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The abscisic acid signaling negative regulator *OsPP2C68* confers drought and salinity tolerance to rice

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Clade A type 2 C protein phosphatases (PP2CAs) are core regulatory factors in the plant abscisic acid (ABA) signaling pathway. They play crucial roles in response to abiotic stress. However, a comprehensive understanding of the functions of individual members of rice PP2CA gene families remains limited. This study investigates the role of *OsPP2C68* in response to abiotic stress. Our findings indicated that the *OsPP2C68* is highly expressed in the embryo and endosperm, and is subcellular localized in the nucleus. *OsPP2C68* knockout mutants reduced seed germination and root and stem lengths under ABA treatment. The mutants also exhibited higher stomatal closure rates, indicating increased sensitivity to ABA. In addition, the *OsPP2C68* knockout mutants exhibited altered synthesis of osmolytes and antioxidant enzymes under drought and high salinity stress, along with the differential expression of genes associated with drought and salt stress responses, enhancing rice tolerance to drought and salt. These results collectively identify *OsPP2C68* as a negative regulator in the rice ABA signaling pathway. It is responsive to drought and salt stress, and involved in regulating stomatal movement.

In natural environments, plants are frequently subjected to various abiotic stresses and other adverse factors, resulting in growth inhibition, developmental delays, reduced yields and loss of ecological value[1]. The manner in which plants respond to stress factors is intricately complex and initiated by the activation of an effective signaling system[2]. This condition triggers a series of efficient and intricate signal transduction mechanisms at the physiological, biochemical, and molecular levels to defend against various adversities[3].

Abscisic acid (ABA) is a stress hormone in plants that mediates responses to environmental stresses[4]. When adversity occurs, plants initiate ABA-mediated signaling responses that affect plant growth and survival[5]. The ABA-dependent signaling pathway includes the following components: ABA receptors PYL/RCARs[6], clade A type 2 C protein phosphatases[7,8], SnRK2[9], bZIP transcription factors[10] and ABA response cis-element(ABRE). In the absence of ABA, PP2CAs interact with SnRK2s to inhibit their activities, blocking ABA signal transduction[11]. In the presence of ABA, PP2CAs bind to the ABA receptor PYR1/PYL/RCAR to form a complex. This interaction leads to the activation of SnRK2s, initiating ABA-induced physiological responses[12]. The PP2CA gene families consists of serine/threonine protein phosphatases that play a crucial role in regulating ABA signal transduction through various mechanisms across different plant organs and tissues. They are integral components of the ABA signaling pathway, primarily exerting negative regulation. PP2CA proteins participate extensively in ABA-mediated processes, such as seed germination, dormancy, stomatal closure, and responses to environmental stresses[13,14].

Rice is one of the crucial staple crops worldwide. However, adverse environmental conditions, such as drought, high salinity, and high temperatures, contribute significantly to reduced crop yields, posing a serious threat to food security. As climate change intensifies, these challenges are becoming increasingly detrimental to rice production[15,16]. At present, numerous PP2C genes have been identified in plants, such as *Arabidopsis*, rice, wheat, and tomato, which respond to abiotic stresses, such as ABA, drought, salt, and low temperatures[17,18]. The expression of *AtPP2CG1* in *Arabidopsis* is induced by salt, drought, and ABA treatments. *AtPP2CG1* positively regulates salt stress in an ABA-dependent manner[19]. Rice PP2CA gene families have 10 members[20], and the functions of some gene family members have been initially clarified. Some genes have been found to respond

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to abiotic stresses, but the biological function of *OsPP2C68* in rice remains unclear, and a comprehensive understanding is lacking at present.

Our current study focuses on the *japonica* rice variety, Nipponbare, as the research material. It utilizes biological techniques, such as Cas9 editing, to study the functions of the *OsPP2C68*. The results indicate that *OsPP2C68* functions as a negative regulator in the rice ABA signaling pathway, responding to stresses, such as drought and salinity. Additionally, *OsPP2C68* is involved in regulating ABA-mediated stomatal movement. This study enriches our understanding of the biological functions of PP2CA gene families.

Result

Isolation and generation of knockout variants of *OsPP2C68*

OsPP2C68 (*LOC_Os09g15670*) is located on rice chromosome 9, with a total length of 1407 bp, a CDS length of 1077 bp, and no intronFig. . 1a). It encodes 358 amino acids. OsPP2C68 has a PP2C structural domain, which is located in amino acid interval 92–352Fig. . 1b). To analyze the function of *OsPP2C68*, we constructed loss-of-function mutants of the OsPP2C68 transporter protein by using CRISPR-assisted genetic analysis in the background of Nipponbare. We obtained two knockout mutants, *pp2c68-1* and *pp2c68-2*, where single base insertions occurred at the target sites, resulting in an altered amino acid sequence and a premature termination of translationFig. . 1c). To comprehensively study the function of *OsPP2C68*, we constructed overexpression lines and obtained seven positive plants. However, the overexpression lines exhibited slow growth, yellowing leaves during growth, and then gradual death, making them unsuitable for further research.

Expression pattern and subcellular localization of *OsPP2C68*

Through RT-qPCR, we examined the expression level of *OsPP2C68* and found that OsPP2C68 was expressed in roots, stems, flowers, leaves, nodes, shells, embryos, and endosperm. Its expression was highest in embryos and

