

Osmotic stress-induced CsRCI2E endosomal trafficking promotes the redistribution of aquaporin CsPIP2 at the plasma membrane of *Camelina sativa* L.

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Research Article

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Abstract

Rare Cold Inducible 2s (RCI2s) are proteolipids that travel from the plasma membrane (PM) to the endomembrane. The expression of RCI2s is induced by abiotic stresses such as cold, heat, drought, and salinity and affects abiotic stress tolerance in plants. It has been reported that CsRCI2E interacts with the water transport protein CsPIP2;1 to reduce its abundance at the PM during NaCl stress. Therefore, CsRCI2E is considered a potential factor affecting the endocytosis of CsPIP2s for maintaining cell homeostasis; however, its exact function in membrane trafficking remains unclear. In this study, we observed the rapid internalization of CsRCI2E and CsPIP2s under both mannitol- and NaCl-induced osmotic stresses using a sucrose density gradient. The transcription of *CsRCI2E* increased markedly 3 h after treatment with mannitol or NaCl. The over-expression of *CsRCI2E* enhanced stress tolerance and reduced cell damage from reactive oxygen species accumulation in germinating *Camelina*. Interestingly, the subcellular distribution of CsRCI2E and CsPIP2s shifted rapidly from the PM to the endomembrane within 0.5 h after both osmotic shocks even though the *CsRCI2E* gene expression had not been changed by the stresses at that time. Additionally, *CsRCI2E* overexpression caused the internalization and subcellular redistribution of CsRCI2E and CsPIP2 under osmotic stress conditions as well as no stress conditions. These results suggest that the internalization of CsRCI2E is a sensing mechanism in the early stages of osmotic shock. Furthermore, an increased amount of CsRCI2E promotes the membrane trafficking of CsPIP2 from the PM to the endomembrane to maintain water homeostasis in *Camelina*.

Key message

Osmotic stress-induced CsRCI2E contributes to fine-tuning of the CsPIP2s population from the plasma membrane to the endomembrane further promote osmotic stress tolerance in *Camelina*

Introduction

Water is the main solvent for nutritional elements that become substrates for cellular components or regulate cellular metabolism (Osakabe et al. 2014). In addition, the water in plant cells is essential for the concentration and transport of solutes and ions. Thus, water homeostasis is important for basal metabolism. However, changes in water potential due to ion ratios affect the water regulation in plant cells (Wakeel et al. 2011). High concentrations of salts such as NaCl cause a severe osmotic imbalance called osmotic shock (Munns 2002; Kim et al. 2019). Therefore, water and ions are in a complementary relationship and have a complex system for proper regulation. Higher plants have transcriptionally and translationally regulated water and ion transport proteins in their plasma membrane (PM). These proteins are regulated by kinase signaling pathways and act as major defense mechanisms for drought stress tolerance in plants (Zhu 2016; Lozano-Juste et al. 2020). Nevertheless, studies on the post-translational regulation of major membrane transporters are important to understand the multiple triggers for optimizing water and ion homeostasis, which is regulated by phosphorylation, protein–protein interactions, and recycling processes through membrane trafficking (Zhu 2016; Wang et al. 2020).

When sensing an osmotic imbalance, plants adjust stomatal closure and water transport activity to prevent water loss due to osmotic stress (Granot and Kelly, 2019). In cellular metabolism, the activity and abundance of major transporter proteins are reorganized for recycling to adapt to disadvantageous conditions. These are the typical strategies of stress tolerance mechanisms in plant cells. Under osmotic stress, the internalization of PM is regulated by two pathways—clathrin-mediated and sterol-raft-mediated endocytosis (Li et al. 2011). However, Rare cold inducible 2 (RCI2) proteins are candidates in novel pathways for the endocytosis of PM proteins in *Camelina* under NaCl stress (Kim et al. 2019). In particular, the CsRCI2E protein interacts with the aquaporin CsPIP2;1 to reduce water transport and protein abundance in the PM. Therefore, the changes in the membrane trafficking of CsRCI2E and CsPIP2;1 during osmotic stress should be observed at the plant cell level.

RCI2 genes encode 5–8 kDa proteins that respond to drought, temperature, and salinity stresses. Eight *RCI2* genes (*RCI2A* to *H*) have been identified in *Arabidopsis* and *Camelina*. These genes have been subdivided into two subgroups. One group (*RCI2D*, *RCI2E*, *RCI2F*, and *RCI2G*) has hydrophilic amino acids and the other group (*RCI2A*, *RCI2B*, *RCI2C*, and *RCI2H*) does not have a tail at the C-terminal end (Medina et al. 2007). The protein structure of *RCI2* is highly hydrophobic; therefore, *RCI2* is also called plasma membrane proteolipid 3 (PMP3) or low temperature induced 6 (LTI6). The expression of *RCI2*/PMP3/LTI6 is dramatically increased when the plant is exposed to various stresses; however, the degree of the stress response is different for each gene. Unlike its name, *RCI2* is not rare but enriched in the PM and is useful as a tracer for studying PM endocytosis (Li et al. 2011; Kim et al. 2021).

*RCI2*s are the typical abiotic stress indicators and tolerance factors in many plant species. *RCI2* overexpression enhances abiotic stress tolerance in plants such as *Arabidopsis*, *Camelina*, maize, rice, and tomato (Kim et al. 2007; Mitsuya et al. 2006; Sivankalyani et al. 2015; Kim et al. 2020). In contrast, *RCI2* knockout reduces salt tolerance in *Arabidopsis* (Medina et al. 2001; Mitsuya et al. 2005). Studies on the expression of *RCI2*s have clearly indicated that they are related to abiotic stress tolerance. However, the translational functions of *RCI2*s are not fully understood. The post-translational functions of *RCI2*s are considered to influence membrane trafficking to reorganize PM proteins for adapting to environmental changes (Kim et al. 2021). In particular, membrane trafficking of *RCI2*s was observed when plants were exposed to osmotic stresses such as drought and NaCl and mannitol treatments (Kim et al. 2020; Kim et al. 2021; Kim et al. 2022). In addition, most of the abiotic stresses disrupt water homeostasis when plants fail to adapt to environmental changes. Thus, *RCI2* is speculated to be specialized in membrane trafficking to maintain cell homeostasis under stressful conditions. On the other hand, it has been reported that, depending on its protein structure, each *RCI2* shows different responses under different abiotic stresses. Kim et al. (2022) reported that a tail-type *RCI2*, *CsRCI2D*, is induced in *Camelina* under heat stress. Moreover, *CsRCI2D* moved from the PM to the endomembrane fraction in *Camelina* under heat stress. However, the other tail-type *RCI2*s (*CsRCI2E*, *CsRCI2F*, and *CsRCI2G*) did not move to the endomembrane (Kim et al. 2022). Furthermore, the overexpression of *RCI2A*, *RCI2D*, and *RCI2H* in *Arabidopsis* and *Camelina* enhanced their NaCl tolerance. Moreover, *RCI2A*-overexpressing *Arabidopsis* plants showed decreased Na⁺ accumulation and increased K⁺ accumulation compared to

wild-type (WT) plants (Mitsuya et al. 2006). Although these reports indicate that RC12 is involved in ion transport during ionic imbalances in the environment, its function has not yet been cross-confirmed with the membrane trafficking of RC12. Therefore, identifying the protein interactions of RC12 with water and ion transporter proteins in the PM is important for further studies on the functions of RC12.

