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CmERF5-CmRAP2.3 transcriptional cascade positively regulates waterlogging tolerance in *Chrysanthemum morifolium*

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Summary

Waterlogging stress affects plant growth by limiting root respiration and reducing yield and economic value. Therefore, identifying genes involved in regulating waterlogging stress is vital. This study reports the ethylene-responsive VII transcription factor (*CmRAP2.3*) in the chrysanthemum. Subcellular localization and transactivation assay analyses revealed that CmRAP2.3 was localized in the nucleus and possessed transactivation activity. Overexpression of *CmRAP2.3* in chrysanthemum was found to enhance waterlogging tolerance by decreasing reactive oxygen species (ROS) levels. Furthermore, we found that the transcription factor CmERF5 binds to GCC-like motifs in the *CmRAP2.3* promoter region and activates *CmRAP2.3* expression. Additionally, *CmERF5* overexpression maintained a low ROS level and improved chrysanthemum waterlogging tolerance. Taken together, this study shows a molecular mechanism by which *CmERF5* transcriptionally activates *CmRAP2.3* to reduce waterlogging stress via the ROS pathway in the chrysanthemum.

Introduction

Waterlogging stress leads to hypoxia and ethylene accumulation in plants (Loreti *et al*., 2016), and severely prevents plant growth by limiting root respiration (Buchanan-Wollaston *et al*., 2003), resulting in yield loss or quality decline. Thus, it is essential to study the mechanisms of plant waterlogging tolerance in order to maintain productivity. Various mechanisms occur in plants in response to waterlogging stress. These include morphological changes (Fukao and Bailey-Serres, 2008), reactive oxygen species (ROS) scavenging (Yuan *et al*., 2017) and the regulation of hypoxia response genes (Licausi *et al*., 2011).

Ethylene-responsive factor (ERF) is one of the most prominent transcription factor families in plants. It has been reported that group VII ERFs (ERF-VIIs) are essential for regulating hypoxic responses (Bailey-Serres *et al*., 2012). Additionally, protein sequence analysis showed that ERF-VIIs have an APETALA2 (AP2) domain and a conserved N-terminal MCGGAI (I/L) motif [termed the Met-Cys motif (MC motif)] (Nakano *et al*., 2006). The cysteine residue (C in the second position) in the MC motif is essential for the oxygen-triggered degradation of ERF-VIIs via the N-degron pathway in normoxia. In the presence of oxygen, this process begins with the oxidation of cysteine residue in the MC motif, forming cys-sulfonic or cys-sulfinic acid. Then, ERF-VIIs are modified by the Arg-tRNA protein transferase and further degraded by the proteolysis-6 ubiquitin ligase (Bailey-Serres *et al*., 2012). Previous studies have shown that ERF-VIIs from different species, including petunia [*Petunia juss* (Yin *et al*., 2019)], kiwi fruit [*Actinidia deliciosa* (Liu *et al*., 2022)] and barley [*Hordeum vulgare* (Luan *et al*., 2020)], participate in the waterlogging stress response. One of the ERF-VIIs, related to *AP2.3* (*RAP2.3*), accumulates in the nucleus and regulates the expression of hypoxia-responsive genes (Papdi *et al*., 2015). Additionally, *RAP2.3* is involved in the heat stress response (Ogawa *et al*., 2005), disease response (Kim *et al*., 2018) and cold-induced sweetening (Shi *et al*., 2021). However, the molecular mechanisms of *RAP2.3* in waterlogging stress response remain poorly understood.

Other ERFs are also involved in the waterlogging stress response. For example, *MaRAP2.4* regulates *AtSWEET10* (a bidirectional sugar transporter) to modulate abiotic stresses, including waterlogging (Phukan *et al*., 2018). Furthermore, the *ethylene-responsive transcription factor 5* (*ERF5*) is required for osmotic stress adaptation in *Arabidopsis thaliana* (Dubois *et al*., 2013) and drought stress regulation in *Solanum lycopersicum* (Zhu *et al*., 2018). Therefore, *ERF5* is involved in stress response in different plant species. However, the role of *ERF5* in waterlogging stress and its molecular basis in stress response remains unclear.

Chrysanthemum is one of the most important economic plants globally due to its ornamental and medicinal values. However, it is a shallow-rooted crop susceptible to waterlogging stress. Therefore, understanding how chrysanthemum responds to waterlogging stress at the molecular level is essential for its genetic improvement. Previous studies have shown that a low ROS level and a high antioxidant enzyme activity cause plants resistance to waterlogging stress (Yin *et al*., 2009). Moreover, the overexpression of salt overly sensitive 1 (*SOS1*) in chrysanthemum improves tolerance to waterlogging stress (Wang *et al*., 2020).

A previous study reported that the *CmRAP2.3* (CL4854.Contig1) gene in chrysanthemum was up-regulated by waterlogging stress and down-regulated after 2 h of re-oxygenation (Zhao

et al., 2018), indicating its potential role in waterlogging response. Nevertheless, the detailed molecular mechanism of how *CmRAP2.3* mediates waterlogging stress remains to be elaborated. Here, we demonstrated that CmERF5 regulates *CmRAP2.3*, and both genes improve plant waterlogging tolerance by regulating ROS homeostasis. Overall, this work establishes a mechanism by which the CmERF5 activates *CmRAP2.3* expression to modulate waterlogging stress via the ROS pathway in chrysanthemum.

Results

The expression of *CmRAP2.3* is induced by waterlogging

To validate the expression of *CmRAP2.3* in response to waterlogging stress, we performed RT-qPCR test using roots from the chrysanthemum cultivar 'Jinba'. We found that the *CmRAP2.3* expression was up-regulated after 12 h of waterlogging treatment and then down-regulated after 2 h of re-oxygenation (Figure 1a), consistent with the transcriptional profiles generated from the transcriptome data (Zhao *et al*., 2018).

We then cloned *CmRAP2.3* from the chrysanthemum cultivar 'Jinba'. Protein sequence analysis showed that CmRAP2.3 contains an AP2 domain and an MC motif (MCGGAI/L) involved in the N-degron pathway, indicating that CmRAP2.3 belongs to the ERF-VIIs (Figure 1b). Phylogenetic analysis revealed that CmRAP2.3 clustered with *Tanacetum cinerariifolium* TcRAP2.3 (Figure 1c).

CmRAP2.3 is a transcription activator

To investigate the subcellular localization of CmRAP2.3, we transiently expressed *CmRAP2.3* in *Nicotiana benthamiana*, by infiltrating plant leaves with *Agrobacterium tumefaciens* suspension carrying the *35S::CmRAP2.3-GFP* expression construct or the

