



The pepper MAP4K kinase CaMAP4K3 negatively regulates drought resistance

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ABSTRACT

Mitogen-activated protein kinase (MAPK) cascades are crucial in plant responses to various stresses. While the positioning of MAP4Ks within the canonical MAPK signaling module in plants remains controversial, emerging research continues to shed light on their functional roles. However, information on the MAP4K gene family in pepper (*Capsicum annuum*) is still limited. In this study, seven putative MAP4K genes (designated as CaMAP4K1–CaMAP4K7) were identified from the pepper genome, each containing a conserved serine/threonine kinase domain. These genes were differentially expressed across various pepper organs, with CaMAP4K3 exhibiting consistently high expression in all organs and significant induction under drought stress. Kinase assays revealed that CaMAP4K3 is an active kinase whose activity is enhanced by drought and salt stress. Functional studies showed that silencing CaMAP4K3 enhanced drought resistance in pepper plants, reducing transpirational water loss and increasing leaf temperatures. Conversely, CaMAP4K3 overexpression in tobacco and Arabidopsis reduced drought tolerance, as evidenced by increased wilting and transpirational water loss. Additionally, CaMAP4K3-overexpressing Arabidopsis plants exhibited reduced sensitivity to abscisic acid (ABA) during seed germination and seedling growth. Collectively, these results suggest that CaMAP4K3 impairs drought resistance in pepper plants and potentially affects seed germination and seedling growth through the regulation of ABA signaling.

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1. Introduction

The mitogen-activated protein kinase (MAPK) cascade is a critical signaling pathway in plants that regulates responses to various biotic and abiotic stresses, as well as key processes in growth and development [1–4]. Drought is one of the most significant abiotic stresses affecting crop productivity worldwide, leading to substantial yield losses due to water deficit and associated physiological changes, including stomatal closure and altered transpiration rates. The ability of plants to sense and respond to drought stress is critical for their survival and adaptation. Extensive studies have shown that MAPK pathways play pivotal roles in plant responses to drought stress [5–7]. As a member of the Solanaceae family, pepper (*Capsicum annuum*) is an economically important vegetable crop cultivated globally; however, it is known to be highly sensitive to water deficit, particularly that triggered by low temperatures, salinity, and drought [8–10]. Understanding the molecular mecha-

nisms underlying drought tolerance, particularly those involving the MAPK pathway, is crucial for improving pepper crop resilience.

The MAPK cascade typically comprises at least three sequential kinases: MAPK (also known as MPK), MAPKK (MAP2K/MKK), and MAPKKK (MAP3K) [11]. In this pathway, the MAP4K family sometimes serves as upstream activators of MAP3Ks, initiating a series of phosphorylation events that ultimately influence the activity of MAPKs. Since the discovery of the kinase Sterile 20 protein (Ste20p) in yeast (*Saccharomyces cerevisiae*) as a MAP4K that phosphorylates MAP3K Ste11 in the mating signaling pathway [12,13], Ste20p-like MAP4K genes have been identified across various organisms, including mammals, fruit fly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), and human [14]. For instance, mammalian Ste20p homologs, such as hematopoietic progenitor kinase 1 (HPK1) [15,16] and p21-activated kinase 2 (PAK2) [17], have been shown to activate the MAP3Ks MEKK1 and Raf-1, respectively, through direct phosphorylation. In plants, following the first identification of MAP4Ks in *Brassica napus* [18], MAP4K genes have been identified in various plant species [19], including *Arabidopsis thaliana* [11], *Salvia miltiorrhiza* [20], *Triticum aestivum* [21], *Vitis vinifera* [22], and *Zea mays* [23]. Notably, the Arabidopsis

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genome contains 10 MAP4K genes, which are classified into three clades (I, II, and III), with clade III further divided into four subclades (IIIA, IIIB, IIIC, and IIID) [19]. However, little is known about MAP4K genes and their roles in pepper plants, and to date, no evidence has suggested the MAP4K-mediated phosphorylation of MAP3Ks in plants.

Recently, MAP4Ks have attracted attention in plant biology due to their involvement in various biological processes, including development and stress signaling. In Arabidopsis, BLUE LIGHT SIGNALING1 (BLUS1)/MAP4K10 plays a crucial role in facilitating blue-light-triggered stomatal opening following phosphorylation by phototropins [24]. Meanwhile, MAP4K4, MAP4K7, and MAP4K8 are reportedly expressed in stomatal cells; nevertheless, their precise functions remain to be elucidated [25]. SERINE/THREONINE KINASE 1 (SIK1)/MAP4K3 is critical for cell proliferation and expansion during organ growth [26]. Alongside MAP4K4, it plays key roles in flg22-triggered immunity by modulating the immune signaling component BOTRYTIS-INDUCED KINASE1 (BIK1) [27]. Furthermore, TARGET OF TEMPERATURE 3 (TOT3)/MAP4K4, in conjunction with TOT3-INTERACTING PROTEIN 4 (TOI4)/MAP4K6 and TOI5/MAP5K5, modulate thermomorphogenesis in Arabidopsis and wheat [28]. Despite the evolutionary conservation of MAP4K genes across plant species, their functional roles, particularly in drought stress responses, remain poorly understood in pepper plants.

In this study, we focused on isolating and characterizing MAP4K genes from the pepper genome, with a particular emphasis on their involvement in responses to drought stress. We identified seven putative pepper MAP4K genes (designated as CaMAP4K1 to CaMAP4K7) and characterized their expression patterns in response to drought stress. Among these, we focused on CaMAP4K3, which exhibited high expression levels in various plant organs and significant induction under drought conditions. Through a series of functional analyses, we explored the role of CaMAP4K3 in modulating drought resistance in pepper plants. Our findings suggest that CaMAP4K3 acts as a negative regulator of drought resistance, with its kinase activity being essential for this modulation.

2. Materials and methods

2.1. Plant materials and growth conditions

In this study, pepper (*C. annuum* L. 'Nockwang'), tobacco (*Nicotiana benthamiana*), and Arabidopsis (*A. thaliana*, ecotype Columbia-0) were used. For germination, the seeds of pepper and tobacco plants were sown in steam-sterilized compost soil mix containing peat moss, perlite, and vermiculite (5:3:2, v/v/v), as well as sand and loam soil (1:1:1, v/v/v). Arabidopsis seeds were sown on Murashige and Skoog (MS) salt (Duchefa Biochemie, Haarlem, Netherlands) supplemented with 1% sucrose and 0.8% Microagar (Duchefa Biochemie). The plates were incubated at 4 °C for 2 d to synchronize germination and then moved to the growth room. All plant seedlings were grown in a growth room at 24 ± 1 °C under white fluorescent light (130 μmol photons m⁻² s⁻¹; 16 h per day).

2.2. Virus-induced gene silencing (VIGS) and virus-mediated overexpression (VOX) of CaMAP4K3

To silence the CaMAP4K3 gene in pepper plants, VIGS was conducted as described previously [29]. Using the SGN VIGS tool (<https://vigs.solgenomics.net>), two target sequence regions without off-target effects were predicted, as shown in Fig. S1, followed by amplification with specific primers (Table S1) using PCR. Subse-

quently, the two amplified fragments were cloned into tobacco rat-tle virus (TRV) pTRV2 vectors, and the final construct was introduced into *Agrobacterium tumefaciens* strain GV3101.

VOX for transient CaMAP4K3 expression in tobacco plants was conducted as described previously [30]. Full-length coding sequences of CaMAP4K3, tagged with 3× FLAG, were cloned into the TRV-RNA2-pPEBV-MCS vector (SPDK3876, #149276; Addgene, Watertown, MA, USA) digested with *Bam*HI and *Sac*I. The final pTRV2-CaMAP4K3-OX vector was introduced into *A. tumefaciens* strain GV3101.

For agroinfiltration, GV3101 cells harboring pTRV1 and pTRV:00 (a negative control) or pTRV2:CaMAP4K3 for VIGS were mixed in a 1:1 ratio (OD₆₀₀ = 0.5 for each construct). For VOX experiment, pTRV2-CaMAP4K3-OX and pTRV2-CaMAP4K3^{K46R}-OX were used in the place of pTRV2:CaMAP4K3. The cell mixtures were co-infiltrated into the fully expanded cotyledons of 7-day-old pepper and 10-day-old tobacco seedlings.

2.3. Generation of CaMAP4K3-overexpressing (OX) Arabidopsis transgenic plants

The full-length coding regions of CaMAP4K3, lacking a stop codon, were cloned into a pCR8/GW/TOPO vector (Thermo Fisher Scientific, Waltham, MA, USA), followed by the LR reaction with a p326GFP binary vector. The final construct, Pro35S:CaMAP4K3-GFP, was integrated into *A. tumefaciens* GV3101, and this cell line was used for floral dip transformation [31] of Arabidopsis plants (Col-0 background). Putative CaMAP4K3-OX Arabidopsis transgenic plants were selected on solid 1/2 MS medium supplemented with 25 μg mL⁻¹ phosphinothricin, and only homozygous T₃ transgenic seeds were used for further analysis.