Aquaporins are passive water channel transporters that transport water across the cell membrane. Plasma membrane intrinsic protein 2s (PIP2s) form the major water channels at the PM. The function of PIP2s is to transport H₂O₂, CO₂, ions, solutes, and water (Verdoucq et al. 2014). In previous studies, NaCl and polyethylene glycol treatments inhibited root hydraulic conductivity due to the downregulation of PIP protein accumulation in the root (Paudel et al. 2017). In *Arabidopsis*, PIP2;1 is phosphorylated and internalized when the plants are subjected to NaCl and H₂O₂ treatments (Wudick et al. 2015; Ueda et al. 2016). The phosphorylation of PIP2;1 regulates its activity and abundance in the PM (Prak et al. 2008; Moeller et al. 2014). Under stressful conditions such as drought and high salinity, the abundance of PIPs at the PM is regulated by changes in translation and endomembrane trafficking. For example, PIPs were endocytosed to the vacuole or early endosome nearby the trans-Golgi network (TGN) for recycling. Endocytosed PIPs are either redistributed to the PM or transported to multivesicular bodies (MVBs) for recycling or degradation (Hachez et al. 2014). These recycling and degradation pathways are mediated by protein–protein interactions through clathrin-mediated (Rodriguez-Furlan et al. 2019) and clathrin-independent sterol-raft pathways (Li et al. 2011). In addition, protein interaction between PIP2;1 and RC12E was observed under NaCl stress (Kim et al. 2019). Our previous study reported that the interaction of RC12E and RC12F with PIP2;1 decreases water permeability in *Camelina* (Kim et al. 2019). Therefore, the function of RC12s is expected to be the redistribution of PIPs at the PM for adaptation to salt stress.

Recently, it was reported that RC1A and RC12B undergo intercellular endocytosis under NaCl stress (Kim et al. 2021). RC12D is localized in the PM, endoplasmic reticulum (ER), and Golgi apparatus (Medina et al. 2007; Kim et al. 2020). Moreover, CsRC12D-overexpressing *Camelina* plants showed increased lipid vesicle accumulation in the root compared to WT plants (Kim et al. 2022). In yeast, mutations in PMP3 (RC12 homolog) resulted in reduced endocytosis and turnover of proteins from the PM (De Block et al. 2015). In maize, the expression of ZmPMP3g (RC12B homolog) was associated with the DELLA protein signaling pathway, which is related to the membrane trafficking of PM proteins (Lei et al. 2023). Therefore, RC12s potentially regulate key transporters in the PM to maintain cellular homeostasis during abiotic stresses.

In this study, we further investigated the membrane trafficking of CsRC12E—a protein that responds rapidly to osmotic shock in *Camelina*. Furthermore, we demonstrated that CsRC12E promotes the endocytosis of CsPIP2s and enhances osmotic stress tolerance in transgenic plants overexpressing CsRC12E.

Material and methods

Plant materials and treatment

Camelina sativa L. cv. CAME seedlings were grown hydroponically on half strength of Hoagland nutrient solution for 1 or 3 wks at 22 °C in a growth chamber with an 8:16 h day/night cycle. Subsequently, 1-wk-old seedlings were immediately treated with or without 300 mM mannitol or 150 mM NaCl to sample total RNA or total membrane fractions. Seed germination was tested on an agar media containing Hoagland nutrient solution with or without 300 mM mannitol or 150 mM NaCl supplement. Root length was measured at 0, 1, 2, and 4 d after sowing on the medium. The *CsRCI2E* (JQ814753.1) coding sequence was cloned into the pCB302-3 cloning vector containing a CaMV 35S promoter using gateway cloning system (Invitrogen). The binary plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101. *Camelina* plants were transformed using the floral dip method. Transgenic T₃ plants were used in the experiments. Four-week-old transgenic *Camelina* plants were treated with mannitol or NaCl for 0.5 h to sample total membrane fractions. Information on the primers for gateway system is provided in Supplemental Table 1.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

One-week-old seedlings were treated with 300 mM mannitol or 150 mM NaCl. The seedlings were harvested at 0, 0.5, 1, 3, 6, 9, 12, and 24 h after treatment and frozen using liquid nitrogen to get snapshots of the RNA transcription in the plant. Total RNA was extracted from the shoot and root of the seedlings using a Clear-S Total RNA Extraction Kit (Invirustech, South Korea). The total RNA samples were treated with DNA-free DNA Removal Kit (Invitrogen, USA). The cDNA was synthesized from 1 µg of total RNA using a first-strand cDNA synthesis kit (Bioneer, South Korea). The cDNA (100 ng) was amplified using AccuPower 2X Greenstar qPCR Master Mix (Bioneer, South Korea) with gene-specific primers. The housekeeping gene *CsActin* served as the reference gene. Information on the primers used is provided in Supplemental Table 1.

Subcellular localization of CsRCI2E in tobacco leaves

The *CsRCI2E* coding sequence was cloned into a pFAST cloning vector containing a CaMV 35S promoter. The binary plasmid was transformed into *Agrobacterium* strain GV3101. Tobacco (*Nicotiana benthamiana*) leaves were infiltrated with transformed GV3101 for 36 h. The fluorescence signals of the YFP-CsRCI2E fusion proteins in tobacco leaves were observed using laser confocal scanning microscopy (TCS SP5, Leica, Germany). The primer information is provided in Supplemental Table 1.

Isolation of microsomal membranes from Camelina

A modified membrane vesicle isolation method based on the method described by Palmgren (1990) was used. Briefly, mannitol- and NaCl-treated *Camelina* seedlings were harvested and immediately rinsed twice using distilled water. The samples were ground in ice-cold homogenization buffer (pH 7.5, 50 mM MOPs-BTP, 330 mM sucrose, 5 mM ethylenediaminetetraacetic acid, 5mM dithiothreitol, 0.2% bovine serum albumin, and 0.5% polyvinylpyrrolidone) using a mortar and pestle. The ground samples were filtered through a four-layered Miracloth and centrifuged at 10,000 × g for 15 min at 4 °C. The

supernatants were collated and then re-centrifuged at $80,000 \times g$ for 45 min at 4 °C. The membrane pellets were dissolved in 3 mL buffer (pH 7.8, 5 mM potassium phosphate buffer, 5 mM KCl, 330 mM sucrose, 1 mM ethylenediaminetetraacetic acid, and 100 μ M dithiothreitol).

PM isolation using an aqueous two-phase system

The microsomal membrane samples were mixed in a two-phase aqueous partitioning system containing 6.5% (w/w) dextran (MW 45,000–65,000), 6.5% polyethylene glycol (PEG 4000), 250 mM sucrose, 4 mM KCl, and 5 mM KH_2PO_4 (pH 7.8) and then flipped twice. The gently mixed samples were centrifuged at 2,000 rpm for 10 min at 4 °C. The upper phase of the centrifuged samples was collected and mixed with the final buffer (pH 7.5, 50 mM MOPs-BTP, 330 mM sucrose, and 5 mM KCl). The samples were centrifuged at $100,000 \times g$ for 1 h at 4 °C. The pelleted membranes were resuspended in the final buffer (Kim et al. 2016). The protein contents of the collected PM samples were quantified using the Bradford method (Bradford, 1976) before use in the western blotting experiment.