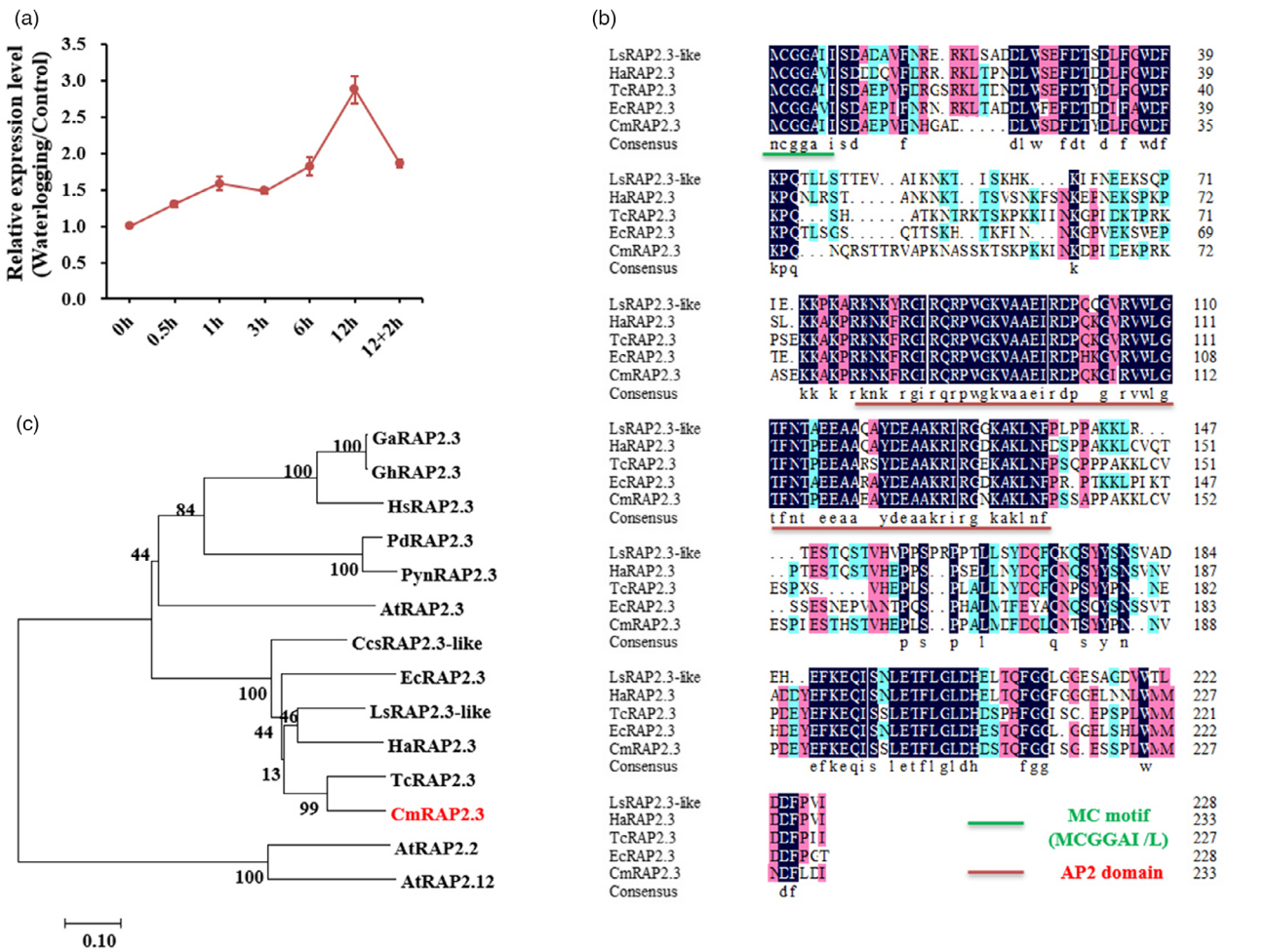

Figure 1 Expression pattern, sequence alignments and phylogenetic analysis of *CmRAP2.3*. (a) *CmRAP2.3* expression levels in the wild-type (WT) chrysanthemum roots after 0, 0.5, 1, 3, 6 and 12 h of waterlogging stress and 2 h of re-oxygenation. The data shown are presented as mean ± standard errors ($n = 3$). (b) Multiple alignments of the CmRAP2.3 and homologous ERF-VII proteins of other species. The MC motif and AP2 domain were marked with green and red lines. (c) Phylogenetic tree of the CmRAP2.3 (marked in red) and other ERF-VII proteins of *Hibiscus syriacus* (prefixed with Hs, XP_039048699.1), *Gossypium arboreum* (Ga, XP_017649944.1), *Gossypium hirsutum* (Gh, XP_016677360.1), *Lactuca sativa* (Ls, XP_023768721.1), *Helianthus annuus* (Ha, XP_021969959.1), *Tanacetum cinerariifolium* (Tc, GEV78540.1), *Cynara cardunculus* var. *scolymus* (Ccs, XP_024994163.1), *Prunus dulcis* (Pd, XP_034209096.1), *Erigeron canadensis* (Ec, XP_043640030.1), *Prunus yedoensis* var. *nudiflora* (Pyn, PQQ08082.1) and *Arabidopsis thaliana* [*AtRAP2.3* (AT3G16770), *AtRAP2.12* (AT1G53910.1) and *AtRAP2.2* (AT3G14230.1)].

35S::GFP control vector, together with the nuclear marker D53-mCherry. Results showed that CmRAP2.3 is localized in the nucleus (Figure 2a).

Furthermore, we fused the CmRAP2.3 with the GAL4-binding domain (BD) and then expressed the chimeric protein in the Y2H yeast strain to examine the transcriptional activity. The yeast cells transformed with BD-CmRAP2.3 vector survived on a synthetic defined medium without adenine and histidine (SD/Ade-His- medium), suggesting that CmRAP2.3 has transcriptional activity (Figure 2b). To further investigate the specific region (s) that contribute to transcription activation, we analysed truncated forms of CmRAP2.3, either lacking the C-terminal sequence (BD-CmRAP2.3ΔC, 1–146 aa) or the N-terminal sequences (BD-CmRAP2.3ΔN, 83–234 aa). We found that only BD-CmRAP2.3ΔN exhibited a high transcriptional activation level (Figure 2b), indicating that CmRAP2.3 is a transcription activator and functions via its C-terminal region.

CmRAP2.3 positively regulates waterlogging tolerance in chrysanthemum

To investigate the biological function of *CmRAP2.3* in chrysanthemum, we generated overexpressing lines of *CmRAP2.3* (*CmRAP2.3*-OX) by introducing the plasmid 35S:CmRAP2.3 (Figures S1 and 3a). Eight- to ten-leaf-old transgenic and wild-type (WT) plants were used for waterlogging treatment, and leaf yellowing rate was analysed at 8 days. Results showed that the *CmRAP2.3*-OX lines were less damaged by waterlogging than WT plants (Figure 3b,c). A low relative electric conductivity is generally correlated with reduced plant injury under stress conditions (Arvin and Donnelly, 2008). Analogously, *CmRAP2.3*-OX plants exhibited lower relative electric conductivity values than those of WT plants (Figure 3d). After waterlogging treatment, plants were recovered under normal growth conditions for 18 days, and the recovery rate was calculated. We found that the recovery rate of *CmRAP2.3*-OX plants was at least two-fold higher than that of WT plants (Figure 3e). Additionally, the phenotype severity was correlated with the up-regulation of *CmRAP2.3* in transgenic lines (Figure 3). Taken together, these results indicate that *CmRAP2.3* positively regulates waterlogging tolerance in the chrysanthemum.

CmRAP2.3-mediated ROS scavenging is involved in the positive regulation of chrysanthemum waterlogging tolerance

To further investigate the mechanisms of *CmRAP2.3*-mediated waterlogging tolerance, RNA-seq analysis was performed on the

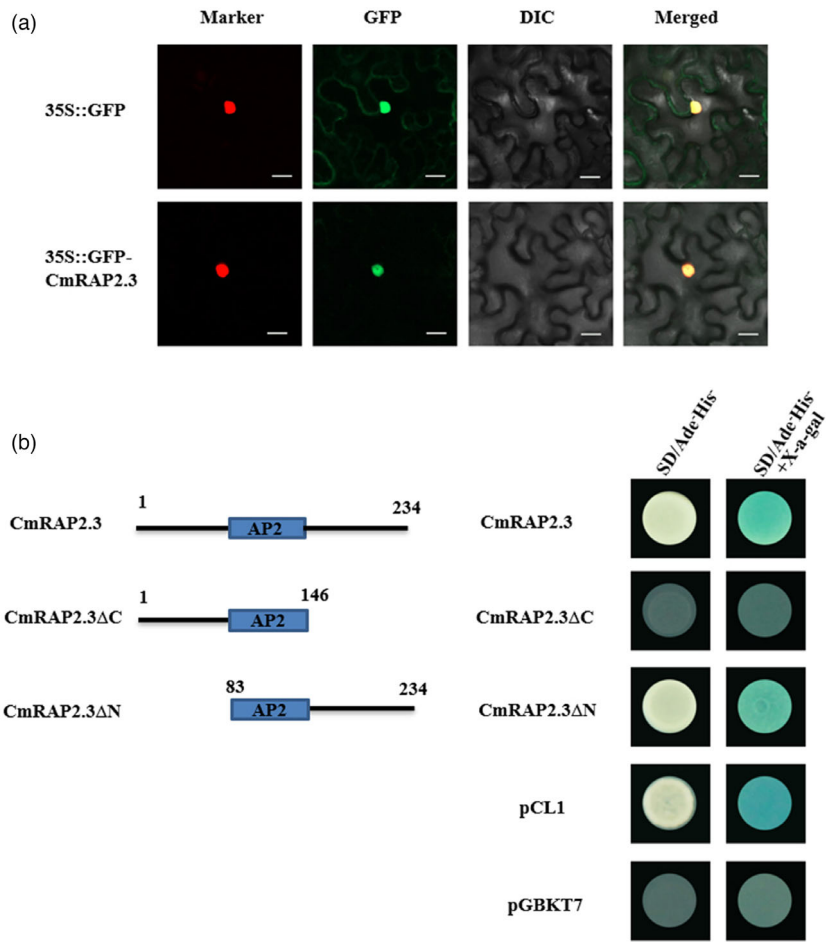

Figure 2 CmRAP2.3 subcellular localization and transcriptional activation. (a) Subcellular localization of CmRAP2.3 in tobacco (*Nicotiana benthamiana*) leaves. The co-expressed 35S::D53-RFP construct was used as a nuclear marker. Marker: images taken in the red fluorescence channel; GFP: images taken in the green fluorescence channel; DIC: images taken in the bright light channel; merged: both overlay plots. Bars = 20 μm. (b) Amino acid segmentation of CmRAP2.3 based on the AP2 domain and the transcriptional activity in yeast. Synthetic defined medium without adenine and histidine (SD/Ade-His-:); SD/Ade-His- + X-α-gal: SD/Ade-His- medium containing 20 mg/mL X-α-gal.

