Calmodulin HvCaM1 Negatively Regulates Salt Tolerance via Modulation of HvHKT1s and HvCAMTA4\[OPEN\]

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Calcium (Ca\(^{2+}\)) signaling modulates sodium (Na\(^{+}\)) transport in plants; however, the role of the Ca\(^{2+}\) sensor calmodulin (CaM) in salt tolerance is elusive. We previously identified a salt-responsive calmodulin (HvCaM1) in a proteome study of barley (Hordeum vulgare) roots. Here, we employed bioinformatic, physiological, molecular, and biochemical approaches to determine the role of HvCaM1 in barley salt tolerance. CaMs are highly conserved in green plants and probably originated from ancestors of green algae of the Chlamydomonadales order. HvCaM1 was mainly expressed in roots and was significantly up-regulated in response to long-term salt stress. Localization analyses revealed that HvCaM1 is an intracellular signaling protein that localizes to the root stele and vascular systems of barley. After treatment with 200 mM NaCl for 4 weeks, HvCaM1 was significantly up-regulated in response to long-term salt stress. HvCaM1 knockdown (RNA interference) lines showed significantly larger biomass but lower Na\(^{+}\) concentration, xylem Na\(^{+}\) loading, and Na\(^{+}\) transportation rates in shoots compared with overexpression lines and wild-type plants. Thus, we propose that HvCaM1 is involved in regulating Na\(^{+}\) transport, probably via certain class I high-affinity potassium transporter (HvHKT1;5 and HvHKT1;1)-mediated Na\(^{+}\) translocation in roots. Moreover, we demonstrated that HvCaM1 interacted with a CaM-binding transcription activator (HvCAMTA4), which may be a critical factor in the regulation of HKTI\(_{5}\) in barley. We conclude that HvCaM1 negatively regulates salt tolerance, probably via interaction with HvCAMTA4 to modulate the down-regulation of HvHKT1;5 and/or the up-regulation of HvHKT1;1 to reduce shoot Na\(^{+}\) accumulation under salt stress in barley.

Salt stress is a major abiotic factor restricting crop growth and productivity, which is exacerbated by global climate change and human activities (Munns and Tester, 2008; Munns et al., 2020). Excess sodium (Na\(^{+}\)) causes ion toxicity and also ion imbalance through competitively inhibiting the uptake of some mineral nutrients such as potassium (K\(^{+}\)). Maintaining lower Na\(^{+}\) accumulation in shoots is crucial for salt-tolerant plant species and genotypes under salt stress (Munns, 2005; Munns and Tester, 2008; Horie et al., 2012; Deinlein et al., 2014; Shen et al., 2016, 2017). Lower shoot Na\(^{+}\) accumulation in plants is attributed to either shoot Na\(^{+}\) exclusion or vacuolar sequestration, both of which are regulated by membrane transporters, such as some members of high-affinity potassium transporters (HKTs), Na\(^{+}\)/H\(^{+}\) exchangers (NHXs), and Salt Overly Sensitive1 (SOS1; Apse et al., 1999; Shi et al., 2000; Zhang and Blumwald, 2001; Davenport et al., 2007; Barragán et al., 2012; Huang et al., 2020). Activation of membrane transport systems in roots plays a key role in reducing shoot Na\(^{+}\) accumulation and enhancing salt tolerance in many plant species (Zhu, 2002; Shabala and Cuin, 2008; Ismail and Horie, 2017). However, the mechanisms underlying Na\(^{+}\) exclusion and translocation and the link to calcium (Ca\(^{2+}\)) signaling require further investigation.

Ca\(^{2+}\)-mediated signal transduction plays a regulatory role in plant salt tolerance (Zhu, 2002, 2016; Demidchik et al., 2018). For instance, SOS3 (calcineurin-like protein [CBL4]) is a Ca\(^{2+}\) sensor, which perceives...
the Na\textsuperscript{+}-induced cytosolic Ca\textsuperscript{2+} rise via the interaction with SOS2 (calcineurin-interacting protein kinase [CIPK24]), causing phosphorylation and activation of the plasma membrane SOS1/NHX1 (Shi et al., 2000; Zhu, 2002; El Mahi et al., 2019). The activation of SOS1 in root epidermal cells and xylem parenchyma cells leads to the extrusion of Na\textsuperscript{+} from roots to rhizosphere (Shi et al., 2002; Zhu, 2016). Meanwhile, a rapid elevation of cytosolic Ca\textsuperscript{2+} under Na\textsuperscript{+} stress can also be captured by other Ca\textsuperscript{2+} sensors, including calmodulin (CaM), calmodulin-like proteins, and calcium-dependent protein kinases (Galon et al., 2010; Cho et al., 2016). Multitomics techniques have revealed that CaM exhibits salt-induced expression changes at the transcriptional and translational levels in seedlings or roots, suggesting that these Ca\textsuperscript{2+} sensors participate in salt stress signaling (Zhang et al., 2012; Wu et al., 2014; Zhu et al., 2015; Shen et al., 2018; El Mahi et al., 2019). However, regulation of the complex Ca\textsuperscript{2+} signaling by CaMs to function in plant salt tolerance is still elusive.