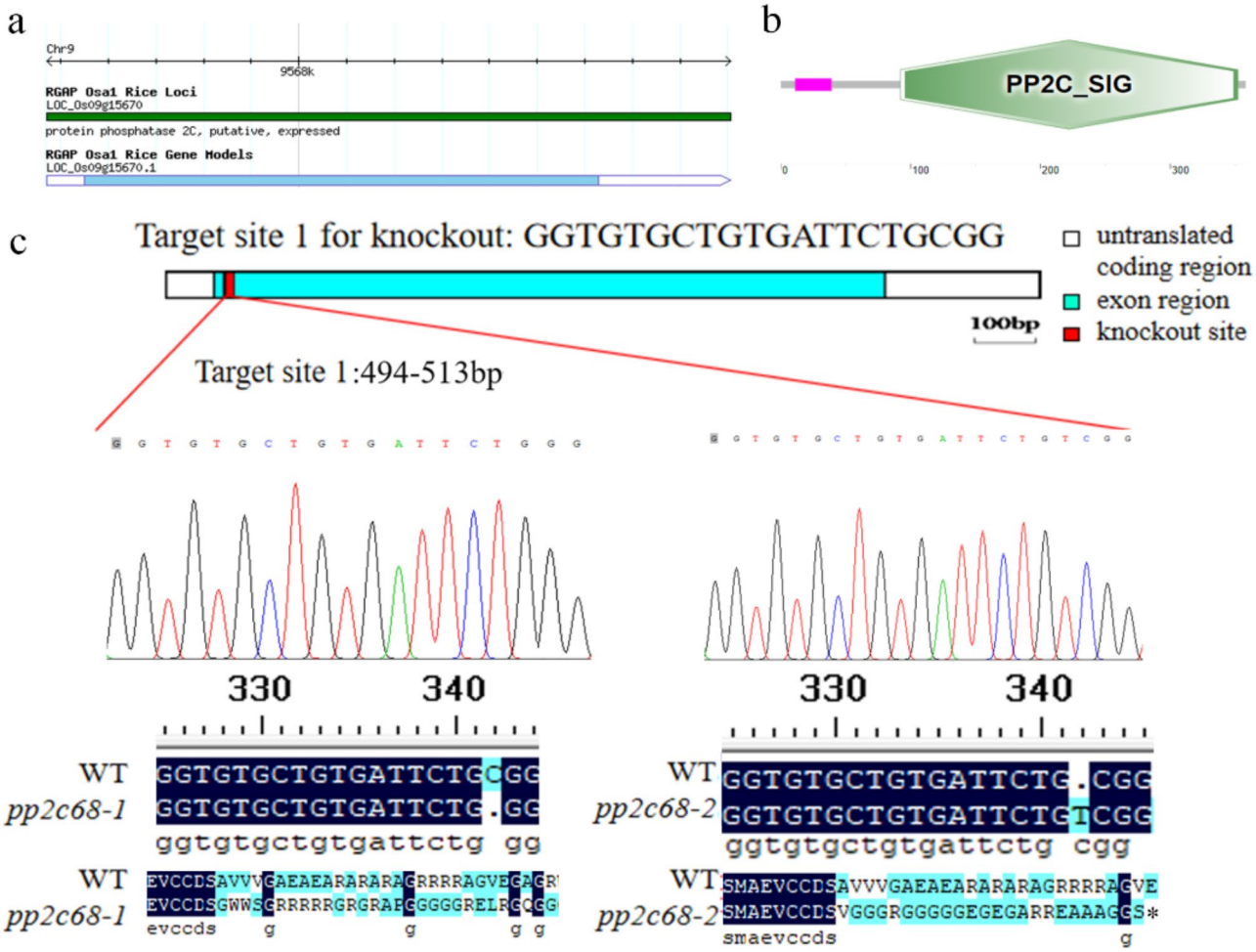

Fig. 1. The gene structure of *OsPP2C68* and CRISPR/Cas9 induced mutation of the *OsPP2C68*. (**a**) The gene structure of *OsPP2C68*. (**b**) Prediction of protein conserved domain. (**c**) The *OsPP2C68* mutation site is shown on the gene structure. Comparison of base and amino acid sequences of *pp2c68-1* and *pp2c68-2* with WT. Premature stop codons in the deduced amino acid sequences of the mutant OsPP2C68 proteins are indicated with asterisks.

endosperm, while lowest in roots and stem tissues (Fig. 2a). Experiments in which the obtained homozygous *OsPP2C68* knockout mutants that contained the *proOsPP2C68:GUS* vector were stained revealed that *OsPP2C68* was expressed in the roots and leaves of seedlings. GUS staining was also observed in the nodes and leaves of mature plants, and in the embryos and endosperm of seeds, with deeper staining observed in embryos and endosperm (Fig. 2b). To determine the subcellular localization of OsPP2C68, the *OsLC2-RFP* nuclear marker plasmid was co-transferred with *OsPP2C68-GFP* into rice protoplasts. Observation via laser confocal microscopy indicated that OsPP2C68 was localized in the cell nucleus (Fig. 2c).

Analysis of the expression levels of *OsPP2C68* under different abiotic stress conditions

The promoter analysis results showed that the promoter region of *OsPP2C68* contained common cis-regulatory elements, such as CAAT-box and TATA-box, and specific regulatory elements that were responsive to ABA and MeJA (Supplementary Table S1). This result suggested that *OsPP2C68* might be involved in responding to various abiotic stresses. To investigate the expression pattern of *OsPP2C68* under different abiotic stresses, three-leaf stage seedlings of Nipponbare were treated with 100 μM ABA, 20% polyethylene glycol (PEG), 100 mM NaCl, and low temperature(4℃) at varying durations to analyze the expression level of *OsPP2C68*. The results indicated that the expression of *OsPP2C68* was induced by ABA, PEG, NaCl, and low temperature (Fig. 3a-d).

OsPP2C68 negatively regulates ABA response

To investigate the sensitivity of the *OsPP2C68* knockout mutant to ABA, the germination rates of the *OsPP2C68* knockout mutant and wild-type seeds under exogenous ABA treatment were statistically analyzed. The germination rates of the mutants *pp2c68-1* and *pp2c68-2* were the same as those of the wild type on 1/2 MS medium without ABA. On the 1/2 MS medium that contained 1, 2, 5, and 10 μM ABA, the germination rates of mutant seeds were significantly lower than that of the wild-type control (Fig. 4). The difference between the

