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Overview of RING gene family in maize (*Zea mays* L.): *ZmRING-93* enhances drought tolerance in transgenic *Arabidopsis*

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Abstract

Background RING-type E3 ligases are critical regulators of diverse plant processes, yet their roles in maize remain poorly defined, particularly in drought responses. To address this knowledge gap, we conducted an integrative analysis of maize RING genes, combining evolutionary profiling, and candidate-gene association studies to identify drought-associated candidates. Key genes were further characterized via quantitative expression profiling, subcellular localization, in vitro ubiquitination assay, and functional validation in transgenic *Arabidopsis*. This study aimed to provide new insights into the maize RING gene family's role in drought stress adaptation.

Results We employed three distinct methods and identified a total of 590 proteins. Phylogenetic analysis revealed that these proteins could be grouped into 11 separate clusters. Our findings suggested that the expansion of the RING family in maize was likely due to gene duplication events. Notably, genetic variations *in ZmRING-93* were significantly associated with drought tolerance, and its expression was up-regulated under various abiotic stress conditions and hormone treatments. We further discovered that ZmRING-93 was a functional ubiquitin E3 ligase that localized to the nucleus, cytoplasm, and parts of the endoplasmic reticulum. Transgenic *Arabidopsis* plants overexpressing *ZmRING-93* exhibited enhanced drought tolerance, with a lower water loss rate, further supporting the importance of *ZmRING-93* in drought tolerance.

Conclusions These findings revealed that *ZmRING-93* contributed to drought tolerance in maize and provided a basis for further investigation of the role of RING domain-containing proteins.

Keywords Maize, E3 ubiquitin ligase, RINGs family gene, Drought tolerance, *ZmRING-93*

Introduction

Ubiquitins (Ubs) are a group of stable and highly conserved proteins that can be divided into three different types: E1 ubiquitin-activating enzymes (E1s), E2 ubiqui-tin-conjugation enzymes (E2s), and E3 ubiquitin-ligase enzymes (E3s). These proteins together form the ubiquitin–proteasome system (UPS), which is involved in important post-translational modification processes during ubiquitination [1, 2]. Ubiquitination plays vital roles in plant growth and development, as well as in plant responses to biotic and abiotic stress [2–9]. The process of ubiquitination begins with the activation of Ub by E1, followed by the transfer of Ub

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to E2, resulting in the formation of an E2-Ub intermediate linked by a thioester. E2-Ub then interacts with the E3-Ub ligase, leading to the transfer of Ub to the target protein. This process is repeated multiple times, allowing the 26S proteasome system to recognize and degrade the substrate proteins [10]. The Ub molecule is then recycled for further rounds of ubiquitination [11].

Compared with E1 and E2, E3 enzymes play an important role in ubiquitination, determining its overall specificity [6]. Based on their structural similarities and catalytic domains, E3 ubiquitin ligases can be divided into three types: HECT (Homologous to the E6AP Carboxyl Terminus), RING (Really Interesting New Gene), and U-box [2, 7, 8, 12]. RING-type ubiquitin ligases possess a RING domain composed of 40–60 amino acids [12], making them the largest class of E3s, and a RING-type ligase is characterized by eight conserved metal ligand (ML) residues that coordinate with two zinc ions, creating a unique cross-support structure [8]. Within this structure, ML1-ML2 and ML5-ML6 bind to one zinc ion, while ML3-ML4 and ML7-ML8 bind to the other [13]. Depending on which residue is situated with the ML, RING proteins can be further divided into two main types (RING-H2 and RING-HC) and five modification types (RING-v, RING-C2, RING-D, RING-S/T, and RING-G) [8].

Maize is an important cereal crop, but it is also sensitive to environmental stresses [14, 15]. There have been numerous studies on stress tolerance genes in maize [16–18], among which the RING gene family is a research hotspot. Recent studies have shown that RING-type ubiquitin ligases play essential roles in various biological processes in plants, such as tolerance to abiotic and biotic stresses, hormone signaling, and growth and development. For example, *KEG* regulates plant growth and development [19]; *SDIR1* affects seed germination and the salt stress response [20]; *AtAIRP* is a positive regulator of ABA-dependent drought tolerance [21–24]; *ATLs* participate in the abiotic stress response [25]; *SpRING* is related to salt stress, acting as an active regulator of salt tolerance [26]; *CHYR1* regulates stomatal movement and the drought response capacity in *Arabidopsis* [27]; The family of proteins termed BnaSINAs in Brassica napus possessed a conserved C-terminal SINA domain and were uniformly responsive to salt and osmotic stress, with *BnaSINA17* capable of restoring resistance to these stresses when expressed in the *Arabidopsis sina2* mutant [28]; whereas in potato, *StRFP2* affects plant architecture, as well as regulating the content and activity of ROS in the cells, and improves drought tolerance by regulating plant height, stem diameter, root length, fresh weight, and root/shoot ratio [29]. To date, the RING gene family has been identified in *Arabidopsis*, rice, cabbage, tomato, soybean, apple, and wheat [8, 30–37].

Although a number of RING family genes have been identified and characterized in *Arabidopsis* and several other species, the RING-type protein family has yet to be comprehensively and systematically studied in maize. In addition, it remains unclear which one of the *ZmRINGs* could contribute to drought resistance. To address these questions, we comprehensively analyzed the RING gene family in the maize genome and examined its molecular function under drought tolerance. To accomplish this, phylogenetic relationships, duplication events, and association analysis, expression patterns, in vitro self-ubiquitination assay, subcellular location, and candidate gene overexpression were performed. Collectively, these results provide a foundation for further analyses of the biological functions of RING domain-containing proteins in maize.

Results

The maize genome contains 590 RING family members

In the current study, 590 RING family members were retrieved from the maize genome using three different methods. These proteins were named based on their genomic physical locations. The proteins were classified into six categories based on amino acid residues between MLs and their positional distance: RING-H2 ($n=331$), RING-HC ($n=181$), RING-v ($n=27$), RING-C2 ($n=46$), RING-G ($n=1$), and RING-S/T ($n=4$). No RING-D type proteins were detected. RING-H2 was the most abundant, accounting for 56.1% of the total, followed by RING-HC at 30.68%. Meanwhile, RING-H2 and RING-C2, which have two cysteine residues instead of histidine at ML4 and ML5, made up only 7.8% (46). Only one RING-G protein, with a glycine residue at ML5, was identified (Table 1).

Spacing and amino acid number between metal ligands of RING domain-containing proteins in maize

RING domain-containing proteins are defined by eight ML. Of these, the ML pair ML1-ML2 chelates one zinc ion with ML5-ML6, while ML3-ML4 chelates the second with ML7-ML8 [38]. In this study, the number of amino acid residues between ML1-ML2, ML3-ML4, ML4-ML5, ML5-ML6, and ML7-ML8 was relatively conserved (Table 2), compared to varying numbers between ML2-ML3 and ML6-ML7 (Fig. 1).

