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Functional Analysis of the Pepper RING-Type E3 Ligase CaANKR1 Involved in Drought Stress Tolerance via Modulation of Absciscic Acid Signaling

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ABSTRACT

Plants have developed a diverse array of mechanisms that facilitate survival under stress conditions, among which the ubiquitin-proteasome system (UPS) is a post-translational system used to modulate abiotic stress responses at the molecular level. Within the UPS, E3 ligase plays a key role in determining substrate specificity and has been implicated in the abscisic acid (ABA) signaling pathway during drought stress. In this study, we isolated CaANKR1, an ankyrin repeat-containing C3HC4-type RING E3 ligase, from pepper and characterized its functions in plants subjected to drought stress. *CaANKR1* expression was induced by various abiotic stresses, including dehydration, salinity, and mannitol. CaANKR1 was found to be localized in the nucleus and to possess E3 ligase activity. Additionally, we generated *CaANKR1*-silenced peppers and *CaANKR1*-overexpressing (OE) *Arabidopsis* transgenic plants to analyze the functional roles of *CaANKR1* in response to drought stress. *CaANKR1*-silenced peppers exhibited enhanced drought tolerance, which was associated with reduced transpirational water loss and increased ABA sensitivity. In contrast, *CaANKR1*-OE *Arabidopsis* transgenic plants showed reduced drought tolerance and decreased sensitivity to ABA. Collectively, these findings suggest that CaANKR1 functions as a negative regulator in drought stress responses.

1 | Introduction

During their lifespan, plants have to contend with a range of abiotic stresses, such as those associated with drought, low temperatures, heavy metals, and high salinity (Hirayama and Shinozaki 2010; Zhu 2016). By inhibiting growth and developmental processes, such stresses can have a number of detrimental effects on plants, among which drought stress is considered to have the most damaging effects (Anjum et al. 2011). Under drought conditions, plants experience water deficits, thereby limiting growth and ultimately reducing productivity. To

enable survival under stress conditions, plants have developed a broad range of molecular and physiological mechanisms, including the regulation of stomatal apertures and water balance (Yamaguchi-Shinozaki and Shinozaki 2006; Zhu 2002), and it has been established that these mechanisms are associated with the plant hormone abscisic acid (ABA), which is synthesized and accumulates in plants when these are exposed to drought conditions (Finkelstein et al. 2002; Lee and Luan 2012).

The phytohormone ABA plays roles in a range of processes and is particularly implicated in the regulation of stress tolerance

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(Cutler et al. 2010; Giraudat et al. 1994). Under drought conditions, ABA induces stomatal closure and the accumulation of osmolytes, thereby contributing to the maintenance of water capacity (Ng et al. 2014). The ABA signaling pathway comprises three key elements, namely, the ABA receptors pyrabactin resistance/pyrabactin-resistance-like/regulatory component of ABA receptor (PYR/PYL/RCAR), group A 2C-type protein phosphatases (PP2Cs), and SNF1-related protein kinases 2 (SnRK2s) (Ma et al. 2009; Park et al. 2009). Under normal conditions, PP2Cs bind to SnRK2s and thereby inhibit their activity via interaction with and dephosphorylation of target proteins (de Zelicourt et al. 2016). However, under stress conditions, ABA binds to ABA receptors, which in turn bind to PP2Cs, thus inhibiting their activity. As a consequence of PP2C inactivation, SnRK2s phosphorylate substrates such as ABA-responsive promoter elements (ABREs) and ABA-binding factors, thus promoting the expression of ABA-responsive genes (Cutler et al. 2010; de Zelicourt et al. 2016; Soma et al. 2021).

In eukaryotes, ubiquitination is a widespread post-translational modification process that primarily functions in the elimination of specific proteins (Lyzenga and Stone 2011). This process is mediated via the activities of the three enzymes ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). Initially, E1 activates ubiquitin using ATP, and the activated ubiquitin is then transferred to E2, forming the E2-ubiquitin complex. Thereafter, the E3 ligase interacts with both E2 and the substrate protein, thereby attaching ubiquitin to the substrate (Metzger et al. 2014; Yee and Goring 2009). These interactions are subsequently repeated, and the substrate is eventually degraded by the 26S proteasome system (Smalle and Vierstra 2004). Among these components of the ubiquitination system, the E3 ligase is considered the key enzyme for identifying and recruiting specific substrates (Ciechanover and Schwartz 1998). Plant E3 ligases are mainly categorized into two groups, namely, the single-subunit and multi-subunit types (Shu and Yang 2017), the former of which comprises Homology to E6-AP C-terminus (HECT), Plant U-BOX (PUB), and Really interesting new gene (RING) proteins, which are classified based on structure. Multimeric E3 ligases can be further subdivided into the four subtypes CUL1-based E3 ligases or SKP1-like Cul1 F-box (SCF), CUL3-RING E3 ligases (CRL3), CUL4-based E3 ligases (CRL4), and the anaphase-promoting complex/cyclosome (APC/C) E3 ubiquitin ligase complex (Chen and Hellmann 2013), which play roles in a range of physiological processes in plants. Notably, E3 ligases have emerged as regulators of abiotic stress responses, particularly those associated with drought stress (Lyzenga and Stone 2011; Stone 2014).

It has been established that the genome of *Arabidopsis* encodes in excess of 1400 E3 ligases, among which, RING-type E3 ligases account for more than 470 (Stone et al. 2005; Vierstra 2009). RING-type E3 ligases are classified into two canonical types (C3H2C3 and C3HC4) and five modified types (RING-v, RING-D, RING-S/T, RING-G, and RING-C2) (Stone et al. 2005). In eukaryotes, the ankyrin repeat is a highly conserved sequence consisting of 30 or 34 amino acid residues (Sedgwick and Smerdon 1999). The ankyrin repeat is the most prevalent motif and is primarily associated with protein-protein interactions (Li et al. 2006). Among the RING-type E3 ligases, ankyrin repeat-containing C3HC4 types have been established to function in a diverse range of

physiological processes in plants. For example, in *Oryza sativa*, XA21 binding protein 3 (XB3), a C3HC4-type RING E3 ligase containing an ankyrin repeat, plays roles in plant immunity via its association with XA21 (Wang et al. 2006), whereas in *Arabidopsis*, there are five ankyrin repeat C3HC4-type RING E3 ligases referred to as XBATs (Nodzon et al. 2004), among which, XBAT32 is involved in the regulation of lateral root formation and XBAT35 is associated with ethylene signaling in apical hook curvature (Carvalho et al. 2012; Nodzon et al. 2004; Yuan et al. 2013). As a consequence of alternative splicing, XBAT35 can occur as two isoforms, XBAT35.1 and XBAT35.2, which have been demonstrated to be characterized by different cellular localization (Carvalho et al. 2012; Liu et al. 2017). Of these two isoforms, XBAT35.2 has been revealed to be involved in the regulation of abiotic stress responses via interaction with Accelerated cell death 11 (ACD11) (Li et al. 2020).

In this study, we isolated and characterized CaANKR1, a C3HC4-type RING E3 ligase with ankyrin repeats, in pepper, which is nuclear localized and exhibits E3 ligase activity. To assess the biological function of CaANKR1 in drought stress, we used *CaANKR1*-silenced pepper and *CaANKR1*-overexpressing (OE) *Arabidopsis* plants, the former of which showed increases in ABA sensitivity and drought tolerance, whereas, in contrast, the latter were characterized by reductions in ABA sensitivity and drought tolerance. These findings accordingly indicate that CaANKR1 functions as a negative modulator of the drought stress response.