Membrane fractionation using a sucrose density gradient system

A sucrose density gradient was stacked with 6 mL of a five-phase buffer (pH 7.5, 5 mM MOPs-BTP, and 5 mM KCl) containing 15, 22.5, 30, 37.5, and 45% (w/w) sucrose solutions. The gradients were frozen at -20 °C for 4 h and then melted overnight at 4 °C. The microsomal membranes were loaded on continuous gradients and centrifuged at $120,000 \times g$ for 16 h at 4°C. Sixty membrane fractions of 500 μ L each were collected from the top to the bottom. The collected samples were used for western blot analysis.

Western blot analysis

The PM samples (10 μ g) and fractionated membrane samples (5–10 μ L) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane. The membranes were incubated in a 1:1,000 dilution of PBST-20 buffer containing antibodies. Proteins were detected using anti-RCI2E (synthetic peptide C-QNREGNHELGAPLNSA, positions 58–73 of CsRCI2E), anti-PIP2;1, anti-PIP2;2 (Agrisera, AS09 488), anti-PM H^+ -ATPase (obtained from Dr. Hasegawa, Okayama University, Japan), anti-BiP (obtained from Dr. Lee, Chonnam National University, South Korea), and anti-HMA3 (synthetic peptide C-GLLQKNEETSKKT, positions 734–746 of CsHMA3). The antibody-bound membranes were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G. The fluorescence signals were photographed using a G:BOX gel doc system (Syngene, UK).

Reactive oxygen species (ROS) content analysis

Camelina seedlings were transplanted to a nutrition solution containing 300 mM mannitol or 300 mM NaCl for 24 h. *Camelina* roots were stained overnight in the dark using 3,3'-diaminobenzidine (DAB) solution (1 mg/mL, pH 3.8) at room temperature. The root samples were rinsed using distilled water. Photographs were taken using a stereo microscope (DMS1000, Leica). The treated *Camelina* roots (500

mg) were harvested for H₂O₂ content measurement. The snap-frozen root samples were ground and then mixed with 2 mL (0.1%, v/v) of trichloroacetic acid on ice. The homogenates were centrifuged at 4 °C for 10 min at 12,000 rpm. The supernatants were mixed with 0.5 mL of 100 mM potassium phosphate buffer (pH 7.0) and 1 mL of KI solution (1 M). The mixtures were incubated in the dark for 1 h. The absorbance of the samples was measured at 410 nm and the H₂O₂ contents were calculated using a standard curve.

Statistical analysis

Data were analyzed for statistical significance using SPSS version 25 (IBM, Chicago, IL, USA). Analysis of variance was used to compare statistical differences based on Duncan test. $P < 0.05$ was considered statistically significant.

Results

CsRCI2E overexpression enhances seed germination under mannitol and NaCl stresses in *Camelina*

To observe the expression levels of *CsRCI2E* under osmotic and salt stresses, we conducted qRT-PCR analyses of 7-d-old *Camelina* seedlings. The plants were treated with mannitol or NaCl for 0, 0.5, 1, 3, 6, 9, 12, and 24 h (Fig. 1a). Mannitol treatment did not significantly change *CsRCI2E* expression in the shoot and root samples after 0.5 h but dramatically increased *CsRCI2E* expression compared to the control (0 h) at 3 h after treatment. The *CsRCI2E* expression increased to 133.7 and 16.8 folds in the shoot and root at 6 and 9 h, respectively. Similarly, NaCl treatment did not change *CsRCI2E* expression in the shoot and root at 0.5 h but the *CsRCI2E* expression peaked at 6 h after treatment (Fig. 1b). Thus, neither mannitol nor NaCl induced the expression of *CsRCI2E* at as early as 0.5 h of osmotic stress. Additionally, the fold changes in *CsRCI2E* expression differed based on the tissue and type of stress. Particularly, the *CsRCI2E* expression induced after NaCl treatment for 24 h was lower than that induced by mannitol treatment. These results suggested that both mannitol and NaCl induce osmotic stress in *Camelina* at an early stage, but the response of the plant differs based on the type of stress treatment.

To investigate the effects of *CsRCI2E* expression on stress tolerance, we generated transgenic CaMV35S:*CsRCI2E* overexpression lines. Under NaCl stress, the transgenic *Camelina* plants Eox#3 and Eox#8 showed 8- and 12-fold *CsRCI2E* expression, respectively, compared to the WT plants (Fig. S1). Germination and root growth under mannitol or NaCl stress conditions were analyzed in both WT and *CsRCI2E* overexpression lines of *Camelina*. Under control conditions, the germination rate of the Eox lines was higher than that of the WT plants at 12 h; however, after 24 h, the difference in germination rates between the Eox and WT lines was not significant. When treated with 300 mM mannitol, the germination rates of both Eox lines were higher than that of the WT plants at 12 h. Likewise, when treated with 150 mM NaCl, the Eox lines showed increased germination compared to the WT plants (Fig. 2a). Under control conditions, the root growth of Eox lines was higher than that of WT plants at 2 d and similar to that of WT plants at 4 d. After mannitol or NaCl treatment, the transgenic lines showed significantly higher root growth compared to the WT plants (Fig. 2b). These results indicated that *CsRCI2E* overexpression

enhances germination in the early stage of mannitol or NaCl stress. In addition, under control conditions, the higher root growth in Eox lines at 2 d was due to the faster germination of Eox lines compared to the WT plants.

In the plant cell, osmotic and salinity stresses induce increased ROS production for signaling and metabolism (Lim et al. 2015). We investigated ROS accumulation in the roots under mannitol or NaCl stress using the DAB staining method. Dose-dependent cell damage and degree of DAB staining were pretested using WT plants (Fig. S2). Under control conditions, ROS accumulation was not detected in the Eox and WT lines. However, under mannitol or NaCl stress conditions, the DAB staining observed in Eox lines was lesser than that observed in WT plants (Fig. 2d). Moreover, the H₂O₂ content in the WT plants was higher than that in the Eox lines under both stresses (Fig. 2e). These results suggested that CsRCI2E overexpression reduces ROS accumulation under mannitol and NaCl stresses in *Camelina*.

CsRCI2E is mainly localized in the PM but travels to the endomembrane regions in *Camelina*

We observed the subcellular localization of CsRCI2E by transiently expressing the eYFP-CsRCI2E fusion protein in tobacco leaves. The eYFP-CsRCI2E fusion protein was mainly localized at the PM. Intracellular vesicles also observed eYFP signals (Fig. 3a). This result showed that CsRCI2E is localized at the PM but also travels to the intracellular region.