CaMs usually encode a single peptide protein consisting of a pair of unique Ca\textsuperscript{2+}-binding EF-hands and have no other functional domains or motifs (Reddy et al., 2011). Meanwhile, CaM protein sequences are highly conserved in green plants, with an average 90% identity of 65 members detected in 15 representative plant species from Chlorophyceae to angiosperms (Zhu et al., 2015). Moreover, CaMs have less variation and fewer family members than other Ca\textsuperscript{2+} sensors (Xu et al., 2015; Zhu et al., 2015). However, little attention has been paid to the origin and evolution of CaMs in green plants. The recent advancement of assembled genomes (Kersey, 2019) and transcriptome databases (Leebens-Mack et al., 2019) will likely unlock the evolution of CaMs, providing insights into their roles in stress tolerance of plants.

CaM may function through posttranscriptional modification similar to that in SOS3 via the regulation of downstream receptors, such as CaM-binding proteins (CBPs; Galon et al., 2010; Batistić and Kudla, 2012; El Mahi et al., 2019). In different plants, CaM can adjust the affinity of the Ca\textsuperscript{2+}/CaM complex for the selection of special receptors (e.g. phosphodiesterase) and targets (e.g. Cyclic Nucleotide-Gated Channel12) after recognizing the cytosolic Ca\textsuperscript{2+} change for a specific stress response (Gifford et al., 2013; DeFalco et al., 2016). In Arabidopsis (Arabidopsis thaliana), AtCaM1/4 shows an upregulated expression under salt stress, resulting in enhanced salt tolerance via binding with S-nitrosoglutathione reductase in nitric oxide signaling (Zhou et al., 2016). CaM-binding protein kinases (AtCaM2/3/5) modulate the activity of heat shock factors through phosphorylation of a CaM-binding protein kinase (AtCBK3) in heat stress signaling (Zhang et al., 2009), while AtCaM6 and AtCaM7 are CaM-binding protein kinase (AtCBK3) in heat stress signaling (Zhang et al., 2009), while AtCaM6 and AtCaM7 are activated by the transcriptional factor AtCAMTA6 regulates the expression of a series of salt-responsive genes, including a key Pro biosynthetic enzyme, P5CS1, in soybean (Glycine max); Yoo et al., 2005; Rao et al., 2014). Moreover, many transcription factors, including CaM-binding transcription activators (CAM-TAs), CBP60s, MYBs, WRKYs, bZIPs, bHLHs, NACs, and GTs, have been identified as CBPs, suggesting that CaMs might play important roles in the transcriptional regulation of salt stress tolerance in plants (Galon et al., 2010; Xi et al., 2012; Shkolnik et al., 2019). For example, AtCAMTA6 regulates the expression of AtHKT1 via the binding sites of CGCG-core or CGTGT-core, contributing to Na\textsuperscript{+} homeostasis during seed germination (Shkolnik et al., 2019). Therefore, the roles of CaMs in the salt stress response have been reported, but their function in the regulation of Na\textsuperscript{+} transport remains to be revealed.

As one of the most salt-tolerant diploid crop species with a large and complex genome, barley (Hordeum vulgare) has been used as a model plant to decipher the physiological and molecular mechanisms of salinity tolerance in plants (Chen et al., 2005; Munns and Tester, 2008; Nevo and Chen, 2010; Mascher et al., 2017; Dai et al., 2018). However, there are limited reports on CaM members and their functions in barley. We have previously identified one salt-responding CaM protein (protein identifier A0MMD0) in the root proteome of wild barley genotypes (Wu et al., 2014; Shen et al., 2018). We hypothesized that HvCaM1 plays a critical role in the establishment of higher salt tolerance in barley via the regulation of Ca\textsuperscript{2+} signaling transduction, transcriptional factors, and membrane transport. Here, we cloned HvCaM1 (gene identifier HORVU0Hr1G001270) and comprehensively analyzed its function through overexpression (OE) and RNA interference (RNAi) in barley with a range of physiological and molecular techniques. We found that HvCaM1 negatively regulates salt tolerance in barley and participates in Na\textsuperscript{+} transport from roots to shoots. Furthermore, we also detected that HvCaM1 regulates two HvHKT1 members via the differential interaction with the transcriptional activator HvCAMTA4.

RESULTS

Evolutionary Bioinformatics and Phylogenetic Analysis of HvCaM1

We first cloned the full-length cDNA and genome DNA of HvCaM1 from the barley ‘Golden Promise’. HvCaM1 contains two exons and one intron encoding a peptide consisting of 149 amino acids, with two typical EF-hands (Supplemental Fig. S1A). No difference in coding sequences (CDs) and protein sequences was found between the Tibetan wild barley accessions and barley cultivars (Supplemental Table S1). Sequence similarity analysis showed that HvCaM1 exhibits, on average, 98.8% identity with the CaM members in rice.
and Arabidopsis with the four conserved Ca$^{2+}$-binding sites (Supplemental Fig. S1B). Phylogenetic analysis showed that HvCaM1 is relatively close to OsCaM1-1 and AtCaM1 (Supplemental Fig. S1C).

Evolutionary analysis further indicated that CaM1 likely originates from the ancestors of the Volvocales order of chlorophyte algae (Chlamydomonadaceae; Fig. 1A). CaM1s are particularly conserved in angiosperms, with up to 87% identity detected in the CaM1 proteins of 60 representative plant species (Supplemental Table S2). Moreover, sequence analysis showed that each CaM1 protein contains two conserved EF-hand motifs and the ubiquitous presence of a consensus sequence of DDGDGE and DXDXNE in the protein structure of Ca$^{2+}$-binding sites in major lineages (Fig. 1B). However, the difference of a couple of amino acids in the protein sequences of different chlorophyte algae indicated that CaM1s likely evolved from Chlamydomonadaceae but are conserved from streptophyte algae (e.g., Klebsormidium subtile) and then diversified in their function during the adaptation of land plants (Fig. 1; Supplemental Fig. S1).