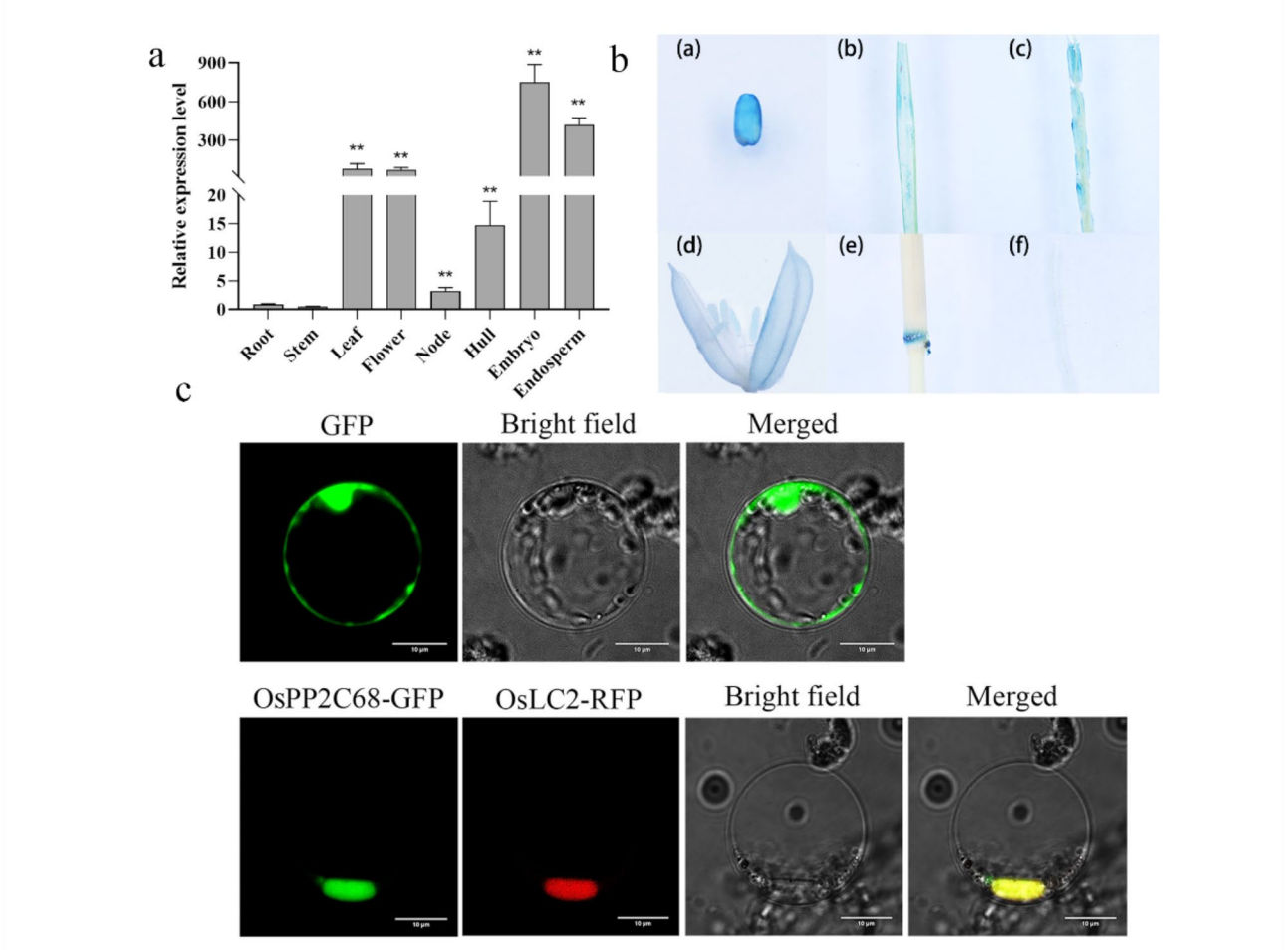

Fig. 2. *OsPP2C68* expression profile in different rice tissues and OsPP2C68 subcellular localization. (**a**) *OsPP2C68* expression levels in mature rice tissues (root, stem, leaf, flower, node, hull, embryo and endosperm) by RT – qPCR. Data represent mean ± SD ($n = 3$). Asterisks indicate statistically significant differences (Student's t-test; * $p < 0.05$, ** $p < 0.01$). (**b**) Histochemical GUS staining analysis of *proOsPP2C68:GUS* transgenic rice seedlings and plants. ((a), seed (b), leaf (c), hull (d), flower (e), node (f), root.) (**c**) Subcellular localization of *OsPP2C68 – GFP* in a rice protoplast. bars = 10 μm.

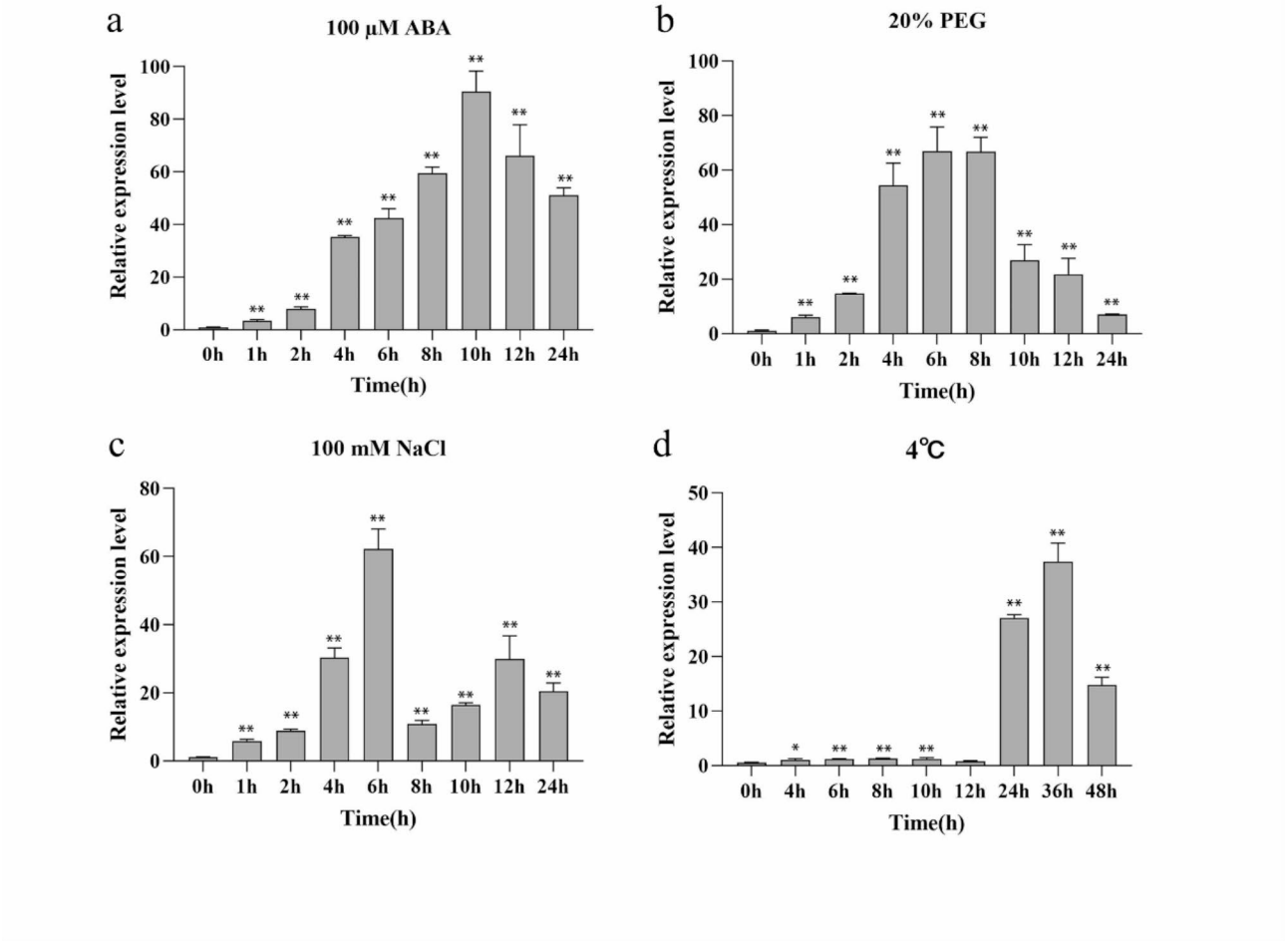

Fig. 3. Expression analysis of *OsPP2C68* in different abiotic stresses in *OsPP2C68* Knockout Mutants. (**a**) Expression under 100 μM ABA treatment. (**b**) Expression under 20% PEG treatment. (**c**) Expression under 100 mM NaCl treatment. (**d**) Expression under low temperature treatment. Data represent mean ± SD ($n = 3$). Asterisks indicate statistically significant differences (Student's t-test; * $p < 0.05$, ** $p < 0.01$).

germination rates of the mutant and wild-type seeds gradually increased with an increase in exogenous ABA concentration.