2.4. Total RNA isolation and quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) assay

Total RNA isolation from pepper, tobacco, and Arabidopsis plants was conducted using a Ribospin Plant RNA isolation kit (GeneAll, Seoul, Republic of Korea) in accordance with the manufacturer's protocol. To investigate gene expression, six-leaf stage pepper plants were treated with 500 mmol L⁻¹ mannitol, 100 μmol L⁻¹ abscisic acid (ABA), 200 mmol L⁻¹ NaCl, 100 μmol L⁻¹ H₂O₂, low temperature (10 °C), or drought stress as described previously [29]. For drought stress treatment, pepper (2 weeks after agroinfiltration), tobacco (3 weeks after agroinfiltration), and Arabidopsis (2-week-old) plants were uprooted and then dried. RNA samples were incubated with RNase-free DNase to ensure the absence of genomic DNA contamination, followed by cDNA synthesis using a Transcript First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). Semi-quantitative and quantitative RT-PCR were conducted with gene-specific primers (Table S1). As internal control genes, pepper CaACT1, tobacco NbACT, and Arabidopsis AtACT8 were amplified in parallel. In RT-qPCR analysis, the relative expression level of each gene was determined using the 2^{-ΔΔCT} method [32].

2.5. Subcellular localization assay

The subcellular localization of CaMAP4K3 protein was analyzed as previously described [33]. Briefly, *Agrobacterium* cells harboring Pro35S:CaMAP4K3-GFP were mixed with the silencing suppressor p19 at a 1:1 ratio (OD₆₀₀ = 0.5) and infiltrated into the abaxial side of tobacco plant leaves. Green fluorescent protein (GFP) signals were detected 2 d post-agroinfiltration using a confocal microscope (510 UV/Vis Meta; Carl Zeiss, Oberkochen, Germany) with LSM Image Browser software (Carl Zeiss).

2.6. In vitro and in-gel kinase assays

An *in vitro* kinase assay was performed as previously described [34,35]. Briefly, total proteins were extracted from tobacco leaves transiently expressing CaMAP4K3-GFP through agroinfiltration. The CaMAP4K3-GFP and its kinase mutant CaMAP4K3^{K46R}-GFP proteins were immunoprecipitated using GFP magnetic beads (Chromotek, Munich, Germany). Subsequently, they incubated at 30 °C for 1 h with a kinase buffer (1 mmol L⁻¹ CaCl₂, 2.5 mmol L⁻¹ MgCl₂, 2.5 mmol L⁻¹ MnCl₂, 1 mmol L⁻¹ DTT, and 20 mmol L⁻¹ Tris-HCl, at pH 7.5) supplemented with 7.5 µCi [γ -³²P]-ATP. The reaction was stopped by adding 5× SDS sample buffer, followed by boiling at 97 °C. Subsequently, the samples were resolved on a 10% SDS-PAGE gel, and radiolabeled signals were detected using a Personal Molecular Imager (Bio-Rad Laboratories, Hercules, CA, USA).

The in-gel kinase assay was performed according to previously described methods [36,37]. Tobacco leaves were transiently transformed via agroinfiltration with *Pro35S::CaDSK2-1-GFP*. The tobacco plants were then exposed to drought stress by removing the roots and then drying, treated with 100 µmol L⁻¹ ABA via foliar spray, subjected to salt stress by applying 200 mmol L⁻¹ NaCl solution, and exposed to heat stress by incubating at 42 °C. As described above, proteins were isolated from tobacco leaves and immunoprecipitated using GFP magnetic beads. Half of the samples were resolved on 10% SDS-PAGE gels for Coomassie Brilliant Blue (CBB) staining, and the other half on 10% SDS-PAGE gels containing Myelin Basic Protein (0.1 mg mL⁻¹; MyBioSource, San Diego, CA, USA) as a substrate for radioactive analysis.

2.7. Plant phenotypic analysis

To assess drought stress resistance, *CaMAP4K3*-silenced pepper (2 weeks post-agroinfiltration), *CaMAP4K3*-OX tobacco (3 weeks post-agroinfiltration), and *CaMAP4K3*-OX (3-week-old) Arabidopsis plants were compared with their respective control plants. Drought stress treatment was conducted by withholding watering for 10 d for pepper and tobacco and 14 d for Arabidopsis, respectively. Survival rates for each plant line were calculated after rehydration for 3 d. Thermographic images were obtained using a FLIR T420 infrared camera (FLIR Systems, Wilsonville, OR, USA) before and after 7 d treatment to evaluate changes in leaf surface temperature in pepper and tobacco plants under drought stress. The leaf temperatures of each plant line were analyzed using FLIR Tools + version 5.13 (FLIR Systems).

Leaf samples were collected from the *CaMAP4K3*-silenced pepper (2 weeks post-agroinfiltration) and *CaMAP4K3*-OX tobacco (3 weeks post-agroinfiltration) plants to determine transpirational water loss, and aerial parts were collected from the *CaMAP4K3*-OX (3-week-old) Arabidopsis plants. Thereafter, the samples were randomly arranged in individual plastic dishes, and their fresh weights were recorded at 1 h intervals during the drying period.

The germination rate and seedling growth of *CaMAP4K3*-OX Arabidopsis plants were evaluated as described previously [30,35]. Briefly, seeds ($n = 100$) of each Arabidopsis plant line were sown in two pairs in 1/2 MS media containing 0 and 0.3 µmol L⁻¹ ABA, with one set of plates oriented vertically and the other horizontally. The number of seeds with radicle emergence was counted daily to determine the germination rate. After 7 d, seedlings with fully expanded cotyledons were recorded to determine cotyledon greening rates, and root lengths were measured from the horizontally placed plates. Additionally, 3-day-old seedlings were transferred to 1/2 MS media containing either 0 or 7.5 µmol L⁻¹ ABA to evaluate root elongation during the post-germinative stage. Primary root lengths were measured after 5 d.

3. Results

3.1. Isolation of putative MAP4K genes in pepper genome

Although *MAP4K* genes are evolutionarily conserved across plant species, little is known about the *MAP4K* gene family in pepper plants, particularly their functions in response to abiotic stresses. To address this, we identified *MAP4K* genes from the pepper genome (*C. annuum* cv. CM334 version 1.55) using amino acid sequences of Arabidopsis *MAP4K* genes as a query. A BLASTP search in the Sol Genomics Network (<https://solgenomics.net/>) identified seven putative *MAP4K* gene loci. These genes were designated *CaMAP4K1* to *CaMAP4K7* according to their chromosomal order and were further subjected to multiple sequence alignment and phylogenetic tree analyses. As shown in Fig. 1A, one or two pepper *MAP4K* proteins clustered with Arabidopsis *MAP4Ks*, which are classified into three distinct classes (I, GCK-III; II, GCK-II; III, GCK-VI) [19,38]. Notably, Class III is further divided into four subclasses; however, none of the pepper *MAP4Ks* were found in Class II. Domain analysis revealed that the pepper *MAP4K* proteins possess a serine/threonine kinase domain [528–725 amino acids (aa)] in their N-terminal region. Particularly, subdomain VIII of this kinase domain contains the conserved *MAP4K* signature motif (GTPyW-MAPEv, where lowercase letters represent less conserved residues) [14] (Fig. 1B). RT-qPCR analysis was used to further characterize these genes and assess their expression in various pepper organs, including flowers, leaves, stems, and roots. All pepper *MAP4K* genes, except *CaMAP4K5*, exhibited differential expression patterns across these organs (Fig. 1C). *CaMAP4K3* exhibited consistently high expression across all examined organs, with notably higher expression in stems and roots compared with that of the other *MAP4K* genes. Additionally, *CaMAP4K1* and *CaMAP4K4* were highly expressed in leaves and flowers, respectively, while *CaMAP4K7* was mainly expressed in stems. These findings suggest potential organ-specific roles for the *CaMAP4K* gene family in pepper plants.

3.2. Induction of *CaMAP4K* genes in pepper leaves by drought stress

Given the functional involvement of the MAPK cascade in abiotic stress responses, particularly drought stress [5–7], we sought to determine how drought stress affects the expression of *CaMAP4K* genes. To investigate this, we conducted an RT-qPCR assay to analyze the expression patterns of the seven *CaMAP4K* genes in pepper leaves in response to drought stress. As a positive control, we amplified the drought-induced marker gene *CaOSR1* [29,39] in parallel, and its expression increased gradually after drought stress treatment (Fig. 2). Among the seven *CaMAP4K* genes, *CaMAP4K5* gene expression was not detected in the leaves, consistent with the data shown in Fig. 1C. The expression levels of six genes, excluding *CaMAP4K7*, were significantly induced by drought stress, particularly 6 h after treatment, when the first notable induction was observed in all the genes. These data suggest a potential role for *CaMAP4K* genes in response to drought stress.