We isolated plant membrane fractions from whole plant samples using sucrose density gradient centrifugation. To compare the changes in CsRCI2E trafficking, the resolution of the membrane fractions was checked using low (14 fractions) and high (60 fractions) resolution sucrose density gradient systems (Fig. S3). Furthermore, we identified the localization of the reference endomembrane using marker proteins such as PM H⁺-ATPase, HMA3 (tonoplast), and BiP (endoplasmic reticulum, ER). When we used the high-resolution sucrose density gradient system, HMA3 observed strong signals in fractions 21–27, whereas BiP signals were localized in fractions 39–49 (Fig. 3b). CsRCI2E was observed in two broad signal regions—the ER and endomembrane fractions (fractions 33–45) and the PM-enriched fractions (fractions 47–57). This result showed that although CsRCI2E was mainly localized at the PM, it also traveled to the intracellular membrane region.

CsRCI2E and CsPIP2 are rapidly endocytosed and redistributed under mannitol and NaCl shock in *Camelina*

To observe the subcellular redistribution of CsRCI2E, we isolated cell microsomal membranes from whole plants treated with NaCl solution for 0.5 to 6 h. At first, we investigated the time-dependent changes in the localization of RCI2E and PIP2 under NaCl stress using a low-resolution system (Fig. S4 and 5). In the control sample, the CsRCI2E signals were detected in a broad range of membrane fractions overlapping the ER and PM regions. After NaCl treatment for 0.5 h, CsRCI2E shifted to the low-density fraction, localizing in the ER and early endosome fractions (Fig. S4). In the same sample, CsPIP2 signals shifted to the low-density fraction 1 h after NaCl treatment. However, PIP2 showed strong signals in the PM-enriched fraction after NaCl treatment for 24 h (Fig. S5). Although the density shifting of CsRCI2E and

CsPIP2 was confirmed, the 14-phase gradient system had limitations in clearly confirming membrane shifting. To extend the high-resolution fraction, we minutely separated the continuous sucrose density gradient fraction. We observed that the CsRCI2E signals were strongly detected in fractions 33–57 under no stress conditions but shifted to lower-density fractions (fractions 33–47) when exposed to NaCl for 0.5 h (Fig. 4a). Moreover, after NaCl treatment for 24 h, the strongest CsRCI2E signals were detected in the intracellular region (fractions 27–31). After mannitol treatment, the CsRCI2E signals showed a greater shift toward the ER-enriched regions (fractions 35–49) after 0.5 h and toward the lower-density fractions (fractions 31–37) after 24 h, compared to that under no stress conditions. Furthermore, the CsRCI2E trafficking patterns were similar under both salinity and osmotic stresses. On the other hand, CsPIP2 was localized in the membrane fraction (fractions 45–53) but shifted to two phases of distribution under mannitol and NaCl stresses. CsPIP2 shifted to fractions 43–49 and 53–57 at 0.5 h after mannitol treatment and to fractions 41–49 and 55–57 at 0.5 h after NaCl treatment. Moreover, the redistribution of CsPIP2 was widely spread toward the lower-density fractions at 24 h than at 0.5 h after treatment. These results indicated that CsRCI2E and CsPIP2 traveled from the PM to the endomembrane when exposed to osmotic shock using mannitol or NaCl treatments. In addition, the redistribution to lower or higher density suggested that CsRCI2E travels rapidly to the endomembrane even within 0.5 h after stress induction. Moreover, the multiple-phase redistribution of CsPIP2 owing to changes in osmolarity indicates protein recycling, which involves the removal or degradation of PM components.

CsRCI2E overexpression and osmotic stresses influence the internalization and redistribution of CsPIP2

To investigate the role of CsRCI2E in CsPIP2 redistribution, we conducted a high-resolution sucrose density gradient assay using *CsRI2*-overexpressing *Camelina* plants (Eox#3). Under control conditions, Eox#3 plants showed a significant shift of CsRCI2E toward lower density fractions (37 to 41) compared to WT plants (Fig. 5a). This result indicated that the amount of CsRCI2E influenced the membrane trafficking of CsRCI2E from the PM to the endomembrane region. In the same samples, Eox#3 plants showed a greater shift of CsPIP2 toward higher density fractions (fractions 49–55) than WT plants (fractions 43–51) under control conditions. This result indicated that the overexpression of CsRCI2E reduced the distribution of CsPIP2 at the endomembrane. However, mannitol treatment for 30 min reduced the distribution of CsPIP2 in fractions 39–43 in WT plants, although Eox#3 plants showed increased distribution of CsPIP2 in the same region. CsPIP2s were localized at both the endomembrane (fractions 39–47) and the PM (fractions 51–53) of WT plants after NaCl treatment for 30 min; however, Eox#3 plants showed a lower number of CsPIP2s at the endomembrane and a higher number of CsPIP2s at the PM (fractions 47–55) (Fig. 5b). These results suggested that CsPIP2s are redistributed when CsRCI2E is overexpressed, and osmotic shocks caused rapid internalization of CsPIP2s from the PM to the endomembrane region.

Discussion

CsRCI2E overexpression helps in rapid stress detection and PIP2 redistribution under mannitol and NaCl stresses in *Camelina*

In most plant species, the expression of RCI2/PMP3/LTI6 is induced by low temperature, drought, and salinity stresses (Rocha 2016; Kim et al. 2021). RCI2s have individual characteristics in terms of protein structure and response to abiotic stresses. Many studies have reported the expression of RCI2s in various plant species such as *Arabidopsis*, maize, rice, *Camelina*, and wheat (Capel et al. 1997; Imai et al. 2005; Morsy et al. 2005; Kim et al. 2016). In our previous report, the expression of *CsRCI2E* in *Camelina* was induced by low temperature, drought, and NaCl stresses (Kim et al. 2016). In this report, we investigated the expression of *CsRCI2E* in the shoot and root of *Camelina* after mannitol and NaCl treatments. The samples showed remarkable *CsRCI2E* expression under both stresses. On the other hand, *CsRCI2E* expression did not increase markedly 1 h after mannitol or NaCl treatment (Fig. 1). The expression of *CsRCI2E* clearly showed that both osmotic stresses induced transcription. Thus, the expression of *CsRCI2E* is expected to be related to the tolerance to mannitol and NaCl stresses. To confirm this, we generated transgenic *CsRCI2E*-overexpressing *Camelina* plants and analyzed their stress tolerance. The transgenic plants showed increased root growth under mannitol and NaCl stresses at the seedling stage (Fig. 2). These results indicated that *CsRCI2E* is a tolerance-promoting factor under osmotic and salt stresses. Additionally, the overexpression of *CsRCI2E* promoted the earlier germination of transgenic plants compared to WT plants, resulting in the increased root elongation of transgenic seedlings on day 2 despite the absence of stress (Fig. 2b). In *Arabidopsis*, Medina et al. (2001) reported that the expression of *AtRCI2A* was highly induced on the second day after seed germination. In *Camelina*, the overexpression of *CsRCI2D* and *CsRCI2D* in C-terminal tail-deleted transgenic *Camelina* increased the germination rate (Kim et al. 2021). The *CsRCI2D* protein structure belongs to the same tail group as *CsRCI2E*, and the germination rate was increased when the C-terminal tail was removed from the transgenic *Camelina*. Meanwhile, ROS accumulation was reduced when *CsRCI2E* was overexpressed in *Camelina* (Fig. 2d and e). These results suggest that the function of RCI2s is related to the reduction of ROS damage during the germination stage. The enhanced seed germination is probably due to the redistribution of PIP2 through its interaction with RIC2s. In a previous report, NaCl induced the interaction of *CsRCI2E* with *CsPIP2* to reduce water permeability and protein abundance at the PM (Kim et al. 2019). The reduced number of PIP2s reduced water loss and ion channel activity. Thus, *CsRCI2E* was presumed to trigger PIP2 endocytosis to prevent Na⁺ ion uptake and water loss. In addition, Wudick et al. (2015) reported the redistribution of PIP2 in response to H₂O₂ treatment. Therefore, the overexpression of RCI2s can enhance the tolerance to osmotic and NaCl stresses by contributing to the rapid and bulk sensing of osmotic imbalance and membrane protein redistribution for maintaining cell homeostasis. Therefore, we hypothesized that the function of *CsRCI2E* is to regulate PIP2 abundance under osmotic stress.