Two amino acids were observed between the majority of ML1-ML2, ML4-ML5, ML5-ML6, and ML7-ML8 pairs, but only one was located between ML3-ML4 (Table 2). Meanwhile, the number between ML2-ML3 ranged from 10 to 20, while that between ML6-ML7 ranged from 5

Table 1 Types of RING domain-containing proteins in maize

RING domain		Consensus Sequence													
Type	**No**	**ML1**		**ML2**		**ML3**		**ML4**		**ML5**		**ML6**		**ML7**		**ML8**
RING-H2	331	C	$X_{2\text{-}2}$	C	$X_{11\text{-}38}$	C	$X_{1\text{-}3}$	H	X_2	H	X_2	C	$X_{7\text{-}64}$	C	X_2	C
RING-HC	181	C	$X_{2\text{-}2}$	C	$X_{9\text{-}39}$	C	$X_{1\text{-}3}$	H	$X_{2\text{-}3}$	C	$X_{2\text{-}4}$	C	$X_{4\text{-}37}$	C	$X_{2\text{-}4}$	C
RING-v	27	C	$X_{2\text{-}2}$	C	$X_{11\text{-}63}$	C	X_1	C	X_7	H	X_2	C	$X_{12\text{-}15}$	C	X_2	C
RING-C2	46	C	$X_{2\text{-}2}$	C	$X_{10\text{-}16}$	C	$X_{1\text{-}2}$	C	X_4	C	X_2	C	$X_{10\text{-}16}$	C	X_2	C
RING-S/T	4	C	$X_{2\text{-}2}$	C	X_{14}	C	X_1	H	X_3	C	X_2	S	$X_{17\text{-}19}$	C	X_2	C
RING-G	1	C	$X_{2\text{-}2}$	C	X_{16}	C	X_1	H	X_2	G	X_2	C	X_{14}	C	X_2	C

ML Metal–ligand, *X(n)* Number of amino acids between metal ligands, *C* Cysteine, *H* Histidine, *G* Glycine, *S* Serine

Table 2 Spacing variation of Metal Ligand (ML) pairs in RING domain-containing proteins in maize

ML pairs	ML1-Xn-ML2	ML3-Xn-ML4			ML4-Xn-ML5				ML5-Xn-ML6			ML7-Xn-ML8		
No. of amino acid(n)	**2**	**1**	**2**	**3**	**2**	**3**	**4**	**7**	**2**	**3**	**4**	**2**	**3**	**4**
RING-H2	331	329	0	2	331	0	0	0	331	0	0	331	0	0
RING-HC	181	170	1	10	139	42	0	0	173	6	2	166	3	12
RING-v	27	27	0	0	0	0	0	27	27	0	0	27	0	0
RING-C2	46	4	42	0	0	0	46	0	46	0	0	46	0	0
RING-S/T	4	4	0	0	0	4	0	0	4	0	0	4	0	0
RING-G	1	1	0	0	1	0	0	0	1	0	0	1	0	0
Total No	590	535	43	12	471	46	46	27	582	6	2	575	3	12

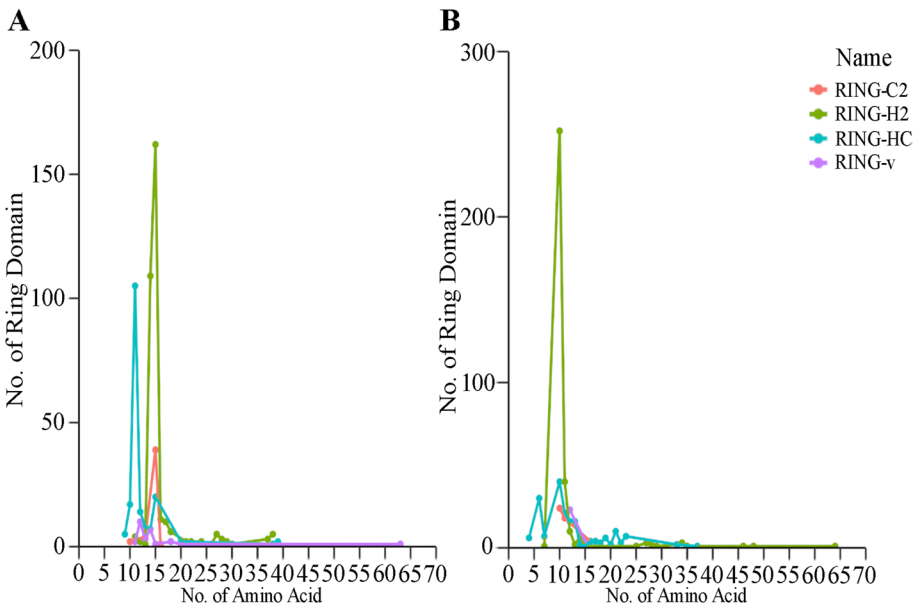


Fig. 1 Comparison of amino acid number between metal ligand ML2-ML3 and metal ligand ML6-ML7 in maize. **A** metal ligands ML2-ML3; (**B**) metal ligands ML6-ML7

to 15. The number of amino acids between ML2-ML3 in RING-H2 and RING-HC was 15 (162/590) and 11 (105/590), respectively, while the majority of RING-H2 and RING-HC possessed 10 amino acids between ML6-ML8.

Conservative sequences between the metal ligands

Sequence logos of the representative domain types were constructed to determine the number of conserved amino acid sequences between ML in each

type of RING domain (Fig. 2). All RING domains, except for RING-S/T, were preceded by the amino acids Ile or Val at ML2. In RING-H2, more than 80% of the amino acids preceding ML5 were Phe or Tyr, while in RING-HC, Phe frequently appeared before ML5, and in RING-v, Ala, and Val frequently preceded ML5. Meanwhile, Pro consistently followed ML7 in RING-H2, RING-HC, RING-C2, RING-S/T, and RING-G, whereas in RING-v, the motif C-X3-[W]-X3-[KG]-X6-C was observed between ML6 and ML7. In the majority of RING-v, an Arg residue followed ML1, and a Lys residue followed ML4.

Duplicated RINGs in maize, rice, and sorghum genome

To determine the evolutionary relationships among RING genes in maize, rice, and sorghum, whole-genome gene duplication and collinearity data were downloaded from the PGDD database [39]. A search for duplication information revealed 340 *ZmRINGs* sharing one or two duplicated copies in the rice and sorghum genomes (Fig. 3). These duplication events were divided into four types: Os-Zm-Sb ($n=206$), Os-Zm-Sb×2 ($n=81$), 2×Os-Zm-2×Sb ($n=30$), and 2×Os-Zm-Sb ($n=28$) (Fig. 3E). Although 590 RING genes were identified in the maize genome, only 340 gene duplication events were found. At the same time, the Ka/Ks of duplicate gene pairs were calculated, of which the Ka/Ks values were less than 1 (Table S2), suggesting a purifying selection.