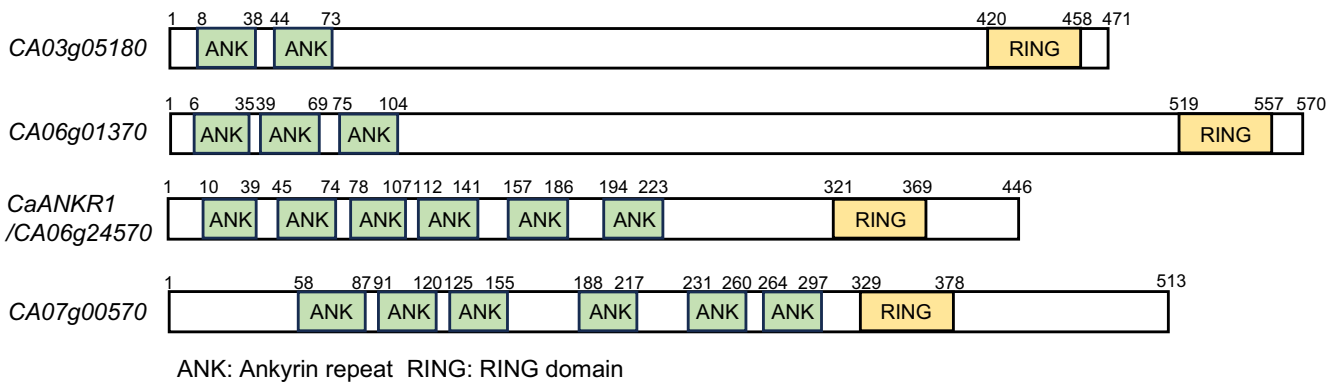
2 | Results

2.1 | Isolation of CaANKR1 and Sequence Analysis

In previous studies, ankyrin repeat C3HC4-type RING proteins have been shown to function in various plant physiological processes, including responses to abiotic stress (Yuan et al. 2013). These RING proteins are referred to as XB3-like proteins due to their structural similarity to the XA21-binding protein (XB3), and 187 such proteins have been identified among 29 species (Yuan et al. 2013). Although in *Arabidopsis*, these RING proteins, referred to as XBATs, have been widely studied as modulators of different molecular mechanisms, ankyrin repeat C3HC4-type RING proteins remain largely uncharacterized in other species. In this study, we sought to determine whether ankyrin repeat C3HC4-type RING proteins play a role in the abiotic stress responses of pepper. Using the amino acid sequences of XB3-like proteins from pepper, we performed a BLASTP search and accordingly identified four RING proteins (CA06g24570, CA03g05180, CA06g01370, and CA07g00570). Initially, we analyzed the domain structures of these four proteins using the SMART web tool (Figure 1A). They possess two distinctive domains. All four proteins possess two conserved domains: an N-terminal ankyrin repeat domain comprising 2 to 6 repeat units, and a C-terminal C3HC4-type RING domain of approximately 50 amino acids.

Next, to identify dehydration stress-responsive RING genes, we conducted RT-qPCR analysis using pepper leaves subjected to dehydration for 0, 3, and 9 h (Figure 1B). The dehydration-responsive marker gene *CaOSR1* was strongly induced under

(A)



(B)

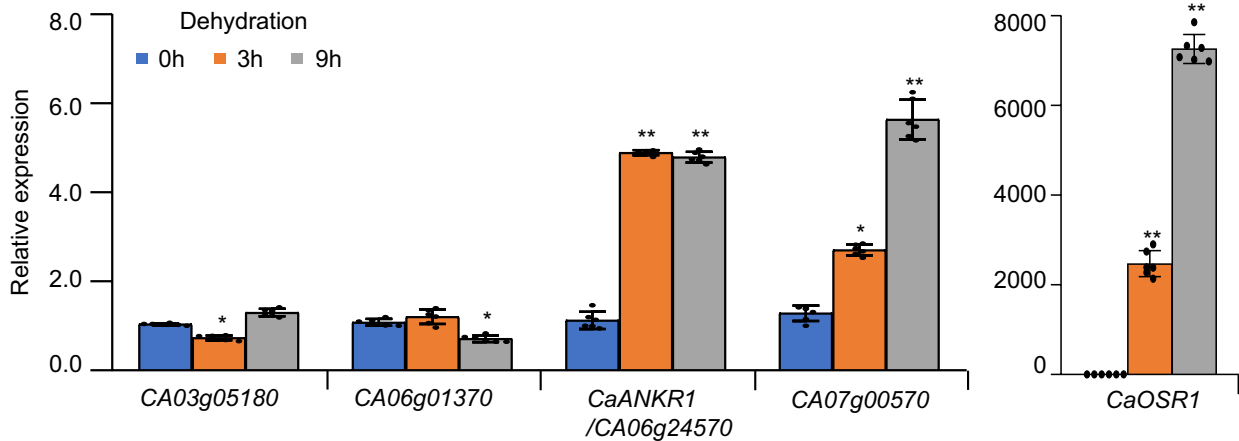


FIGURE 1 | Characterization of pepper ankyrin repeat C3HC4-type RING genes. (A) Domain organization of the pepper ankyrin repeat C3HC4-type RING genes. The amino acid sequences were obtained from SGN (<http://solgenomics.net/>), and the domains were predicted using SMART (Simple Modular Architecture Research Tool; <http://smart.embl-heidelberg.de/>). (B) Expression of ankyrin repeat C3HC4-type RING genes in response to abiotic stresses. Pepper plants were subjected to dehydration, with the *CaACT1* gene being used as an internal control (Student's *t*-test; **p* < 0.05, ***p* < 0.01).

stress conditions, validating the experimental setup. Among the four RING candidates, *CA06g24570* and *CA07g00570* showed statistically significant induction in response to dehydration, while the expression levels of the other two genes remained largely unchanged. Based on its early and robust induction, *CA06g24570* was selected for further characterization and designated *CaANKR1*.

2.2 | Molecular Characterization of Pepper *CaANKR1*

To characterize the function of the *CaANKR1* gene in pepper plants, we first examined its expression in various organs, including leaves, roots, stems and flowers (Figure 2A). Notably, leaves, roots, and stems were collected from pepper plants at the 6-leaf stage and used for RNA isolation. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis revealed that the *CaANKR1* gene was expressed in all organs, but significantly lower expression of *CaANKR1* was observed in the stem compared to the other tissues (Figure 2A). We also conducted RT-qPCR analysis to investigate the expression patterns of the *CaANKR1* gene in pepper leaves following exposure to abiotic stresses and stress-related signaling molecules

(Figure 2B). Upon treatment with ABA and H₂O₂, the expression level of *CaANKR1* gradually increased up to 12 h, but then decreased markedly by 24 h, returning to levels similar to those at 2 h. Similarly, mannitol treatment induced a gradual increase in *CaANKR1* expression up to 24 h. In contrast, NaCl treatment did not result in a statistically significant difference in *CaANKR1* expression.

Next, to determine the subcellular localization of *CaANKR1*, we generated a green fluorescent protein (GFP)-fused *CaANKR1* construct, which was transiently expressed in *Nicotiana benthamiana* (Figure 2C). Co-localization of the fluorescent signal of *CaANKR1*-GFP with DAPI nuclear stain indicated that the *CaANKR1* protein is localized in the nucleus. These findings suggest that *CaANKR1* may be involved in the response to various abiotic stresses, potentially functioning within the nucleus.

2.3 | *CaANKR1* Has E3 Ligase Activity

Given that *CaANKR1* possesses a C3HC4-type RING domain in its C-terminus (Figure 1A), we hypothesized that *CaANKR1* may function as an E3 ligase. As shown in Figure 3A, multiple sequence alignment analysis revealed that the RING domain of