Analysis and comparison of growth differences between the *OsPP2C68* knockout mutants and wild-type seedlings after different exogenous ABA treatments during the seedling stage were conducted. The results showed that in the hydroponic solutions without ABA, no significant difference in growth was found between the wild-type and mutant *pp2c68-1* and *pp2c68-2* seedlings. However, in the hydroponic solutions with varying concentrations of ABA, the root length, stem length, and fresh weight of the mutants *pp2c68-1* and *pp2c68-2* were smaller than those of the wild type, and the differences became more evident with increasing ABA concentration. This finding suggests that the knockout of the *OsPP2C68* gene enhances rice sensitivity to ABA, indicating that *OsPP2C68* negatively regulates response to ABA (Fig. 5a-d).

We further analyzed stomatal status in the leaves of the wild-type and *OsPP2C68* knockout mutant plants. No differences were noted in stomatal density and length between the knockout mutant and wild-type plants (Fig. 5f–g). Comparing stomatal aperture between the seedling-stage mutants and wild types under normal conditions, the percentage of closed stomata was slightly higher in the mutants, while the percentage of fully open stomata was lower compared with that in the wild type. In addition, the differences in the percentages of completely closed and fully open stomata between the knockout mutants and wild type became more significant after ABA treatment (Fig. 5h–i). These results indicate that *OsPP2C68* can inhibit ABA signal transduction and further regulate stomatal movement mediated by ABA.

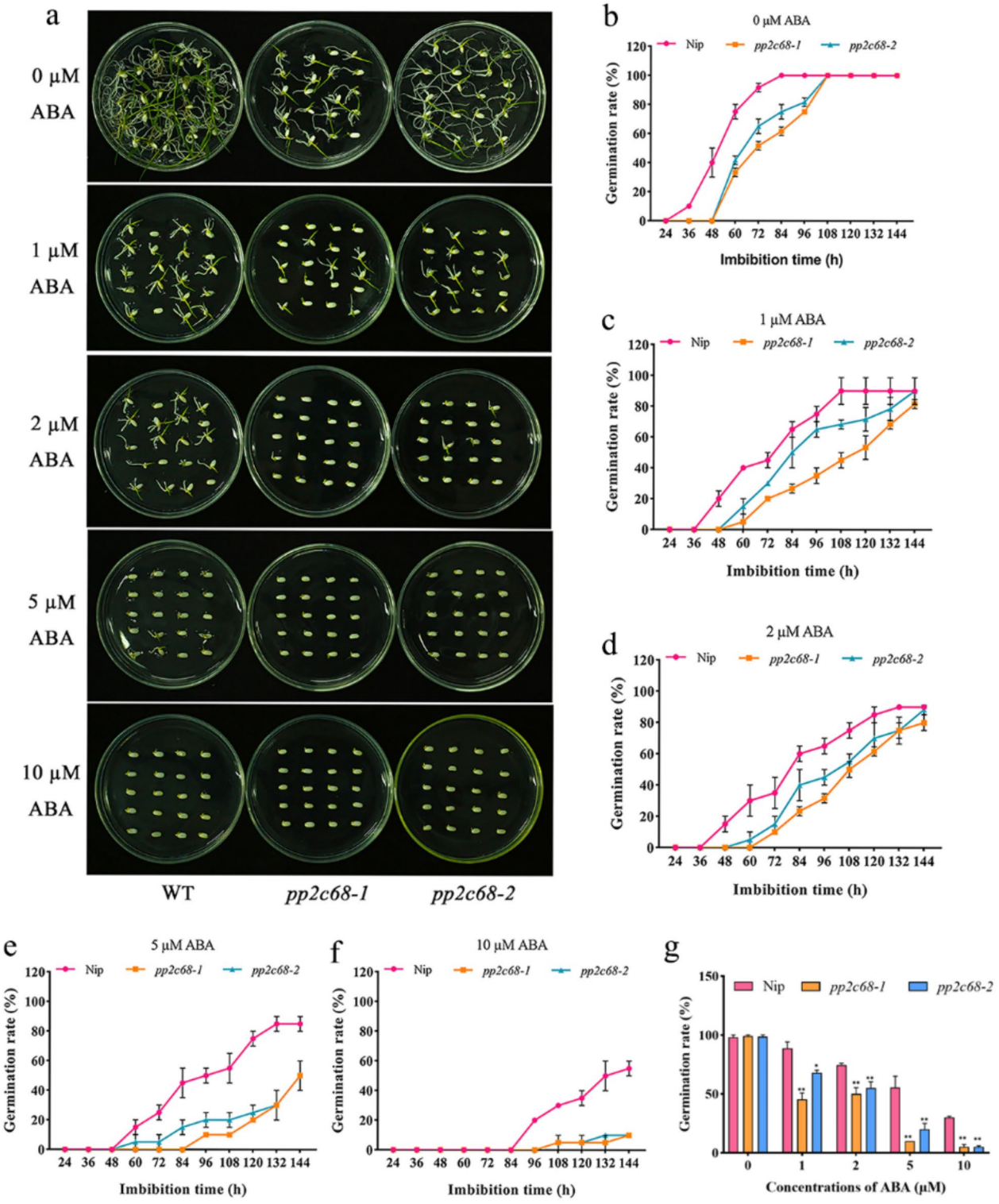

Fig. 4. Analysis of germination rate of *OsPP2C68* knockout mutants and wild type under ABA treatment. (**a**) Germination of *OsPP2C68* knockout mutants and WT in 0, 1, 2, 5 and 10 μM ABA medium for 6 days. (**b**-**f**) Germination rates of *OsPP2C68* knockout mutants and WT in media containing 0 μM ABA (b), 1 μM ABA (c), 2 μM ABA (d), 5 μM ABA (e) and 10 μM ABA (f). (**g**) Seed germination rate of *OsPP2C68* knockout mutants and wild type after treatment with 0, 1, 2, 5 and 10 μ m ABA for 108 h. Data represent mean ± SD ($n = 3$). Asterisks indicate statistically significant differences (Student's t-test; * $p < 0.05$, ** $p < 0.01$).