ZmRING-93 was an important candidate gene for drought tolerance

To determine whether natural variations in any of the *RINGs* are associated with drought tolerance in maize, candidate-gene-based association analysis was conducted using previously reported methods and data [40]. Using the general linear method (GLM) and a *P*-value cutoff of 0.01, 44 of the 590 *ZmRINGs* were found to possess at least one significant marker-trait association (Fig. 4). These 44 *ZmRINGs* were therefore selected for further analysis (Fig. 4B). Using the mixed linear model (MLM) method with P-values of 0.01 and 0.0001 [41], *ZmRING*-93 was found to be consistently significantly associated with drought tolerance under both thresholds (Fig. 4C, D). These findings suggested

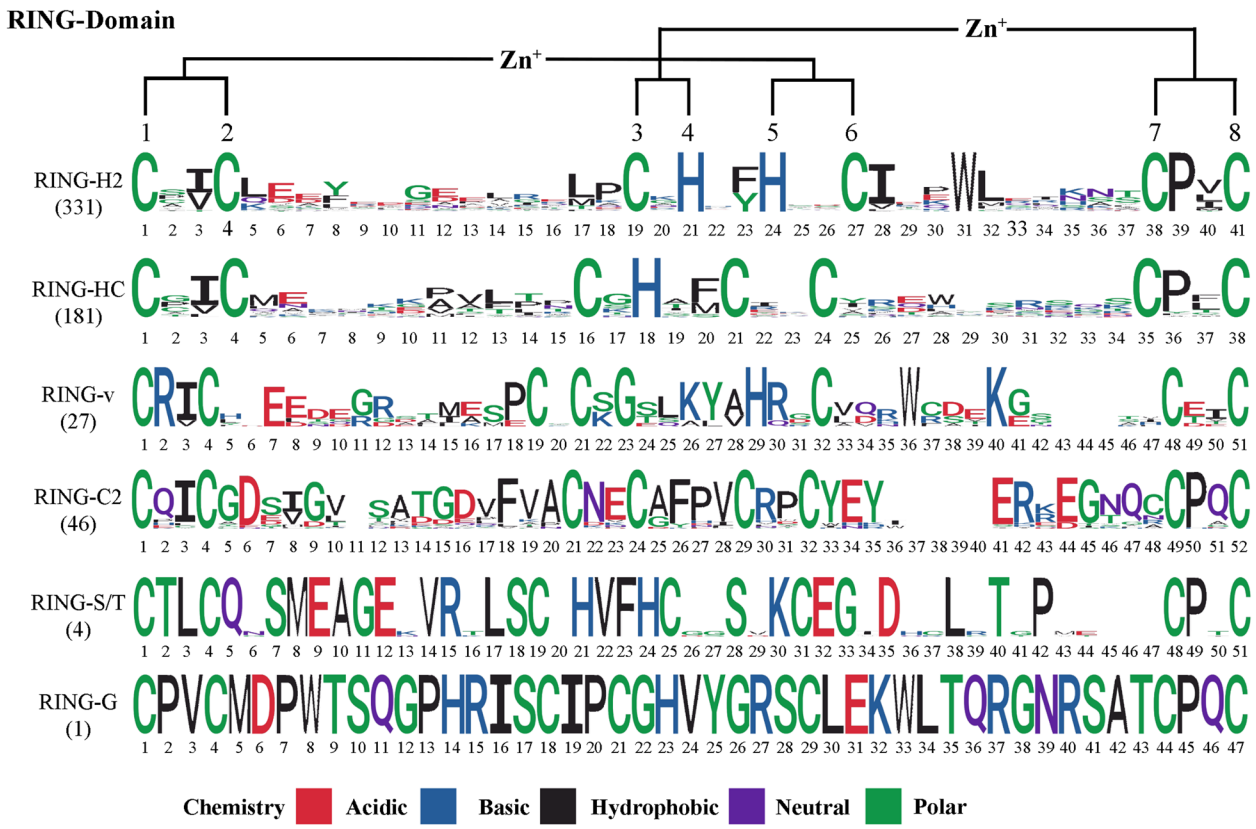
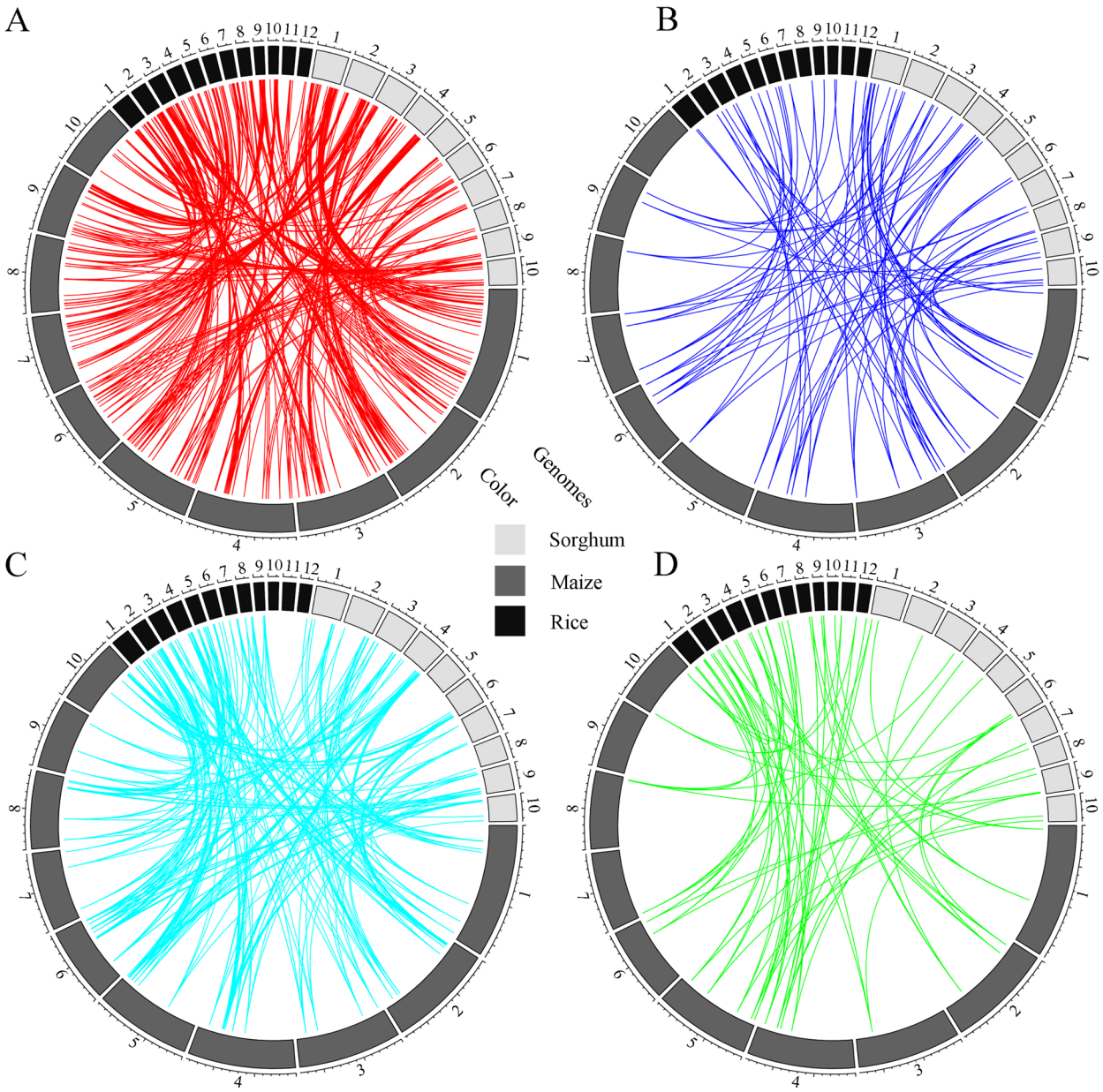


Fig. 2 Positional distribution of amino acid residues among the metal ligands in maize RING domain. Full-length sequences of maize RING family proteins were analyzed for multiple sequence comparisons using the Toparslan, E method [38]. The different RING type proteins were classified according to the different proteins and the amino acid variations between the different proteins were compared



E

Color	Type	RING number Os	RING number Zm	RING number Sb	Total number
	Os-Zm-Sb	1	1	1	206
	2×Os-Zm-Sb	2	1	1	81
	Os-Zm-2×Sb	1	1	2	30
	2×Os-Zm-2×Sb	2	1	2	28