3.3. Molecular characterization of *CaMAP4K3* gene

Among the seven *CaMAP4K* genes in pepper plants, we selected *CaMAP4K3* (gene locus CA02g29630) for further studies due to its high expression across various pepper organs compared to other *MAP4K* genes and its strong induction under drought stress. As shown in Fig. 1B, *CaMAP4K3* contains a serine/threonine kinase domain (from 17 to 278 aa) in its N-terminal region, where in addition to the *MAP4K* signature motif, key kinase features are found, including a glycine-rich loop (from 24 to 29 aa), an invariant lysine residue (46th aa), an HRD motif (from 138 to 140 aa), and a DFG

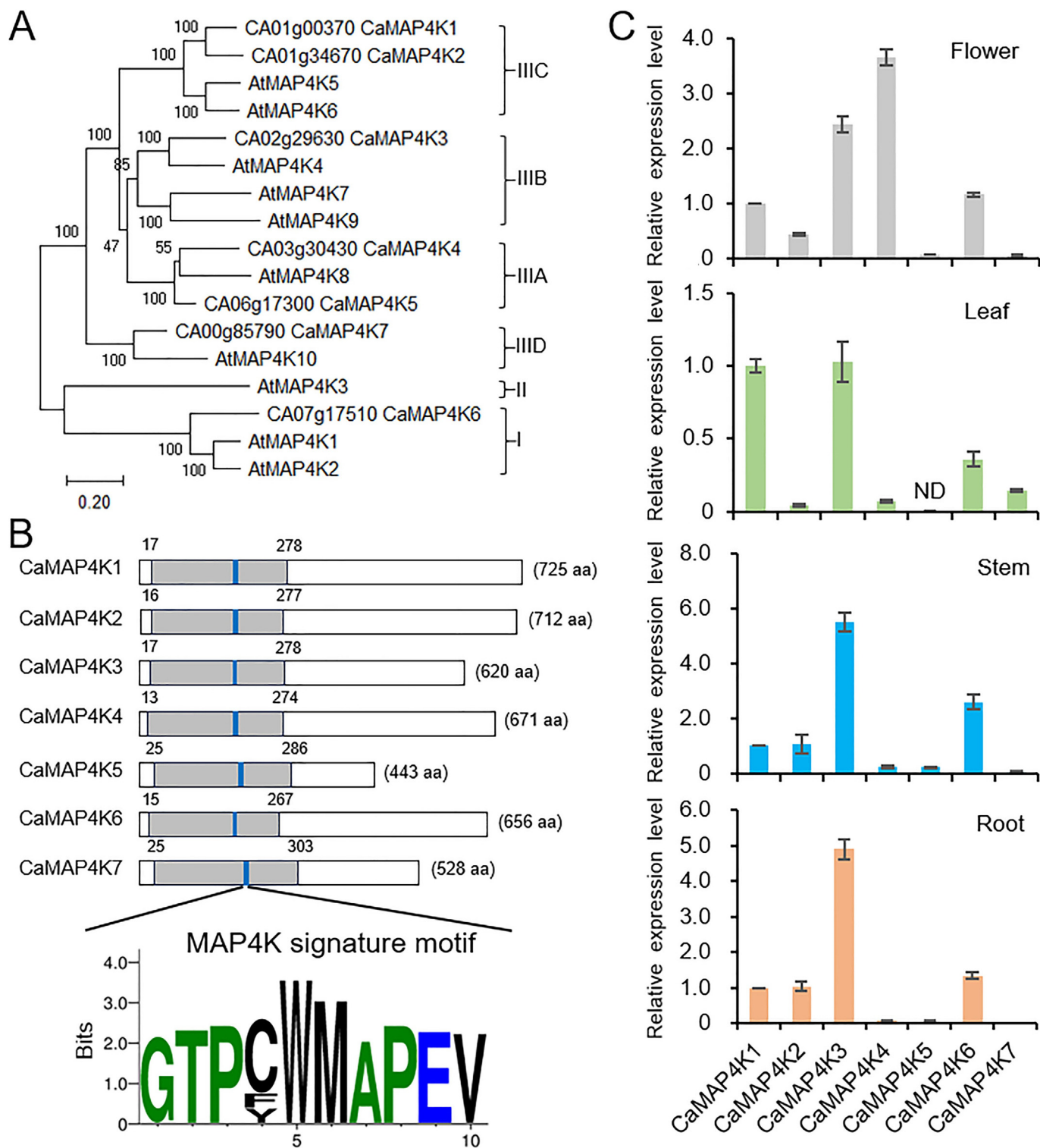


Fig. 1. Identification of MAP4K in pepper plants. (A) Phylogenetic tree analysis of pepper MAP4Ks. Putative MAP4K proteins from pepper were aligned with Arabidopsis MAP4K proteins using the web tool Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>), and a phylogenetic tree was constructed using the neighbor-joining method in MEGA X software. (B) Domain analysis of pepper MAP4Ks. Serine/threonine-protein kinase domains were predicted using the Simple Modular Architecture Research Tool (<http://smart.embl-heidelberg.de/>). The MAP4K signature motif in pepper MAP4Ks was visualized through sequence logos, where the height of each residue represents its relative frequency in the alignment. (C) Expression analysis of MAP4K genes in different organs from 4-leaf stage pepper plants. As an internal control, the pepper *Actin1* gene (*CaACT1*) was amplified, and its levels were used for normalization. The expression level of *CaMAP4K1* in different organs was set to 1.0.

motif (from 158 to 160 aa) (Figs. 3A, S1) [27,38,40]. To gain deeper insight into the expression pattern of *CaMAP4K3* in pepper plants, we conducted RT-qPCR analysis using cDNA samples from 10-day-old seedlings and mature plants at the six-leaf stage (Fig. 3B).

CaMAP4K3 was expressed in all examined organs, with the highest expression in the tap roots of mature plants. However, the differences in expression levels among the other organs did not reach statistical significance. We further examined the effects of various

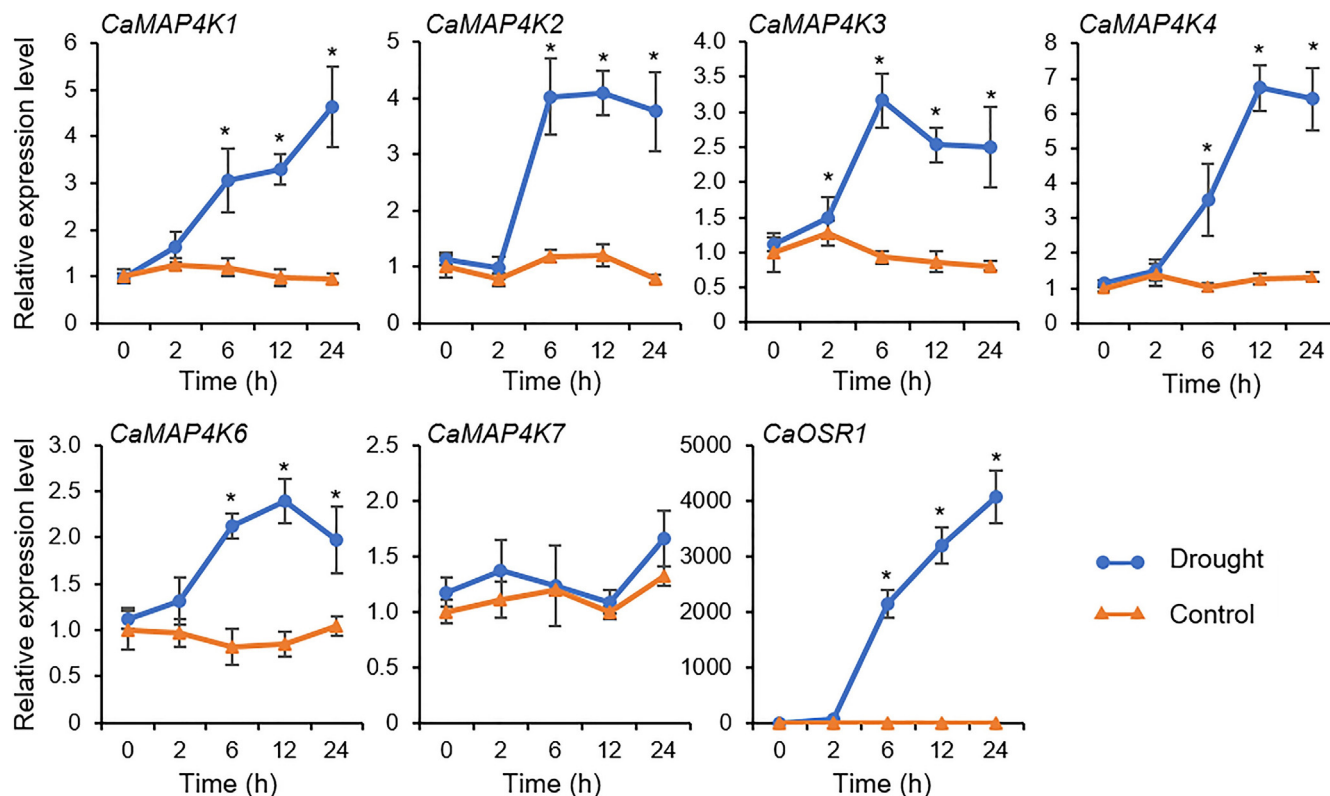


Fig. 2. Expression patterns of *CaMAP4K* genes in response to drought stress. RT-qPCR analysis was conducted on cDNA derived from the leaves of pepper plants subjected to drought stress by removing soil from their roots. As an internal control, the pepper *Actin1* gene (*CaACT1*) was amplified, and its levels were used for normalization. The expression levels at 0 h for each gene in control samples were set to 1.0. Values are means $\pm$ standard deviation of three independent experiments (Student's *t*-test; *, $P < 0.05$). Asterisks indicate significant differences compared with the control (0 h).

environmental stimuli on *CaMAP4K3* expression in pepper leaves and tap roots. In the leaves, osmotic stress induced by mannitol treatment led to a marked increase in *CaMAP4K3* expression, with significant induction observed 6 h after treatment, peaking at 12 h, similar to its response under drought stress (Fig. 3C). Salt stress also triggered a transient induction of *CaMAP4K3* expression 6 h after treatment. Temperature changes influenced *CaMAP4K3* expression, with heat stress (42 °C) causing a transient increase 2 h after treatment, whereas chilling stress (10 °C) decreased its expression levels. Additionally, exogenous ABA application significantly increased *CaMAP4K3* expression 3 h after treatment, whereas H₂O₂ treatment reduced its expression levels. Notably, apart from the response to heat stress, stress-induced alterations in *CaMAP4K3* expression were not observed in the tap roots.