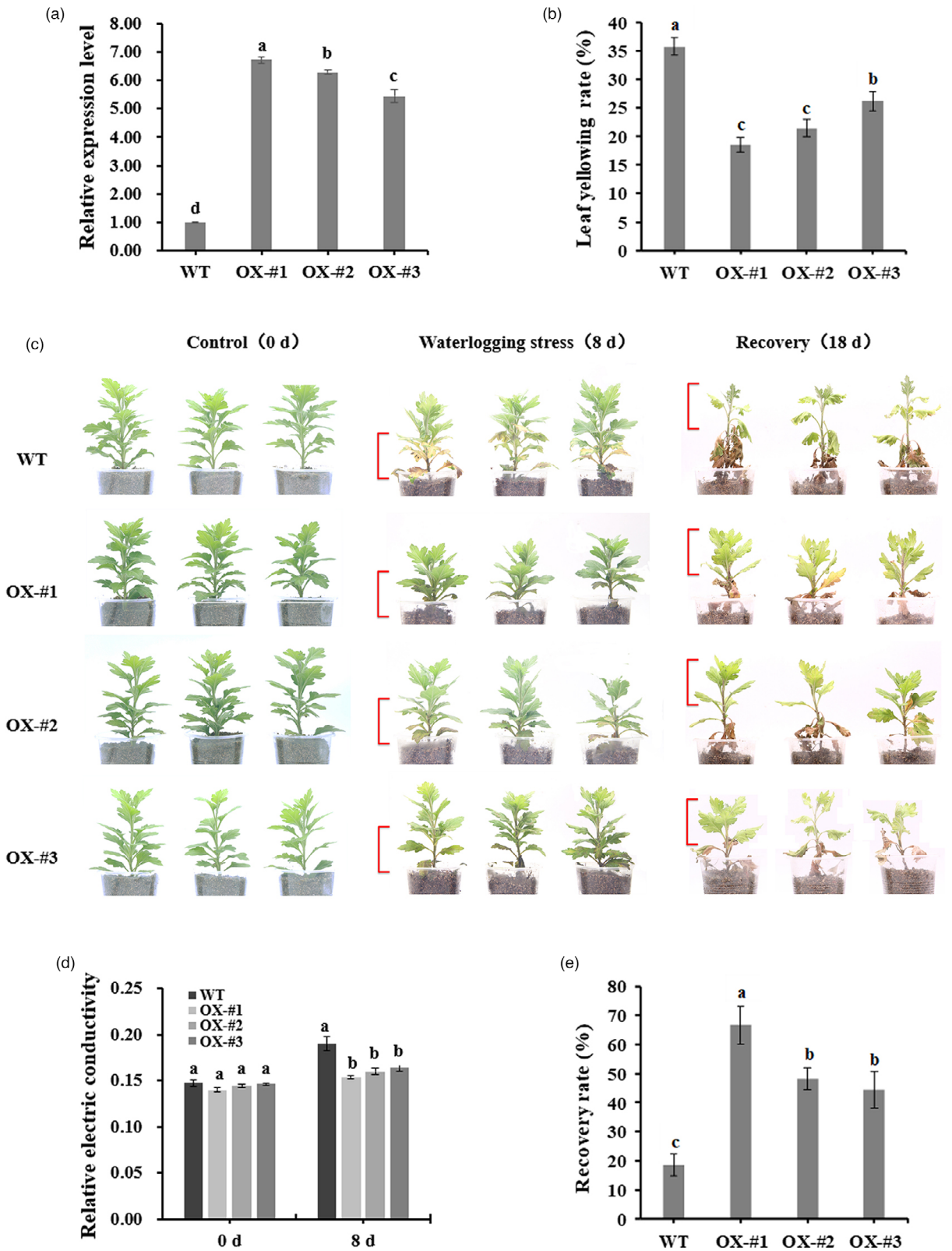

Figure 3 Phenotypic observation of *CmRAP2.3* overexpression lines in response to waterlogging. (a) *CmRAP2.3* relative expression levels in *CmRAP2.3*-OX lines and WT plants. (b) Leaf yellowing rate of *CmRAP2.3*-OX lines and WT plants after 8 days of waterlogging stress. (c) The phenotype of three *CmRAP2.3*-OX (OX-#1, OX-#2 and OX-#3) lines and WT plants in waterlogging stress after 0 and 8 days of waterlogging and recovery for 18 days. Phenotype differences are indicated with red brackets. (d) The relative electric conductivity of *CmRAP2.3*-OX lines and WT plants on the 0 and 8th days of waterlogging stress. (e) The recovery rate of *CmRAP2.3*-OX lines and WT plants after recovery for 18 days. The data shown in the figure are presented as mean ± standard errors ($n = 3$). The letters above the bars indicate significant differences according to Duncan's multiple-range test at $P < 0.05$.

roots of OX-*CmRAP2.3* lines (OX-#1) and WT plants. Pearson's correlation coefficient analysis showed good reproducibility among three biological replicates (Figure S2a). A *Q*-value < 0.05 and |log2 Ratio| ≥ 0.5 were used to identify differentially expressed genes (DEGs). Among these DEGs, 2954 were up-regulated and 1983 were down-regulated (Figure 4b). Gene ontology (GO) analysis revealed that DEGs were significantly enriched in the oxidative stress processes, including oxidoreductase activity and hydrogen peroxide catabolic process (Table S1, Figures 4a and S2b). To validate the gene expression in RNA-seq data, the RT-qPCR was used to measure the relative expression levels of *CmSOD*, *CmCAT* and *CmAPX6* in the OX-*CmRAP2.3* chrysanthemum lines and WT plants. Results indicated that the expression patterns of three genes were consistent with the expression levels obtained from RNA-seq analysis (Figure 4c). Thus, we speculated that *CmRAP2.3* may be involved in the scavenging of ROS induced by waterlogging stress in the chrysanthemum.

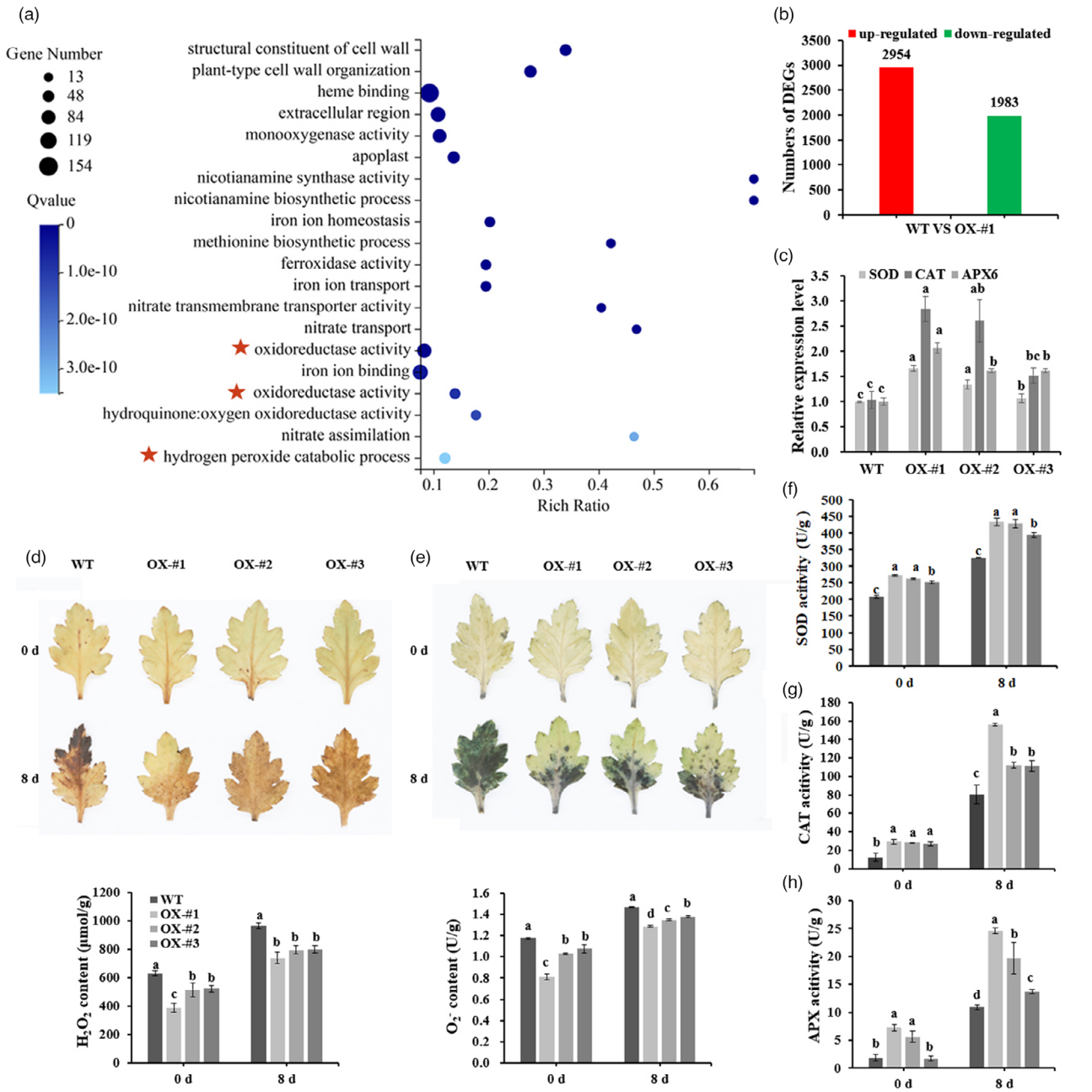

Figure 4 RNA-seq of WT and OX-*CmRAP2.3* plants implicate *CmRAP2.3* is involved in chrysanthemum waterlogging stress by affecting the genes related to ROS. (a) Top 20 terms from a gene ontology (GO) enrichment analysis of the DEGs of GO category. GO terms related to oxidative stress processes were marked by a red star. (b) Number of up- or down-regulated genes in the comparison between the wild-type and *CmRAP2.3*-OX-#1 transgenic plants. (c) A RT-qPCR validation of the genes selected from DEGs involved in ROS pathway in WT and *CmRAP2.3*-OX lines. (d) Diaminobenzidine staining and H_2O_2 content. (e) Nitro tetrazolium blue chloride staining and O_2^- content. (f–h) Reactive oxygen species scavenger activity in leaves of WT and *CmRAP2.3*-OX plants. The values are presented as mean ± standard errors (n = 3). Significant differences between ROS levels and scavenger activity in WT and *CmRAP2.3*-OX plants were analysed using Duncan's multiple-range test at P < 0.05.

To verify this hypothesis, we conducted the diaminobenzidine [DAB (dark brown)] and nitrotetrazolium blue chloride [NBT (dark blue)] staining in transgenic and WT plants with or without waterlogging treatment to examine ROS accumulation. A higher ROS level was observed in WT plants than in *CmRAP2.3*-OX transgenic plants (Figure 4d,e). Next, we measured the H_2O_2 and O_2^- contents in these plants. The H_2O_2 and O_2^- levels in WT plants were higher than those in *CmRAP2.3*-OX plants (Figure 4d,e), consistent with the DAB and NBT staining. Furthermore, we examined the activity of ROS scavengers. Results showed that superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) activities in *CmRAP2.3*-OX plants were higher than those in WT after being exposed to waterlogging stress treatment for 0 and 8 days (Figure 4f–h). Collectively, all these results indicate that *CmRAP2.3* positively regulates waterlogging tolerance by elevating ROS scavenging ability.