HvCaM1 Is Up-Regulated in Roots under Salt Stress and Mainly Localized in Stele and Vascular Cells of Roots

We investigated the expression of HvCaM1 in roots and shoots of barley seedlings. Quantitative PCR (qPCR) analysis showed that HvCaM1 is expressed in both roots and shoots, with significantly higher expression in roots (Fig. 2A). Moreover, the expression of HvCaM1 in roots was greatly affected by salt concentration, with the highest up-regulated value occurring at 200 mM NaCl treatment (Fig. 2B). Interestingly, the expression of HvCaM1 was significantly up-regulated by 3.04-fold after 4 weeks of long-term salt stress compared with the control value (Fig. 2C).

The subcellular localization detected by GFP showed that HvCaM1 appears to be expressed in the nucleus and plasma membrane but not in the cytosol of onion (Allium cepa) epidermal cells, while it is expressed in the nucleus, cytosol, and plasma membrane in barley leaf mesophyll protoplasts, suggesting that it may be an intracellular signal protein (Fig. 2, D and E). We performed in situ PCR to determine the cellular localization of HvCaM1 transcripts in cross sections of roots and leaves. In roots, the HvCaM1 transcripts were mainly detected in phloem and xylem parenchyma cells of the stele (Fig. 2F). In leaves, the transcripts were found predominantly in phloem vessel and mesophyll cells and to a lesser extent in stomatal cells (Fig. 2G). Additionally, observation of fluorescence signals from GFP fused with the 2.1-kb promoter of HvCaM1 in the transformed barley seedlings also confirmed that HvCaM1 mainly localizes in the stele of roots (Supplemental Fig. S2A) and in stomatal guard cells,

![Figure 1](https://plantphysiol.org)

**Figure 1.** Phylogenetic tree and sequence analysis of HvCaM1. A, Evolution analysis of predicted CaM1 candidates in 60 representative species of the major lineage of green plants. All sequences were downloaded from the 1,000 Plant Transcriptome and EnsemblPlants databases. B, Sequence alignment of the two conserved EF-hand domains among nine representative species of major lineages. Hv, Hordeum vulgare; Ks, Klebsormidium subtile; Mp, Marchantia polymorpha; Mt, Microcachrys tetragona; Pg, Polypodium glycyrrhiza; Ph, Paraphymatoceros hallii; Pp, Pyramimonas parkeae; Sm, Selaginella moellendorfii; Tl, Takakia lepidiozoides. Arrowheads indicate conserved amino acids in the four Ca$^{2+}$-binding sites.
Figure 2. Expression pattern analysis of HvCaM1. A, Absolute quantification of HvCaM1 transcript in roots and shoots under the normal condition. B, Relative expression of HvCaM1 in roots exposed to salt concentration gradients within 2 h. C, Relative HvCaM1 expression within 4 weeks of salt exposure (200 mM). Asterisks represent highly significant and significant differences determined by independent Student’s t-test (*P < 0.05 and **P < 0.01; n = 4; error bars indicate SE). D and E, Subcellular localization of HvCaM1 in onion epidermal cells (D) and barley leaf protoplasts (E). F and G, In situ PCR analysis of HvCaM1 transcript in root (F) and leaf (G) cross sections of 14-d-old seedlings. bs, Bundle sheath; c, cortex; en, endodermis; ms, mesophyll; p, phloem; pe, pericycle; pv, phloem vessel; s, stomatal guard cell; xp, xylem parenchyma; xv, xylem vessel. Bars = 50 μm (D), 5 μm (E), and 100 μm (F and G).
subsidiary cells, and leaf mesophyll cells (Supplemental Fig. S2B).

HvCaM1 Negatively Regulates Salt Tolerance via the Modulation of Na\(^+\) Transport in Barley

To examine the function of HvCaM1, we generated 46 and 52 independent knockdown (RNAi) and OE barley lines, respectively. Three RNAi lines and three OE lines were examined by PCR verification via specific primers (Supplemental Fig. S3A), showing the significant average decrease and increase in the expression levels of HvCaM1 by 59% and 2.41-fold, respectively, in comparison with the wild-type plants (Supplemental Fig. S3B). The phenotypic difference between the genetically transformed lines and the wild-type plant was evident 3 weeks after 200 mM salt exposure (Fig. 3A). RNAi lines showed significantly higher salt tolerance than OE and wild-type seedlings 4 weeks after salt stress, as reflected by shoot and root dry weight (Fig. 3B). Only the dry weight of OE73 was significantly lower than that of the wild type in response to salt stress. Meanwhile, shoots of RNAi lines had higher relative water content than those of OE lines and wild-type plants at 4 weeks of salt stress (Supplemental Fig. S4A). When planted in the saline soil, these RNAi lines were less affected in grain yield traits (spikes per plant, seeds per spike, setting rate, and kernel weight) in comparison with wild-type plants (Supplemental Fig. S5). These results indicate that HvCaM1 may function as a negative regulator of salt tolerance in barley.