Considering the association between gene function and subcellular localization, we investigated the localization of *CaMAP4K3* protein in plant cells. Using the web-based prediction tool WoLF PSORT (<https://wolfpsort.hgc.jp>), we predicted that *CaMAP4K3* protein localizes to both the nucleus and cytoplasm. To verify this experimentally, a GFP-tagged *CaMAP4K3* construct was transiently expressed in tobacco leaves through agroinfiltration. Confocal microscopic analysis revealed GFP fluorescence predominantly in the cytoplasm (Fig. 3D). Notably, GFP signals near the nucleus did not appear to overlap with the DAPI signal (blue), indicating the localization of *CaMAP4K3*-GFP outside the nucleus. To further distinguish GFP fluorescence between the plasma membrane and cell wall, we induced plasmolysis by incubating infiltrated leaves in 800 mmol L⁻¹ mannitol solution for 30 min. Plasma membrane localization was confirmed by FM4-64 staining (red). After plasmolysis, GFP signals were observed in both the cytoplasm and plasma membrane.

3.4. Activation of *CaMAP4K3* protein kinase by drought stress

To investigate whether *CaMAP4K3* is an active kinase and whether its activity is affected by environmental stimuli such as drought stress, we conducted an *in vitro* kinase assay using *CaMAP4K3*-GFP proteins expressed in tobacco leaves. *CaMAP4K3* possesses an invariant lysine (K) at position 46 and an active site aspartate at position 140, which are responsible for its catalytic activity [19]. Since mutation of these residues leads to loss of kinase function [27,38,40], lysine 46 and aspartate 140 were replaced by arginine (R) and glutamic acid (E), respectively, creating the kinase-dead mutants, *CaMAP4K3*^{K46R} and *CaMAP4K3*^{D140E}, which were used as negative controls in the kinase assay. As expected, the intact *CaMAP4K3* protein was auto-phosphorylated in a dose-dependent manner, while *CaMAP4K3*^{D140E} displayed a reduced phosphorylation signal, and *CaMAP4K3*^{K46R} showed no phosphorylation (Figs. 4A, S2). These data indicate that the *CaMAP4K3* protein is an active kinase.

Based on the transcriptional induction of *CaMAP4K3* in response to drought stress, NaCl, heat, and ABA treatments (Fig. 3C), we further investigated how *CaMAP4K3* kinase activity is affected by these environmental stimuli. To assess this, *CaMAP4K3*-GFP protein and its kinase-dead mutant were transiently expressed in tobacco leaves as described above. The infiltrated tobacco plants were subsequently exposed to drought stress, NaCl (200 mmol L⁻¹), heat (42 °C), and ABA (100 μ mol L⁻¹) treatments. An in-gel kinase assay revealed that *CaMAP4K3* phosphorylation was enhanced by drought, NaCl, and heat stresses but not by ABA treatment (Fig. 4B–E). These findings suggest that *CaMAP4K3* kinase activation may be triggered by drought stress and is not dependent on ABA.

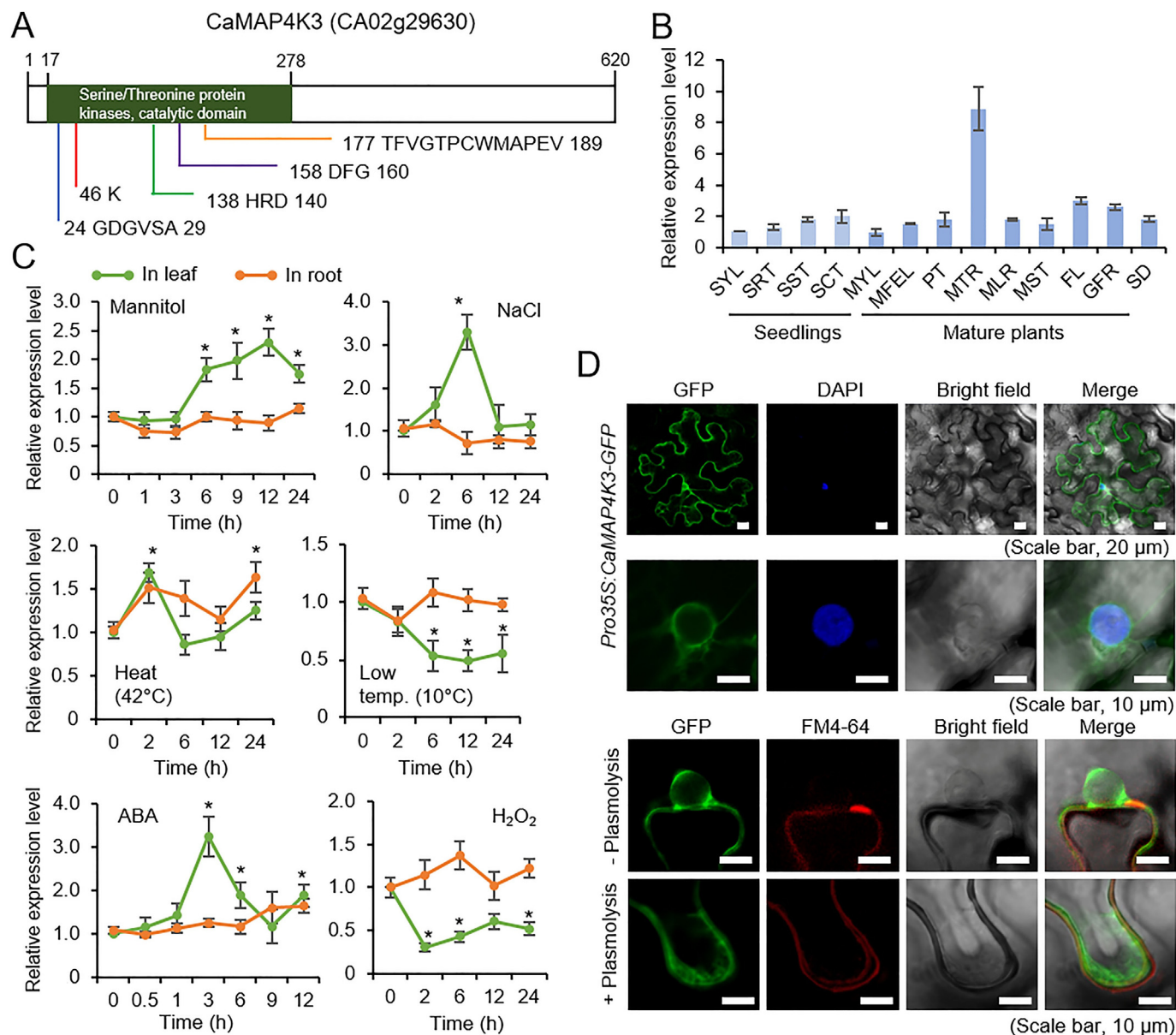


Fig. 3. Molecular characterization of CaMAP4K3. (A) Consensus sequences relevant to a MAP4K in the kinase domain of CaMAP4K3 protein. (B) Organ-specific expression of CaMAP4K3 in pepper plants at the seedling (S) and mature (M) stages. The gene level in young leaves (YL) was set to 1.0. CT, cotyledon; FEL, fully expanded leaf; FL, flower; FRT, fruit; LR, lateral root; PT, petiole; RT, root; SD, seed; ST, stem; TR, tap root; GFR, green fruit. (C) Expression patterns of CaMAP4K3 in the leaves and tap roots of pepper plants in response to treatment with 500 mmol L⁻¹ mannitol, 100 µmol L⁻¹ abscisic acid (ABA), 200 mmol L⁻¹ NaCl, 100 µmol L⁻¹ H₂O₂, or low temperature (10 °C). As an internal control, the pepper Actin1 gene (*CaACT1*) was amplified, and its levels were used for normalization. The expression levels at 0 h for each gene were set to 1.0. Values are means ± standard deviation of three independent experiments (Student's *t*-test; *, *P* < 0.05). Asterisks indicate significant differences compared with the control (0 h). (D) Subcellular localization of CaMAP4K3 protein. Through agroinfiltration, green fluorescent protein (GFP)-tagged CaMAP4K3 protein was transiently expressed in the leaves of *Nicotiana benthamiana* plants. Plasmolysis was induced by incubating leaf disks with 800 mmol L⁻¹ mannitol solution for 30 min. 4',6-Diamidino-2-phenylindole (DAPI) was utilized to label nuclei, while FM4-64 (50 µmol L⁻¹) was employed as a plasma membrane marker.

3.5. Enhanced drought resistance of CaMAP4K3-silenced pepper plants

Given that drought stress induced CaMAP4K3 expression and its kinase activation (Figs. 2, 4B), we analyzed the potential role of CaMAP4K3 in response to drought stress. To this end, we employed the VIGS technique to knockdown CaMAP4K3 expression in pepper plants. Within the coding region of CaMAP4K3, two fragments were selected for VIGS: TRV2:CaMAP4K3 #1 (from 832 to 1131 bp) and TRV2:CaMAP4K3 #2 (from 1476 to 1755 bp) (Fig. S3A). We assessed CaMAP4K3 expression levels in the gene-silenced pepper plants two weeks after agroinfiltration. RT-qPCR analysis revealed that compared with those of the control TRV2:00, both CaMAP4K3-

silenced pepper plant lines showed significantly reduced CaMAP4K3 expression levels (Fig. S3B). The CaMAP4K3-silenced pepper plants were subsequently used for a drought resistance assay (Fig. 5). We observed no phenotypic differences between TRV2:00 and TRV2:CaMAP4K3 pepper plants grown under well-watered conditions for 2 weeks (Fig. 5A, top). The pepper plants were next exposed to drought stress by withholding watering for 10 d. Compared with those of TRV2:00, TRV2:CaMAP4K3 plant lines showed less wilting (Fig. 5A, middle), and their survival rates were much higher after 3 d of re-watering (Fig. 5A, bottom).