Fig. 3 Synteny analysis of the maize RING gene family members in rice and sorghum. **A**, **B**, **C**, **D** Chromosome distribution and gene replication of RINGs family members in maize, rice, and sorghum; (**E**) Type and number of RINGs gene replication in maize, rice, and sorghum genomes

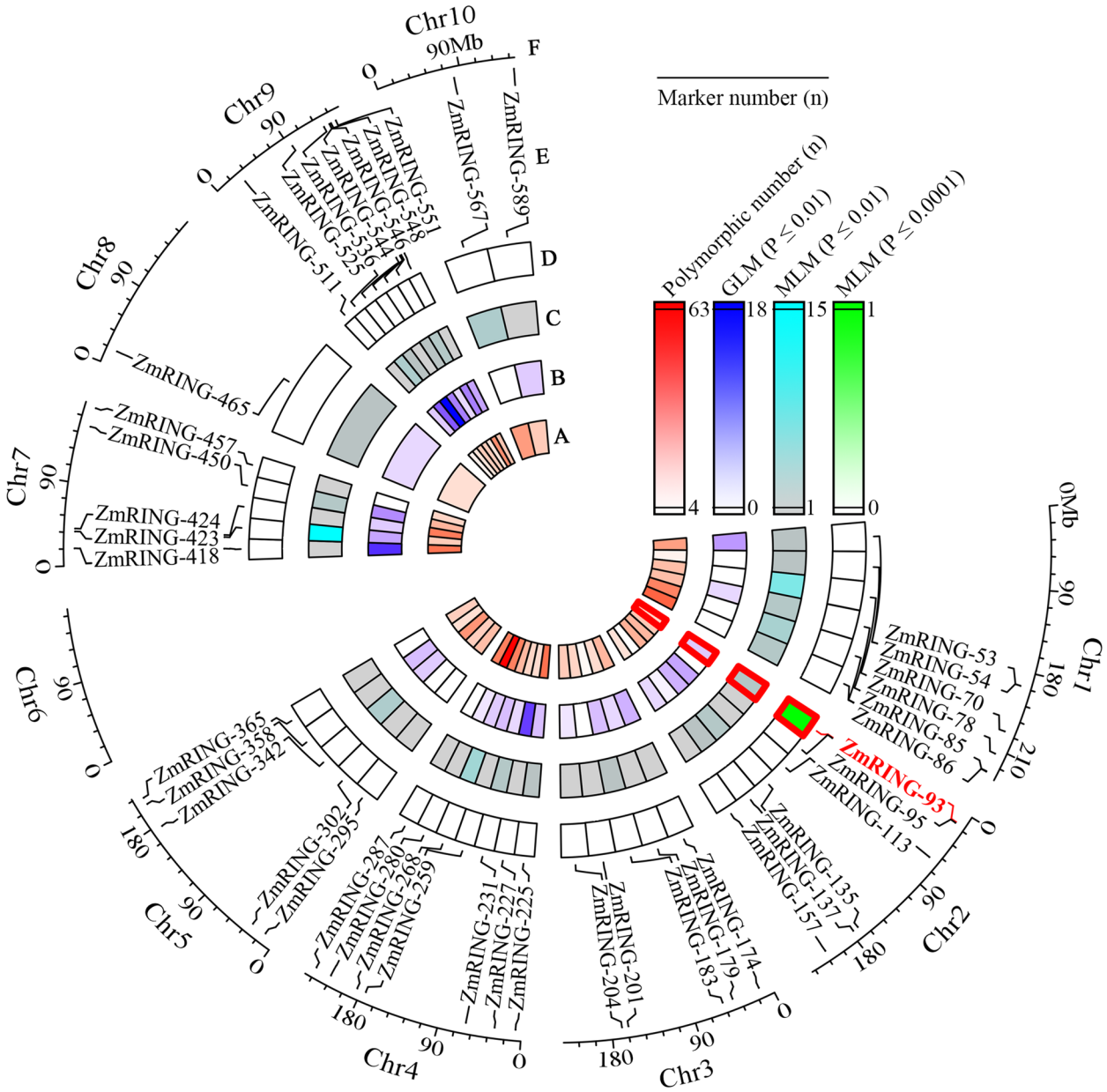


Fig. 4 Association analysis of maize RING genes with drought tolerance. **A** The number of polymorphism markers in *ZmRINGs*; (**B**) Significant associated *ZmRINGs* and the number of significantly associated markers under GLM and $P \leq 0.01$; (**C**) MLM and $P \leq 0.01$; (**D**) MLM and $P \leq 0.0001$; (**E**) *ZmRINGs* gene name; (**F**) Chromosome size

that *ZmRING*-93 is an promisin candidate gene for drought tolerance.

Relative expression level of ZmRING-93 were up-regulated under abiotic stress and hormone treatments

Candidate-gene based association analysis revealed that *ZmRING-93* was a promising gene under drought stress. Thus, the expression of *ZmRING-93* under abiotic stress and exogenous hormone treatment was subsequently examined (Fig. 5). Accordingly, the expression levels were significantly up-regulated under each stress treatment, consistent across different treatments and time points. Under drought, high temperature, and H_2O_2 treatments, the up-regulation of *ZmRING-93* was greater in the shoots than in the roots. In contrast, under hydrogen peroxide and ABA treatment, up-regulation was more significant in the roots than in the shoot (Fig. 5E, I). These results suggest that the intragenic expression of *ZmRING-93* was