After 4 weeks of salt treatment, RNAi lines had a significantly lower shoot Na\(^+\) concentration (52 mg g\(^{-1}\) dry weight) than OE (81.4 mg g\(^{-1}\) dry weight) and wild-type (78.6 mg g\(^{-1}\) dry weight) plants, but such differences were not found in roots (Fig. 3C). RNAi lines showed a significantly higher K\(^+\) concentration in both roots and shoots (1.52- and 1.45-fold higher) than in wild-type plants, respectively. The OE lines showed significant NaCl-induced reduction of shoot K\(^+\) in comparison with the wild type (Fig. 3D). Consistently, RNAi lines showed a significant 144% and 109% higher shoot K\(^+\)/Na\(^+\) ratio than the wild-type and OE lines under salt stress, respectively (Fig. 3E). Thus, we hypothesized that HvCaM1 negatively regulates salt tolerance in barley, probably due to root-to-shoot Na\(^+\) translocation.

To validate the hypothesis that the involvement of HvCaM1 in the regulation of salt tolerance is related to Na\(^+\) transport, we examined the responses of RNAi lines (R1, R2, and R3) and wild-type seedlings to 200 mM (S200) and 250 mM (S250) salt stress solutions (Fig. 4). After 4 weeks of salt stress, three RNAi lines had significantly larger root and shoot dry weight than the wild-type plant under both S200 and S250 treatments, but there was no difference under S300

Figure 3. Phenotypic differences and ion responses of wild-type (WT), RNAi, and OE seedlings under salt stress. A, Phenotypes under 200 mM salt treatment and the control condition. Observation was conducted every week from the 14-d-old seedling (referred as the 0 time point) until 4 weeks. Bars = 10 cm. B to E, Dry weight (B), Na\(^+\) concentration (C), K\(^+\) concentration (D), and K\(^+\)/Na\(^+\) ratio (E) of barley genotypes under salt stress for 4 weeks. RNAi lines, R1, R2, and R3; OE lines, OE73, OE74, and OE15; WTN, transgenically negative line; 4C, 4 weeks under the control condition from 14-d-old seedlings. Data are means of six replicates ± s.e. (n = 6). Different lowercase letters above the columns represent significant differences at P < 0.05 determined by one-way ANOVA. DW, Dry weight.
treatment (Fig. 4, A and B; Supplemental Table S3). Furthermore, wild-type plants had significantly higher shoot Na\textsuperscript{+} concentrations than RNAi lines under S250 treatment. Such a difference was not detected in roots (Fig. 4C). Meanwhile, three RNAi lines and wild-type plants showed little difference in the total Na\textsuperscript{+} accumulation per plant (Fig. 4D), but the RNAi lines had much lower Na\textsuperscript{+} transport rates than the wild-type plants at S200 treatment, while differences at S250 were not significant (Fig. 4E). Clearly, the higher salt stress tolerance of RNAi lines is closely associated with less Na\textsuperscript{+} transport from roots to shoots, which was mediated by HvCaM1. On the other hand, RNAi lines had higher K\textsuperscript{+} concentration and accumulation in plant tissues in comparison with wild-type plants under salt treatment (Fig. 4, F–H). Therefore, our results indicate that HvCaM1 negatively regulates salt tolerance, indirectly resulting in reduced Na\textsuperscript{+} transport from roots to shoots under salt stress.

Silencing of HvCaM1 Modulates HKT1 Expression in Roots

To determine whether HvCaM1 regulates Na\textsuperscript{+} transport, qPCR analysis of the well-known genes associated with Na\textsuperscript{+} and K\textsuperscript{+} transport (HKT1;1–HKT1;5 except HKT1;2, HKT2;1–HKT2;3, NHX1–NHX6, and SOS1–SOS3 members) was performed for roots of the wild type and RNAi lines under the normal condition and salt treatment (Fig. 5). The relative expression (salt stress/control) of all examined Na\textsuperscript{+} and K\textsuperscript{+} transporter genes showed the dramatic difference between the two RNAi lines and wild-type plants at 3 weeks after salt treatment (Fig. 5; Supplemental Fig. S6). Moreover, all eight genes were greatly down-regulated in the two RNAi lines in comparison with the wild-type plants, except for HvHKT1;1, which was clearly up-regulated in the RNAi lines. Interestingly, over the 3-week salt treatment, relative expression of HvHKT1;5 showed a similar pattern to that of HvCaM1, while HvHKT1;1 showed a completely opposite response, suggesting the involvement of HvCaM1 in the regulation of both HvHKT1;1 and HvHKT1;5 expression (Fig. 5, A–C). On the contrary, four NHX genes (HvSOS1/HvNHX7, HvNHX1, HvNHX3, and HvNHX5) showed different patterns of relative expression from that of HvCaM1, although these genes were also greatly up-regulated in the wild-type plants under salt treatment (Fig. 5, D–G). HvSOS2 and HvSOS3 showed a similar expression pattern to that of the NHX genes (Fig. 5, H and I). Therefore, the involvement of HvCaM1 in regulating salt stress tolerance may influence the expression of HvHKT1;1 and HvHKT1;5 responsible for Na\textsuperscript{+} transport.