We hypothesized that the enhanced resistance of TRV2:CaMAP4K3 plants to drought stress might be associated with

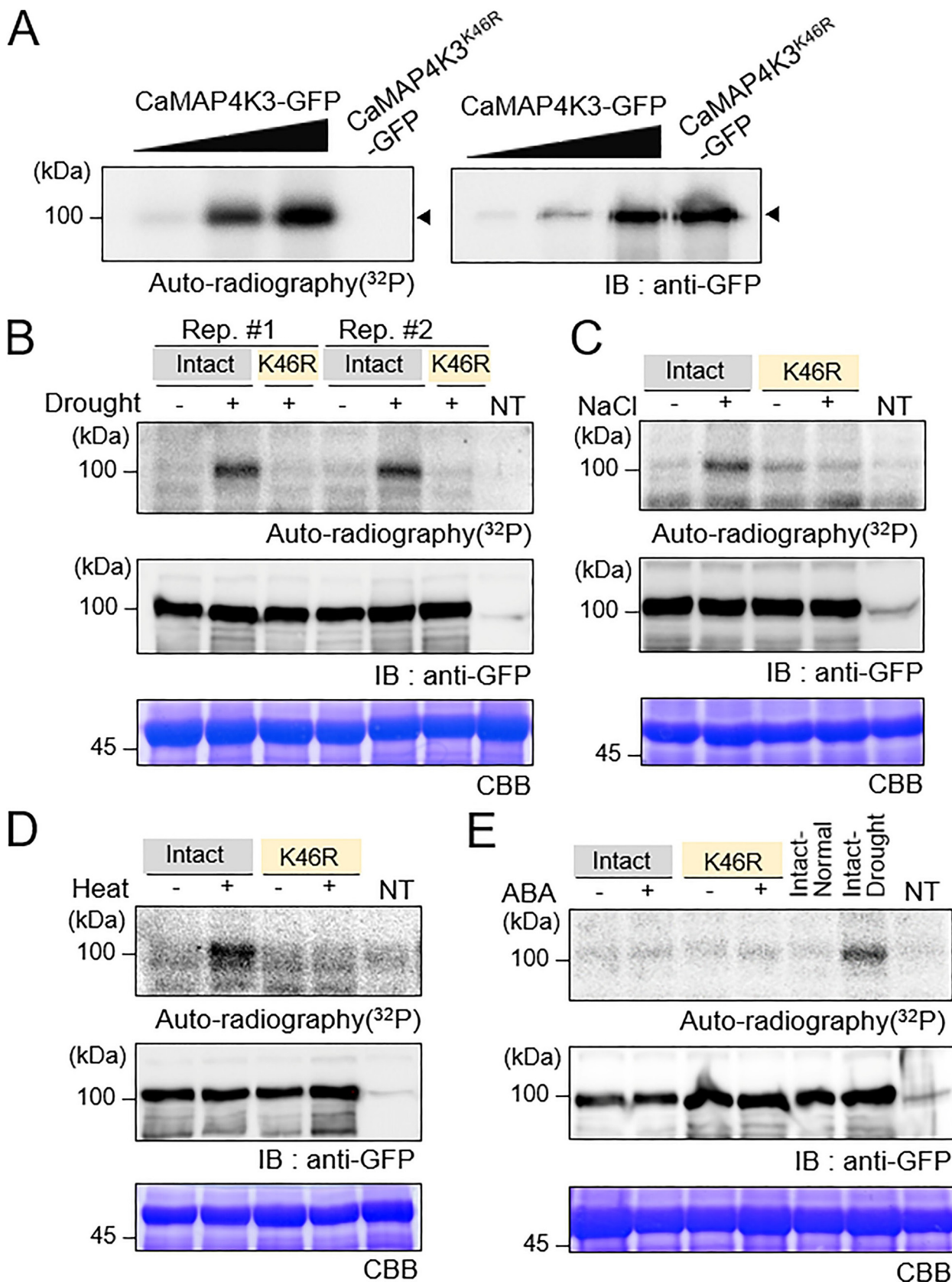


Fig. 4. Activation of CaMAP4K3 kinase activity in response to abiotic stresses and abscisic acid (ABA). (A) *In vitro* autophosphorylation of CaMAP4K3 protein. Total proteins were extracted from tobacco leaves expressing either CaMAP4K3-GFP or its kinase-deficient mutant CaMAP4K3^{K46R}-GFP, followed by immunoprecipitation with anti-GFP antibodies. *In vitro* kinase assays with [γ -³²P]-ATP were conducted on half of the immunoprecipitates (left), and the rest were subjected to immunoblotting with anti-GFP antibodies (right). (B–E) *In-gel* kinase assay of CaMAP4K3. Tobacco plants expressing either CaMAP4K3-GFP or its kinase-deficient mutant CaMAP4K3^{K46R}-GFP were subjected to drought stress treatment by removing soil from their roots (B), 200 mmol L⁻¹ NaCl (C), heat stress (42 °C) (D), and 100 μ mol L⁻¹ ABA (E). Leaf samples were harvested after treatment with drought and NaCl for 6 h, heat for 30 min, and ABA for 3 h, and used for protein extraction. After equal amounts of proteins (CBB; lower) were immunoprecipitated with anti-GFP antibodies. *In vitro* kinase assays with [γ -³²P]-ATP were conducted on half of the immunoprecipitates (upper), and the rest were subjected to immunoblotting with anti-GFP antibodies (middle). Myelin Basic Protein was used as a substrate. NT, non-transgenic plant.