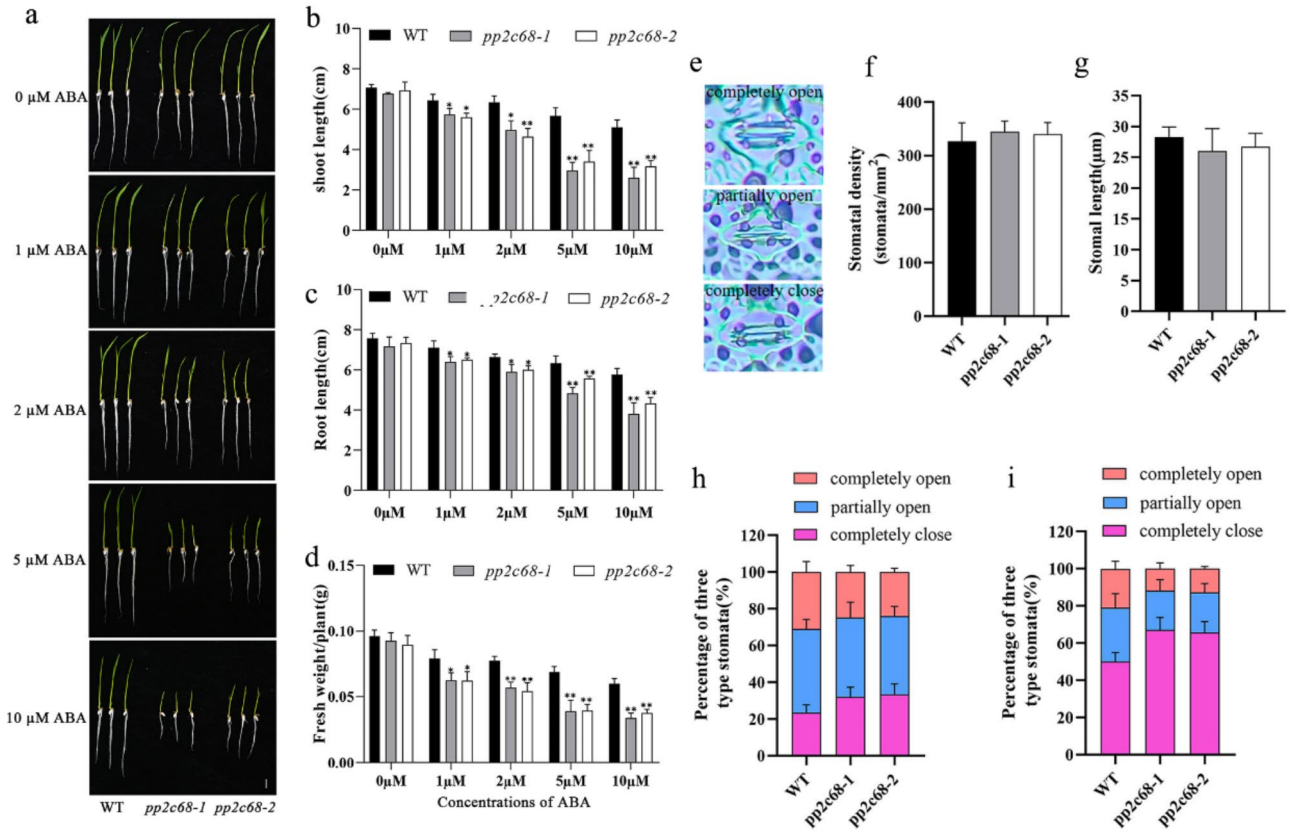

Fig. 5. Phenotype and stomatal analysis of wild-type and *OsPP2C68* knockout mutants under ABA treatment. (**a**) Growth of WT and *OsPP2C68* knockout mutants in 0, 1, 2, 5, and 10 μM ABA hydroponic solution for 5 days. (**b–d**) Shoot length (b), root length (c), and fresh weight (d) of WT and *OsPP2C68* knockout mutants in hydroponics containing 0, 1, 2, 5, and 10 μM ABA. (**e**) three stomatal types (**f**) Comparison of leaf stomatal density between WT and *OsPP2C68* knockout mutants under normal conditions. (**g**) Comparison of leaf stomatal length between WT and *OsPP2C68* knockout mutants under normal conditions. (**h**) Percentage of three stomatal types of WT and *OsPP2C68* knockout mutants under normal conditions. (**i**) Percentage of three stomatal types of WT and *OsPP2C68* knockout mutants under ABA treatment. Bars = 1 cm Data represent mean ± SD ($n = 3$). Asterisks indicate statistically significant differences (Student's t-test; * $p < 0.05$, ** $p < 0.01$).

Analysis of drought tolerance in *OsPP2C68* knockout mutant plants

To study the response of *OsPP2C68* to drought stress, *OsPP2C68* knockout mutant materials were subjected to mannitol osmotic stress during the seedling stage, and the root length, shoot length, and fresh weight were measured. The results showed that on 1/2 MS medium that contained 0 mM and 100 mM mannitol, no significant differences occurred in the shoot length, root length, and fresh weight between the mutant lines *pp2c68-1* and *pp2c68-2*, and the wild-type seedlings. However, on 1/2 MS medium that contained 200 mM and 300 mM mannitol, the shoot length, root length, and fresh weight of the mutant lines *pp2c68-1* and *pp2c68-2* were significantly higher than those of the wild-type seedlings (Fig. 6). This result indicates that *OsPP2C68* is involved in rice response to drought stress.

Further experiments were conducted during the seedling stage by using the *OsPP2C68* knockout mutant materials under PEG-simulated drought conditions. Wild-type and *OsPP2C68* knockout mutant materials were grown in normal hydroponic solution until the three-leaf stage. Then, they were subjected to stress treatment with a hydroponic solution that contained 25% PEG6000 to induce severe leaf dehydration, followed by a recovery period of 10 days in normal hydroponic solution. The results showed that after recovery growth, the *OsPP2C68* knockout mutant seedlings retained more green leaves and exhibited increased plant height (Fig. 7a). The survival rate of the *pp2c68-1* and *pp2c68-2* mutant rice seedlings was significantly higher than that of the wild type (Fig. 7b), indicating that mutation of the *OsPP2C68* gene enhanced drought tolerance in rice.

When the wild-type and *OsPP2C68* knockout mutant seedlings were grown under soil conditions until the reproductive stage, drought stress was initiated by withholding water until the leaves curled, wilted, and turned yellow, followed by rewatering. As shown in Fig. 7c, no significant difference was found between the *OsPP2C68* knockout mutant and wild-type plants before drought stress. After rehydration, significant differences in growth were observed between the *OsPP2C68* knockout mutants and wild-type plants. The mutant plants *pp2c68-1* and *pp2c68-2* had more green leaves, smooth leaves, and sturdy stems, while the wild-type plants exhibited severe leaf curling and even death. These results indicate that the *OsPP2C68* knockout mutant plants exhibit drought tolerance, suggesting that *OsPP2C68* plays a negative regulatory role in the drought tolerance of rice.

