, A., Li, J., Yan, J., Yang, X. et al. (2016) Genetic variation in ZmVPF1 contributes to drought tolerance in maize seedlings. *Nat. Genet.* 48, 1233–1241.

Watanabe, K., Takahashi, H., Sato, S., Nishuchi, S., Oomori, F., Malik, A.I., Colmer, T.D. et al. (2017) A major locus involved in the formation of the radial oxygen loss barrier in adventitious roots of teosinte Zea nicaraguensis is located on the short-arm of chromosome 3. *Plant Cell Environ.* 40, 304–316.

Xiang, Y., Sun, X., Gao, S., Qin, F. and Dai, M. (2017) Deletion of an endoplasmic reticulum stress response element in a ZmPP2C-A gene facilitates drought tolerance of maize seedlings. *Mol. Plant.* 10, 456–469.

Xu, K., Xu, X., Fukao, T., Canlas, P., Maghirang-Rodriguez, R., Heuer, S., Ismail, A.M. et al. (2006) Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature* 442, 705–708.

Yoo, S.D., Cho, Y.H. and Sheen, J. (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.* 2, 1565–1572.

Yu, J., Pressor, G., Briggs, W.H., Vroh Bi, I., Yamasaki, M., Doebly, J.F., McMullen, M.D. et al. (2005) A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. *Nat. Genet.* 38, 203–208.

Yu, F., Han, X., Geng, C., Zhao, Y., Zhang, Z. and Qiu, F. (2015) Comparative proteomic analysis revealing the complex network associated with waterlogging stress in maize (Zea mays L.) seedling root cells. *Proteomics* 15, 135–147.

Yu, F., Liang, K., Zhang, Z., Du, D., Zhang, X., Zhao, H., Li Haq, B. et al. (2018) Dissecting the genetic architecture of waterlogging stress-related traits uncovers a key waterlogging tolerance gene in maize. *Theor. Appl. Genet.* 131, 2299–2310.

Zaidi, P.H., Maniselvan, P., Srivastava, A., Yadav, P. and Singh, R.P. (2010) Genetic analysis of waterlogging tolerance in tropical maize (Zea mays L.). *Maydica* 55, 17–26.

Zaidi, P.H., Rashid, Z., Vinayan, M.T., Almeida, G.D., Phagana, R.K. and Babu, R. (2015) QTL mapping of agronomic waterlogging tolerance using recombinant inbred lines derived from tropical maize (Zea mays L.) germplasm. *PLoS ONE* 10, e0124350.

Zhai, L., Liu, Z., Zou, X., Jiang, Y., Qiu, F., Zheng, Y. and Zhang, Z. (2013) Genome-wide identification and analysis of microRNA responding to long-term waterlogging in crown roots of maize seedlings. *Physiol. Plant.* 147, 181–193.

Zhang, Z., Ersoz, E., Lai, C.Q., Todhunter, R.J., Tiwari, H.K., Gore, M.A., Bradbury, P.J. et al. (2010) Mixed linear model approach adapted for genome-wide association studies. *Nat. Genet.* 42, 355–360.

Zhang, Z., Zheng, X., Yang, J., Messing, J. and Wu, Y. (2016) Maize endosperm-specific transcription factors OsZ and PBF network the regulation of protein and starch synthesis. *Proc. Natl. Acad. Sci. USA* 113, 10842–10847.

Zou, X., Jiang, Y., Liu, L., Zhang, Z. and Zheng, Y. (2010) Identification of transcriptome induced in roots of maize seedlings at the late stage of waterlogging. *BMC Plant Biol.* 10, 189.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Position of 19 ZmERF-VIIs genes on the maize chromosome.

**Figure S2** Characterization of gene structure and putative conserved motif of ZmERF-VIIs.

**Figure S3** Phenotypic distribution of survival rate (SR) in the association panel.

**Figure S4** Phenotype of ectopic expression of ZmEREB180 in Arabidopsis under waterlogging stress.

**Figure S5** Physiological characteristics of overexpression ZmEREB180 Arabidopsis lines.

**Figure S6** Dynamic phenotypes of overexpressing ZmEREB180 maize lines under waterlogging stress.

**Figure S7** Characteristics of adventitious roots of overexpressing ZmEREB180 maize lines under waterlogging stress.

**Figure S8** Transcriptomic analysis of overexpressing ZmEREB180 maize lines under normal conditions.

**Table S1** Gene identification information for 19 group VII EREB genes in versions 3 and 4 of the B73 reference genome.

**Table S2** Primers used for resequencing and expression analysis of ZmEREB180.

**Table S3** qRT-PCR primers used for up-regulation gene validation under waterlogging stress in RNA-seq analysis.

**Table S4** Up-regulated genes in OE240 seedling roots compared to C01 under normal conditions.

**Table S5** Down-regulated genes in OE240 seedling roots compared to C01 under normal conditions.

**Table S6** Up-regulated genes in OE240 seedling roots compared to C01 under waterlogging stress.

**Table S7** Down-regulated genes in OE240 seedling roots compared to C01 under waterlogging stress.