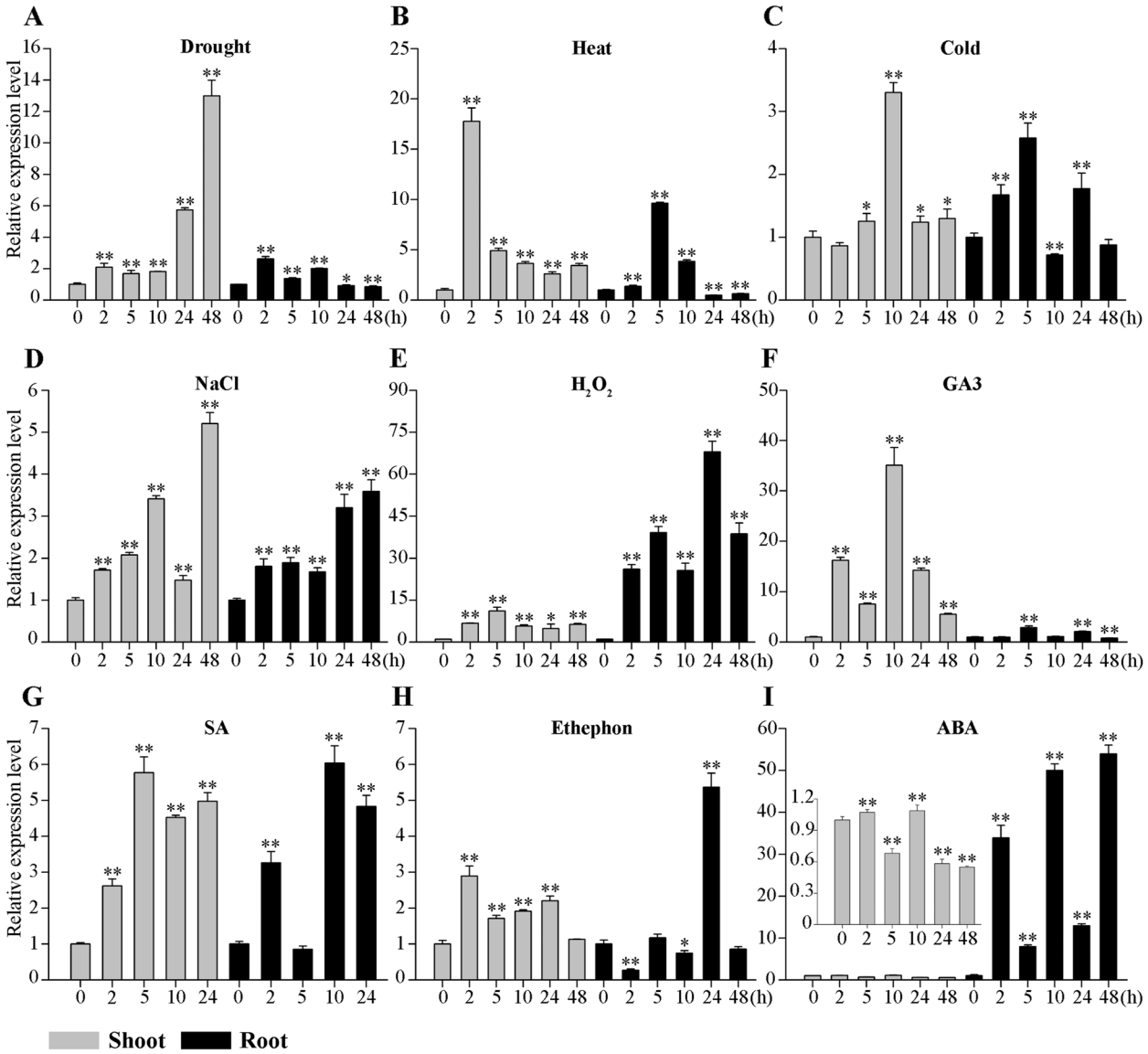


Fig. 5 Analysis of the expression patterns of *ZmRING-93* in maize under drought. **A**, high temperature (**B**), low temperature (**C**), NaCl (**D**), H_2O_2 (**E**), GA3 (**F**), SA (**G**), Ethephon (**H**), and ABA (**I**). Bar shows values representing mean ± SD. A two-sided *t*-test determined statistical significance: $^{*}P < 0.05$, $^{**}P < 0.01$

induced by multiple stresses, highlighting its potential role in the molecular response to stress in maize.

ZmRING-93 exhibited E3 ubiquitin ligase activity

ZmRING-93 contained a domain of RING (Fig. S1). To test whether ZmRING-93 had an E3 ubiquitin ligase activity or no, an in vitro ubiquitination assay was conducted. All or any three of E1, E2, HA-Ubi, and GST-ZmRING-93 were used to perform the ubiquitination assay, and the reaction products were subsequently examined by western blotting with anti-GST or anti-HA antibody. The results showed that the high molecular mass bands appeared in the presence of HA-Ubi, E1, E2, and GST-ZmRING-93. However, no polyubiquitinated bands were detected when any one of the E1, E2, HA-Ubi, and GST-ZmRING-93 was excluded (Fig. 6). Collectively, these data suggested that ZmRING-93 functioned as an active E3 ubiquitin ligase.

Subcellular localization analysis revealed that ZmRING-93 was distributed across the nucleus, cytoplasm, and a subset of the endoplasmic reticulum

The vacant GFP protein was ubiquitously distributed in the protoplasts, whereas in host protoplasts transfected

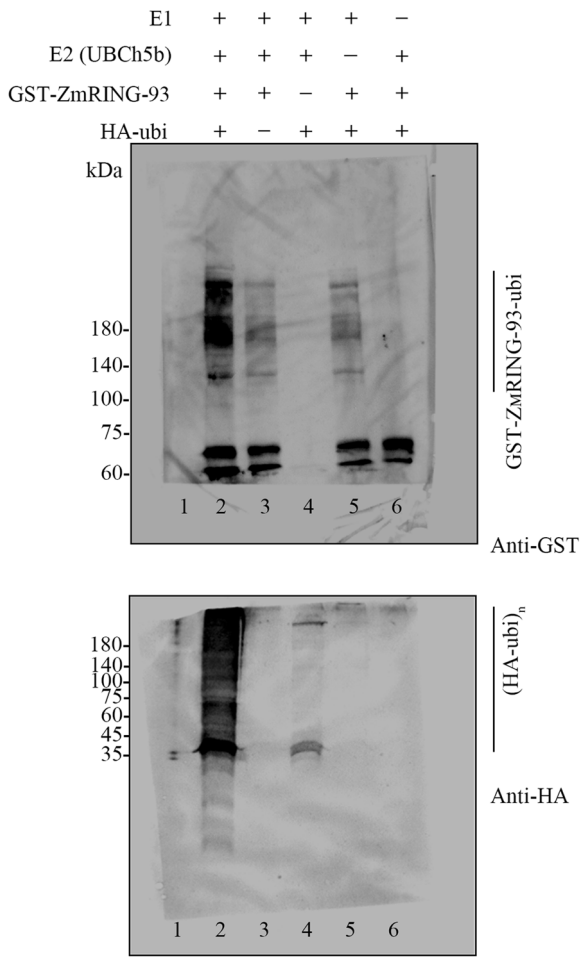


Fig. 6 In vitro self-ubiquitination assay of ZmRING-93. "+" and "-" represent the presence or absence of this protein in the reaction system. The protein marker was placed on the left

with ZmRING-93-GFP, GFP fluorescence signals were observed only in the nucleus and cytoplasm (Fig. 7A).

The fluorescence of ZmRING-93 in the cytoplasm showed a reticular pattern similar to that of the endoplasmic reticulum. To further localize the subcellular location of ZmRING-93-GFP, *pGreenII-ZmRING-93-GFP* was co-transfected with *35S:HDEL-mCherry* in maize protoplasts. The green fluorescence in the cytoplasm of ZmRING-93-GFP was found to co-localize with the red fluorescence of mCherry partially. These findings suggest that ZmRING-93-GFP was present in the nucleus, cytoplasm and part of the endoplasmic reticulum (Fig. 7B).

Enhancement of drought tolerance in *Arabidopsis* by overexpressing of *ZmRING-93*

To verify the function of *ZmRING-93*, *ZmRING-93* was transformed into *Arabidopsis*. The expression levels of *ZmRING-93* in the transgenic lines were validated using semi-quantitative PCR. As a result, a higher expression level were found in p35S:Ω-OE8 (OE8) and p35S:Ω-OE30 (OE30) than that in VC (Fig. 8B, and Supplemental Fig. 4). Therefore, OE8 and OE30 were selected for further analysis.

The seedlings of the VC, OE8, and OE30 grew 22 days before being subjected to drought treatment. Each line showed an identical growth stage before drought treatment (Fig. 8A). By withholding water for 14 days and rewatering for 3 days, the survival rate was 4.17%, 86.11%, and 90.28% for VC, OE8, and OE30, respectively (Fig. 8C). Coincidently, the water loss rate was lower in OE8 and OE30 than in VC (Fig. 8D). These results suggest that, under drought stress, drought tolerance increased significantly in OE8 and OE30 than that in VC (Fig. 8A, C, D) [27, 42, 43]. Together, these findings suggest that the overexpression of *ZmRING-93* improved drought tolerance in transgenic *Arabidopsis*.