CmERF5 directly regulates *CmRAP2.3*

To gain insight into the mechanism of *CmRAP2.3*-mediated waterlogging tolerance, we cloned a 924-bp promoter sequence of CmRAP2.3 (CmRAP2.3pro; Figure S3) and identified the direct upstream activator(s) using yeast one-hybrid (Y1H) screening using a chrysanthemum complementary DNA library. A total of 187 sequences were isolated from screening, including six transcriptional factors [*CmHSFB2a*, *CmTGA2.2*, *CmERF5*, *CmABF4*, *CmASIL1* and *CmERF4* (Table S2)]. We found that *CmERF5* expression was induced by waterlogging and recovered by re-oxygenation, consistent with the *CmRAP2.3* expression pattern (Figures 5a and S6), and only *CmERF5* directly bound to the *CmRAP2.3* promoter (Figures 5b, S4 and S5). These results indicate that CmERF5 is a potential upstream transcription factor controlling *CmRAP2.3* gene expression.

Previous reports have shown that AP2/ERF proteins bind to the sequences containing the GCC motif [AGCCGCC (Hao *et al.*, 1998)], and the positions A-1 and C-6 appeared to be less critical for binding in *Arabidopsis* (Fujimoto *et al.*, 2000). The current study identified a GCC-like (TGCCGCC) motif in the *CmRAP2.3* promoter region (Figure S3). The CmERF5 binding to the GCC-like motif was confirmed by the electrophoretic mobility shift assay (EMSA; Figure 5c) and ChIP-PCR (Figure 5d). We found that CmERF5 was localized in the nucleus and had transcriptional activity (Figure 5e–h). These results indicate that *CmERF5* is a direct upstream activator of the *CmRAP2.3*.

CmERF5 positively regulates waterlogging tolerance in chrysanthemum

Given that the *CmERF5* expression is induced by waterlogging and is a direct upstream activator of *CmRAP2.3*, we generated plants overexpressing *CmERF5* (*CmERF5*-OX) to confirm the role of *CmERF5* in waterlogging stress (Figures 6a and S7). We found that *CmRAP2.3* expression in *CmERF5*-OX lines was higher than that in WT plants (Figure 6b), consistent with the transcriptional regulation of *CmRAP2.3* by CmERF5. *CmERF5*-OX lines exhibited less damage than that in WT plants after waterlogging stress treatment (Figure 6c). Relative electric conductivity and recovery rate showed that plants with overexpression of *CmERF5* exhibited enhanced resistance to waterlogging (Figure 6d,e), indicating that *CmERF5* positively regulates waterlogging tolerance in chrysanthemum.

Considering that *CmERF5* and *CmRAP2.3* positively regulate waterlogging tolerance in chrysanthemum, and CmERF5 is a direct upstream activator of *CmRAP2.3*, we speculate that both of them regulate waterlogging tolerance in ROS scavenging pathway. To test this hypothesis, we measured the ROS levels in *CmERF5*-OX lines and WT control plants. Results showed that ROS accumulation in WT is higher than that in *CmERF5*-OX lines after waterlogging treatment (Figure 7a,b). Meanwhile, measurements of SOD, CAT and APX activities suggested that the activity of ROS scavengers was higher in *CmERF5*-OX lines than that in WT plants (Figure 7c–e). These results indicate that CmERF5, together with CmRAP2.3, positively regulates waterlogging tolerance by controlling ROS accumulation in the chrysanthemum.

Discussion

CmRAP2.3 and *CmERF5* positively regulate waterlogging tolerance by maintaining a low ROS level in chrysanthemum

ROS accumulated in plant cells under waterlogging stress disrupts cellular homeostasis. However, the activity of ROS scavengers acting against the imbalance was simultaneously enhanced (Wrzaczek *et al.*, 2013). In chrysanthemum, a higher activity of antioxidant enzymes (SOD, APX and CAT) was observed in tolerant chrysanthemum cultivars than that in sensitive cultivars (Yin *et al.*, 2009). Furthermore, the increase in enzyme activity was associated with ROS accumulation leading to the differences in waterlogging tolerance between the two chrysanthemum cultivars (Zhao *et al.*, 2018). Therefore, the transcriptional factors related to the ROS pathway in chrysanthemum should be investigated further.

Transcription factors of the ERF-VII family regulate ROS accumulation in response to waterlogging. For example, *ZmEREB180* enhances the growth of adventitious roots and regulates antioxidant levels to confer waterlogging tolerance in maize seedlings (Yu *et al.*, 2019). Moreover, the activity of ROS scavengers (SOD, CAT and POD) was higher in *HvERF2.11* overexpression lines than in WT plants (Luan *et al.*, 2020). Additionally, a previous study revealed that other groups of the ERF family are also involved in waterlogging stress. For example, ERF-III and IX transcription factors are major regulators of waterlogging stress in sesame [*Sesamum indicum* (Wang *et al.*, 2021)]. In addition, *PhERF2* plays a vital role in petunia's response to waterlogging stress (Yin *et al.*, 2019). Although many ERF genes are associated with waterlogging stress, it is essential to investigate the specific mechanisms of them in response to waterlogging. In previous studies, *ERF5* was involved in drought stress regulation (Zhu *et al.*, 2018) and disease resistance (Li *et al.*, 2021). However, its function in waterlogging response in the chrysanthemum is still unclear.

In the current study, *CmRAP2.3* and *CmERF5* accumulated in the nucleus (Figures 2a and 5e), conferring waterlogging tolerance in chrysanthemum (Figures 3 and 6), similar to *AtRAP2.3* in *Arabidopsis* (Papdi *et al.*, 2015). The transcriptomics profiling in *CmRAP2.3* transgenic lines and WT plants (Figure 4) suggested that the genes involved in the ROS pathway were significantly enriched in *CmRAP2.3* transgenic lines in comparison with WT plants, reflecting that *CmRAP2.3* was involved in ROS pathway in response to chrysanthemum waterlogging stress. The result was identical to that of the subsequent analysis of ROS accumulation in *CmRAP2.3* and *CmERF5* transgenic lines and WT plants (Figures 4 and 7). In addition, the other pathways related to waterlogging were also enriched, including the methionine biosynthetic process, nitric oxide biosynthetic process and

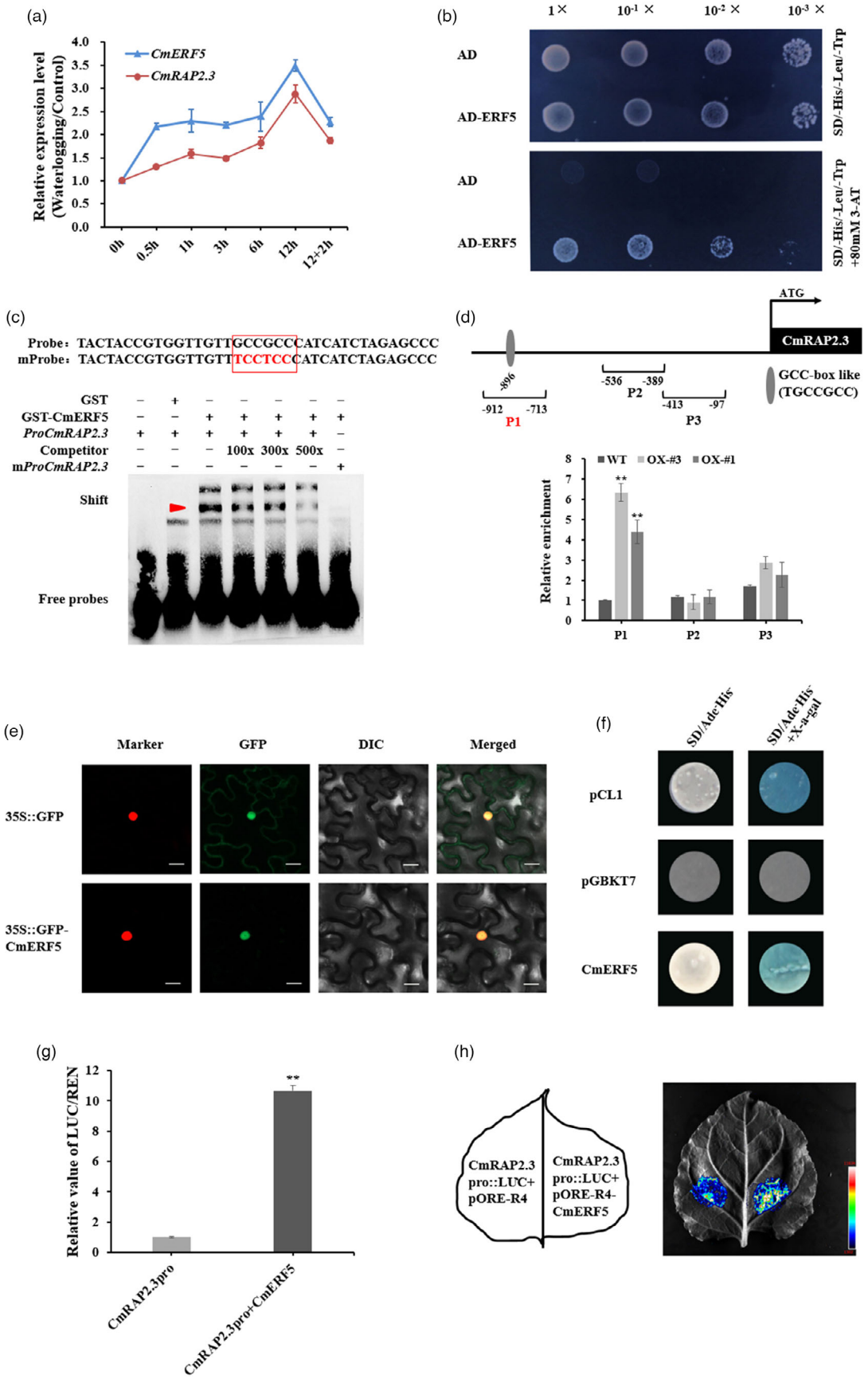
(a)
Relative expression level (Waterlogging/Control)
CmERF5
CmRAP2.3
0h
0.5h
1h
3h
6h
12h
12+2h
(b)
1×
10-1 ×
10-2 ×
10-3 ×
AD
AD-ERF5
SD/-His/-Leu/-Trp
SD/-His/-Leu/-Trp +80mM 3-AT
(c)
Probe: TACTACCGTGGTTGTTGCCGCCCATCATCTAGAGCCC
mProbe: TACTACCGTGGTTGTTTCCTCCCATCATCTAGAGCCC
GST
GST-CmERF5
ProCmRAP2.3
Competitor
mProCmRAP2.3
100x 300x 500x
Shift
Free probes
(d)
ATG
CmRAP2.3
GCC-box like (TGCCGCC)
-896
-912 -713
P1
-536 -389
P2
-413 -97
P3
WT OX-#3 OX-#1
Relative enrichment
P1
P2
P3
(e)
Marker
GFP
DIC
Merged
35S::GFP
35S::GFP-CmERF5
(f)
SD/Ade-His-
SD/Ade-His- +X-a-gal
pCL1
pGBKT7
CmERF5
(g)
Relative value of LUC/REN
CmRAP2.3pro
CmRAP2.3pro+CmERF5
(h)
CmRAP2.3 pro::LUC+ pORE-R4
CmRAP2.3 pro::LUC+ pORE-R4-CmERF5