Membrane trafficking of *CsRCI2E* in *Camelina* is a defense mechanism against mannitol and NaCl stresses

Membrane trafficking of the RCI2 protein *CsRCI2D* was observed under NaCl (Kim et al. 2021) and high-temperature (Kim et al. 2022) stresses. Therefore, we hypothesized that *CsRCI2E* can travel from the PM

to the endomembrane system under osmotic stress. Transient expression of the GFP-CsRCI2E fusion protein in tobacco leaves revealed that CsRCI2E was not only localized in the PM but also in the intracellular region (Fig. 3A). The membrane trafficking of CsRCI2E was analyzed in detail using a sucrose density gradient with membrane protein markers such as PM H⁺-ATPase, HMA3, and BiP (Fig. 3b). In previous studies, we tested the redistribution of CsRCI2s using fourteen membrane fractions in a sucrose density gradient (Kim et al. 2021; Kim et al. 2022); however, the test had a limited resolution for comparing the shift in membrane fractions, especially for PIP2 (Fig. S3). Therefore, we used an extended gradient system divided into 60 fractions for wider resolution. The results showed that the PM fractions were present at a higher-density region compared to the tonoplast fractions, whereas the ER fractions showed a broad range covering the PM region. However, CsRCI2E was localized in a midrange of density fractions, covering both ER and PM regions. This result indicated that CsRCI2E can localize at the PM and the endomembrane, including the ER region. In addition, RCI2D has been reported to be present at the ER membrane in *Arabidopsis*, while CsRCI2D has been reported to be localized at both the PM and the ER in *Camelina* (Medina et al. 2007; Kim et al. 2020). This means that the RCI2 family can function in multiple membrane systems in the plant cell. Meanwhile, the early response of *CsRCI2E* to mannitol and NaCl stresses resulted in a similar internalization in *Camelina*. This might be predominantly affected by osmotic shock before Na⁺ ion toxicity in the early stress phase (Shavrukov, 2013). Plant gene expression under NaCl stress can be divided into the ABA (abscisic acid)-dependent and ABA-independent pathways. Several studies have reported a slight increase in ABA accumulation during the early stages of NaCl stress (Fàbregas et al. 2020). This means that the trafficking of CsRCI2E is predominantly a response to osmotic shock at the early stage of NaCl stress. However, RCI2s have a multiple-family response to drought and NaCl stresses. Therefore, it is necessary to compare the membrane trafficking of the RCI2 family at the same stage under various stress conditions to understand the complex functions of RCI2s.

Membrane trafficking of CsRCI2E is a quicker response than novel CsRCI2E transcription under osmotic shock

Previously, Rocha (2016) reported that the transcriptional regulation of RCI2s reinforces stress tolerance factors. However, our results showed the rapid trafficking of CsRCI2E to lower-density membrane fractions in a sucrose density gradient assay when treated with a high concentration of mannitol and NaCl for 0.5 h. Furthermore, a more significant shift was observed 24 h after inducing osmotic stresses (Fig. 4). The change in the distribution of CsRCI2E without a marked increase in *CsRCI2E* transcription indicated the trafficking of CsRCI2E from a large membrane system such as the PM to the endomembrane (Fig. 1). These results suggest that the resident CsRCI2E undergo membrane trafficking. Subsequently, we tested the hypothesis that CsRCI2E trafficking is related to PIP2 trafficking under osmotic shock. In the same samples, the distribution of CsPIP2s shifted to a lower-density region and protein localization was relatively lower in the PM region when exposed to osmotic shock (Fig. 4). This result indicated that CsPIP2s were internalized to the endomembrane in response to osmotic stress, showing the same direction of membrane trafficking as CsRCI2E. PIP2 can transport not only water but also various ions and solutes, including Na⁺. Moreover, aquaporins can transport water to extracellular

regions through osmotic potential gradation. Therefore, limiting aquaporin abundance is a reasonable mechanism for maintaining water and ion homeostasis (Byrt et al. 2017). Our previous report showed that the interaction of CsRCI2E and CsPIP2;1 promoted NaCl stress tolerance by limiting the activity of CsPIP2;1. However, the question of whether PIP2 internalization was RCI2-mediated remained unsolved (Kim et al. 2019). Therefore, we tested the membrane trafficking of PIP2 in CsRCI2E-overexpressing *Camelina* plants. Under normal conditions, the distribution of CsRCI2E in the transgenic plants shifted to a lower-density region than that in the WT plants. This result indicated that the expression of CsRCI2E directly affects its membrane trafficking and subcellular localization at the membrane. Moreover, the distribution of CsPIP2 in the WT and transgenic *Camelina* plants differed even in the absence of stress. Furthermore, the abundance of CsPIP2 diminished more rapidly after mannitol and NaCl treatment for 0.5 h (Fig. 4). These results clearly showed that CsRCI2E internalization promotes PIP2;1 internalization to reduce water and ion channel activity. Therefore, it is assumed that the PM-resident CsRCI2E is endocytosed by osmotic stresses and that the rapid response is a function of CsRCI2E under osmotic shock.

PM-resident CsRCI2E regulates the activity and abundance of PIP2 by promoting its endocytosis and redistribution

RCI2 proteins are too small to have a channel or pump function. However, these hydrophobic proteins have a suitable structure for traveling from the PM to the endomembrane system (Kim et al. 2016). Previous studies have shown the membrane trafficking of RCI2s from the PM to early endosomes and vacuoles (Li et al. 2011; Ueda et al. 2016; Kim et al. 2021). Our results showed that CsRCI2E overexpression promotes CsPIP2 redistribution from the PM to the endomembrane region. In addition, PIP2 membrane trafficking shifted to a lower-density membrane region at the early stages of stress but then returned to the PM or arranged themselves widely at membranes of various densities (Fig. 3b). This redistribution is a typical phenomenon during protein recycling under abiotic stresses (Wang et al. 2020). The major membrane transport proteins often have multiple triggers for activity changes such as PM H⁺-ATPase recycling (Xia et al. 2019). RCI2 expression is observed under normal conditions (due to the circadian rhythm); however, RCI2 expression increases gradually when there are osmotic stimulations (Kim et al. 2016). A comparison of the subcellular distribution of CsRCI2E in WT and transgenic *Camelina* plants showed that the constitutive overexpression of CsRCI2E causes it to localize in the endomembrane (Fig. 5). This suggests that the increased amount of CsRCI2E might be a factor for membrane trafficking from high-density regions such as the PM to low-density regions. After NaCl treatment, the transgenic *Camelina* plants had higher relative water contents than the WT plants (Fig. S6). This result supports our hypothesis that RCI2E-mediated PIP2 redistribution prevents water loss under osmotic shock conditions.