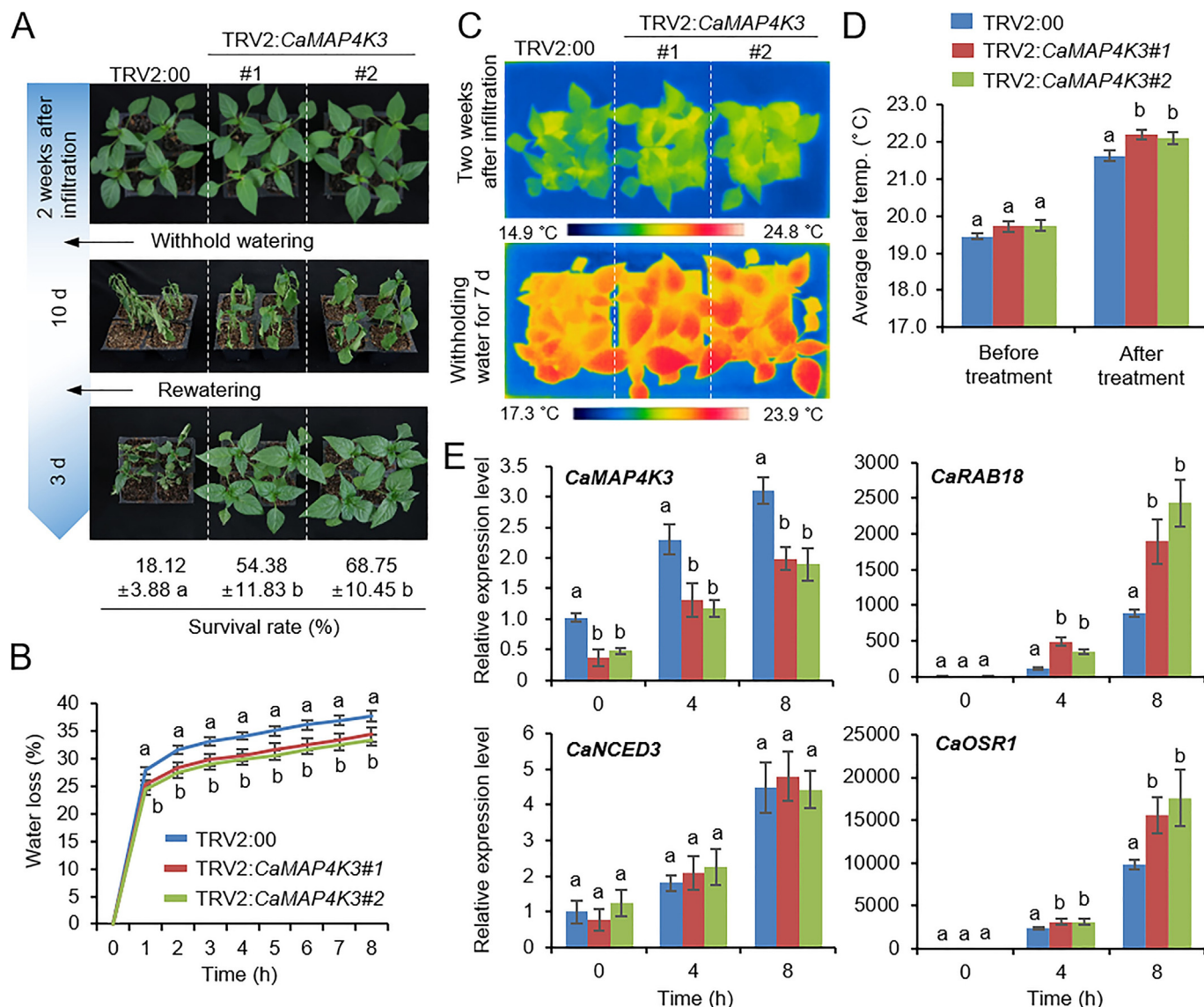


Fig. 5. Enhanced resistance of *CaMAP4K3*-silenced pepper plants to drought stress. (A) Drought sensitivity of *CaMAP4K3*-silenced pepper plants. Following 2 weeks of agroinfiltration, TRV2:00 and TRV2:*CaMAP4K3* plants grown under well-watered conditions (upper) were exposed to drought stress by withholding watering for 10 d (middle). The plants were then rehydrated for 3 d (bottom), and their survival rates were measured. ($n = 36$ per plant line) (B) Transpirational water loss from the leaves of TRV2:00 and TRV2:*CaMAP4K3* pepper plants. Following leaf detachment, the leaf fresh weights of each plant line were measured at the indicated time points. (C, D) Effect of drought stress on leaf surface temperatures of TRV2:00 and TRV2:*CaMAP4K3* pepper plants. Representative thermographic images were obtained after 7 d of drought stress (C). Parallelly, the mean temperatures were measured from the 1st and 2nd leaves of each plant line (D). (E) RT-qPCR analysis of stress-responsive genes in the leaves of TRV2:00 and TRV2:*CaMAP4K3* pepper plants subjected to drought stress. As an internal control, the pepper *Actin1* gene (*CaACT1*) was amplified, and its levels were used for normalization. The expression level of each gene in control plants at 0 h was set to 1.0. Values are means $\pm$ standard error of three independent experiments. Different letters indicate statistically significant differences between TRV2:00 and TRV2:*CaMAP4K3* plants (ANOVA; $P < 0.05$).

reduced transpirational water loss. To investigate this, we detached the 1st and 2nd leaves from TRV2:*CaMAP4K3* and TRV2:00 plant lines and measured their fresh weights hourly to monitor leaf transpiration. As shown in Fig. 5B, transpirational water loss was significantly lower in TRV2:*CaMAP4K3* than in TRV2:00 plants. Additionally, we assessed changes in leaf surface temperature following drought stress treatment (Fig. 5C, D). Before treatment, TRV2:*CaMAP4K3* and TRV2:00 plant lines exhibited similar leaf temperatures. However, 7 d after drought stress treatment, leaf temperatures in TRV2:*CaMAP4K3* were higher than in TRV2:00.

We also evaluated the effect of *CaMAP4K3* knockdown on the expression patterns of stress-responsive marker genes in response to drought stress. RT-qPCR analysis was conducted to examine *CaNCED3*, *CaRAB18*, and *CaOSR1* expression levels in pepper leaves. Drought stress increased *CaMAP4K3* expression, but its levels were much lower in TRV2:*CaMAP4K3* than in TRV2:00 plants (Fig. 5E).

The stress-responsive marker genes *CaRAB18* and *CaOSR1* were highly expressed in TRV2:*CaMAP4K3* than in TRV2:00 plants at all examined time points following drought stress treatment. However, *CaNCED3* expression levels between the two plant lines did not significantly differ. These results suggest that *CaMAP4K3* may negatively modulate transpirational water loss and the expression of stress-responsive marker genes, thereby reducing drought resistance in pepper plants.

3.6. Reduced drought resistance of *CaMAP4K3*-overexpressing tobacco plants

To further analyze the functional role of *CaMAP4K3* in response to drought stress, we utilized a TRV-mediated gene overexpression system to induce *CaMAP4K3* overexpression in tobacco plants, which, similar to pepper, are members of the *Solanaceae* family.

As shown in Fig. S3C, the full-length coding sequence of *CaMAP4K3* and its kinase-dead mutant *CaMAP4K3^{K46R}* were cloned into the TRV2-SPDK3888 vector, generating TRV2:*CaMAP4K3*-OX and TRV2:*CaMAP4K3^{K46R}*-OX constructs, respectively. These constructs were introduced into tobacco plants via agroinfiltration. Three weeks after agroinfiltration, *CaMAP4K3* and *CaMAP4K3^{K46R}* transcriptional levels were assessed in the infiltrated tobacco leaves, revealing high expression of both constructs, whereas no expression was detected in the control TRV2:00 plants (Fig. S3D). Moreover, western blot analysis confirmed the robust accumulation of *CaMAP4K3* protein in the overexpressing tobacco plants (Fig. S3E), corroborating the successful overexpression of *CaMAP4K3* at the protein level.

To assess the impact of *CaMAP4K3* overexpression on the drought resistance of tobacco plants, TRV2:*CaMAP4K3*-OX, TRV2:*CaMAP4K3^{K46R}*-OX, and control TRV2:00 plants were subjected to drought stress 3 weeks after agroinfiltration by withholding watering for 10 d. While no significant phenotypic differences among the plant lines were observed at the onset of drought stress (Fig. 6A, top), TRV2:*CaMAP4K3*-OX exhibited more wilting 7 d after drought treatment than that in TRV2:00 plants (Fig. 6A, middle). After re-watering for 3 d, the survival rate of TRV2:00 plants was 67.16%, whereas only 18.61% of TRV2:*CaMAP4K3*-OX plants recovered and resumed growth (Fig. 6A, bottom). This reduced drought resistance in TRV2:*CaMAP4K3*-OX may be attributed to the kinase activity of *CaMAP4K3*. Overexpression of the kinase-dead mutant *CaMAP4K3^{K46R}* did not result in any discernible differences relative to the control plants. Further analysis showed that TRV2:*CaMAP4K3*-OX leaves exhibited a higher rate of transpirational water loss than that in TRV2:*CaMAP4K3^{K46R}*-OX and TRV2:00 leaves. Following drought stress treatment, leaf surface temperatures were consistently higher in TRV2:*CaMAP4K3*-OX plants, with no significant differences observed among the three plant lines before treatment (Fig. 6C, D). We also analyzed the expression of drought-responsive marker genes in tobacco plants, including *NbNCED3*, *NbRAB18*, *NbSnRK2.3*, and *EARLY RESPONSIVE TO DEHYDRATION (NbERD)*. RT-qPCR analysis revealed that the expression levels of these genes were lower in TRV2:*CaMAP4K3*-OX leaves compared with those in TRV2:*CaMAP4K3^{K46R}*-OX and TRV2:00 leaves after drought stress treatment (Fig. 6E). These findings indicate that *CaMAP4K3* contributes to reduced drought resistance in tobacco plants, similar to its effect in pepper plants, and this function is dependent on its kinase activity.

3.7. Negative effects of *CaMAP4K3* overexpression on ABA sensitivity during seed germination and seedling growth, as well as on drought stress in Arabidopsis plants

We additionally generated transgenic Arabidopsis plants overexpressing the *CaMAP4K3* gene, *Pro35S:CaMAP4K3*, and selected two independent lines (#1 and #10) based on high expression levels (Fig. S3F). In 2-week-old seedlings, gene overexpression did not result in phenotypic differences compared with wild-type plants, consistent with the observation in TRV2:*CaMAP4K3*-OX tobacco plants. Next, these lines were exposed to drought stress by withholding watering for 14 d (Fig. 7A). After re-watering for 3 d, *Pro35S:CaMAP4K3* exhibited significantly lower survival rates (25.93%–33.46%) than those of wild-type plants (61.20%). This reduced drought resistance was also associated with a higher transpirational rate in *Pro35S:CaMAP4K3* at all examined time points (Fig. 7B). Consistently, the expression levels of drought-responsive marker genes, including *RD29B*, *RAB18*, and *NCED3* [41–44], were relatively lower in *Pro35S:CaMAP4K3* plant lines after drought stress relative to those in wild-type plants (Fig. 7C).

Given the expression of *CaMAP4K3* in pepper seeds (Fig. 3B), we hypothesized that this gene may play a role in seed germination

and seedling establishment. However, studying its function in seeds is challenging due to the transient nature of *CaMAP4K3* knockdown in pepper plants. Therefore, we employed the seeds of *Pro35S:CaMAP4K3* transgenic Arabidopsis plants for further analysis. When seeds from wild-type and *Pro35S:CaMAP4K3* plants were germinated on 1/2 MS media, *Pro35S:CaMAP4K3* seeds exhibited a slightly faster germination rate 1 d after sowing compared with that of wild-type seeds (Fig. 7D).

Considering the role of ABA in regulating seed germination, we wondered whether *CaMAP4K3* overexpression affects ABA signaling during seed germination. To clarify this, seeds from wild-type and *Pro35S:CaMAP4K3* plants were germinated on 1/2 MS media containing 0.3 $\mu\text{mol L}^{-1}$ ABA. The application of ABA significantly inhibited the germination rate of wild-type seeds but not that of *Pro35S:CaMAP4K3* seeds (Fig. 7D). This reduced ABA sensitivity of *Pro35S:CaMAP4K3* seeds was also observed when seeds were germinated and grown vertically (Fig. 7E). Compared with wild-type plants, *Pro35S:CaMAP4K3* seedlings exhibited relatively longer root lengths when grown in the presence of ABA, whereas, in the absence of exogenous ABA, no discernible phenotypic differences between plant lines were observed (Fig. 7E, F). Additionally, *Pro35S:CaMAP4K3* seedlings displayed a higher percentage of fully expanded green cotyledons 7 d after sowing (Fig. 7G, H).

To further assess whether *CaMAP4K3* overexpression affects root elongation after germination, 3-day-old *Pro35S:CaMAP4K3* and wild-type seedlings were transferred onto 1/2 MS media containing 0 and 7.5 $\mu\text{mol L}^{-1}$ ABA. After five additional days of growth, the root length of *Pro35S:CaMAP4K3* seedlings was longer than that of wild-type seedlings (Fig. 7I, J). These results suggest that *CaMAP4K3* negatively affects seed germination and seedling establishment by modulating ABA signaling and contributes to compromised drought resistance in Arabidopsis plants.