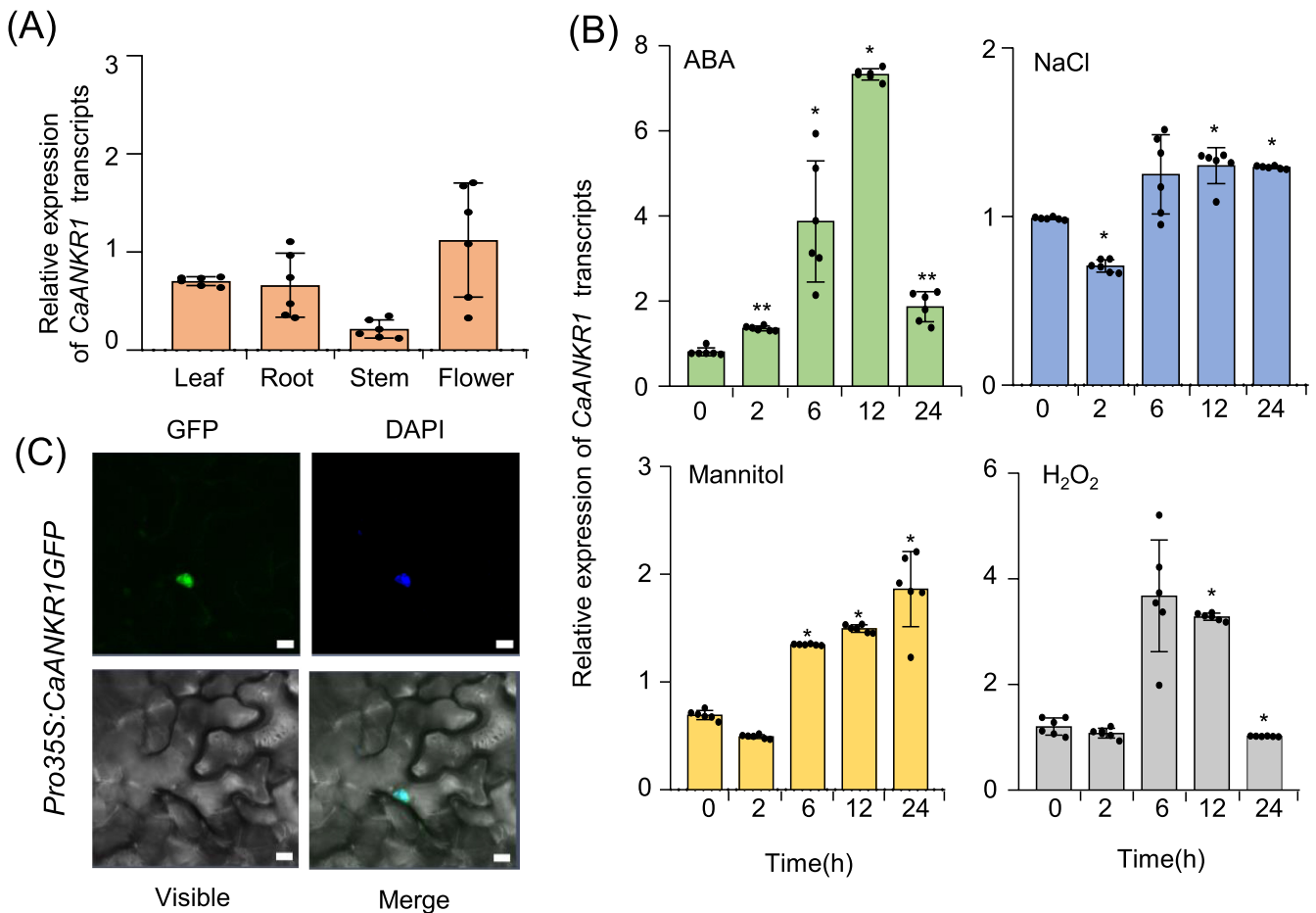


FIGURE 2 | Molecular characterization of pepper *CaANKR1*. (A) Organ-specific expression of *CaANKR1*. (B) Relative expression of *CaANKR1* transcripts in response to different abiotic stresses. Pepper plants were treated with abscisic acid (ABA) (100 μ M), NaCl (200 mM), mannitol (250 mM), or H₂O₂ (100 mM), and the expression of *CaANKR1* was validated by RT-qPCR analyses using cDNA derived from the first and second leaves. The pepper *PP2A* (*CaPP2A*) gene was used as an internal control. All values shown are the means $\pm$ standard error of three independent experiments, and asterisks indicate statistically significant differences compared with the value at 0 h (Student's *t*-test; *p* < 0.05). (C) Subcellular localization of *CaANKR1* proteins in *Nicotiana benthamiana* leaves.

CaANKR1 shares high sequence homology with those of its homologs from other plant species. To investigate the E3 ligase activity of *CaANKR1*, we conducted a self-ubiquitination assay by incubating maltose-binding protein (MBP)-tagged *CaANKR1* with various combinations of ubiquitination components, including ubiquitin, UBE1 (E1), and UBC5b (E2) (Figure 3B). When incubated with all components, a polyubiquitinated ladder of *CaANKR1* proteins was detected, indicating that *CaANKR1* protein possesses E3 ligase activity. Since the highly conserved cysteine and histidine residues in the RING domain are essential for coordinating two zinc ions and maintaining structural integrity, these amino acid substitutions are designed to disrupt the function of the RING domain (Garcia-Barcena et al. 2020). We generated a mutant, *CaANKR1*^{H338Y/C341S}, in which histidine 338 and cysteine 341 were substituted with tyrosine and serine, respectively. In contrast to the intact *CaANKR1* protein, the MBP-*CaANKR1*^{H338Y/C341S} mutant failed to produce polyubiquitinated ladders, indicating that the integrity of the RING domain is critical for the E3 ligase activity of *CaANKR1*.

To assess the effect of dehydration stress on *CaANKR1* activity in planta, an in vivo ubiquitination assay was conducted using *N. benthamiana* leaves transiently expressing *CaANKR1*-GFP.

Under dehydration stress, the *CaANKR1*-GFP protein level gradually declined with increasing stress duration, which was accompanied by a concomitant decrease in its autoubiquitination (Figure 3C).

2.4 | *CaANKR1* Expression Is Associated With Drought Tolerance in Pepper Plants

To determine the biological roles of *CaANKR1*, we generated *CaANKR1*-silenced pepper plants using a virus-induced gene silencing (VIGS) system (Figure 4). The efficiency of VIGS was confirmed by performing RT-PCR analysis using mRNA extracted from leaves of *CaANKR1*-silenced pepper (TRV2:*CaANKR1*) and the control (TRV2:00) pepper plants (Figure 4A). Compared with the TRV2:00 plants, TRV2:*CaANKR1* plants exhibited markedly reduced *CaANKR1* transcript levels. These two plant lines were subsequently used for further analysis.

To evaluate the role of *CaANKR1* in drought response, we subjected both TRV2:00 and TRV2:*CaANKR1* plants to drought stress by withholding water for 14 days, followed by re-watering for 3 days (Figure 4B). Although we observed no significantly

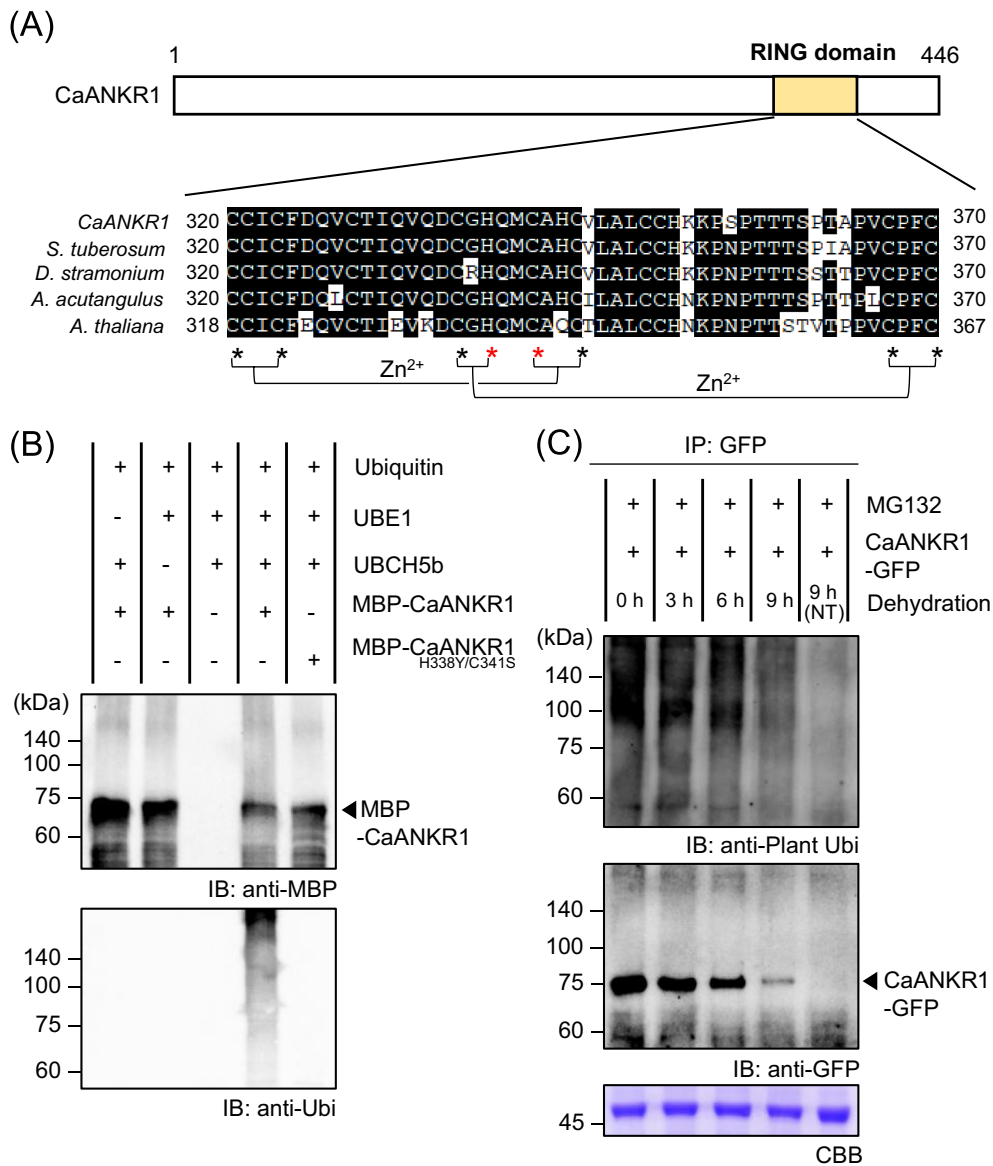


FIGURE 3 | CaANKR1 has E3 ligase activity. (A) Amino acid sequence alignment of RING domain in CaANKR1 and homologous proteins. The MSA analysis was performed by GeneDoc version 2.7.000. (B) Autoubiquitination of CaANKR1. The MBP-CaANKR1 fusion proteins were detected using anti-MBP and anti-ubiquitin antibodies. (C) In vivo ubiquitination assay of CaANKR1 E3 ligase under dehydration stress. Total leaf protein extracts served as the input, and immunoprecipitated samples were analyzed by western blotting with anti-GFP and anti-plant ubiquitin antibodies. Coomassie brilliant blue (CBB) staining confirmed equal protein loading across samples.

phenotypic differences between the two plant lines under normal or drought conditions (Figure 4B, upper and middle images), TRV2:00 plants exhibited more severe wilting than TRV2:CaANKR1 plants after rewatering (Figure 4B, lower image). Notably, the survival of TRV2:CaANKR1 plants (66.67%) was substantially higher than that of TRV2:00 plants (35.83%). This enhanced drought tolerance of TRV2:CaANKR1 plants appeared to be linked to differences in water retention. When the rate of water loss was assessed by measuring the weight of detached first and second leaves over 8 h, TRV2:CaANKR1 plants showed a significantly lower rate of water loss compared to controls (Figure 4C). Since transpirational water loss is influenced by ABA-mediated stomatal closure (Muhammad Aslam et al. 2022). We next examined leaf temperature and stomatal aperture in both TRV2:00 and TRV2:CaANKR1 plants following ABA treatment (Figure 4D,E). Prior to ABA treatment,

there were no significant differences between the plant lines. However, after ABA application, TRV2:CaANKR1 plants exhibited higher leaf temperatures (Figure 4D) and consistently smaller stomatal apertures (Figure 4E) than TRV2:00 plants. These findings suggest that the enhanced drought tolerance of CaANKR1-silenced peppers may be attributed to increased sensitivity to ABA, particularly in relation to stomatal regulation.