Figure 5 CmERF5 binds to the *CmRAP2.3* promoter and activates the *CmRAP2.3* expression. (a) *CmERF5* and *CmRAP2.3* relative expression levels after 0, 0.5, 1, 3, 6 and 12 h of waterlogging stress and re-oxygenation for 2 h. (b) CmERF5 binding to the *CmRAP2.3* promoter in the yeast one-hybrid assay. AD: the pGADT7 vector and pHIS2-*CmRAP2.3pro* combination in Y187 cells (negative control). AD-ERF5: the pGADT7-*ERF5* and pHIS2-*CmRAP2.3pro* combination in Y187 cells. (c) CmERF5 binding to the *CmRAP2.3* promoter in EMSA assay. From left to right: biotin-labelled probe; GST protein and biotin-labelled probe; GST-CmERF5 protein and biotin-labelled probe; GST-CmERF5 protein and biotin-labelled probe with 100× unlabelled probe; GST-CmERF5 protein and biotin-labelled probe with 300× unlabelled probe; GST-CmERF5 protein and biotin-labelled robe with 500× unlabelled probe and GST-CmERF5 protein and mutant probe. (d) CmERF5 binding to the *CmRAP2.3* promoter in ChIP-PCR assay. TGCCGCC: a combination of components (GCC-box like) in the Cm*RAP2.3* promoter; P1–P3: various segments of the promoter sequence, of which P1 contain the GCC box like. (e) Subcellular localization of CmERF5 in tobacco leaves. The co-expressed 35S::D53-RFP construct was used as a nuclear marker. Bars = 20 μm. (f) The CmERF5 transcriptional activity in yeast. SD/Ade-His-: SD medium lacking adenine and histidine; SD/Ade-His- + X-α-gal: SD/Ade-His- medium containing 20 mg/mL X-α-gal. (g) Luciferase assay in Arabidopsis protoplasts. Luciferase/Renilla (LUC/REN) ratio of the mixture of CmRAP2.3pro-0800-LUC and R4 vector was used as the control. (h) Luciferase assay in tobacco leaves. Student's *t*-test determined significant differences between relative expression levels at $^{**}P < 0.01$.

glycolytic process (Figure S2b). The result showed that *CmRAP2.3* probably modulated various pathways to deal with the adversities associated with waterlogging stress, and the ROS pathway played a leading role. *AtRAP2.3* (Papdi *et al*., 2015) and *AdRAP2.3* (Pan *et al*., 2019) mainly affected the *alcohol dehydrogenase-1* gene in response to waterlogging stress. The different *RAP2.3* mechanisms between chrysanthemum and *Arabidopsis* may be associated with the low similarity in AA sequences (38.20%; Figure 1). The results indicate that the ERF-VII homologous genes of different species might be involved in the waterlogging response via a different pathway and reveal the function of *CmERF5*. Overall, it provides the basis for understanding the mechanism of chrysanthemum waterlogging tolerance, important for the molecular breeding of tolerant chrysanthemum lines.

CmERF5 directly activates the *CmRAP2.3* at the transcriptional level

A previous study showed that transcription factors of the ERF-VII family, including *RAP2.3*, were degraded in the N-degron pathway as previously described (Bailey-Serres *et al*., 2012). Furthermore, *RAP2.2* was the direct target of *WRKY33* and *WRKY12* in *Arabidopsis* (Tang *et al*., 2021); however, the upstream regulators of ERF-VII factors remain unknown. To analyse the regulatory relationship of *CmRAP2.3* at the transcriptional level, the *CmRAP2.3* promoter was used for screening in the yeast one-hybrid screening assay, and *CmERF5* was subsequently screened. The EMSA and ChIP-PCR results showed that CmERF5 binds to the GCC-like motif in the *CmRAP2.3* promoter region (Figure 5c,d) at a similar binding site to other ERF family factors (Fujimoto *et al*., 2000).

ERF is one of the most prominent families of transcription factors and plays a significant role in response to abiotic and biotic stress. Additionally, the correlation between the different ERF factors, including protein–protein and protein–DNA interactions, can effectively regulate abiotic and biotic stresses in plants. For example, the interaction between ORA59 and RAP2.3 enhanced the response to ethylene in *Arabidopsis* (Kim *et al*., 2018). Additionally, AtERF95 interacts with AtERF97 to enhance heat stress tolerance (Huang *et al*., 2021). In transgenic soybean, drought tolerance was improved by the interaction between GmDREB1 and GmERF008 or GmERF106 (Chen *et al*., 2022). However, the protein–DNA interactions among different ERF factors have not been explored.

In this study, the analysis of the relationship between *CmERF5 and CmRAP2.3* indicates that CmERF5 binds to the *CmRAP2.3* promoter and activates its gene expression (Figure 5), providing an example of protein–DNA interaction between genes of the ERF family (Figure 8). Furthermore, the CmERF5-CmRAP2.3 transcriptional cascade paves the road for investigating other ERF-VII factors at the transcriptional level.

Methods

Plant materials and waterlogging treatment

A common commercial disbud-type chrysanthemum cultivar 'Jinba' susceptible to waterlogging was used in this study. The transgenic and WT plants were preserved at the Chrysanthemum Germplasm Resource Preserving Center, Nanjing Agricultural University, Nanjing, China. The waterlogging treatment methods used in the experiment were as described by Su *et al*. (2016). Briefly, rooted cuttings with eight to ten leaves were subjected to waterlogging stress by submerging them in water up to 3 cm above the soil surface. After continuous waterlogging treatment for 8 days, water was drained and plants were kept under normal growth conditions. The experiment was conducted in a greenhouse with a 16 h light (25°C) and 8 h darkness (18°C) photoperiod.

Isolation and sequence analysis

The *CmRAP2.3* and *CmERF5* ORF sequences were amplified using primer pairs CmRAP2.3-F/-R and CmERF5-F/-R (Table S3) using the reverse transcription amplification as a template. Additionally, the amplicons were inserted into the pMD19-T vector (Takara Bio, Tokyo, Japan) for sequencing.

We used DNAMAN 5.2.2 software to perform multiple-sequence alignment among the RAP2.3/ERF5 protein homologues obtained from the GenBank (https://www.ncbi.nlm.nih.gov). A neighbour-joining phylogeny tree was constructed using MEGA 7.0 software with 1000 bootstrap replications.

Subcellular localization

Agrobacterium injection was carried out to transform the combinations of p35S::GFP-*CmRAP2.3*(CmRAP2.3-R4) and 35S::D53-RFP constructs (nuclear markers) into tobacco leaves. The pORE-R4 vector (TAIR: CD3-932) and nuclear marker mixture were used as controls. Furthermore, confocal microscopy was performed to observe GFP activity between 48 and 72 h. The CmERF5 subcellular localization was identified by repeating the above procedure. Primer pairs used are listed in Table S3.