Recently, Tyerman et al. (2021) reported the endocytosis mechanism of PIP2s under osmotic stress conditions. PIP2 trafficking is regulated by two kinds of pathways—clathrin-mediated or sterol-raft-mediated endocytosis pathways. These endocytosis mechanisms lead to the removal, recycling, and degradation of PIP2s to establish water and ion homeostasis. However, clathrin is a basic component of

cells and is encoded by a housekeeping gene; thus, it is not considered to be a stress-induced tolerance factor. Therefore, further studies on novel RCI2-mediated pathways of PIP2 regulation are important to characterize the reorganization of the PM components. In addition, overexpressed endocytosis factors such as sorting nexin proteins and PM-localized deubiquitylating enzymes contribute to endosomal trafficking for modulating hormonal balance and fine-tuning membrane proteins (Liang et al. 2022; Vogel et al. 2022). This evidence suggested that the expression level of endocytosis factors plays a role in membrane protein redistribution. However, the pathway of RCI2-mediated endocytosis did not show the destination of the CsPIP2s for temporal storage or degradation for reusing amino acids. In *Arabidopsis*, Ueda et al. (2016) reported that, when exposed to NaCl, AtRCI2A was stored in the vacuole with AtPIP2;1. Thus, CsRCI2E and CsPIP2s are also possibly stored at the vacuole; however, our result did not show the membrane shifting to the vacuole based on comparisons to the HMA3 localized fractions (Ueda et al. 2016). This suggests that short-term osmotic shock does not affect the vacuolar storage of CsRCI2E and CsPIP2s. Therefore, tracking RCI2-associated PM protein degradation or ubiquitination is important for further studies on time-dependent osmotic stress. Furthermore, finding PM components that interact with RCI2s will be useful in understanding PM protein redistribution in abiotic stress tolerance mechanisms at the cellular level. Meanwhile, the function of aquaporin is necessary for plant survival even under constitutive osmotic stress conditions. Gómez-Méndez et al. (2023) reported that increased aquaporin abundance is crucial for NaCl tolerance in a halophyte. Moreover, selective channel activity was observed in heteromerization studies of PIP1 and PIP2 (Byrt et al. 2016; Tyerman et al. 2021). Therefore, further studies on the changes in the abundance of the appropriate form of PIPs under osmotic stress are required.

In summary, CsRCI2E is a constitutive resident at the PM in the absence of an osmotic imbalance. However, when it rapidly senses osmotic changes, CsRCI2E is endocytosed from the PM to the endomembrane system during the initial phase of osmotic stress. During the same phase, CsRCI2E interacts with CsPIP2s to promote their endocytosis from the PM to prevent water loss under osmotic shock. This is because PIP2s are a potential path for water loss when the osmotic potential is inverted. Thus, the distribution of this protein at the PM needs to be restricted (Meinild et al. 1998). Subsequently, stress-induced CsRCI2E contributes to the fine-tuning of the CsPIP2s population at the PM to further promote osmotic stress tolerance (Fig. 6). In addition, PIP2 is composed of homo- or heterotetramers, and various combinations of PIP1 and PIP2 tetramers show individual channel activity of water and ion transport (Tyerman et al. 2021). Heteromerization facilitates selective channel activity and direction of PIP2s under various conditions. Therefore, further study on RCI2s and PIP2s should consider the heterotetramerization-dependent changes in protein interaction.

Declarations

Author contribution statement

Jung-Ho Shin and Hyun-Sung Kim conceived and designed research, Jung-Ho Shin, Hyun-Sung Kim and Sehee Kim conducted plants incuataion and experiments. Jung-Ho Shin and Hyun-Sung Kim wrote the

manuscript. Sung-Ju Ahn supervised the entire study and manuscript editing. All authors read and agree to publish the manuscript.

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Conflict of interest : The authors declare no conflict of interest.

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Figures

Figure. 1

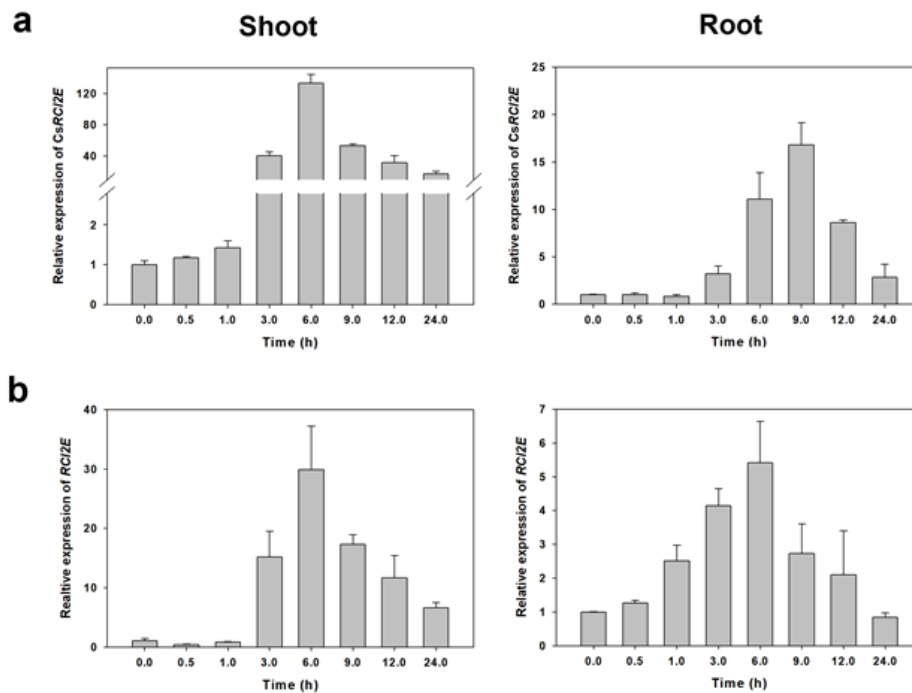


Figure 1

Expression of *CsRC12E* under mannitol and NaCl stresses. *Camelina* seedlings were grown in a hydroponics system for 7 d. Then, they were transplanted to a nutrient solution containing 300 mM mannitol (A and B) or 150 mM NaCl (C and D) for 24 h. The quantitative expression of *CsRC12E* was analyzed in the shoot (left) and root (right) samples. Bars and error bars indicate mean $\pm$ standard error (SE).