4. Discussion

Plant MAP4Ks have recently attracted much research interest due to their involvement in multiple processes, including development and stress responses [27,28,38,40,45]. However, most MAP4K members remain uncharacterized, and little is known about the gene family in pepper plants. In the present study, we aimed to isolate MAP4K genes from the pepper genome and investigate their potential involvement in drought stress responses. The MAP4K gene family exhibits a high degree of conservation across plant species, and 10 genes have been identified in Arabidopsis as members of the MAP4K family [11,38]. We identified seven putative homologs in pepper plants, all possessing a conserved serine/threonine kinase domain with the MAP4K signature motif. Among these, *CaMAP4K3* was functionally characterized due to its high expression in various pepper organs and its significant induction in leaves under drought stress. The current study demonstrated that *CaMAP4K3* functions as a negative regulator of drought resistance in pepper plants.

CaMAP4K3 was found to be an active kinase, and its kinase activity was activated under drought conditions. Mutation of lysine 46, a critical residue for kinase activity, abolished *CaMAP4K3* phosphorylation under drought stress. This loss of activity mirrors findings in Arabidopsis MAP4K genes, where mutation of aspartate 139 in AtMAP4K4, the closest homolog of *CaMAP4K3* in subgroup IIIB, resulted in reduced phosphorylation [27]. Similarly, mutation of the corresponding aspartate 140 in *CaMAP4K3* resulted in a comparable loss of kinase activity, suggesting that lysine 46 is a key residue for its kinase activity. In addition to kinase activity, *CaMAP4K3* expression was upregulated under drought conditions. However, this expression pattern is not unique to *CaMAP4K3*; all other *CaMAP4K* genes, except *CaMAP4K7*, also showed significant

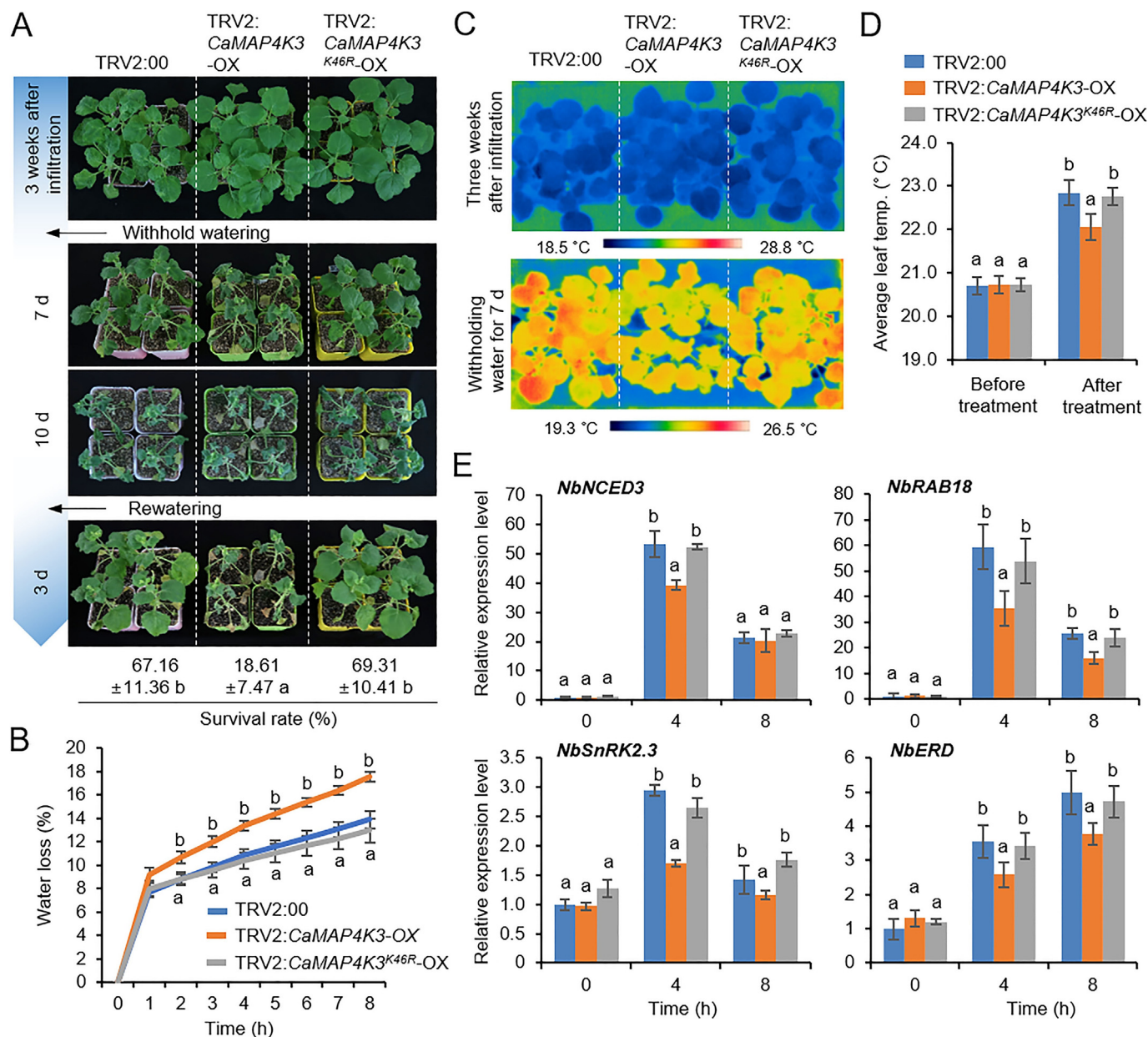


Fig. 6. Reduced drought stress resistance of *CaMAP4K3*-overexpressing tobacco plants. (A) Drought sensitivity of *CaMAP4K3*-overexpressing tobacco plants. Following 2 weeks of agroinfiltration, TRV2:00, TRV2:CaMAP4K3-OX, and TRV2:CaMAP4K3^{K46R}-OX tobacco plants grown under well-watered conditions (upper) were exposed to drought stress by withholding watering for 10 d (middle). The plants were then rehydrated for 3 d (bottom), and their survival rates were measured. (n = 36 per plant line) (B) Transpirational water loss from the leaves of TRV2:00, TRV2:CaMAP4K3-OX, and TRV2:CaMAP4K3^{K46R}-OX tobacco plants. Following leaf detachment, the leaf fresh weights of each plant line were measured at the indicated time points. (C, D) Effect of drought stress on leaf surface temperatures of TRV2:00, TRV2:CaMAP4K3-OX, and TRV2:CaMAP4K3^{K46R}-OX tobacco plants. Representative thermographic images were obtained after 7 d of drought stress (C). Parallely, the mean temperatures were measured from the 1st and 2nd leaves of each plant line (D). (E) RT-qPCR analysis of stress-responsive genes in the leaves of TRV2:00, TRV2:CaMAP4K3-OX, and TRV2:CaMAP4K3^{K46R}-OX tobacco plants subjected to drought stress. As an internal control, the tobacco *Actin1* gene (*NbACT1*) was amplified, and its levels were used for normalization. The expression level of each gene in control plants at 0 h was set to 1.0. Values are means ± standard error of three independent experiments. Different letters indicate statistically significant differences compared with the control (ANOVA; *P* < 0.05).

upregulation under drought stress treatment, suggesting a broader involvement of *CaMAP4K* genes in drought responses. A recent study reported extensive co-expression and heterodimerization among *Arabidopsis* MAP4Ks [25], with MAP4K4/TOT3, MAP4K5/TOI5, and MAP4K6/TOI6 playing a role in temperature-mediated growth [28]. These findings suggest that *CaMAP4K3* may not function alone in regulating drought resistance, and other *CaMAP4K*s may contribute to its phosphorylation and overall function in drought stress responses. Further research into the interactions and co-regulation of drought-activated MAP4Ks with *CaMAP4K3*

will provide deeper insights into MAP4K-mediated drought resistance mechanisms in pepper.