Transcriptional activity analysis

The *CmRAP2.3* full-length ORF and CmRAP2.3ΔC (1–146 aa) and CmRAP2.3ΔN (83–234 aa) were inserted into the pGBKT7 vector (Waryong, Beijing, China; Table S3). These plasmids were used to transform into Y2H yeast cells in a synthetic defined medium without tryptophan, including the negative control

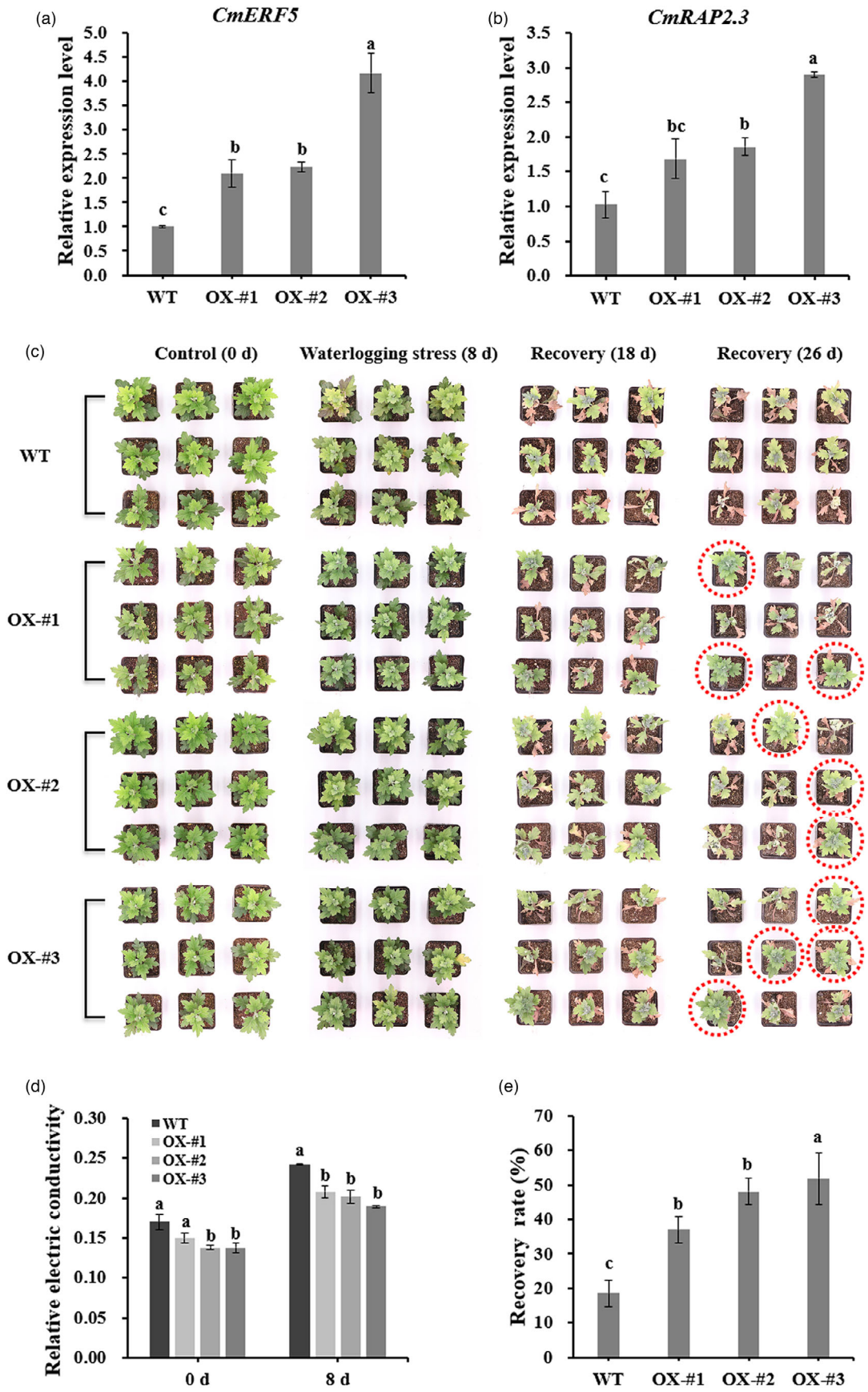
(a)
CmERF5
Relative expression level
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c
b
b
a
WT
OX-#1
OX-#2
OX-#3
(b)
CmRAP2.3
Relative expression level
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c
bc
b
a
WT
OX-#1
OX-#2
OX-#3
(c)
Control (0 d)
Waterlogging stress (8 d)
Recovery (18 d)
Recovery (26 d)
WT
OX-#1
OX-#2
OX-#3
(d)
Relative electric conductivity
0.30
0.25
0.20
0.15
0.10
0.05
0.00
WT
OX-#1
OX-#2
OX-#3
a
a
b
b
a
b
b
b
0 d
8 d
(e)
Recovery rate (%)
70
60
50
40
30
20
10
0
c
b
b
a
WT
OX-#1
OX-#2
OX-#3

Figure 6 The phenotype of *CmERF5* overexpression lines under waterlogging and recovery. (a) *CmERF5* relative expression levels in *CmERF5*-OX (OX-#1, OX-#2 and OX-#3) lines and WT plants. (b) *CmRAP2.3* relative expression levels in *CmERF5*-OX lines and WT plants. (c) Phenotype observation of *CmERF5*-OX lines and WT plants after 8 days of waterlogging stress and 18 and 26 days of recovery. The plants sprouted new leaves in red circles. (d) The relative electric conductivity of *CmERF5*-OX lines and WT plants on the 0 and 8th days of waterlogging stress. (e) The recovery rate of *CmERF5*-OX lines and WT plants on the 26th day of recovery. The data shown in the figure are presented as mean ± standard errors ($n = 3$). Letters above the bar indicate significant differences at $P < 0.05$ according to Duncan's multiple-range test. Nine plants were used per replicate.

(pGBKT7 empty vector). The yeast cells transformed with pCL1 (positive control) were cultivated on a synthetic defined medium without leucine. After 3 days at 30°C, the yeast cells were cultured on the SD/Ade-His- medium and SD/Ade-His- supplemented with 20 mg/mL X-α-Gal. Growth and colour changes of Y2H yeast cells in the medium were observed to determine the transcriptional activity of the plasmids. The above assay was repeated to analyse the transcriptional activity of full-length ORF for *CmERF5*.

Chrysanthemum transformation and phenotype analysis

To obtain the transgenic lines, the pORE-R4-CmRAP2.3/CmERF5 plasmid was transformed into *Agrobacterium* (*EHA105*) for chrysanthemum transformation using the Agrobacterium-mediated leaf disc transformation method as previously described (Guan *et al.*, 2021). A rapid plant genomic DNA Isolation kit (Sangon Biotech, Shanghai, China) was used to extract the DNA of transgenic lines, and the primer pair 35S-F/gene-R was used to detect positive plants (Table S3). The newly generated transgenic plants were transferred from MS medium to the soil, and cultivated in a greenhouse till 15–20 fully expanded leaf stage. These plants were propagated by stem cuttings. Briefly, the new cuttings were induced by removing the apexes. Then, the cuttings were collected and cultivated to the 8- to 10-leaf stage, which was further subjected to waterlogging stresses by partially submerging them into water. RT-qPCR was performed to identify the relative expression levels using the primer pairs listed in Table S3.

The phenotype images of overexpressing lines and WT plants were taken on the 0 and 8th days after waterlogging stress and the 18th day of recovery. Recovery images of the *CmERF5*-OX lines and WT plants were taken on the 26th day to distinguish between tolerant and susceptible lines. Leaf yellowing rate of *CmRAP2.3*-OX and WT was calculated on the 8th day of

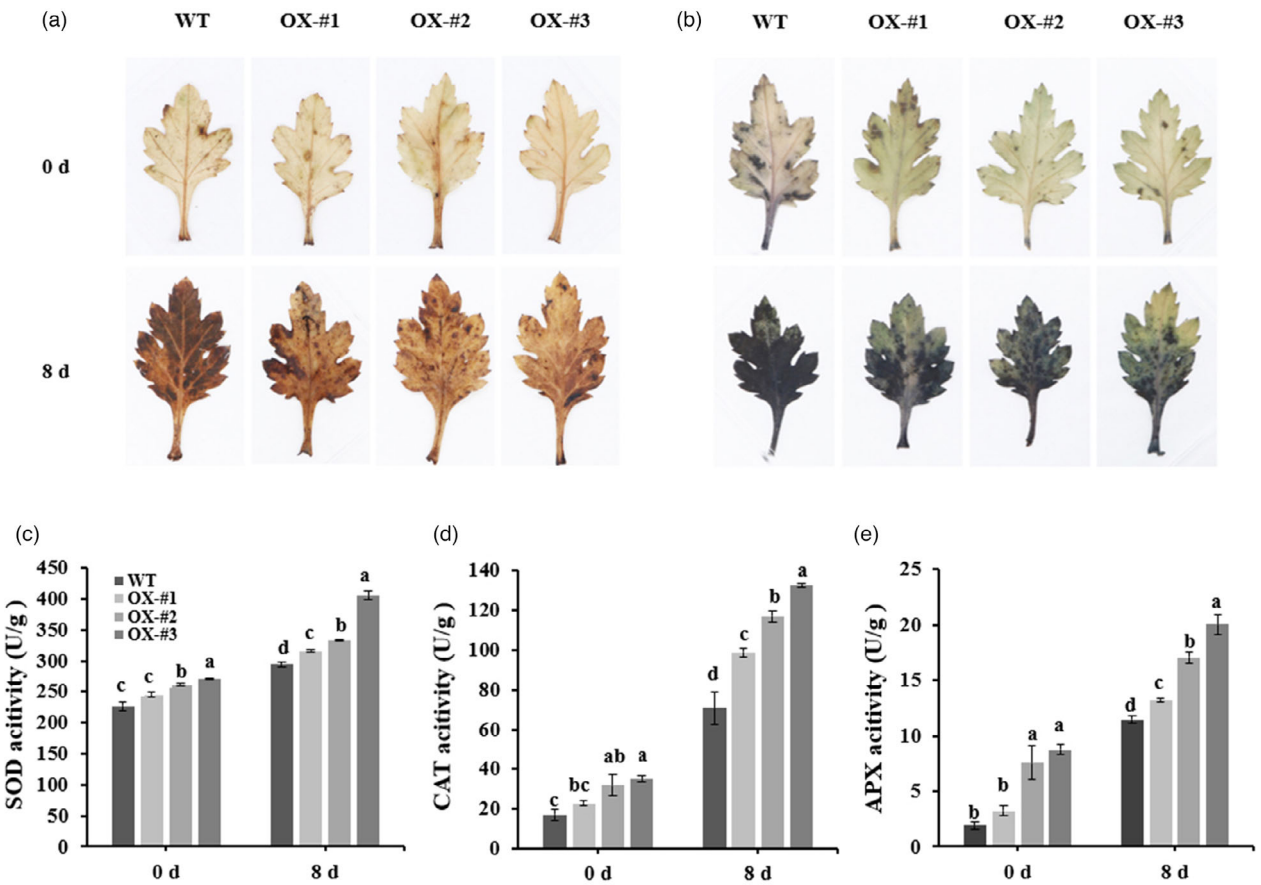

Figure 7 ROS levels and scavenger activities in WT and *CmERF5*-OX lines. (a) Diaminobenzidine staining. (b) Nitro tetrazolium blue chloride staining. (c–e) Reactive oxygen species scavenger (superoxide dismutase, catalase and ascorbate peroxidase) activities in leaves of WT and *CmERF5*-OX lines. Significant differences between ROS levels and scavenger activities in WT and *CmERF5*-OX lines were analysed by Duncan's multiple-range test at $P < 0.05$ and values were presented as mean ± standard errors ($n = 3$).