Figure. 2

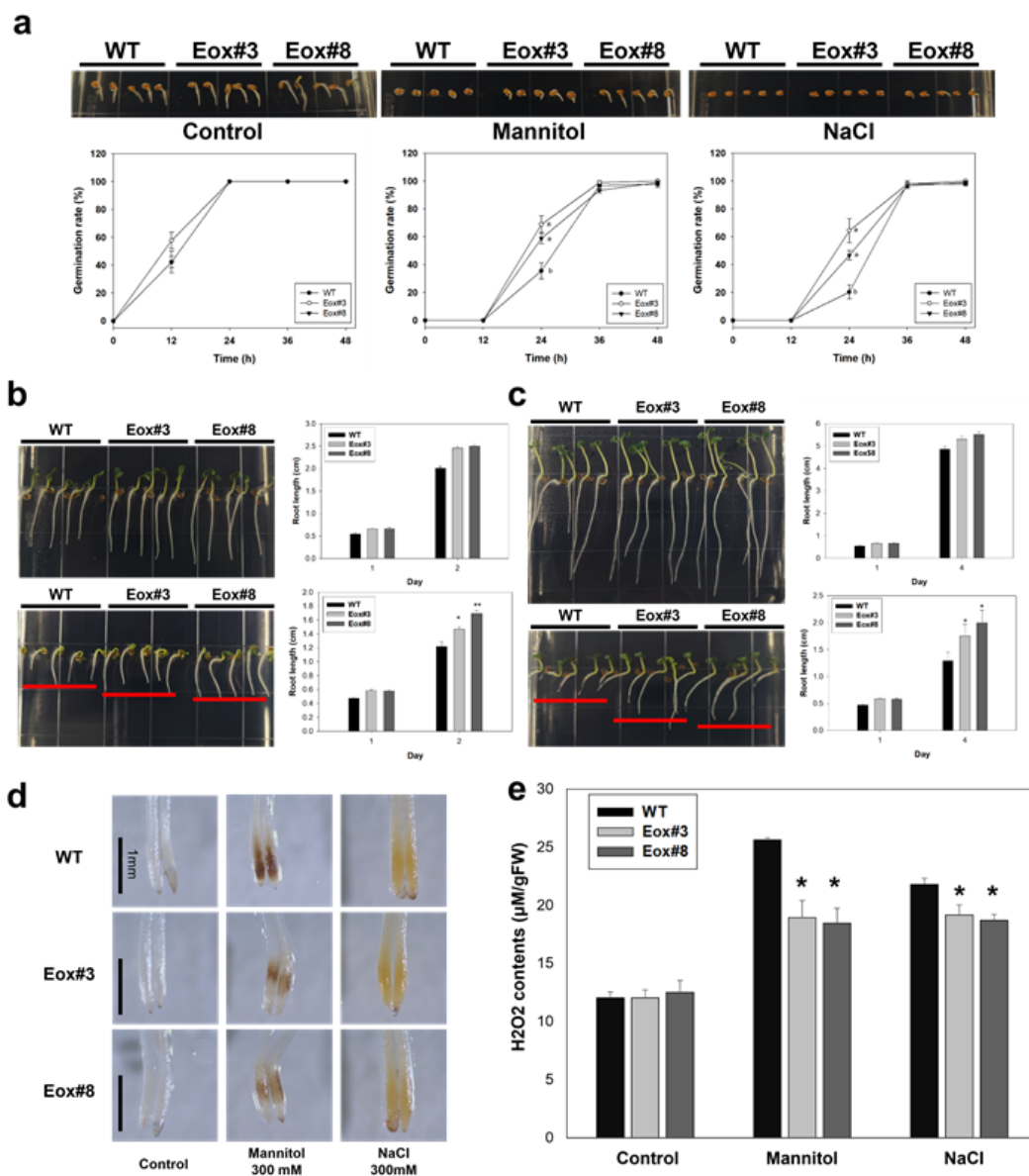


Figure 2

The phenotype of *CsRCI2E*-overexpressing *Camelina* plants under mannitol and NaCl stresses. Wild-type (WT) and transgenic *Camelina* seeds were sown on a nutrient medium containing 300 mM mannitol or 150 mM NaCl. Seed germination ratios were measured for 48 h (A). The root length of the plants was measured for 4 d (B, C). The WT and *CsRCI2E*-overexpressing *Camelina* plants were grown for 7 d and

then transplanted to a nutrient solution containing 300 mM mannitol or NaCl for 24 h. The treated roots were stained using DAB (D) and their H₂O₂ contents were determined (E). Bars indicate 1 mm.

Figure. 3

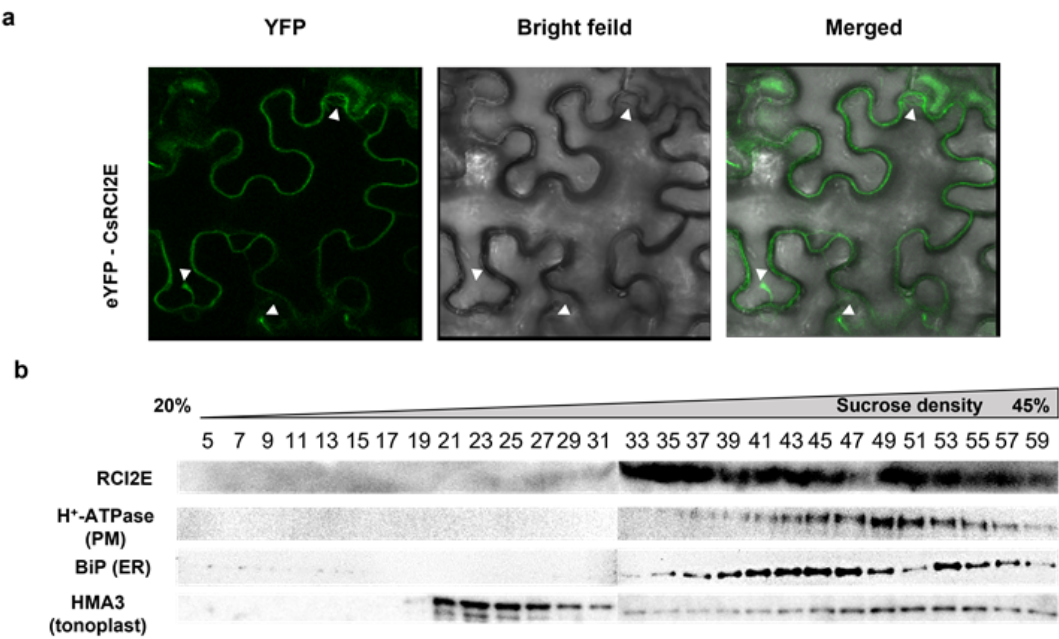


Figure 3

Analysis of the subcellular localization of CsRCI2E. The subcellular localization of eYFP-tagged CsRCI2E was observed through transient expression studies in *Nicotiana benthamiana* leaves (A). *Camelina*

seedlings were grown in a hydroponics system for 7 d. Subsequently, the microsomal membranes were extracted from whole plants. The extracted membranes were separated using a sucrose gradient (concentration range: 20–40% (w/w)). The immunological detection in separated fractions was performed using anti-RCI2E, anti-AHA, anti-BiP, and anti-HMA3 antibodies (B). The RCI2E, H⁺-ATPase, BiP, and HMA3 bands were detected at approximately 8, 105, 74, and 58 kDa, respectively. Three independent experiments were performed, and a representative result is shown.