Under drought stress conditions, *CaMAP4K3* appears to negatively modulate stress resistance in pepper plants. This pattern was also shown in tobacco and *Arabidopsis* plants. In our previous studies, we successfully employed a TRV-mediated gene overexpression system to analyze gene function in pepper plants [30,46]. In addition to silencing, we aimed to generate *CaMAP4K3*-overexpressing peppers to assess their drought resistance. However, we unexpectedly found that *CaMAP4K3* gene expression

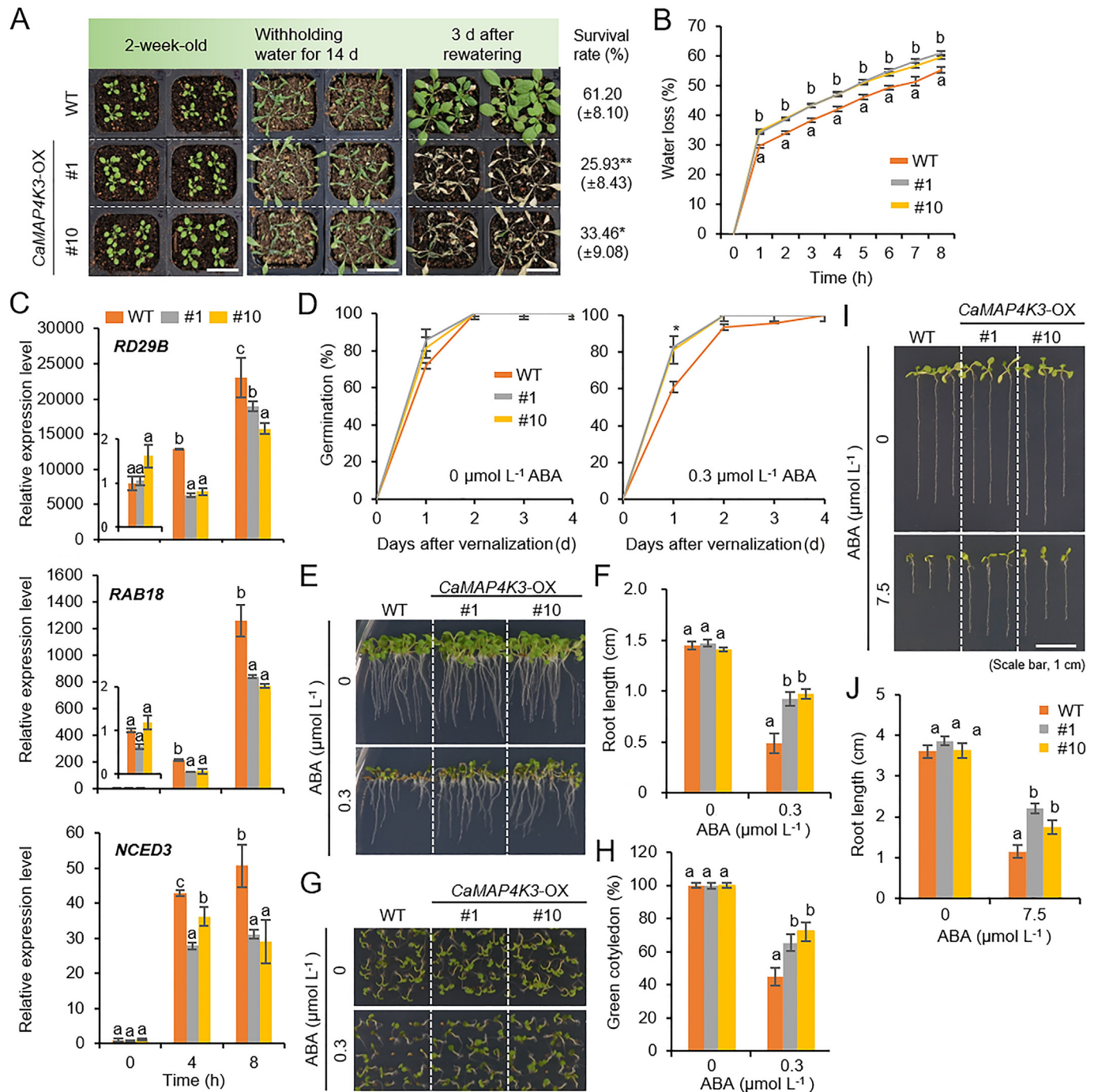


Fig. 7. Reduced drought resistance and abscisic acid (ABA) sensitivity of *CaMAP4K3*-overexpressing Arabidopsis transgenic plants. (A) Drought sensitivity of *CaMAP4K3*-overexpressing Arabidopsis plants. Three-week-old Arabidopsis plants grown under well-watered conditions (upper) were exposed to drought stress by withholding watering for 14 d (middle). The plants were then rehydrated for 3 d (bottom), and their survival rates were measured. Scale bar, 3 cm. ($n = 48$ per plant line) (B) Transpirational water loss from the leaves of *CaMAP4K3*-OX and wild-type plants. Following leaf detachment, the leaf fresh weights of each plant line were measured at the indicated time points. (C) RT-qPCR analysis of stress-responsive genes in the leaves of *CaMAP4K3*-OX and wild-type Arabidopsis plants subjected to drought stress. As an internal control, the Arabidopsis *Actin8* gene (*AtACT8*) was amplified, and its levels were used for normalization. The expression level of each gene in control plants at 0 h was set to 1.0. (D) Germination rates of *CaMAP4K3*-OX and wild-type plants in response to ABA. Seeds of each line were sown on 1/2 MS agar plates containing either 0 or 0.3 $\mu\text{mol L}^{-1}$ ABA, and the number of seeds with radicle emergence was counted. (E, F) Seedling growth of *CaMAP4K3*-OX and wild-type plants in response to ABA. Seeds of each line were sown on 1/2 Murashige and Skoog (MS) agar plates containing either 0 or 0.3 $\mu\text{mol L}^{-1}$ ABA. After the plates were placed vertically for 5 d, representative images were obtained (E), and the root length of each plant was measured (F). (G, H) Cotyledon greening of *CaMAP4K3*-OX and wild-type plants in response to ABA. Seeds of each line were sown on 1/2 MS agar plates containing 0.0 or 0.3 $\mu\text{mol L}^{-1}$ ABA. The percentage of seedlings with green cotyledons was evaluated after 7 d (H), and in parallel, representative images were obtained (G). (I, J) Post-germinative growth of *CaMAP4K3*-OX and wild-type plants in response to ABA. Three-day-old seedlings from each line were transferred to 1/2 MS agar plates with either 0.0 or 7.5 $\mu\text{mol L}^{-1}$ ABA. The primary root lengths were measured after 5 d (J), and representative images were obtained (I). Values are means $\pm$ standard error of three independent experiments, and different letters indicate statistically significant differences between the samples (ANOVA; $P < 0.05$).

levels in the overexpression plants were significantly reduced compared with those in the control, similar to those observed in *CaMAP4K3*-silenced plants. Notably, the TRV system functioned efficiently in tobacco plants, similar to pepper, belonging to the *Solanaceae* family. This discrepancy may be attributed to differences in transformation efficiency between pepper and tobacco. *CaMAP4K3* overexpression in tobacco plants reduced drought resistance compared with that in the control plants. This effect was linked to its kinase activity; overexpression of the *CaMAP4K3* kinase-dead mutant did not alter drought resistance, further supporting the role of its kinase function in modulating drought response. Modulation of drought resistance by *CaMAP4K3* was accompanied by changes in leaf transpiration and stress-responsive gene expression. Notably, water loss through leaf transpiration is known to be critically affected by the regulation of stomatal aperture [47,48]. Notwithstanding, it remains unclear whether *CaMAP4K3* directly regulates stomatal function under drought stress conditions. In *Arabidopsis*, *BLUS1/MAP4K10*, phosphorylated by phototropins, plays a key role in blue-light-induced stomatal opening [24]. Additionally, close homologs of *CaMAP4K3* in *Arabidopsis*—*MAP4K4*, *MAP4K7*, and *MAP4K8*—are expressed in stomata [25], raising the possibility that these genes may play a role in stomatal regulation.

MAPK cascades serve as essential signaling modules in plants, playing critical roles in responses to both biotic and abiotic stresses, as well as in modulating growth and development [1–4]. In a typical MAPK cascade, signal transduction is mediated by sequential phosphorylation and activation of sequential components [49]. In some cases, MAP4Ks are considered upstream components of MAP3Ks in yeast and mammals [14]. For instance, yeast Ste20 functions as a MAP4K, directly phosphorylating MAP3K Ste 11 in the mating pathway [50,51]. Moreover, the mammalian homologs of Ste20p, including HPK1 [15,16] and PAK2 [17], activate MAP3K MEKK1 and Raf-1, respectively, through direct phosphorylation. However, MAP4K-mediated phosphorylation of MAP3Ks in plants is yet to be reported. Conversely, increasing evidence suggests that MAP4Ks in plants can heterodimerize and target proteins besides MAP3Ks, including those outside the MAPK module [25,27,38,40]. In the present study, we found that *CaMAP4K3* interacted with pepper MAP3K *CaMEKK21/CaDIMK1* [52] and *CaMEKK23* [46] in both yeast and plants (Fig. S4A, B). However, *CaMAP4K3* did not induce phosphorylation of either MAP3K (Fig. S4C). Notably, while *CaMAP4K3* negatively regulates drought resistance, *CaMEKK21* and *CaMEKK23* play a positive role in drought resistance in pepper plants. This suggests a complex interaction between these proteins that may go beyond simple phosphorylation events. In response to drought stress, *CaMEKK21* and *CaMEKK23* might function independently of *CaMAP4K3*, or their kinase activity could be modulated through interactions with *CaMAP4K3*, potentially via protein–protein interactions or complex formation. These findings also suggest that *CaMAP4K3* may play a distinct role in stress responses, separately from the classical MAPK cascade.

In conclusion, the current study demonstrates that *CaMAP4K3* negatively regulates drought resistance, with its kinase activity being critical for this modulation. Our findings provide valuable insights into the functional role of *CaMAP4K3* in drought stress regulation and guide future research on the broader MAP4K family and their contributions to plant stress responses. However, the precise mechanisms through which *CaMAP4K3* modulates drought response remain unclear. Future studies should prioritize investigating interactions and co-regulatory roles among MAP4K family members, as well as identifying novel regulatory proteins associated with *CaMAP4K3*. Furthermore, exploring the potential role of *CaMAP4K3* in stomatal regulation will enhance our understanding of MAP4K-mediated signaling pathways.

CRediT authorship contribution statement

Chae Woo Lim: Writing – original draft, Investigation. **Woon-hee Baek:** Investigation. **Junyoung Cho:** Investigation. **Sung Chul Lee:** Writing – review & editing, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data for this article can be found online at <https://doi.org/10.1016/j.cj.2025.03.002>.

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