Furthermore, the physiological role of Na\textsuperscript{+} transporters, mainly for HvHKT1s in Na\textsuperscript{+} transportation...
from roots to shoots, was validated by xylem sap analysis (Fig. 6). Na\textsuperscript{+} concentrations in the xylem sap of HvCaM1 RNAi lines were significantly lower than in the wild-type plants after 7 d of 50 and 100 mM NaCl treatments (Fig. 6B). There was a slight difference in K\textsuperscript{+} concentration in the xylem sap among these barley lines (Fig. 6, C and D).

**DISCUSSION**

**Down-Regulation of HvCaM1 Enhances Salt Tolerance in Barley**

In plants, CaMs are Ca\textsuperscript{2+} sensors responding rapidly to early signaling transduction under salt stress (Zhang et al., 2012; Rao et al., 2014; Zhou et al., 2016). However, the roles of CaM in the long-term response to salt stress remain elusive. Previously, we discovered a salt-responding HvCaM1 in the analysis of the barley root proteome (Wu et al., 2014; Shen et al., 2018). This study shows that HvCaM1 is involved in the regulation of salt...
tolerance via the modulation of gene expression of Na\(^+\) transporters HvHKT1;5 and HvHKT1;1 and HvCAMTA4-mediated transcriptional regulation. In Arabidopsis, the knockout mutants Atcam1 and Atcam4 show hypersensitivity to salt stress (Zhou et al., 2016). Similarly, higher expression of both rice OsCaM1 and soybean GmCaM4 enhances salt tolerance (Chinpongpanich et al., 2012; Rao et al., 2014). However, we found that the knockdown (RNAi) lines of HvCaM1 showed a large increase of salt tolerance, as reflected by their larger biomass.
than wild-type and OE lines under 200 and 250 mM salt treatments (Figs. 3 and 4). There are many reports showing that gene silencing is beneficial for enhanced resistance and production (e.g. AtWRKY11, OsMADS526, and TaDA1), but overexpressing the same gene has negative effects in transgenic plants (Journot-Catalino et al., 2006; Khong et al., 2015; Liu et al., 2020). One possible explanation is that constitutive overexpression of a regulator gene can modulate the expression of downstream stress-associated genes, which could impose a major energy cost to the plants (Munn et al., 2020; Shabala et al., 2020). For example, drought-hypersensitive OsMADS26-overexpressing rice lines modulated 412 differentially expressed genes as compared with 95 differentially expressed genes in the RNAi rice lines, which displayed an enhanced drought tolerance (Khong et al., 2015).

_HvCaM1_ appears to be highly conserved in comparison with its closest homologous genes, _AtCaM1_ and _OsCaM1_ (Fig. 1; Supp. Fig. S1). Why does it show a different function in salt tolerance in barley? Our previous studies on the root proteome found that _HvCaM1_ is significantly down-regulated in the salt-tolerant barley genotypes (XZ16, XZ26, and CM72) under salt stress (Wu et al., 2014; Shen et al., 2018). A plausible explanation is that distinct functions of CaMs among the plants may be driven by natural selection or adaptation favoring a complex survival system in responding to external environments (Zhu et al., 2015; Zhao et al., 2019). barley originated from the Middle East, where drought and high salinity are natural environmental conditions (Nevo and Chen, 2010), in contrast to the natural habitats of Arabidopsis, rice, and soybean (Hyten et al., 2006; Kovach et al., 2009; Beilstein et al., 2010).

Moreover, the expression level of _HvCaM1_ varied greatly with NaCl concentrations and the exposed time in salt treatments (Fig. 2, B and C). As intracellular Ca\(^{2+}\) sensors, the expression level of CaMs could be changed with cytosolic Ca\(^{2+}\) oscillation elicited by Na\(^+\) stress (Reddy et al., 2011; Ismail and Horie, 2017). In comparison with barley, Arabidopsis, rice, and soybean are less tolerant to salt (Munn et al., 2008; Horie et al., 2012), and all CaM1s in these four species are linked to salt tolerance/sensitivity. In addition, there is no difference in the protein sequence of HvCaM1 among those genotypes (Supp. Table S1) and high similarity to CaM1s in Arabidopsis, rice, and soybean, suggesting that its function is probably related to posttranscriptional modification or translational regulatory networks (Batistic and Kudla, 2012; El-Mahi et al., 2019). Therefore, unlike _AtCaM1_ and other CaM1s, _HvCaM1_ may negatively regulate salt tolerance with the function of long-term adaptation to salinity in barley, which has not been previously identified.

_HvCaM1_ May Be Involved in the Modulation of the Long Distance of Na\(^+\) Transport from Roots to Shoots

In general, roots have higher salt tolerance than shoots, and lower shoot Na\(^+\) concentration is mainly associated with less Na\(^+\) transport from roots, which is an important characteristic for higher salt tolerance in most plants, including barley (Shabala et al., 2010; Zahra et al., 2014; Shen et al., 2016). Here, it is obvious that _HvCaM1_ knockdown lines had significantly lower shoot Na\(^+\) concentration than the wild-type or OE lines under both 200 and 250 mM NaCl (Figs. 3B and 4C). Lower shoot Na\(^+\) concentration likely results from either the increase of root Na\(^+\) exclusion or the decrease of root-to-shoot Na\(^+\) transport (Wu et al., 2019; Huang et al., 2020). Interestingly, we confirmed that the lower shoot Na\(^+\) concentration in RNAi lines is closely associated with a lower Na\(^+\) transportation rate from roots to shoots (Fig. 4E; Supplemental Fig. S4B) and a lower xylem sap Na\(^+\) concentration (Fig. 6) but with unchanged Na\(^+\) accumulation per plant (Fig. 4D). These results indicated that _HvCaM1_ is involved in root-to-shoot Na\(^+\) transportation, which is further supported by the preferential expression of _HvCaM1_ in root stele and vascular systems (Fig. 2, A and E). Another important factor that affects the amount of Na\(^+\) transport to the shoot is the rate of xylem Na\(^+\) loading. The xylem Na\(^+\) loading is thermodynamically active (Shabala, 2013) and is also mediated by either SOS1 or CCC transporters operating at the xylem parenchyma interface (Colmenero-Flores et al., 2007; Ishikawa et al., 2018). Here, we showed that transcript levels of SOS genes are significantly down-regulated in RNAi lines (Fig. 5G), which may restrict the amounts of xylem Na\(^+\) loading, thus reducing Na\(^+\) transport between roots and shoots. However, this requires a detailed investigation in the future.