The negative role of CaANKR1 in drought tolerance was further validated through virus-induced overexpression in both pepper (Figure 4F,G) and tobacco, a closely related *Solanaceae* species (Figure 4H,I). In contrast to silenced plants, overexpression of CaANKR1 in both species resulted in reduced drought tolerance compared with the respective controls. Notably, overexpression of a CaANKR1^{H338Y/C341S} mutant lacking E3 ligase activity did not affect drought tolerance, suggesting that the E3 ligase

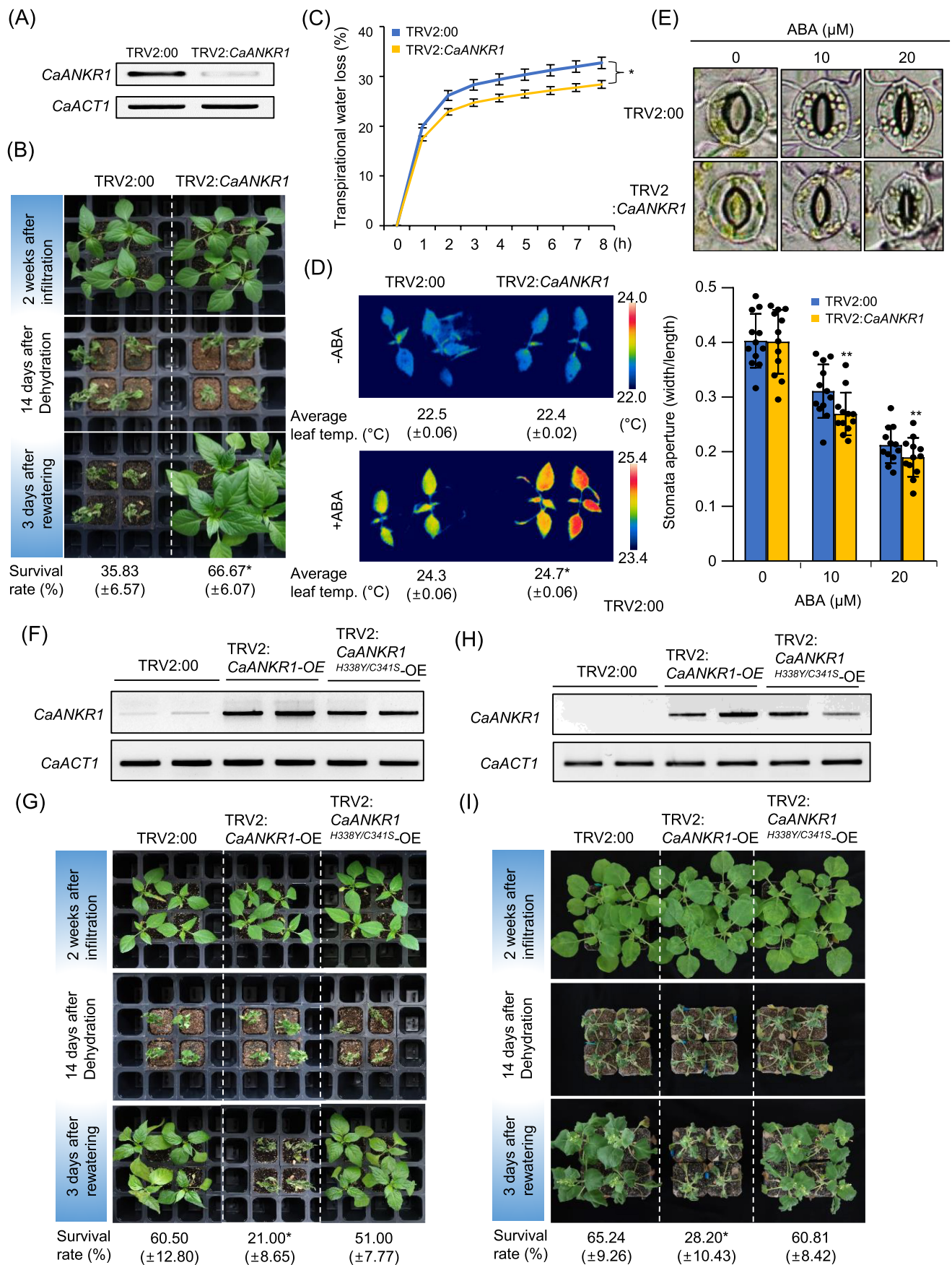


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FIGURE 4 | Increased tolerance of *CaANKR1*-silenced pepper plants to drought stress. (A) Semi-quantitative reverse transcription–polymerase chain reaction analysis of *CaANKR1* expression in the leaves of pepper plants transfected with an empty vector control (TRV2:00) or *CaANKR1*-silenced construct (TRV2:*CaANKR1*). *CaACT1* was used as an internal control gene. (B) Drought tolerance assay of TRV2:*CaANKR1* and TRV2:00 pepper plants. The percentages of surviving plants were calculated after re-watering. (C) Water loss (%) from the leaves of TRV2:*CaANKR1* and TRV2:00 plants at different time points after leaf detachment. Two weeks after agroinfiltration, the first and second leaves from 16 plants per line were used in each experiment. (D) Surface temperatures of the leaves of TRV2:*CaANKR1* and TRV2:00 plants after treatment with abscisic acid (ABA). Representative thermographic images were obtained at 0 h and 3 h after treatment with 100 μ M ABA. Leaf temperatures were measured from the first and second leaves of plants. (E) ABA-induced stomatal closure in TRV2:*CaANKR1* and TRV2:00 plants. Representative images were obtained under a microscope, and the stomatal apertures were measured from 100 randomly selected stomata for each line 3 h after ABA treatment. (F–I) Decreased drought tolerance by virus-mediated *CaANKR1*-overexpression (TRV2:*CaANKR1*-OE) in pepper and tobacco plants. Expression levels of *CaANKR1* in leaves from TRV2:00, TRV2:*CaANKR1*-OE, and its RING-dead mutant TRV2: *CaANKR1*^{H338Y/C341S}-OE plants were analyzed in pepper (F) and tobacco (H). For the drought tolerance assay, those plant lines were used at 2 weeks post-agroinfiltration in pepper (G) and 3 weeks post-agroinfiltration in tobacco (I). Survival rates were determined as described above. All data are presented as the means $\pm$ standard error of three independent biological experiments, each consisting of at least two technical replicates and including a minimum of 24 plants per line. Asterisks indicate significant differences compared to control plants (Student's *t*-test; **p* < 0.05, ***p* < 0.01).

activity of *CaANKR1* is critical for its role in drought response. Collectively, these results suggest that *CaANKR1* functions as a negative regulator of drought tolerance in pepper through its E3 ligase activity.

2.5 | Overexpression of *CaANKR1* Contributes to Reduced ABA Sensitivity During Seed Germination and Seedling Growth in Arabidopsis

Given the increased ABA sensitivity observed in *CaANKR1*-silenced pepper plants, we constructed *Arabidopsis* transgenic lines overexpressing *CaANKR1* to further investigate its role (Figures 5 and 6). RT-PCR analysis revealed that two independent transgenic lines (*CaANKR1*-OE #1 and *CaANKR1*-OE #3) exhibited elevated *CaANKR1* transcript levels compared with wild-type (WT) plants and were selected for analysis (Figure 5A).

To assess whether overexpression of the *CaANKR1* gene affects ABA sensitivity during seed germination and seedling development, we conducted phenotypic analyses using WT and *CaANKR1*-OE plants in the presence or absence of ABA (Figure 5). Under normal conditions, no significant differences were observed between WT and transgenic lines. However, in the presence of 0.4 μ M ABA, both transgenic lines exhibited higher germination and cotyledon greening rates compared to WT plants (Figure 5B,C). Moreover, the primary root lengths of *CaANKR1*-OE plants were significantly longer than those of WT plants when seedlings of both lines were vertically grown for 9 days on MS medium supplemented with 0 or 0.5 μ M ABA (Figure 5D,E). These results indicate that overexpression of the *CaANKR1* gene confers reduced sensitivity to ABA during seed germination and seedling growth.