Figure. 4

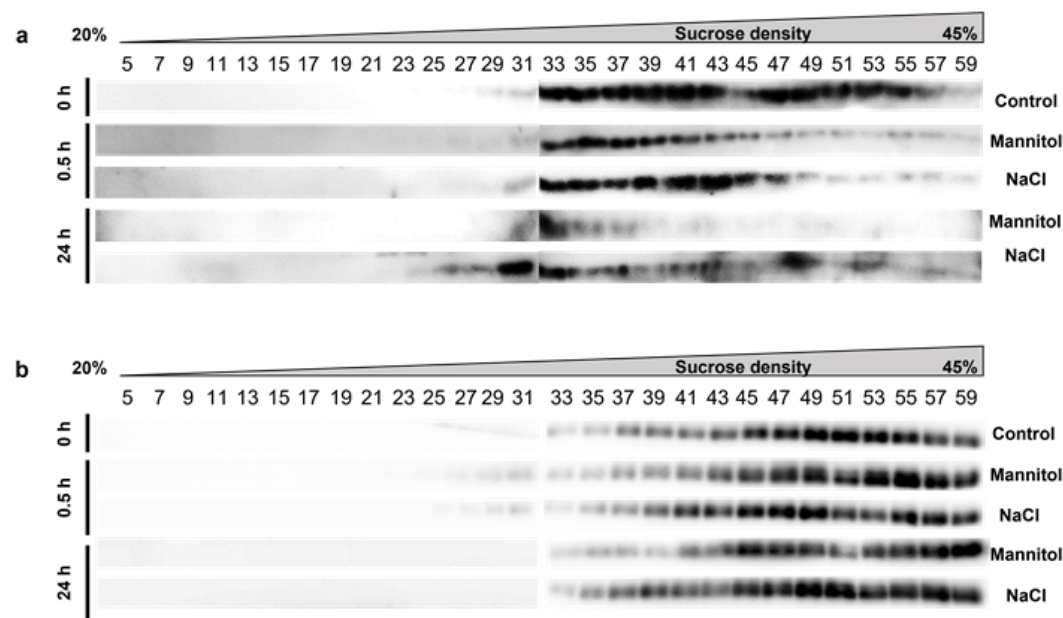


Figure 4

The subcellular redistribution of CsRCI2E and CsPIP2 under mannitol and NaCl stresses. *Camelina* seedlings (7-d-old) were transplanted to a nutrient solution containing 300 mM mannitol or 150 mM NaCl for 0.5 and 24 h. Subsequently, the microsomal membranes from whole plants were separated using a continuous sucrose density gradient. The detection of CsRCI2E (A) and CsPIP2 (B) in each fraction was conducted using anti-RCI2E and anti-PIP2 antibodies. The RCI2E and PIP2 bands were detected at approximately 8 and 25 kDa, respectively. Three independent experiments were performed, and a representative result is shown.

Figure. 5

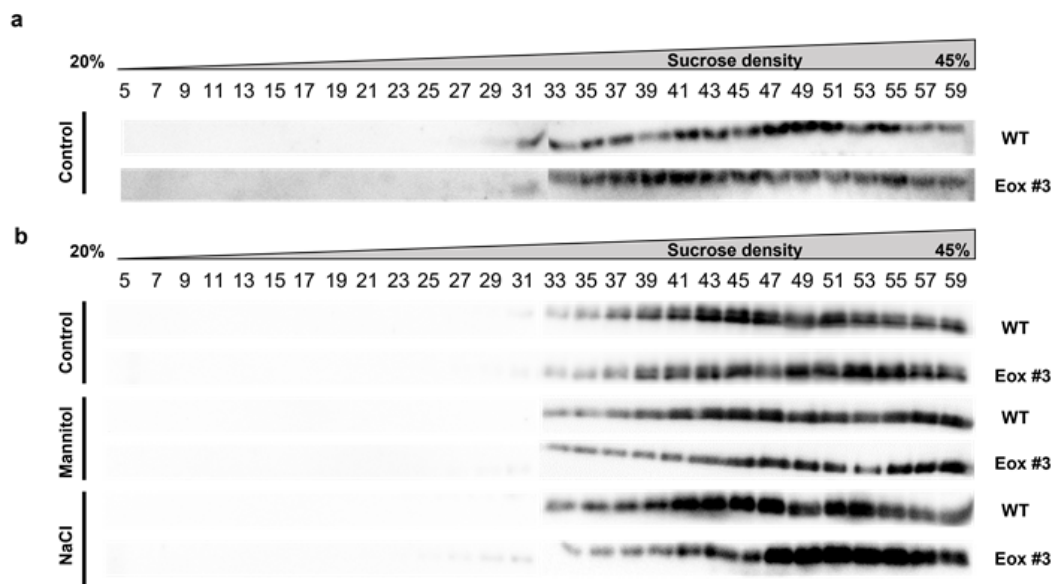


Figure 5

CsRCI2E overexpression influenced the redistribution of CsRCI2E and CsPIP2 on the membrane under mannitol and NaCl stresses. WT and CsRCI2E-overexpressing (Eox) *Camelinaplants* were grown in a hydroponics system for 3 wks and then treated with mannitol and NaCl for 30 min. Subsequently, the microsomal membranes were isolated from whole plants. The samples were separated and then

subjected to western blot analysis using anti-RCI2E (A) and anti-PIP2 (B) antibodies. Three independent experiments were performed, and a representative result is shown.

Figure. 6

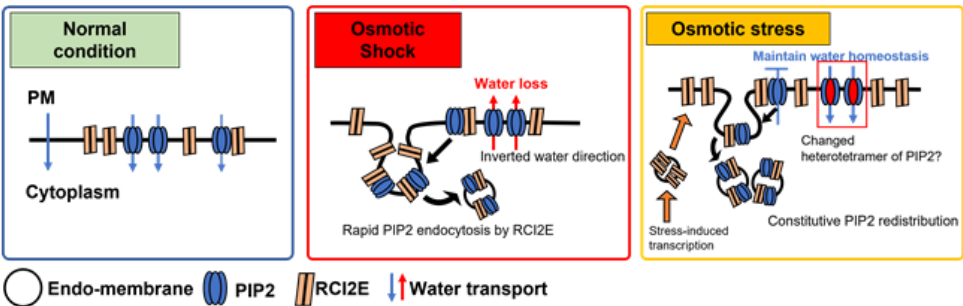


Figure 6

CsRCI2E-induced PIP2 redistribution at the cell membrane. Under non-stress conditions, PIP2 and RCI2E are mostly localized at the PM. Under osmotic shock, the resident RCI2Es in the PM are rapidly

endocytosed to the endomembrane along with PIP2s. The RC12Es redistribute PIP2s to reduce water loss due to the increased osmotic potential outside the cell. Thus, the function of RC12E is to reduce PIP2 channel activity through constitutive redistribution of PIP2 to maintain water homeostasis. However, an increase in PIP2 abundance at the PM was observed after inducing an osmotic shock. Therefore, further investigation is required to analyze whether PIP2 is heteromerized to change the direction of the water channel to overcome the osmotic stress.

Supplementary Files

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