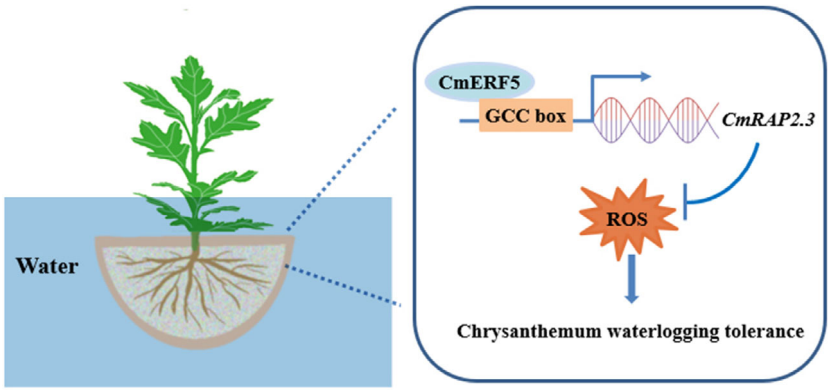

Figure 8 Schematic model of the role of CmERF5-CmRAP2.3 transcriptional cascade in the modulation of chrysanthemum waterlogging tolerance.

waterlogging stress. The recovery rate was the percentage of the plants which sprouted new leaves in the total plants in each treatment. Nine plants were used per replicate.

Relative electric conductivity was evaluated on the 0 and 8th days of waterlogging stress as previously described (Tang *et al.*, 2021). Briefly, before waterlogging, a hole puncher was used to collect small rounds of leaves from all seedlings, and they were placed in tubes with 5 mL of deionized water. Next, the electric conductivity was measured by oscillating on the oscillator for 4 h (recording as R1). Finally, the solutions were boiled for 30 min, the electric conductivity was re-measured (R2) and the R1/R2 ratio was subsequently obtained.

RNA extraction, transcriptome sequencing and bioinformatic analysis

The root samples were collected when transgenic (OX-*CmRAP2.3*-#1) and wild-type (WT) plants were grown to eight- to ten-leaf-old. Each sample contained three biological replicates. RNA was extracted using the plant RNA Isolation kit (Waryong) following the manufacturer's instructions. The RNA was subjected to an Illumina HiSeq™2000 instrument located at the Beijing Genomics Institute (Shenzhen, China; http://www.genomics.cn/index) for sequencing after strict quality testing. Unigene annotation was based on seven functional databases: KEGG, GO, NR, NT, SwissPro, Pfam, KOG and TransDecoder were used to identify candidate CDS regions. In the present analysis, a *Q*-value < 0.05 and |log2 Ratio| ≥ 0.5 were regarded as the criteria for differential genes and fold-change calculation method. Gene ontology (GO) enrichment analysis of the annotated DEGs was performed on the BGI Interactive Reporting System (https://report.bgi.com/ps/login/login.html).

ROS analysis of chrysanthemum plants

Leaves (third from the apex) of chrysanthemum plants were harvested after 0 and 8 days of waterlogging stress for subsequent analysis. The DAB and NBT staining methods were used to observe the ROS production by comparing the stained regions of H_2O_2 and O_2^- accumulation. Briefly, the leaves were immersed in DAB solution (1 mg/mL; pH = 3.8) for 12 h in the dark and in the NBT solution (1 mg/mL; pH = 7.8) for 8 h in the light. The leaves were boiled in 95% ethanol solution for 15 min for initial depigmentation. They were then immersed in 80% ethanol solution and boiled for 15 min and this step was repeated thrice. Next, the leaves were placed in 100% ethanol solution at 4 °C for subsequent imaging. The Commercial Assay kits (Nanjing Jiancheng, Nanjing, China) were used to measure ROS content (H_2O_2 and O_2^-) and SOD, CAT and APX activities, according to the manufacturer's instructions. Each experiment was replicated thrice.

EMSA

The *CmERF5* full-length ORF was constructed into pGEX4T-1 (Waryong; Table S3) and transformed into *Escherichia coli* BL21 (DE3). The protein samples were incubated with 1 mM isopropyl β-D-1-thiogalactopyranoside at 37°C for 6 h and purified using glutathione magnetic (GST) beads (Promega, Madison, WI). An EMSA Probe Biotin Labeling kit (Beyotime, Shanghai, China) was used to label the primer pairs (Table S3). Furthermore, a lightShift™ Chemiluminescent EMSA kit (Thermo Fisher Scientific, Waltham, MA) was used to verify the GST-CmERF5 protein and CmRAP2.3pro interaction.

ChIP-PCR assays

ChIP-PCR assays were performed as previously described (Zhou *et al.*, 2021). Briefly, *35 S::CmERF5*-GFP transgenic chrysanthemum plants (OX-#1 and OX-#3) were subjected to ChIP-PCR assays using Pierce™ ChIP-grade Protein A/G Magnetic Beads (Thermo, Shanghai, China). GFP recombinant rabbit monoclonal antibodies (Thermo) were used. Subsequently, the enriched DNA fragments were examined via RT-qPCR assays using the primer pairs P1, P2 and P3 (Table S3).

Yeast one-hybrid screening

The *CmRAP2.3* (924 bp) promoter was cloned and constructed into the pHIS2 vector (Waryong; Table S3). The pHIS2-CmRAP2.3pro plasmid was used to screen the chrysanthemum yeast library and co-transformed in the *Saccharomyces cerevisiae* Y187 strain. Yeast cells were plated on the SD/-His/-Leu/-Trp medium with 80 mM 3-amino-1,2,4-triazole (3-AT) for subsequent selection and sequencing. BLAST program was used to conduct a homology search of the obtained sequences. Transcription factors (TFs) listed in Table S2 were selected and cloned into the pGADT7 vector (Waryong; AD-TFs; Table S3). Furthermore, the AD-TFs and pHIS2- CmRAP2.3pro were co-expressed in Y187 in the SD/-His/-Leu/-Trp medium at 30°C for 3 days. Yeast cells harbouring AD vector and pHIS2-CmRAP2.3pro were used as the negative control. These yeast cells were incubated in SD/-His-Leu-Trp medium with 80 mM 3-AT at 30°C for 3 days.

Luciferase assays

The primer pair CmRAP2.3pro-F/R (Table S3) was used to clone the *CmRAP2.3pro* and construct the pGreenII0800-LUC-CmRAP2.3pro (CmRAP2.3pro-0800-LUC) plasmid. According to the method from a previous study (Wang *et al*., 2022), a Tanon 5200 multi-imaging apparatus (Tanon, Shanghai, China) was used to observe the luciferase activity in tobacco leaves co-transformed with different plasmid combinations.

LUC/REN ratios were determined by co-transforming CmRAP2.3pro-0800-LUC and CmERF5-R4 plasmids into protoplasts isolated from *Arabidopsis*. The mixture of CmRAP2.3pro-0800-LUC and R4 vector was treated as a negative control. A Dual-Luciferase Reporter Gene Assay kit (YEASEN, Shanghai, China) was used to measure the LUC/REN ratios, following the manufacturer's instructions. All experiments were performed thrice.

Quantitative RT-PCR analysis

To analyse the expression patterns of *CmRAP2.3* and *CmERF5* in different tissues, roots, leaves and stems were harvested at the same stage as the stage when treatments were applied. Then, the roots of WT plants after 0, 0.5, 1, 3, 6 and 12 h of waterlogging stress and 2 h of re-oxygenation were harvested. Re-oxygenation means that the water was removed and the plants were kept under normal growth conditions. Each experiment had three biological replicates. Total RNA was extracted from the above samples and subjected to RT-qPCR analysis. Primers are listed in Table S3.

Statistical analysis

SPSS v19.0 software (SPSS Inc., Chicago, IL) was used for statistical analyses. Duncan's multiple-range test was used to assess significant differences in different tissues, transgenic lines and waterlogging responses at $P < 0.05$. In addition, Student's *t*-test was used to analyse the significant differences in LUC/REN ratios of the *Arabidopsis* protoplasts, which co-transformed different plasmids. The levels of significance were used as $*P < 0.05$ and $**P < 0.01$. All data were presented as mean ± standard errors.

Accession numbers

Sequencing data of this study can be found at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/bioproject/) with the BioProject ID PRJNA861821.

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Conflicts of interest

No conflicts of interest declared.

Author contributions

F.C., J.J. and S.C. supervised the project. F.C. and C.L. conceived and designed the experiments. C.L., J.S., N.Z., L.L., X.O. and Y.Y. performed the experiments. C.L. wrote the study. F.C. and L.W. revised the manuscript. All authors read and approved the final article.

References

Arvin, M. and Donnelly, D. (2008) Screening potato cultivars and wild species to abiotic stresses using an electrolyte leakage bioassay. *J. Agric. Sci. Technol.* **10**, 33–42.

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

Buchanan-Wollaston, V., Earl, S., Harrison, E., Mathas, E., Navabpour, S., Page, T. and Pink, D. (2003) The molecular analysis of leaf senescence–a genomics approach. *Plant Biotechnol. J.* **1**, 3–22.