2.6 | *CaANKR1*-OE *Arabidopsis* Plants Show Reduced Tolerance to Drought Stress

On the basis of our observation of reductions in the ABA sensitivity of *CaANKR1*-OE plants during the germination and seedling stages (Figure 5), we investigated whether this ABA insensitivity influences drought stress responses of *CaANKR1*-OE

plants (Figure 6). We first compared the drought phenotypes of *CaANKR1*-OE and WT plants (Figure 6A). Under normal conditions, there were no significant differences between *CaANKR1*-OE and WT plants (Figure 6A, upper image). However, following drought treatment and subsequent re-watering, *CaANKR1*-OE plants displayed more severe wilting than WT plants (Figure 6A, middle and lower panels). Survival rates after re-watering were significantly lower in *CaANKR1*-OE lines (41.90% and 34.66%) compared to WT (67.50%).

To assess water loss, we measured the fresh weights of detached shoots of WT and *CaANKR1*-OE plants over time (Figure 6B). *CaANKR1*-OE plants exhibited a higher rate of water loss than WT, suggesting impaired water retention. To determine whether this increased transpirational water loss is due to altered stomatal regulation in responses to ABA, we investigated leaf surface temperature and stomatal apertures in both *CaANKR1*-OE and WT plants with or without ABA treatment (Figure 6C,D). Prior to ABA treatment, no significant differences between the two plant lines were observed. However, following ABA treatment, although both WT and *CaANKR1*-OE plants showed increased leaf temperatures, the surface temperature of *CaANKR1*-OE leaves remained significantly lower than that of WT leaves (Figure 6C). Consistently, stomatal apertures in *CaANKR1*-OE plants remained wider than in WT plants following ABA treatment (Figure 6D). These findings suggest that the reduced drought tolerance of *CaANKR1*-OE plants is associated with decreased ABA sensitivity.

To further investigate the molecular basis of these observations, we analyzed the expression of dehydration stress-responsive genes using RT-qPCR (Figure 7). Total RNA samples were extracted from the leaves of *CaANKR1*-silenced pepper and *CaANKR1*-OE plants after 4 h of dehydration stress induced by shoot detachment. As representative stress-responsive genes in pepper plants, *CaOSR1* (*Capsicum annuum* Osmotic Stress Resistance 1), *CaRAB18* (*Capsicum annuum* Response to ABA 18), and *CaDREBLP1* (*Capsicum annuum* Dehydration-Responsive Element Binding factor—Like Protein1) were selected, since these are known to be induced by drought stress in pepper plants and share high sequence homology with *Arabidopsis* *RAB18*, *RD29B*, and *DREB2A*, respectively (Hong and Kim 2005; Lim et al. 2018; Park et al. 2016). Compared with the TRV2:00 control

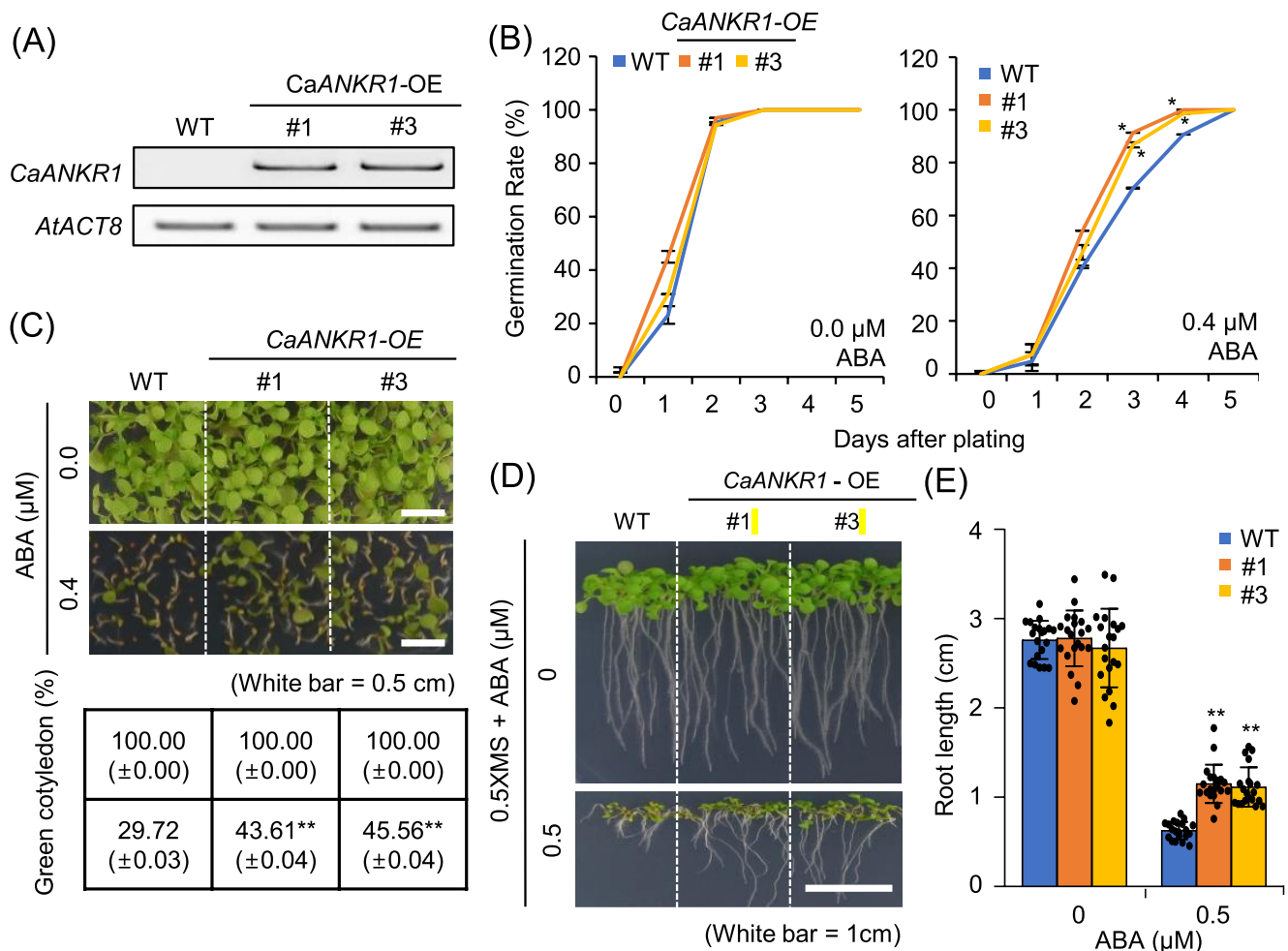


FIGURE 5 | Decreased sensitivity of *CaANKR1*-OE transgenic Arabidopsis lines to abscisic acid (ABA). (A) Semi-quantitative reverse transcription–polymerase chain reaction analysis of *CaANKR1* expression in a wild-type (WT) and *CaANKR1*-OE transgenic plants. *Arabidopsis thaliana* Actin 8 (*AtACT8*) was used as an internal control gene. (B) Germination rates of *CaANKR1*-OE mutants and WT plants. Seeds from each plant line ($n=100$) were germinated on 0.5 $\times$ Murashige and Skoog (MS) medium containing 0.0 or 0.4 μ M ABA. (C) Seedling development of WT and *CaANKR1*-OE plants exposed to 0.0 and 0.4 μ M ABA. Representative photographs were obtained and the percentage of seedlings in each line with green cotyledons was calculated 10 days after plating ($n=36$ per plant line). White bar=0.5 cm. (D and E) Primary root elongation of WT and transgenic lines in response to ABA. Representative photographs were obtained (D) and the lengths of the roots of each plant were measured (E) 9 days after plating ($n=36$ per plant line). All data are presented as the means $\pm$ standard error of three independent biological experiments, each comprising at least two technical replicates. Asterisks indicate significant differences between WT and *CaANKR1*-OE plants (Student's *t*-test; * $p<0.05$, ** $p<0.01$).

plants, we detected the higher expression of *CaOSR1*, *CaRAB18*, and *CaDREBL1* in *CaANKR1*-silenced pepper, whereas in *Arabidopsis*, the levels of *RAB18*, *RD29B*, and *DREB2A* expression were found to be higher in *CaANKR1*-OE plants than in the wild-type plants. These gene expression patterns align with the phenotypic responses observed (Figures 4–6), suggesting that *CaANKR1* acts as a negative regulator of dehydration-responsive gene expression under drought stress conditions, although the precise mechanism by which *CaANKR1* exerts this regulation remains unclear.