High K\(^+\) accumulation and K\(^+\)/Na\(^+\) ratios are beneficial for plants to cope with salt stress (Chen et al., 2007; Shabala et al., 2010). In this study, RNAi lines had higher K\(^+\) concentration and K\(^+\)/Na\(^+\) ratios than wild-type and OE plants (Figs. 3, D and E, and 4, F and G), suggesting that _HvCaM1_ might affect K\(^+\) homeostasis. However, there was no difference in K\(^+\) transportation rate among these barley genotypes under salt stress (Fig. 4H; Supplemental Fig. S4B), indicating that _HvCaM1_ is not involved in the regulation of K\(^+\) transportation. In short, we propose that the preferential expression and cellular localization of _HvCaM1_ makes it possible for barley to modulate long-distance Na\(^+\) transport from roots to shoots (Zhu, 2016).

_HvCaM1_ Regulates Na\(^+\) Transport via Preferential Transcriptional Regulation of _HvHKT1s_

CaMs may bind with quantitatively changed Ca\(^{2+}\) signals across membrane systems (Gifford et al., 2013; DeFalco et al., 2016; Demidchik et al., 2018). The major membrane transporters conferring cellular and whole-plant Na\(^+\) homeostasis are HKTs and NHXs/SOS1 (Zhu, 2002; El-Mahi et al., 2019; Munn et al., 2020; Shabala et al., 2020). Here, only some _HKT1_ members, in particular _HvHKT1;5_ and _HvHKT1;1_, were significantly affected by the knockdown of _HvCaM1_ (Fig. 5,
A–C; Supplemental Fig. S6, A and B). HKTs regulate long-distance Na\(^+\) delivery, depending on root-to-shoot Na\(^+\) translocation and Na\(^+\) exclusion from the reproductive organ (Cao et al., 2020). \(HvHKT1;5\) negatively regulates salt tolerance in barley (Huang et al., 2020). Thus, lower expression of \(HvHKT1;5\) in RNAi roots potentially indicates less Na\(^+\) transport from roots to shoots, which is consistent with the function of \(HvCaM1\) in Na\(^+\) transport. Correspondingly, lower Na\(^+\) loading from roots to shoots via the xylem was confirmed with higher salt tolerance by the down-regulation of \(HvCaM1\) and \(HvHKT1;5\) (Fig. 6, A and B; Huang et al., 2020). By contrast, another Na\(^+\) transporter, \(HvHKT1;1\), plays a role in Na\(^+\) retrieval from shoots to roots, and overexpression of \(HvHKT1;1\) in Arabidopsis reduces Na\(^+\) accumulation (Han et al., 2018). However, there is no report on the overexpression and silencing of \(HvHKT1;1\) in barley, and the level of salt tolerance is very different between barley and Arabidopsis, which requires further investigation. The regulation of \(HvHKT1s\) by \(HvCaM1\) probably occurs at the transcriptional or posttranscriptional level (Reddy et al., 2011; Wang et al., 2015). In general, CaM functions in transcriptional regulation through specific CBP\(^+\) transcription factors, such as CAMTAs (Galon et al., 2010). CAMTA binds to Ca\(^{2+}\)/CaM and participates in transcriptional regulation by recognizing and binding to a specific cis-element, (G/A/C)CGCG(C/G)/T (Yang and Poovaiah, 2002; Shen et al., 2015). AtCAMTA6 negatively regulates salt tolerance via \(HKT1\), probably with the specific CGCG-core or abscisic acid response element-cooperating CGTCT-core motif (Shkolnik et al., 2019). Here, we identified an interacting partner of \(HvCaM1\), \(HvCAMTA4\), through yeast two-hybrid screening, showing transcriptional function in the nucleus (Fig. 7, A and B; Supplemental Table S4). Meanwhile, the expression pattern of \(HvCAMTA4\) was similar to those of \(HvHKT1;5\) and \(HvCaM1\) (Fig. 7C), indicating its possible role as a linker protein for \(HvCaM1\) to transcriptionally regulate the expression of \(HvHKT1;5\). Interestingly both \(HvHKT1;1\) and \(HvHKT1;5\) contain regulatory elements (Fig. 7D) for \(HvCAMTA4\) (Shkolnik et al., 2019), and there are different cis-regulatory element sites in the promoter regions of \(HvHKT1;5\) and \(HvHKT1;1\). Therefore, it may be concluded that \(HvCAMTA4\) regulates \(HvHKT1;5\) independently while it modulates \(HvHKT1;1\) together with regulatory proteins for abscisic acid signaling in response to salt stress (Whalley and Knight, 2013). Moreover, \(HvHKT1;1\) is an important Na\(^+\) transporter that confers salt tolerance (Han et al., 2018), but \(HvHKT1;5\) is a negative regulator of salt tolerance in barley (Huang et al., 2020). Overall expression of the key salt-responsive genes (\(HKT5s\), \(NHXs\), and \(SSOs\)) is down-regulated in the \(HvCaM1\) RNAi lines with the exception of \(HvHKT1;1\). Interestingly, \(HvHKT1;1\) and \(HvHKT1;5\) in the R1 and R2 RNAi lines are similarly highly expressed after salt-induced up-regulation and down-regulation at week 3, respectively (Fig. 5). This could be the explanation for the differential expression of \(HvHKT1;1\) and \(HvHKT1;5\) in \(HvCaM1\) knockdown lines. However, the possibility of other \(HvCaM1\)-interacting proteins, such as CBP60b, participating in the regulation of HKT1 transportation activity by posttranscriptional modification requires future experimentation (Lin et al., 2014).