Under 10% PEG6000 treatment, the MDA content in VC, OE8, and OE30 was significantly lower than that in VC, and the H_2O_2 measurement results showed the same trend under tabletop drought (Fig. 8E, F). In summary, these findings indicated that overexpression *ZmRING-93* enhanced the drought tolerance of transgenic *Arabidopsis*.

Discussion

We conducted a comprehensive analysis of the maize genome (inbred line B73) to identify genes containing the RING domain, as research on this gene family had remained unclear in maize despite extensive studies in other plant species, such as *Arabidopsis* with 469 members, rice with 378, cabbage with 793, and wheat with 135 [8, 32, 33, 37]. In this study, we yielded a total of 590 RING domain-containing genes and characterized a key RING gene associated with drought response, ZmRING-93, which was found to be an active E3 ubiquitin ligase localized in the nucleus, cytoplasm, and a subset of the endoplasmic reticulum. Notably, its expression was induced by stress and hormone treatment, and it contributed to drought stress tolerance, thereby providing insight into its potential role in enhancing maize's resistance to drought tolerance.

Due to the genomes of rice and sorghum are evolutionarily close to the genome of maize, we systematically analyzed their physicochemical property to determine the phylogenetic relationship between rice, maize and sorghum. By analyzing the collinearity data of RING gene family among maize, sorghum and rice, 340 gene duplication events were found (Table S1). The genomes of rice and sorghum were evolutionarily close to the genome of maize, but the number of RING gene families was higher in these species than in maize. This indicateed that the

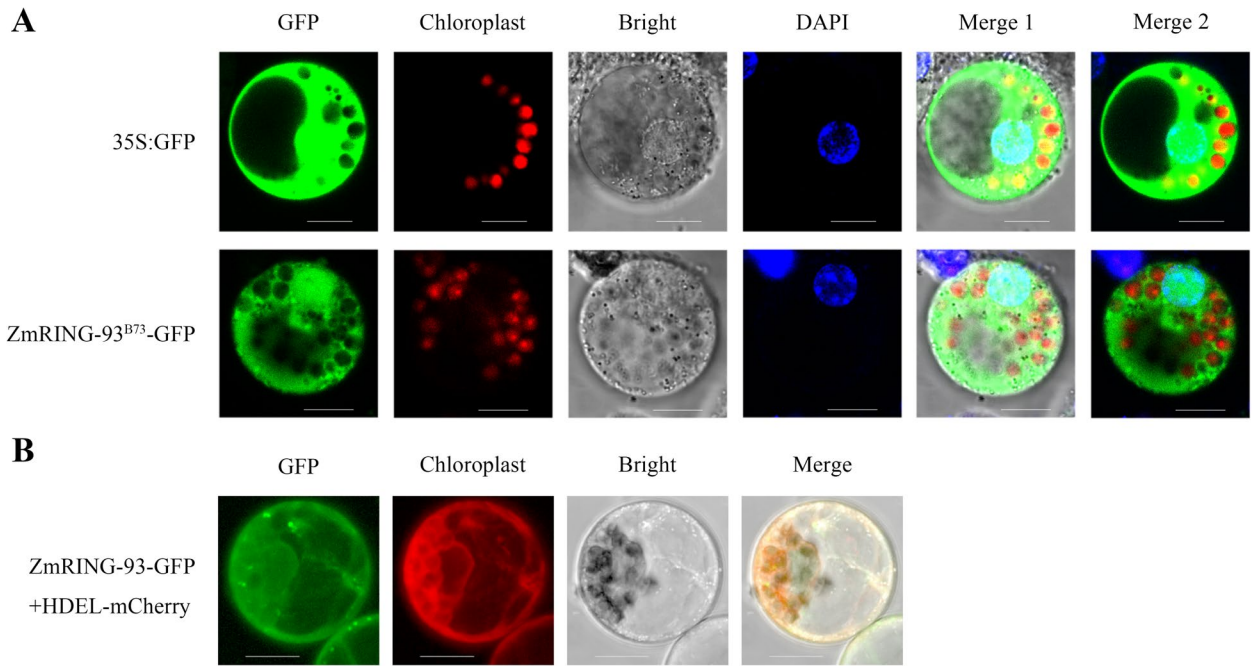


Fig. 7 Subcellular localization of *ZmRING-93* in maize. **A** The subcellular localization of GFP protein in maize protoplast; (**B**) Subcellular co-localization of ZmRING-93-GFP with HDEL-mCherry. *35S:GFP*, *ZmRING-93^{B73}* refers to *pGreenII-GFP*, *pGreenII-ZmRING-93^{B73}-GFP*. HDEL-mCherry is used as the protein localization markers for endoplasmic reticulum. We transformed 2 constructs into maize leaf protoplasts. Bar = 10 μm

RING gene family had further evolved after the differentiation of maize, rice and sorghum. Ka/Ks value of the duplicate gene pairs are less than 1, revealing that the duplicated gene had been purified (Table S2).

Our findings revealed that *ZmRING-93* was significantly associated with drought tolerance in maize (MLM, $P \leq 0.0001$). We found that its expression level was induced by various stresses, including drought, heat, cold, ethephon, NaCl, H_2O_2, and others. This suggested that *ZmRING-93* played a crucial role in responding to abiotic stress inductions across different organs, tissues, and time points [19, 20, 27, 28]. In roots, its expression was significantly up-regulated under ABA treatment, implying that it may have participated in abiotic stress resistance through the ABA pathway [44]. In contrast, its downregulation in shoots may have been involved in mediating stomatal closure or senescence in leaves. Additionally, we found that ZmRING-93's expression was induced by GA3 and SA, indicating that it also participated in gibberellin and salicylic acid pathways. Overall, our results suggested that ZmRING-93 played a key role in the cross-response process of various stresses and hormones in plants. As such, we concluded that analyzing the relationship between ZmRING-93 and ABA would be an important direction for future research.

Most RING domain proteins were found to be localized in the nucleus, while some were located in various intracellular anatomical structures, such as organelles, peripheral cells, cell junctions, and protein-containing complexes [27, 28, 45]. This multifunctional distribution suggested that they played roles in diverse biological processes. Our study revealed that ZmRING-93^{B73} and the ZmRING-93^{CIMBL70} haplotype exhibited similar localization patterns, with signals detected in the nucleus, cytoplasm, and part of the endoplasmic reticulum (Fig. S3). The functional significance of RING domain-containing proteins was highlighted by previous studies, which showed that they enhanced biological and abiotic stress tolerance [2–9]. For example, silencing StRFP1 in potato lines increased susceptibility to pathogen infection, while overexpressing plants displayed slower disease development [46]. Additionally, *Arabidopsis* AtATL78, a plasma-membrane-located RING E3 ubiquitin ligase, was found to mediate ABA-dependent stomatal closure and play a positive regulatory role in drought stress response, H_2O_2 production, and signal transmission [45, 47].