3 | Discussion

Ubiquitination is a key regulatory process involved in modulating the responses of plants to abiotic stress (Lee and Kim 2011). It is mediated by a cascade of three enzymes: ubiquitin-activating

enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), with E3 ligases conferring substrate specificity and thereby ensuring the selective ubiquitination of target proteins (Callis 2014). In *Arabidopsis*, more than 470 RING-type E3 ligases have been reported (Vierstra 2009). Among these, the C3HC4 RING E3 ligases containing ankyrin repeats, referred to as XBATs in *Arabidopsis*, share structural similarity with the XA21-binding protein XB3 in *Oryza sativa* (Yuan et al. 2013). Although XBATs have been implicated in multiple physiological processes and abiotic stress responses in *Arabidopsis* (Li et al. 2020; Prasad et al. 2010; Zhang et al. 2021), there has been comparatively little research focusing on the functional characterization of XB3-like E3 ligases in *Capsicum annuum*. In this study, we isolated and characterized an ankyrin repeat-containing C3HC4 RING E3 ligase, *CaANKR1*, from pepper, and on the basis of the functional analyses of *CaANKR1*-silenced pepper and *CaANKR1*-OE *Arabidopsis* plants, we demonstrated

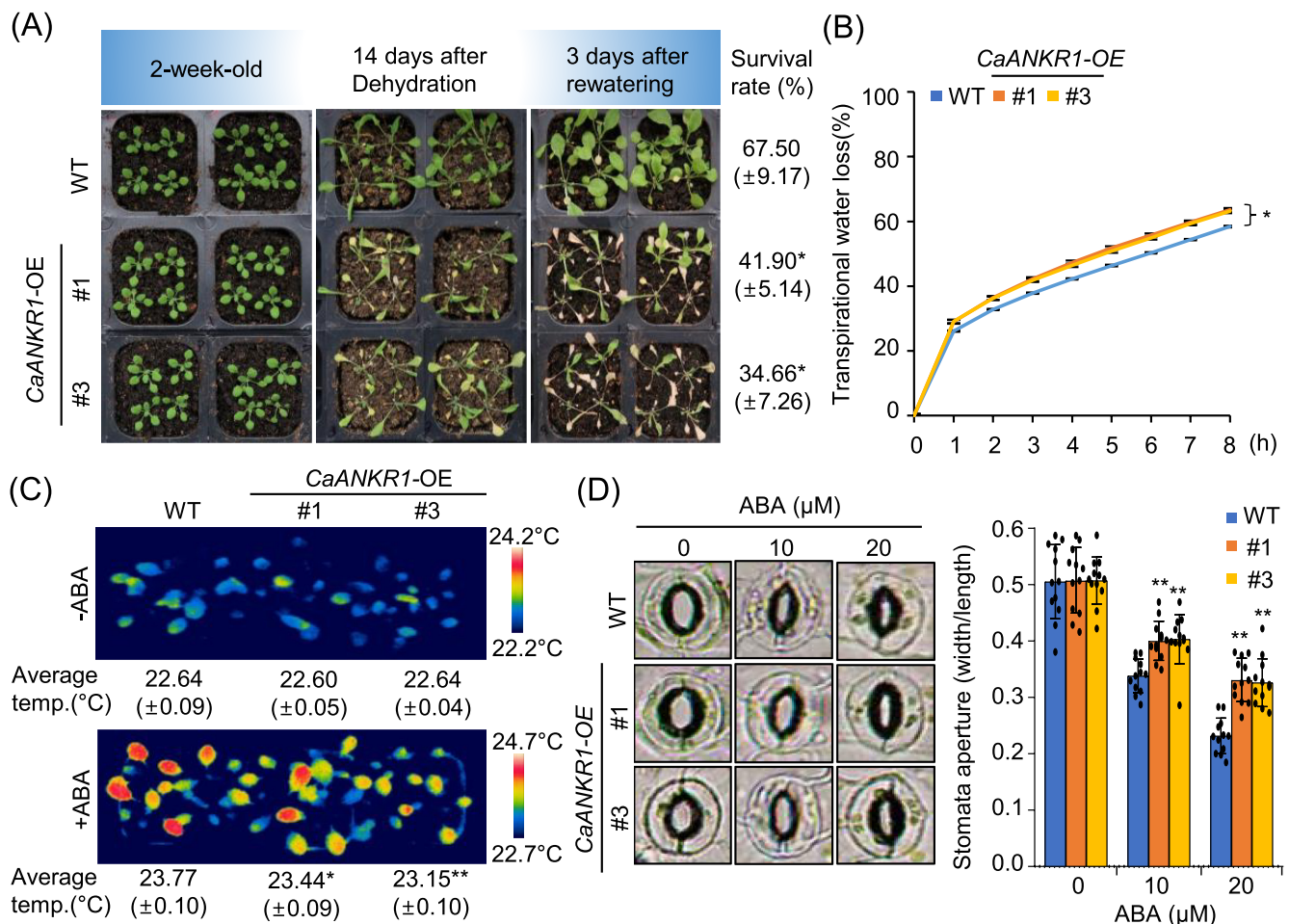


FIGURE 6 | Decreased tolerance of *CaANKR1*-OE transgenic *Arabidopsis* lines to drought stress. (A) Drought tolerance assay of wild-type (WT) and *CaANKR1*-OE transgenic plants. The percentages of surviving plants were calculated after re-watering ($n=32$ per plant line). (B) Water loss (%) from the leaves of WT and transgenic plants at different time points after leaf detachment ($n=36$ per plant line). (C) Surface temperatures of the leaves of WT and transgenic plants with or without treatment of abscisic acid (ABA). Representative thermographic images were obtained at 0 h and 3 h after treatment with 100 μ M ABA ($n=36$ per plant line). (D) Stomatal apertures of WT and *CaANKR1*-OE plants treated with ABA. Leaf peels were harvested from 3-week-old plants of each line ($n=12$) and incubated in SOS buffer containing 0, 10, or 20 μ M ABA. Representative images were obtained under a microscope, and the stomatal apertures were measured from 100 randomly selected stomata for each line 3 h after ABA treatment. All data are presented as the means $\pm$ standard error of three independent biological experiments, each comprising at least two technical replicates. Asterisks indicate significant differences between the WT and *CaANKR1*-OE plants (Student's *t*-test; * $p < 0.05$, ** $p < 0.01$).

that *CaANKR1* acts as a negative regulator of drought responses in pepper plants.

To determine the functional involvement of *CaANKR1* in ABA-mediated drought responses, we first analyzed its expression pattern following treatment with abiotic stresses and stress-related signaling molecules. We observed a significant induction of *CaANKR1* expression in response to drought and ABA treatment, suggesting its involvement in stress adaptation. This is consistent with previous studies showing that many E3 ligases are upregulated under abiotic stresses (Qi et al. 2016; Ryu et al. 2010). Structurally similar E3 ligases, such as XBAT 31 and XBAT 35, have also been reported to participate in abiotic stress responses. XBAT 31 mediates the breakdown of the thermosensor, while XBAT 35 modulates the ABA signaling cascade by ubiquitinating VPS23A (Yu et al. 2020; Zhang et al. 2021). Given the verified involvement of these ligases in stress responses, we hypothesized that *CaANKR1* similarly plays a role in adapting to abiotic stress. To gain further evidence for

this supposition, we generated *CaANKR1*-silenced peppers and *CaANKR1*-OE plants and subjected them to drought stress treatment. *CaANKR1*-silenced peppers exhibited enhanced drought-tolerant phenotypes, whereas *CaANKR1*-OE *Arabidopsis* transgenic plants showed heightened sensitivity to drought, as indicated by differences in stomata aperture and water retention capacity (Figures 4 and 6). Furthermore, *CaANKR1*-OE plants were found to be comparatively insensitive to ABA and were characterized by longer root lengths and enhanced germination rates compared with control plants (Figure 5). These phenotypes collectively suggest that *CaANKR1* negatively regulates ABA-mediated drought responses.