In summary, we found that a transcriptional activator, \(HvCAMTA4\), interacts with \(HvCaM1\) under salt stress to regulate the expression of \(HvHKT1;5\) and \(HvHKT1;1\) at different sites of cis-regulatory elements of the promoter regions. However, the inherent interaction and signal transduction between \(HvCaM1\) and \(HvCAMTA4\) in mediating \(HvHKT1\) activity remain unknown. Meanwhile, the protein sequence of \(HvCaM1\) is quite conserved among plant species. Thus, RNAi or gene editing of \(CaM1s\) may be used to improve the salt tolerance of different crops.

**MATERIALS AND METHODS**

**Growth Conditions and Sampling**

Barley (\( Hordeum vulgare\) ‘Golden Promise’ (cv GP) was used for the experiments. Barley germination and growth conditions were performed as described in previous studies (Wu et al., 2014; Shen et al., 2016). In the hydroponic experiments, salt treatment began in 14-d-old seedlings by adding 100 m\(\text{M}\) NaCl per day to reach a final salt concentration of 100, 200, 250, or 300 m\(\text{M}\), according to experimental purposes. For a screening assay, the seedlings were exposed to the 200 m\(\text{M}\) salt condition and photographed every week. For gene expression analysis, roots and shoots were sampled under normal conditions. Moreover, roots were sampled under 0, 100, 200, and 300 m\(\text{M}\) salt treatments and at 0, 1, 2, 3, and 4 weeks under the 200 m\(\text{M}\) salt level. The normal condition without salt addition was used as the control. The modified one-fifth-strength Hoagland solution was renewed every 3 d. In the soil experiment, seedlings were transplanted into 10-L containers with mixed artificial soil, supplied with the same conditions as described by Shen et al. (2018). Soil salt treatment began at the heading stage by applying 1 L of 200 m\(\text{M}\) NaCl solution to the bottom of the pots every 3 d to a final content of 2% (w/w) NaCl. Seedlings supplied with tap water were used as the control. Yield traits, including spikes per plant, seeds per spike, setting rate, and kernel weight, were recorded. There were six biological replicates in both hydroponic and soil experiments.

**Gene Cloning and Bioinformatics Analysis**

\(HvCaM1\) (HVRUBHr1g0001270) was cloned from cv GP. Single-nucleotide polymorphism and amino acid variation of \(HvCaM1\) were validated among barley genotypes (‘Morex’, ‘CM2’, and ‘GP’ as well as Tibetan wild barley accessions XZ16, XZ26, and XZ169; Wu et al., 2014; Shen et al., 2018). Evolutionary bioinformatics were performed according to Zhao et al. (2019) with some modifications as described by Feng et al. (2020). The candidate sequences of \(CaM1\) members among 60 representative plants were selected from the 1,000 Plant Transcriptome and EnsemblPlants databases by BLAST-P (Zhu et al., 2015; Leebers-Mack et al., 2019; Supplemental Table S2). Multiple sequence alignment and phylogenetic analysis were performed by MAFFT and FastTree via the maximum likelihood method, respectively. The phylogenetic tree was annotated by the Interactive Tree of Life resource (http://itol.embl.de). Jalview was used to perform the alignment of protein sequences. The primers used are listed in Supplemental Table S6.

**Plant Transformation and Verification**

Genetic transformation was mediated by \( Agrobacterium tumefaciens\) strain AGL1 according to Harwood (2014) with some modification. To generate the RNAi construct, a 388-bp specific fragment (primers RNAi\_HvHKT1;1\_F/R) of \(HvCaM1\) transcript was cloned into pDONR-Zeo cloning vector by the Gateway BP Clonase II enzyme mix kit (11789, Invitrogen), according to Miki and Shimamoto (2004) and Miki et al. (2005). The positive cloning plasmid was
then recombined with pANDA vector by Gateway LR reaction (11799; Invitrogen). To generate the OE plasmid, a 450 bp complete coding region (primers OE_HvCaM1_F/R) of HvCaM1 was amplified and then constructed into pBlac214 vector with the similar method of RNAi construction. There were more than 10 independent transgenic events of RNAi and OE. Among them, three RNAi lines (R1, R2, and R3), three OE lines, and one transgenically negative line were used as the materials. Only PCR-positive plants with specific test primers (R_Test_F/R for RNAi and OE_Test_F/R for OE) were used in the following experiments.