Most RING domain-containing proteins are previously reported to be an active ubiquitin ligase [21, 22, 24, 26, 27, 48]. For example, in a recent study, it was discovered that ZmWIPF2 has auto-ubiquitination activity as an E3 ubiquitin ligase [48]. In vitro ubiquitination assays revealed that ZmRING-93 was an E3 ubiquitin ligase, suggesting that RING domain-containing protein might share a conserved function. The RING domain-containing

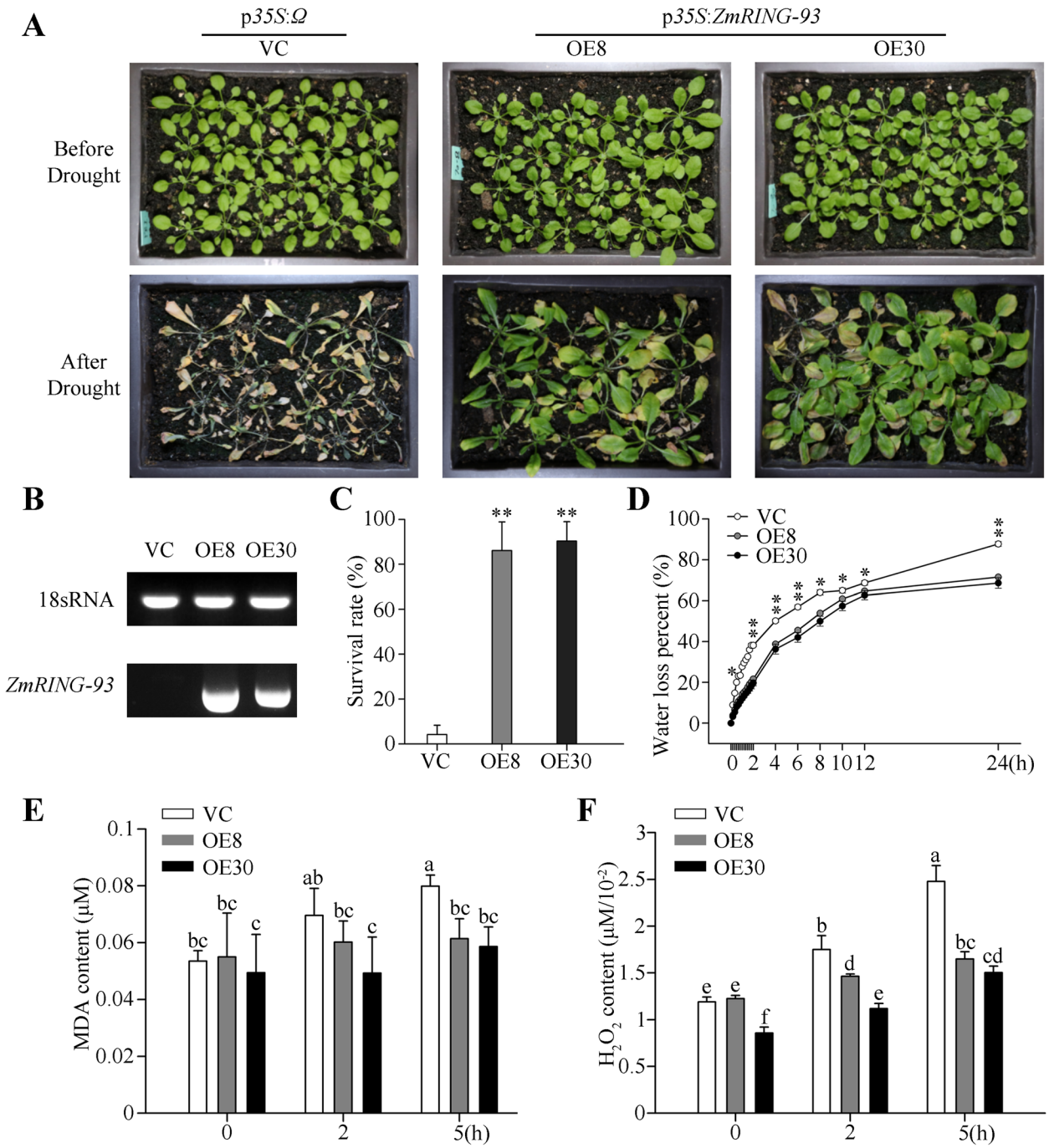


Fig. 8 Analysis of drought tolerance of *ZmRING-93* transgenic *Arabidopsis*. **A** Representative photos for VC, OE8, and OE30 before and after drought stress. VC represents empty pGreenII-35SΩ transformed *Arabidopsis*. **B** Semi-quantitative RT-PCR. **C** Survival rate of *ZmRING-93* transgenic *Arabidopsis*. For VC, OE8, or OR30, the data showed mean ± SD, calculated from a total of 144 plants in three independent experiments. **D** Rate of water loss. Ten plants of each line were measured in each experiment, with three replicates. **E** MDA content determination, more than 20 plants of each line were measured in each experiment, with three replicates. **F** H_2O_2 content determination, more than 20 plants of each line were measured in each experiment, with three replicates. A two-sided *t*-test determined statistical significance: * $P < 0.05$, ** $P < 0.01$

genes were proved to be capable of enhancing biological and abiotic stress tolerance [2–9]. Overexpression of ZmRING-93 could improve drought tolerance in *Arabidopsis*, suggesting the role of ZmRING-93 under drought stress. Taken together, ZmRING-93 is an important gene for drought tolerance in maize.

Conclusions

This study identified 590 maize *ZmRINGs* divided into 6 subclasses and 11 evolutionary groups, and distributed on 10 chromosomes. Gene duplication is thought to have occurred after the differentiation of gramineous plants, acting as the main driving force of RING family evolution. Among *ZmRINGs*, *ZmRING-93* was significantly associated with drought tolerance and was induced in the maize shoot and root under various abiotic stress and hormone treatments. ZmRING-93 has ubiquitin E3 ligase activity and was subsequently localized in the nucleus and cytoplasm. Meanwhile, overexpression of *ZmRING-93* in transgenic *Arabidopsis* improved drought tolerance by reducing the rate of water loss, further suggesting that *ZmRING-93* is an important candidate gene for drought tolerance in maize. Together, these findings provide a foundation for further studies into the roles of RING domain-containing proteins in maize.

Materials and methods

Identification of RING domain-containing proteins in maize

Three methods were used to identify RING domain-containing proteins in the maize genome. First, BLASTp (https://blast.ncbi.nlm.nih.gov/) [49] was used to search against the genome of maize inbred line B73 (AGPv3) using 469 protein sequences of *Arabidopsis* RING domain-containing proteins as a reference. Next, HMM files for PF00097 (zf-C3HC4), PF12906 (RING-v), PF13639 (zf-RING_2), PF13920 (zf-C3HC4_2), and PF15227 (zf-C3HC4_4) from the Pfam [50] database (http://pfam-legacy.xfam.org/) were adopted to search for RING domain-containing proteins in the maize genome using hmmsearch [51]. Finally, regular expressions of RING domains from previous studies [8, 33, 34] were used to search the maize genome. Protein sequences obtained by these three methods were submitted to the CDD [52] database (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) for cross-validation, and only those with an E-value $\leq$ 0.00001 were selected.

Phylogenetic tree construction of ZmRINGs

Evolutionary tree of the full-length protein of the obtained RING genes was constructed using the method described by Toparslan, E [38]. Briefly, the full-length protein sequences were aligned by the function msa in R package msa (https://bioconductor.org/packages/release/bioc/html/msa.html). dis.alignement function in R package seqinr (https://cran.r-project.org/web/packages/seqinr/) was adopted to compute a matrix of pairwise distances from the aligned sequences. Then, the phylogenetic tree was constructed using njs function with 1000 bootstraps in R package ape (https://cran.r-project.org/web/packages/ape/).

Association analysis of ZmRINGs with drought tolerance

The association analysis of *ZmRINGs* was conducted using a maize association mapping population and the corresponding drought tolerant phenotype data from a previous study [40]. With a minor allele frequency (MAF) $\geq$ 0.05, 4506 SNPs were found to be located in the genic region of the 590 ZmRINGs. The general linear model (GLM), and the mixed linear model (MLM) were applied to compute the P-values of marker-trait associations.