The negative regulatory role of *CaANKR1* appeared to be associated with altered expression of stress-responsive genes. ABA plays a pivotal role in enhancing drought tolerance by inducing the expression of stress-responsive genes (Yamaguchi-Shinozaki and Shinozaki 2006; Zhu 2002). To elucidate the role of *CaANKR1* in ABA-mediated drought responses, we

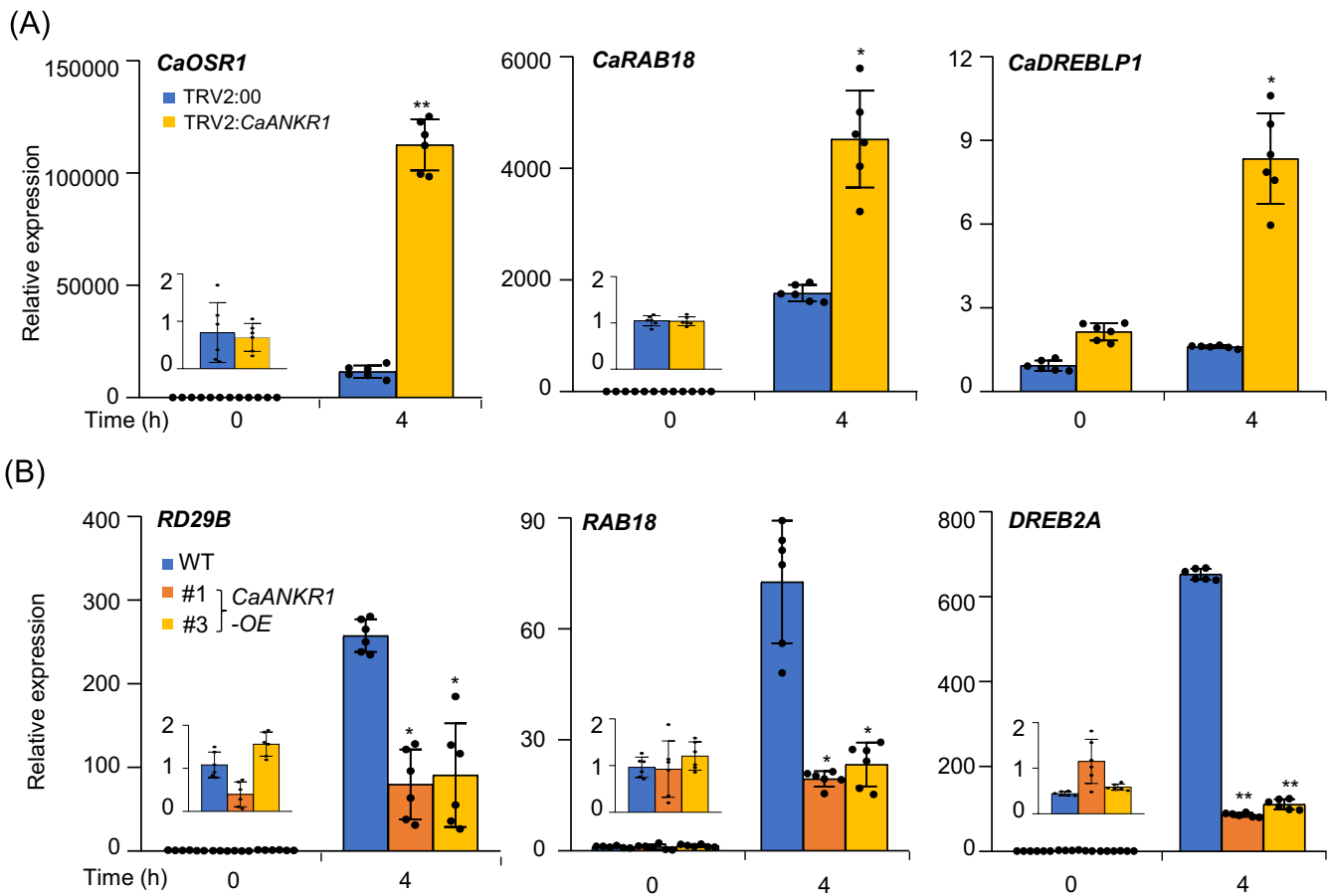


FIGURE 7 | Expression patterns of stress-responsive genes in the leaves of *CaANKR1*-silenced pepper plants and *CaANKR1*-OE transgenic *Arabidopsis*. (A and B) Reverse transcription-quantitative polymerase chain reaction analysis was performed using leaf cDNA samples from *CaANKR1*-silenced pepper plants (A) and *CaANKR1*-OE plants (B) exposed to drought stress for 4 h after detachment. The relative expression ($\Delta\Delta CT$) of each gene was normalized to the expression level of *CaActin1* and *AtActin8* used as an internal control gene. Data represent the means $\pm$ standard error of three independent biological experiments, each comprising at least two technical replicates. Asterisks indicate significant differences compared to the control (Student's *t*-test; **p* < 0.05, ***p* < 0.01).

analyzed the expression of selected stress marker genes, including *RD29B*, *RAB18*, and *DREB2A*, as well as their homologs in pepper plants. *RAB18* and *RD29B* encode dehydrins, which are highly hydrophilic proteins that function in abiotic stress adaptation (Sun et al. 2021), whereas *DREB2A*, a transcription factor induced by drought and salinity stress, regulates downstream stress-responsive genes (Sakuma et al. 2006). Notably, the promoter of *CaOSR1*—a pepper homolog of *RD29B*—is characterized by the presence of DRE/CRT elements and seven ABREs, and is activated by ABA, drought, and NaCl stress (Park et al. 2016). In the present study, *CaANKR1*-silenced pepper plants exhibited increased expression of *CaOSR1*, *CaRAB18*, and *CaDREBLP1*, whereas *CaANKR1*-OE *Arabidopsis* plants showed reduced expression of *RD29B*, *RAB18*, and *DREB2A* (Figure 7). These findings suggest that *CaANKR1* suppresses the expression of stress-related genes, either directly or indirectly, thereby acting as a negative regulator of ABA-mediated drought responses.

Given that *CaANKR1* encodes a RING-type E3 ligase, identification of the substrate proteins targeted by this enzyme is essential for elucidating the precise regulatory mechanisms of *CaANKR1* in stress responses. Notably, the N-terminal

ankyrin repeat domain of *CaANKR1*, consisting of six repeat units, may provide a structural basis for identifying its target substrates. The ANK domain is primarily characterized by its folding structure (Chakrabarty and Parekh 2014) and is known to mediate protein–protein interactions (Li et al. 2006; Michaely and Bennett 1992). In addition, *CaANKR1* contains a RING finger domain within the C-terminal region and shows ligase activity, similar to other RING-type E3 ligases (Figures 1 and 2). The findings of previous studies have revealed that these RING-type E3 ligases are involved in modulating hormone signaling and stress responses via the ubiquitination of specific substrates. For example, AIP2 has been shown to act as a negative regulator of ABA signaling by targeting ABI3 for degradation (Zhang et al. 2005), whereas RGLG1 and RGLG5 ubiquitinate the phosphatase PP2CA, a key component of the ABA signaling cascade (Wu et al. 2016). In addition, RGLG1 and RGLG2 negatively regulate drought stress tolerance by promoting the ubiquitination and degradation of MAPKKK18 in *Arabidopsis* plants (Yu et al. 2021). Under drought stress conditions, RGLG2 also mediated the ubiquitination of AtERF53 transcription factor, a member of the AP2/ERF superfamily, leading to its proteasomal degradation (Cheng et al. 2012). Furthermore, the RING-type E3

ligases DRIP1 and DRIP2 have been established to interact with and ubiquitinate DREB2A, thereby negatively regulating drought responses (Qin et al. 2008). In pepper, CaDSR1, a RING-type E3 ligase, attenuates ABA-mediated drought stress signaling by promoting the degradation of CaDILZ1, a subgroup D bZIP transcription factor, via its E3 ligase function (Lim et al. 2018). Similar to these E3 ligases, CaANKR1 may modulate the stability of kinases and transcription factors involved in ABA-mediated drought stress signaling. Therefore, identifying the transcription factors or other substrates that interact with CaANKR1 will provide key insights into the molecular mechanisms by which CaANKR1 modulates the expression of stress-related genes and contributes to *in vivo* stress responses.

In conclusion, our results suggest that CaANKR1, a RING-type E3 ligase, is associated with the regulation of drought responses in pepper, potentially through interactions with ABA signaling pathways. Silencing of *CaANKR1* resulted in enhanced drought tolerance in pepper, while overexpression in *Arabidopsis* led to ABA hyposensitivity during seedling development and increased drought susceptibility. These results point to a possible link between CaANKR1 and ABA-mediated drought response; however, direct evidence for its modulation of ABA signaling or identification of downstream targets is currently lacking. Further investigation is required to identify CaANKR1-interacting proteins and elucidate the molecular mechanisms underlying its function in drought stress responses.

4 | Materials and Methods

4.1 | Plant Materials and Growth Conditions

In this study, we used pepper (*Capsicum annuum* cv. Nockwang), *Arabidopsis thaliana* (ecotype Columbia-0), and tobacco (*Nicotiana benthamiana*) plants. For germination, seeds of pepper and tobacco plants were soaked in water and incubated in a 28°C growth chamber for 5 days. *Arabidopsis* seeds were vernalized at 4°C on Murashige and Skoog (MS) salt medium for 2 days, then incubated at 24°C under a 16/8-h light/dark cycle for 7 days. Seedlings of pepper, *Arabidopsis*, and tobacco were planted in a steam-sterilized compost mixture of soil (peat moss, perlite, and vermiculite, 5:3:2 ratio, v/v/v), sand, and loam (mixed at 1:1:1 ratio, v/v/v). The plants were grown in a growth room maintained at 24°C ± 1°C with 60% relative humidity under white fluorescent light (130 μmol photons m⁻² s⁻¹).