Gene Expression Analysis

The transcript levels of HvCaM1 and transporter-associated genes were detected by qPCR. Briefly, total RNA of samples was extracted by the MiniBEST kit and reversed by the PrimeScript RT reagent kit (Takara). Then, qPCR was performed with SYBR Green Supermix (Bio-Rad) on a Roche LightCycler 480 instrument. For absolute qPCR analysis, a pDONR plasmid containing the complete CDS region of HvCaM1 was used to quantify the transcript concentration by diluting to 10^9, 10^-1, 10^-2, 10^-3, 10^-4, and 10^-5 ng µg^-1 (a standard curve of R^2 > 0.999), as described by Wu et al. (2016). For relative gene expression analysis, the comparative 2^-△△Ct method was performed using the reference gene a-Tubulin (Wu et al., 2014). The transcripts of wild-type (cv GP) roots under the normal condition were regarded as the controls. The primers of HKT, NHX, and SOS genes were designed following Fu et al. (2018) and Huang et al. (2020). There were four biological replicates and three technical repeats for gene expression analysis.

Element Determination and Analysis

After 4 weeks of salt exposure (200 or 250 mol), the fresh weight (FW) of roots and shoots was determined, and then tissues were dried in an oven at 80°C for 2 d to obtain dry weight (DW). Relative water content (RWC) was calculated using the following formula: RWC (%) = (FW – DW)/FW × 100%. Dried tissues were digested by HNO₃ using microwave digestion equipment (Multimwave 3000; Anton Paar) according to Shen et al. (2018). For element concentration determination, the 447-bp coding region of HvCaM1 without the stop code was amplified and fused with the 5' terminus of the sgFP gene into pCAMBIA1300 vector after linearization digestion using the homologous recombination cloning kit (Clonal). The recombinant and empty vectors driven by the 35S promoter were transformed separately into onion (Allium cepa) epidermis cells by a biolistic PDS-1000/He particle device (Bio-Rad) along with a cell-localized RFP marker, as described previously (Huang et al., 2020). GFP and RFP fluorescence signals were measured using a confocal laser microscope (LSM780; Zeiss) at 488/490 to 540 nm and 561/580 to 630 nm, respectively.


to the color reaction between alkaline phosphate and DIG-labeled antibody.

Yeast Two-Hybrid Interaction Assay

The candidate gene HvCAMTA4 (HORVU7Hr1G090260) was originally screened from a yeast (Saccharomyces cerevisiae) library constructed with the total cDNA of salt-treated barley roots by the CloneMiler II kit (AI1180; Invitrogen). To verify the interaction between HvCaM1 and HvCAMTA4, the cDNA of HvCaM1 was amplified into pGADT7 as prey, while the coding region of HvCaM1 cloned into pGBK T7 was used as a bait according to Cho et al. (2016). The combination of pGBK T7-53 and pGAD T7-T was used as the positive control, while empty vector of pGBK T7-Lam and pGAD T7-T was used as the negative control. The yeast two-hybrid interaction assay was performed after cotransformation of both the bait and prey constructs into Y2HGold yeast competent cells (Clontech). The transformed yeast cells were then screened on synthetic dextrose double dropout medium (lacking Leu and Trp) with or without aureobasidin A addition.

BIFC Assay

For the BIFC interaction assay, the coding regions of HvCaM1 and HvCAMTA4 were separately cloned into pSAt1-EYFP/nEYFP vectors to generate HvCaM1-eEYFP and nEYFP-HvCAMTA4 vectors with the Vazyime recombinant and empty vectors driven by the 35S promoter were transformed separately into onion (Allium cepa) epidermis cells by a biolistic PDS-1000/He particle device (Bio-Rad) along with a cell-localized RFP marker, as the normalized expression data were presented for sequence alignment and gene expression

Statistical Analysis

The significant differences of dry weight, relative water content, element content, yield, and gene expression among genotypes, treatments, or tissues were analyzed by one-way ANOVA or independent Student’s t test using SPSS 20.0 software (IBM SPSS Statistics). The significance levels at P < 0.05 and P < 0.01 were defined as significant and highly significant, respectively.

Accession Numbers

All accession numbers and species for sequence alignment and gene expression are listed in Supplemental Table S2.
Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Cloning and sequence analysis of HvCaM1.

Supplemental Figure S2. Tissue-specific localization of HvCaM1.

Supplemental Figure S3. PCR verification in RNAi and OE lines.

Supplemental Figure S4. Physiological indexes of wild-type, RNAi, and OE seedlings under salt stress.

Supplemental Figure S5. Growth performance and yield traits of wild-type and RNAi plants in the saline soil (n = 6).

Supplemental Figure S6. Expression analysis of Na\(^+\) and K\(^+\) transporters under salt stress (remaining).

Supplemental Table S1. Sequence variation in the HvCaM1 CDS region of barley genotypes.

Supplemental Table S2. Accession numbers for sequence alignment and gene expression.

Supplemental Table S3. Dry weight of wild-type and RNAi seedlings under salt conditions for 4 weeks (n = 4).

Supplemental Table S4. Positive interaction candidates with HvCaM1 by yeast two-hybrid screening assay.

Supplemental Table S5. CAMTA cis-regulatory elements in the 3-kb promoters of HKT genes.

Supplemental Table S6. Primers used in this study.

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