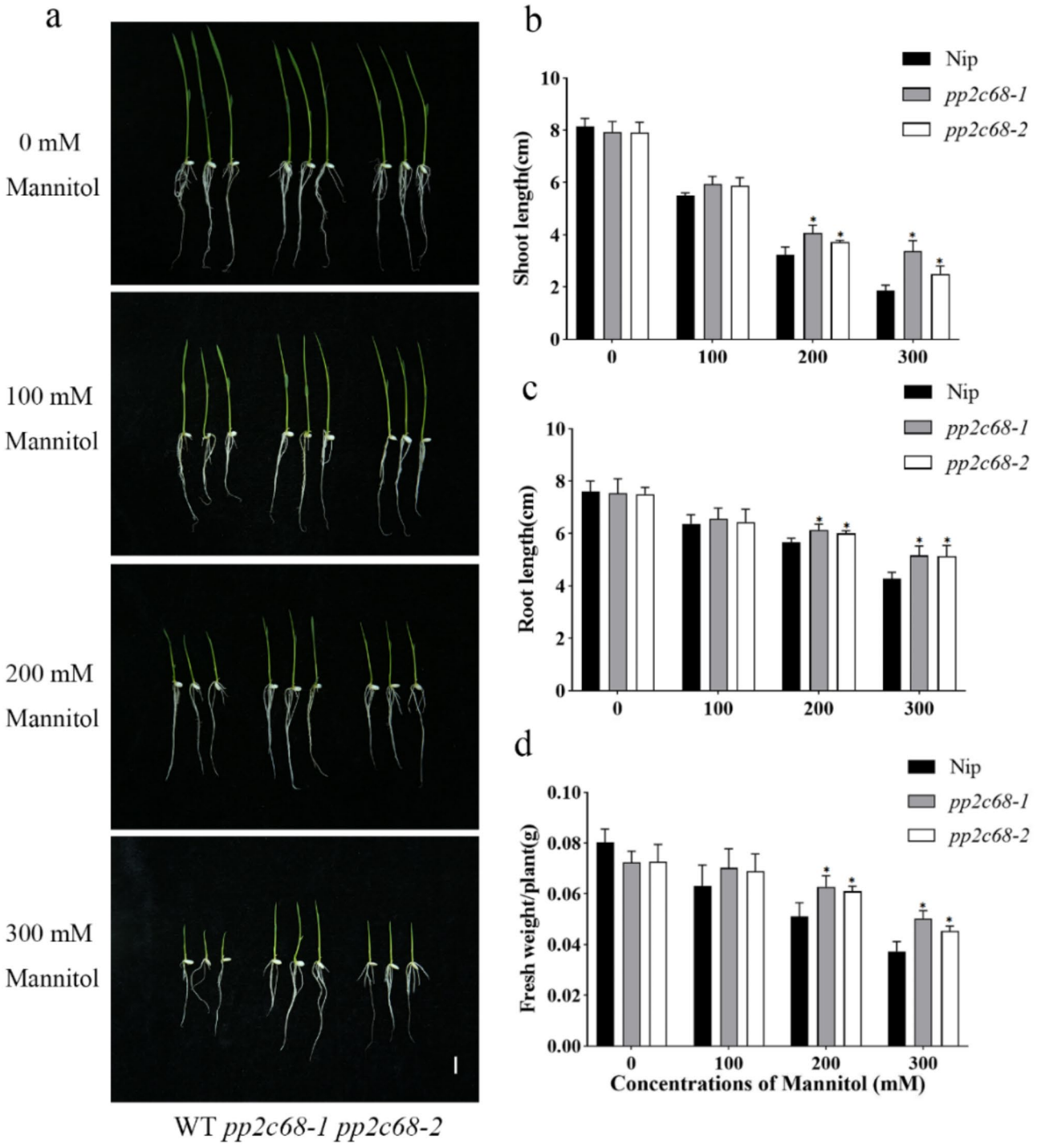

Fig. 6. The phenotype identification of wild type and *OsPP2C68* knockout mutants under different concentration mannitol treatment for 5 days. (**a**) The growth of *OsPP2C68* knockout mutant and wild type under different mannitol treatment for 5 days. (**b-d**) Shoot length, root length and fresh weight statistics of *OsPP2C68* knockout mutant and wild type under different mannitol treatment for 5 days. Bars = 1 cm Data represent mean ± SD ($n = 3$). Asterisks indicate statistically significant differences (Student's t-test; * $p < 0.05$, ** $p < 0.01$).

Changes in physiological indicators under drought stress

The contents of free proline (Pro), malondialdehyde (MDA) and the activities of superoxide dismutase (SOD) and catalase (CAT), in the wild-type and *OsPP2C68* knockout mutant seedlings were measured before and after drought stress. The results showed that after drought treatment, the Pro content and the activities of SOD and CAT significantly increased in the *OsPP2C68* knockout mutants and wild-type seedlings. However, the knockout

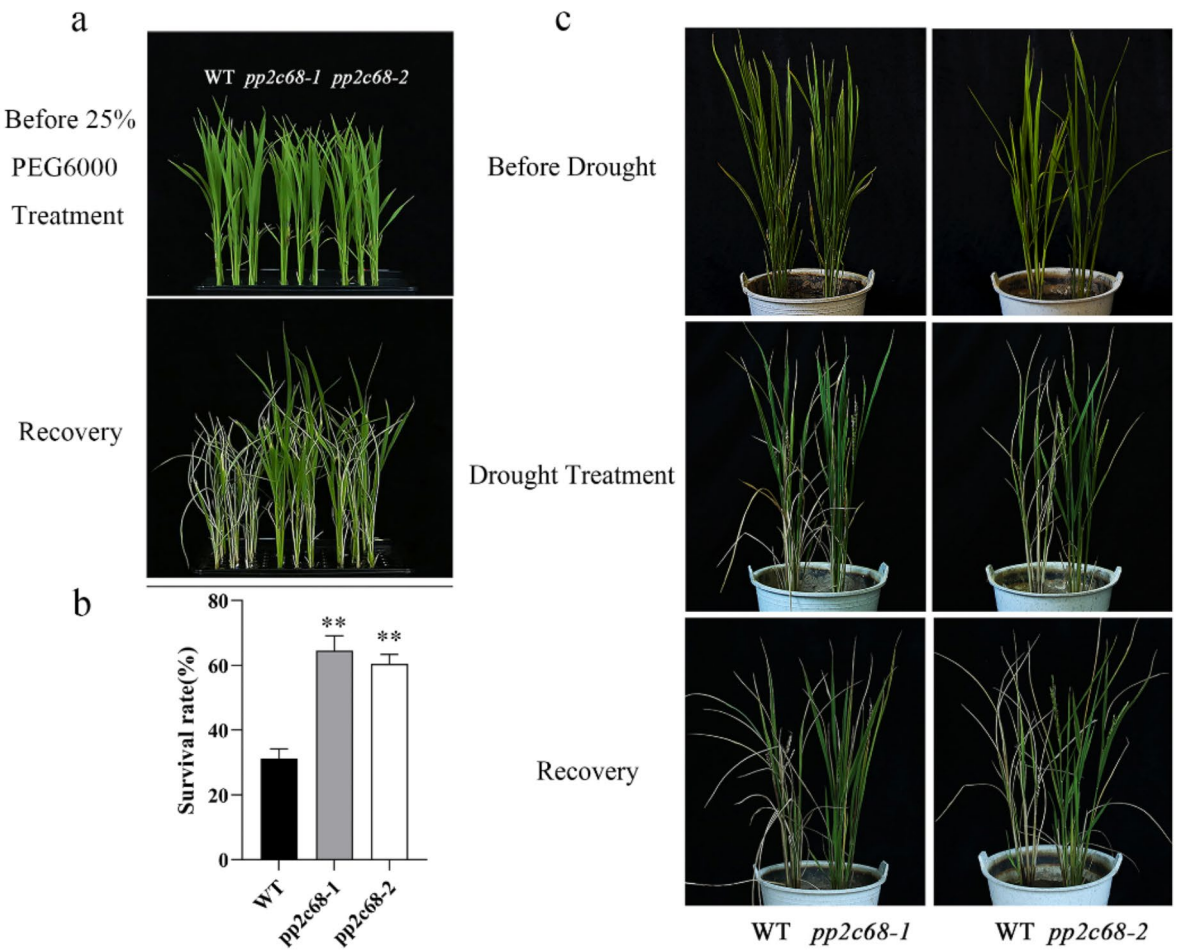

Fig. 7. Phenotype of wild type and *OsPP2C68* knockout mutants under drought stress. (**a**) Growth of wild type and *OsPP2C68* knockout mutants seedlings under 25% PEG6000 stress. (**b**) Survival rate of WT and *OsPP2C68* mutants testing in (**a**). Values are means ± SD ($n = 3$). (**c**) Phenotype of wild type and *OsPP2C68* knockout mutants under soil drought stress. *: Significant level, $0.01 < p < 0.05$.

mutants exhibited significantly higher levels than the wild type. Conversely, the MDA content presented an opposite trend (Fig. 8a–d).