4.2 | RNA Isolation and Reverse Transcription-Quantitative Polymerase Chain Reactions (RT-qPCR)

Total RNA isolation and RT-qPCR analysis were conducted as previously described (Bae et al. 2023; Lim et al. 2021). For RNA isolation, pepper plants at the six-leaf stage were subjected to ABA, NaCl, mannitol, and H₂O₂ treatments as previously described (Bae et al. 2023; Baek et al. 2023; Lim et al. 2021). Plants were sprayed with 100 μM ABA or 100 μM H₂O₂ or irrigated with 200 μM NaCl or 600 μM mannitol solutions, depending

on the respective treatments. Dehydration stress was imposed by removing the roots and exposing the detached shoots to air drying for the indicated time points. The first and second leaves were harvested, immediately frozen in liquid nitrogen, and stored at -80°C until use.

To analyze the expression patterns of stress-responsive marker genes, pepper (2 weeks after agroinfiltration) and *Arabidopsis* (3-week-old) were subjected to dehydration stress as described above. The first and second leaves of the pepper plants and the rosette leaves of the transgenic *Arabidopsis* plants were harvested at 0, 4, and 8 h after treatment.

Total RNA was extracted from the harvested samples using TRIzol Reagent (Molecular Research Center). After DNase I treatment to eliminate genomic DNA contamination, RNA was quantified, and 1 μg of total RNA was used for cDNA synthesis using an iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions. RT-qPCR analysis was conducted on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using iQ SYBR Green Supermix, synthesized cDNA, and gene-specific primers (Table S1). The housekeeping genes *CaACT1* and *AtACT8* were used for normalization in pepper and *Arabidopsis*, respectively, and relative expression levels were calculated following the ΔΔCt method (Livak and Schmittgen 2001).

4.3 | Subcellular Localization Analysis

To investigate the subcellular localization of *CaANKR1*, the full-length coding sequence of *CaANKR1* was fused to the C-terminus of green fluorescent protein (GFP) in the binary vector p326GFP, resulting in the *Pro35S:CaANKR1-GFP* construct. This construct was introduced into *N. benthamiana* leaves by agroinfiltration using *Agrobacterium tumefaciens* strain GV3101 and the silencing suppressor strain p19 (OD₆₀₀ = 0.5 for each, 1:1 ratio). GFP fluorescence was visualized 2 days after infiltration using an LSM700 confocal microscope (Carl Zeiss).

4.4 | Virus-Induced Gene Silencing (VIGS) and Virus-Mediated Overexpression (VOX)

CaANKR1-knockdown pepper plants were generated by a tobacco rattle virus (TRV)-based VIGS system as previously described (Lim et al. 2021). Briefly, a 300-bp sequence targeting the *CaANKR1* gene for VIGS was computationally predicted using the Solanaceae Genomics Network VIGS tool (<https://solgenomics.net/tools/vigs>), based on the *C. annuum* cv. CM334 reference genome. The predicted sequence was cloned into the pTRV2 vector and introduced into *Agrobacterium tumefaciens* strain GV3101. Two-week-old pepper seedlings were co-infiltrated with pTRV1 and either pTRV2:*CaANKR1* (for gene knockdown) or pTRV2:00 (negative control) into the cotyledons of 2-week-old pepper plants.

VOX-mediated transient expression of *CaANKR1* in pepper and tobacco plants was conducted as previously described (Lim et al. 2025). The full-length *CaANKR1* sequence, bearing a 3×FLAG epitope tag, was cloned into the vector (SPDK3888,

#149276; Addgene) following *XbaI* and *SacI* digestion. The resulting construct, pTRV2-*CaANKR1*-OX vector, was introduced into *Agrobacterium tumefaciens* strain GV3101.

4.5 | Generation of Transgenic *CaANKR1*-Overexpressing (OE) *Arabidopsis* Plants

The full-length coding sequence of *CaANKR1* was cloned into the pCR8/GW/TOPO vector (Invitrogen). Using an LR reaction with the pGWB15 vector, the *Pro35S:CaANKR1* construct was generated and introduced into *A. tumefaciens* strain GV3101. *Arabidopsis* plants were then transformed via the *Agrobacterium*-mediated floral-dip method (Clough and Bent 1998). Seeds harvested from the transformed plants were sown on MS medium containing 50 µg/mL kanamycin for the selection of transgenic seeds. For further analysis, two independent T₃ lines (*CaANKR1*-OE) were selected.

4.6 | Plant Phenotypic Analyses

Phenotypic assessments were conducted as previously described (Bae et al. 2023; Lim et al. 2021), using *CaANKR1*-silenced pepper plants (collected 2 weeks after agroinfiltration) and 3-week-old *CaANKR1*-OE lines, along with their respective controls. For the drought resistance assay, the soil in each pot was thoroughly saturated to reach field capacity or higher prior to the initiation of drought stress, ensuring uniform starting conditions. Pepper and *Arabidopsis* plants were subjected to dehydration for 10 and 14 days, respectively, until soil moisture dropped below 30%, and survival rates were evaluated after re-watering for 3 days. Transpirational water loss was assessed using detached first and second leaves from pepper plants and aerial shoots from *Arabidopsis* plant lines. Detached tissues were placed on Petri dishes and weighed at 1-h intervals.

For ABA-induced stomatal closure assays, leaf peels were harvested from both pepper and *Arabidopsis* plants and floated on stomatal opening solution (SOS; pH 6.15, 50 mM KCl, 10 mM MES-KOH, 10 mM CaCl₂) for 3 h to induce stomatal opening. Subsequently, the solution was replaced with fresh SOS buffer containing 0, 10, or 20 µM ABA, and samples were incubated for an additional 3 h. Stomata were observed in randomly chosen fields using a Nikon Eclipse 80i microscope, and stomatal apertures (width/length) were measured for at least 100 stomata per plant line using ImageJ software.

Thermal imaging analysis was performed using pepper plants and *Arabidopsis* plant lines treated with 100 µM ABA by foliar spraying. Leaf surface temperatures were measured using a T420 infrared camera (FLIR Systems) and analyzed with FLIR software.

For the *Arabidopsis* seed germination assay, 36 seeds from both wild-type and *CaANKR1*-OE plants were sown on MS agar medium supplemented with varying concentrations of ABA. After vernalization at 4°C for 2 days, the plates were incubated at 24°C under a 16/8-h light/dark photoperiod. Germination rates were assessed daily by counting seeds exhibiting radicle emergence,

and after incubation for 10 days, the percentage of seedlings with green cotyledons was recorded. To evaluate primary root elongation, sterilized seeds were vertically grown on MS medium containing either 0.0 or 0.5 µM ABA. After 9 days of incubation, root lengths were measured.

4.7 | In Vitro and in Vivo Ubiquitination Assay

Recombinant protein expression and in vitro ubiquitination assay were conducted as previously described (Baek et al. 2025). Briefly, full-length coding sequences of *CaANKR1* and its E3 ligase activity-deficient mutant, *CaANKR1*^{H338Y/C341S}, were cloned into the pMAL-c2X vector (New England Biolabs), and the resulting constructs were expressed in *Escherichia coli* strain BL21 (DE3). Recombinant proteins were purified using amylose resin (New England Biolabs).

For the in vitro auto-ubiquitination assay, purified MBP-*CaANKR1* and MBP-*CaANKR1*^{H338Y/C341S} (500 ng) were mixed with human UBE1 (Boston Biochemicals), His-tagged human UbcH5b (Enzo Life Sciences), bovine ubiquitin (Sigma-Aldrich), and ubiquitination reaction buffer [50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2 mM dithiothreitol, and 2 mM ATP]. These mixtures were incubated for 12 h at 30°C, and reaction products were separated by SDS-PAGE and analyzed by performing immunoblotting with anti-ubiquitin (Santa Cruz Biotechnology) and anti-MBP antibodies (New England Biolabs).

In vivo ubiquitination assay was performed as previously described (Lim et al. 2018) with minor modifications. Three days after agroinfiltration, 50 µM MG132 was infiltrated into *Nicotiana benthamiana* leaves transiently expressing *Pro35S:CaANKR1-GFP*, 12 h before leaf sampling and detachment for dehydration stress treatment. Each leaf sample was ground in native extraction buffer (50 mM Tris-MES pH 8.0, 0.5 M sucrose, 1 mM MgCl₂, 10 mM EDTA pH 8.0, and 5 mM DTT). For co-immunoprecipitation, total leaf protein extracts were incubated with anti-GFP magnetic beads (Chromotek, Planegg, Germany) at 4°C for 3 h. The immunoprecipitated protein complexes were subjected to immunoblot analysis using anti-plant ubiquitin (Agrisera, Vännäs, Sweden) and anti-GFP (Santacruz) antibodies.

Author Contributions

M.K., D.S.K., C.W.L., and S.C.L. conceived the research; M.K., D.S.K., and C.W.L. performed the experiments; M.K., D.S.K., and C.W.L. analyzed the data; and M.K. and S.C.L. wrote the manuscript. All authors have reviewed and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data are available in the manuscript or Supplementary files. The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** ppl70522-sup-0001-Supinfo.pdf.