Abiotic stress treatment

The seeds of maize inbred line B73 [53] were surface-sterilized in 5% H_2O_2 solution for 5 min, then washed with distilled water. The washed seeds were submerged in 5 mM calcium sulfate solution for 12 h. The germinated seeds were then transferred into a nutrient solution for hydroponic cultivation. Seedlings with identical growth stage (V3 stage) were selected and transplanted into a light incubator at 28°C (16 h of light/8 h of darkness). Seedlings at three-leaf stage were subjected for stress treatment, and were exposed for 0 h, 2 h, 5 h, 10 h, 24 h, and 48 h, respectively. The types of stress treatment included drought, heat (42°C), cold (4°C), 100 mM hydrogen peroxide (H_2O_2), 250 mM NaCl, and 100 µM gibberellin (GA3), 100 µM salicylic acid (SA), 100 µM of ethephon, 100 µM abscisic acid (ABA). The treatment method for ABA, ethephon, SA, GA3, NaCl, and H_2O_2 includes transferring maize to a nutritive medium containing the corresponding concentration for 48 h. The drought treatment method includes removing plants from nutritive medium, absorbing excess water from roots, placing them on tabletop, and leaving them at room temperature for 48 h. The cold and heat treatment methods include placing the plant and nutritive medium together in a light incubator. Shoot and root were harvested. The collected roots and shoots were stored in a refrigerator at -80°C for subsequent RNA extraction.

RNA extraction and RT-qPCR analysis

Total RNA was isolated from the frozen samples using TRIZOL reagent (Bio-topped) from no less than three seedlings, treated with RNase-free DNase (Takara), and reverse transcribed into the cDNA library with M-MLV (Takara). The forward and reverse primer in RT-qPCR for *ZmRING-93* was 5'-CCGCTCGTCATCGCTATC AT-3' and 5'-GAGCTGGAACTGAAAGACCCG-3', respectively. RT-qPCR was performed according to Ding et al. [54]. *ZmUBI2* (UniProtKB/TrEMBL; ACC: Q42415) was used as an internal control for RT-qPCR. The PCR reactions were carried out in a 10 µL volume containing 1 µL diluted cDNA, 200 nM gene-specific primers, and 5 µL SYBR Premix Ex Taq II (Takara, China) with the

following conditions: 10 min at 95°C, 40 cycles of 15 s at 95°C, and 30 s at 60°C.

*P*rotein preparation and in vitroubiquitination assays

The coding sequence of *ZmRING-93* was amplified from the cDNA library of maize inbred lines B73 [53]. *pGEX4T-1-ZmRING-93*, a GST tag fused with the RING domain of *ZmRING-93*, was introduced into the BL21 (DE3) strain of *E. coli*. After cultivated at 37°C for 3 h in a shaking incubator, 100 mM IPTG was added to the bacteria which was further cultivated for another 18 h. Both wheat (*Triticum aestivum* L.) E1 (GI:136,632) and human E2 were expressed as a His-tagged protein in the BL21. The protein of wheat E1 and human E2 (His-UBCh5b) [20, 27] were induced with 0.1 mM IPTG at 37°C for 6 h and at 12℃ for 20 h, respectively. The ubiquitination assay was conducted following the protocol developed by Zhao et al. [55]. The assay mixture contained 250 ng of GST-ZmRING-93, 100 ng of wheat E1, 250 ng of human E2, and 2 μg of recombinant human HA-ubiquitin (BIOTECHNOGOLY). The components were mixed with 3 μL of 10×reaction buffer to a final volume of 30 μL. The ultimate concentration of the ubiquitination buffer was 50 mM Tris–HCl pH 7.4, 25 mM $MgCl_2$, 10 mM ATP, and 2 mM DTT. After 20 min incubation at 30°C, the reaction was stopped with 6×SDS loading buffer at 100°C for 5 min. Subsequently, the reaction mixtures were immunoblotted using 8% or 15% SDS-PAGE and the appropriate antibodies. The western blot analysis for the above protein was performed according to the protocol as illustrated by Ding et al. [27].

Subcellular localization of ZmRING-93

Full-length cDNAs of ZmRING-93 were inserted into the vector of *pGreenII-GFP*. Protoplast extraction and transformation of the reconstructed vector into maize protoplast were performed according to Yoo, S.D., et al. [56]. The transformed protoplasts were incubated in darkness at 25°C for 16 h, then they were washed with W5 buffer and placed under a laser confocal microscope (Leica TCSSP8) to observe the fluorescence signal. The excitation wavelengths of GFP and mCherry are 488 nm and 587 nm, respectively, while the emission wavelengths are 507 nm and 610 nm, respectively.

Generation of transgenic plants

The coding sequence of *ZmRING-93^{CIMBL70}*, amplified from maize inbred line CIMBL70, were inserted into the vector *pGreenII-35sΩ* and the reconstructed vector was transferred into Agrobacterium tumefaciens treated with GV3101(*pSoup*). The *Arabidopsis thaliana* ecotype Col was used in this study. Agrobacterium tumefaciens with *pGreenII-35sΩ-ZmRING-93^{CIMBL70}* were subsequently transformed into *Arabidopsis* wild-type Col-0 (WT). Using the kanamycin-based selection, several independent T_2 transgenic lines were obtained, and the relative expression level of *ZmRING-93* transgene lines with that of WT were confirmed by RT-PCR with an internal control 18sRNA [54]. Two T_2 single copy inserted lines, *ZmRING-93^{CIMBL70}-OE8* and *ZmRING-93^{CIMBL70}-OE30* were selected. T3 homozygous seeds were used for subsequent analyses, including: (1) drought survival rate assessment, (2) quantification of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content, and (3) measurement of detached leaf water loss rate.

Measurement of MDA and H_2O_2

VC, OE8 and OE30 grown on 1% MS for 20 days were treated with 10% PEG6000 and tabletop drought respectively, and the content of MDA in VC, OE8 and OE30 under 10% PEG6000 treatment was determined. Meanwhile, the content of H_2O_2 in VC, OE8 and OE30 under tabletop drought was determined. The measurement of MDA and H2O2 was carried out according to Velikova et al. [57].

Abbreviations

CDD	CANdelaStudio Diagnostic Description
PGDD	Plant Genome Duplication Database
Msa	Moth swarm algorithm

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06683-8.

Additional file 1: Table S1: RINGs genes in maize.

Additional file 2: Table S2: Ka/Ks for the paired genes.

Additional file 3: Figure S1: Diagram of the protein domain of ZmRING-93.

Additional file 4: Figure S2: Phylogenetic Tree of ZmRING.

Additional file 5: Figure S3: Subcellular Localization of ZmRING-93^{CIMBL70}.

Additional file 6: Figure S4: Semi-quantitative RT-PCR analysis of *ZmRING93* expression.

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Authors' contributions

XT, HL performed the experiments, analyzed the data, and prepared figures and tables. TW and ZH analyzed the expression pattern of ZmRING-93 under stress treatment. HW gave good advice on the work. SC and HW designed the experiments, wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability

All data generated or analyzed of this study were included in the main text and supplemental files. All maize inbred lines used in this study were requested from maize association mapping population AM368 [53]. The col and the VC of *Arabidopsis* were requested from a published article [27].

Declarations

Ethics approval and consent to participate

This study did not require ethical approval or consent as no animals were involved.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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