The plant tissues and cell membranes were damaged after stress treatment, leading to electrolyte leakage and reduced chlorophyll content. We separately measured changes in conductivity and chlorophyll content in the *OsPP2C68* knockout mutant and wild-type seedlings before and after drought stress. The results showed that after drought treatment, conductivity levels significantly increased while chlorophyll content significantly decreased in the mutant and wild-type seedlings. However, conductivity in the mutant seedlings was significantly lower than that in the wild type, whereas chlorophyll content was significantly higher than that in the wild type (Fig. 8e–f).

The above results indicate that the mutation of *OsPP2C68* enhanced the synthesis of osmotic substances and antioxidant enzymes in rice plant and reduced damage to plant tissues and cell membranes, enhancing the drought tolerance of rice.

Analysis of drought-related gene expression levels in the *OsPP2C68* knockout mutant

To better explain the enhanced drought tolerance of rice at the molecular level due to *OsPP2C68* gene knockout, we selected seven drought-related genes (*OsAP37*, *OsP5CS1*, *OsNCED1*, *OsNCED2*, *OsNCED3*, *OsNCED4*, *OsNCED5* and *OsABI5*). Through RT-qPCR, we measured the expression changes of these genes before and after drought stress in the wild-type and *OsPP2C68* knockout mutant plants. The quantitative results showed

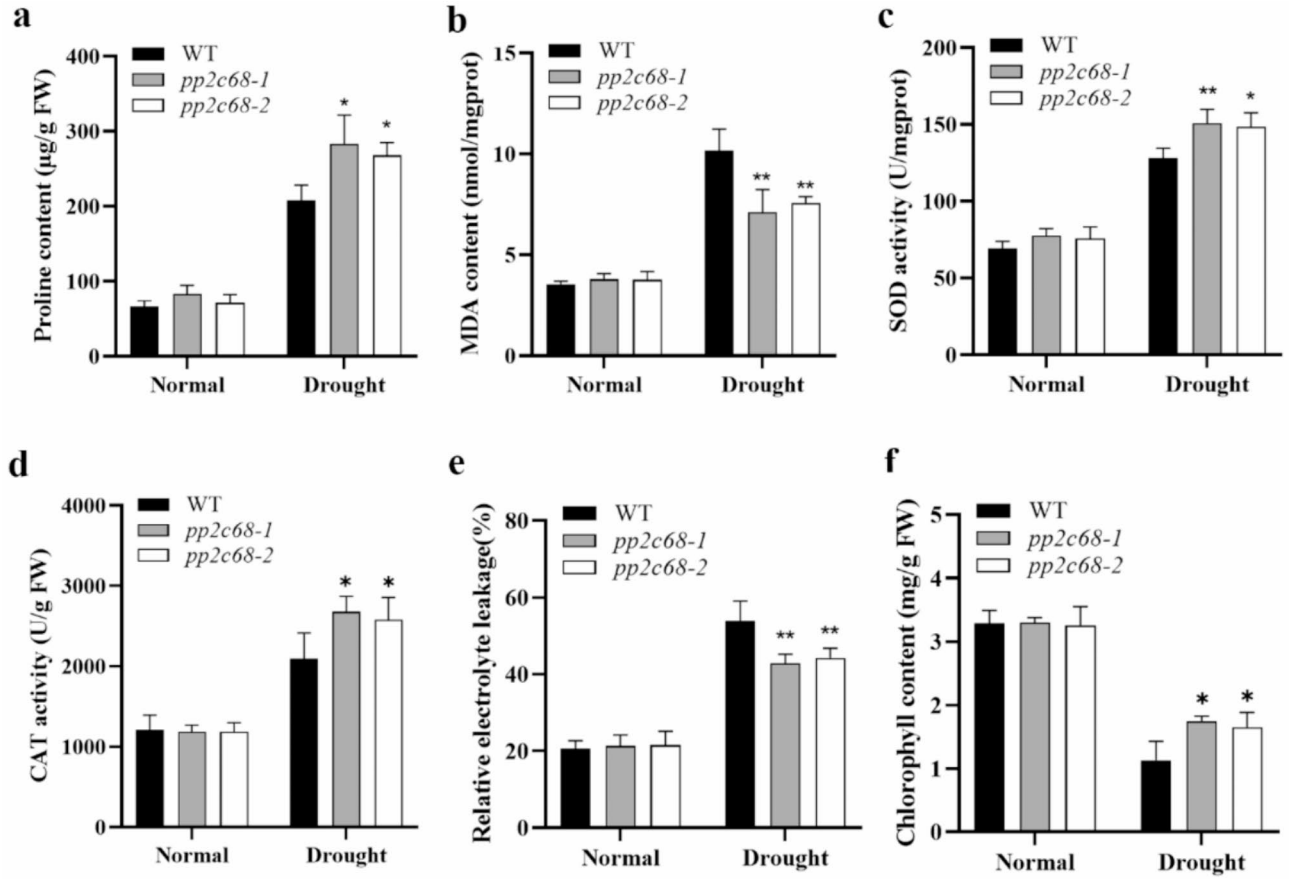

Fig. 8. Analysis of changes in physiological indexes of wild type and *OsPP2C68* knockout mutants under drought tolerance. (**a**) Pro content. (**b**) MDA content. (**c**) SOD activity. (**d**) CAT activity. (e) Relative electrolyte leakage. (f) Chlorophyll content. *Significant level, $0.01< p<0.05$. **Extremely significant level, $p<0.01$.

that before drought stress, no significant difference was found in the expression levels of the seven drought-related genes between the *OsPP2C68* knockout mutants and the wild type. After drought stress treatment, the expression levels of these genes were significantly upregulated in the *pp2c68-1* and *pp2c68-2* mutants and wild type, however, the expression levels of *OsABI5* were significantly downregulated (Fig. 9).