Chen, K., Tang, W., Zhou, Y., Chen, J., Xu, Z., Ma, R., Dong, Y. *et al*. (2022) AP2/ERF transcription factor *GmDREB1* confers drought tolerance in transgenic soybean by interacting with GmERFs. *Plant Physiol. Biochem.* **170**, 287–295.

Dubois, M., Skirycz, A., Claeys, H., Maleux, K., Dhondt, S., De Bodt, S., Vanden Bossche, R. *et al*. (2013) Ethylene Response Factor6 acts as a central regulator of leaf growth under water-limiting conditions in Arabidopsis. *Plant Physiol.* **162**, 319–332.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell*, **12**, 393–404.

Fukao, T. and Bailey-Serres, J. (2008) Submergence tolerance conferred by *Sub1A* is mediated by SLR1 and SLRL1 restriction of gibberellin responses in rice. *Proc. Natl Acad. Sci. USA*, **105**, 16814–16819.

Guan, Y., Ding, L., Jiang, J., Shentu, Y., Zhao, W., Zhao, K., Zhang, X. *et al*. (2021) Overexpression of the *CmJAZ1-like* gene delays flowering in *Chrysanthemum morifolium*. *Hortic. Res.* **8**, 87.

Hao, D., Ohme-Takagi, M. and Sarai, A. (1998) Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. *J. Biol. Chem.* **273**, 26857–26861.

Huang, J., Zhao, X., Bürger, M., Wang, Y. and Chory, J. (2021) Two interacting ethylene response factors regulate heat stress response. *Plant Cell*, **33**, 338–357.

Kim, N.Y., Jang, Y.J. and Park, O.K. (2018) AP2/ERF family transcription factors ORA59 and RAP2.3 interact in the nucleus and function together in ethylene responses. *Front. Plant Sci.* **9**, 1675.

Li, Y., Wang, L., Sun, G., Li, X., Chen, Z., Feng, J. and Yang, Y. (2021) Digital gene expression analysis of the response to *Ralstonia solanacearum* between resistant and susceptible tobacco varieties. *Sci. Rep.* **11**, 3887.

Licausi, F., Kosmacz, M., Weits, D.A., Giuntoli, B., Giorgi, F.M., Voesenek, L.A., Perata, P. *et al*. (2011) Oxygen sensing in plants is mediated by an N-end rule pathway for protein destabilization. *Nature*, **479**, 419–422.

Liu, J., Chen, Y., Wang, W.Q., Liu, J.H., Zhu, C.Q., Zhong, Y.P., Zhang, H.Q. *et al*. (2022) Transcription factors AcERF74/75 respond to waterlogging stress and trigger alcoholic fermentation-related genes in kiwifruit. *Plant Sci.* **314**, 111115.

Loreti, E., van Veen, H. and Perata, P. (2016) Plant responses to flooding stress. *Curr. Opin. Plant Biol.* **33**, 64–71.

Luan, H., Guo, B., Shen, H., Pan, Y., Hong, Y., Lv, C. and Xu, R. (2020) Overexpression of barley transcription factor *HvERF2.11* in Arabidopsis enhances plant waterlogging tolerance. *Int. J. Mol. Sci.* **21**, 1982.

Nakano, T., Suzuki, K., Fujimura, T. and Shinshi, H. (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiol.* **140**, 411–432.

Ogawa, T., Pan, L., Kawai-Yamada, M., Yu, L.H., Yamamura, S., Koyama, T., Kitajima, S. *et al*. (2005) Functional analysis of Arabidopsis ethylene-responsive element binding protein conferring resistance to Bax and abiotic stress-induced plant cell death. *Plant Physiol.* **138**, 1436–1445.

Pan, D.L., Wang, G., Wang, T., Jia, Z.H., Guo, Z.R. and Zhang, J.Y. (2019) *AdRAP2.3*, a novel ethylene response factor VII from *Actinidia deliciosa*, enhances waterlogging resistance in transgenic tobacco through improving expression levels of *PDC* and *ADH* genes. *Int. J. Mol. Sci.* **20**, 1189.

Papdi, C., Pérez-Salamó, I., Joseph, M.P., Giuntoli, B., Bögre, L., Koncz, C. and Szabados, L. (2015) The low oxygen, oxidative and osmotic stress responses synergistically act through the ethylene response factor VII genes *RAP2.12*, *RAP2.2* and *RAP2.3*. *Plant J.* **82**, 772–784.

Phukan, U.J., Jeena, G.S., Tripathi, V. and Shukla, R.K. (2018) MaRAP2-4, a waterlogging-responsive ERF from *Mentha*, regulates bidirectional sugar transporter *AtSWEET10* to modulate stress response in *Arabidopsis*. *Plant Biotechnol. J.* **16**, 221–233.

Shi, W., Song, Y., Liu, T., Ma, Q., Yin, W., Shen, Y., Liu, T. *et al*. (2021) StRAP2.3, an ERF-VII transcription factor, directly activates *StInvInh2* to enhance cold-induced sweetening resistance in potato. *Hortic. Res.* **8**, 82.

Su, J., Zhang, F., Li, P., Guan, Z., Fang, W. and Chen, F. (2016) Genetic variation and association mapping of waterlogging tolerance in chrysanthemum. *Planta*, **244**, 1241–1252.

Tang, H., Bi, H., Liu, B., Lou, S., Song, Y., Tong, S., Chen, N. *et al*. (2021) WRKY33 interacts with WRKY12 protein to up-regulate *RAP2.2* during submergence induced hypoxia response in *Arabidopsis thaliana*. *New Phytol.* **229**, 106–125.

Wang, L., Gao, J., Zhang, Z., Liu, W., Cheng, P., Mu, W., Su, T. *et al*. (2020) Overexpression of *CmSOS1* confers waterlogging tolerance in *Chrysanthemum*. *J. Integr. Plant Biol.* **62**, 1059–1064.

Wang, L., Dossa, K., You, J., Zhang, Y., Li, D., Zhou, R., Yu, J., et al. (2021) High-resolution temporal transcriptome sequencing unravels ERF and WRKY as the master players in the regulatory networks underlying sesame responses to waterlogging and recovery. *Genomics*, **113**, 276–290.

Wang, Y., Zhou, L.J., Wang, Y., Geng, Z., Liu, S., Chen, C., Chen, S. *et al*. (2022) CmMYB9a activates floral coloration by positively regulating anthocyanin biosynthesis in chrysanthemum. *Plant Mol. Biol.* **108**, 51–63.

Wrzaczek, M., Brosché, M. and Kangasjärvi, J. (2013) ROS signaling loops – production, perception, regulation. *Curr. Opin. Plant Biol.* **16**, 575–582.

Yin, D., Chen, S., Chen, F., Guan, Z. and Fang, W. (2009) Morphological and physiological responses of two chrysanthemum cultivars differing in their tolerance to waterlogging. *Environ. Exp. Bot.* **67**, 87–93.

Yin, D., Sun, D., Han, Z., Ni, D., Norris, A. and Jiang, C.Z. (2019) *PhERF2*, an ethylene-responsive element binding factor, plays an essential role in waterlogging tolerance of petunia. *Hortic. Res.* **6**, 83.

Yu, F., Liang, K., Fang, T., Zhao, H., Han, X., Cai, M. and Qiu, F. (2019) A group VII ethylene response factor gene, *ZmEREB180*, coordinates waterlogging tolerance in maize seedlings. *Plant Biotechnol. J.* **17**, 2286–2298.

Yuan, L.B., Dai, Y.S., Xie, L.J., Yu, L.J., Zhou, Y., Lai, Y.X., Yang, Y.C. *et al*. (2017) Jasmonate regulates plant responses to postsubmergence reoxygenation through transcriptional activation of antioxidant synthesis. *Plant Physiol.* **173**, 1864–1880.

Zhao, N., Li, C., Yan, Y., Cao, W., Song, A., Wang, H., Chen, S. *et al*. (2018) Comparative transcriptome analysis of waterlogging-sensitive and waterlogging-tolerant *Chrysanthemum morifolium* cultivars under waterlogging stress and reoxygenation conditions. *Int. J. Mol. Sci.* **19**, 1455.

Zhou, L.J., Geng, Z., Wang, Y., Wang, Y., Liu, S., Chen, C., Song, A. *et al*. (2021) A novel transcription factor CmMYB012 inhibits flavone and anthocyanin biosynthesis in response to high temperatures in chrysanthemum. *Hortic. Res.* **8**, 248.

Zhu, T., Zou, L., Li, Y., Yao, X., Xu, F., Deng, X., Zhang, D. *et al*. (2018) Mitochondrial alternative oxidase-dependent autophagy involved in ethylene-mediated drought tolerance in *Solanum lycopersicum*. *Plant Biotechnol. J.* **16**, 2063–2076.

Supporting information

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

Figure S1 Analysis of *CmRAP2.3* overexpressing lines (*CmRAP2.3*-OX lines).
Figure S2 RNA-seq analysis of WT and OX-*CmRAP2.3* plants.
Figure S3 *CmRAP2.3* promoter with marked GCC-like motif in yellow.
Figure S4 The yeast one-hybrid assay verified screened transcriptional factors (TFs).
Figure S5 CmRAP2.3 does not directly bind to the promoter of *CmERF5*.
Figure S6 Sequence alignments and phylogenetic analysis of *CmERF5*.
Figure S7 Analysis of *CmERF5* overexpressing lines (*CmERF5*-OX lines).
Table S1 DEGs related to oxidative stress processes between the wild-type and OX-*CmRAP2.3* line#1.
Table S2 Transcriptional factors listed via Y1H library screening with the *CmRAP2.3* promoter.
Table S3 Names of primer and sequences used in this study.