Analysis of salt tolerance in *OsPP2C68* knockout mutant plants

Soil salinity is one of the critical factors that threatens rice food security. To investigate the response of *OsPP2C68* to salt stress, *OsPP2C68* knockout mutant materials were subjected to salt stress during the seedling stage. The results showed that on normal 1/2 MS medium without salt, the wild type and mutant lines *pp2c68-1* and *pp2c68-2* exhibited similar growth. However, under treatments of different salt concentrations, the shoot length, root length, and fresh weight of rice seedlings from the mutant lines *pp2c68-1* and *pp2c68-2* were significantly longer and heavier compared with the wild type (Fig. 10a–d). These experimental results indicate that the *OsPP2C68* knockout mutants are more sensitive to salt stress during the germination stage.

After approximately 2 weeks of cultivation in hydroponic solution until the seedlings reached the three-leaf stage, the wild-type and *OsPP2C68* mutant seedlings were subjected to salt stress by replacing the hydroponic solution with 100 mM NaCl for 4 days, followed by a 7-day recovery period. Upon recovery, the *OsPP2C68* knockout mutant seedlings had more green leaves than the wild type (Fig. 10e). In addition, the survival rate of the *pp2c68-1* and *pp2c68-2* rice seedlings was significantly higher than that of the wild type (Fig. 10f). These results indicate that knocking out the *OsPP2C68* gene enhances salt tolerance in rice.

Physiological indicators of *OsPP2C68* knockout mutant seedlings under salt stress

In salt-stressed seedlings during the trefoil stage, physiological indicators (Pro, MDA, SOD, and CAT) were measured before and after stress in the *OsPP2C68* knockout mutants and wild-type japonica rice seedlings. After salt stress treatment, the *OsPP2C68* knockout mutants *pp2c68-1* and *pp2c68-2* exhibited significantly higher Pro content, and SOD and CAT activities compared with the wild type. In addition, MDA content was significantly lower in the knockout mutants compared with the wild type (Fig. 11a–d). Further determination of relative conductivity and chlorophyll content revealed that under 100 mM NaCl stress conditions, the *OsPP2C68* knockout mutant seedlings and wild-type japonica rice seedlings demonstrated increased relative conductivity

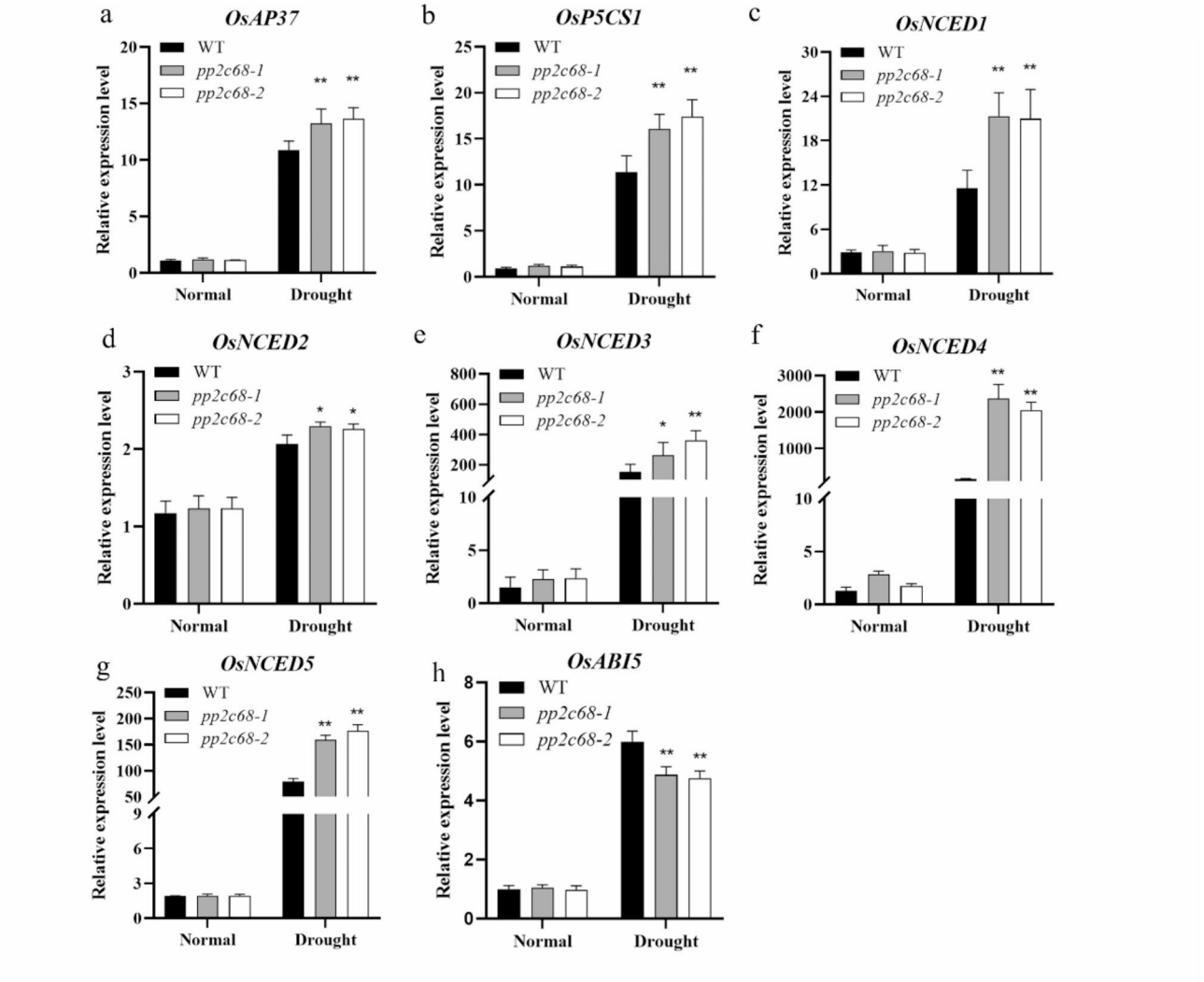

Fig. 9. Analysis of drought relative gene in wild type and *OsPP2C68* knockout mutants. (**a**) Relative expression of *OsAP37*, (**b**) Relative expression of *OsP5CS1*, (**c**) Relative expression of *OsNCED1*, (**d**) Relative expression of *OsNCED2*, (**e**) Relative expression of *OsNCED3*, (**f**) Relative expression of *OsNCED4*, (**g**) Relative expression of *OsNCED5*. (**h**) Relative expression of *OsABI5*. Data represent mean ± SD ($n = 3$). Asterisks indicate statistically significant differences (Student's t-test; $^{*}p < 0.05$, $^{**}p < 0.01$).

and decreased chlorophyll content. However, the knockout mutant seedlings exhibited lower relative conductivity and higher chlorophyll content compared with the wild type (Fig. 11e–f).

Analysis of salt-related gene expression levels in *OsPP2C68* knockout mutants

To validate the improved salt stress tolerance of *OsPP2C68* at the molecular level, we selected nine salt stress-related genes (*OsDREB6*, *OsHKT1*, *OsNAC9*, *OsbZIP23*, *OsbZIP71*, *OsNCED2*, *OsNCED3*, *OsNCED4*, and *OsNCED5*) and detected expression changes in the wild-type and *OsPP2C68* knockout mutant seedlings before and after salt stress. Under normal conditions, no significant difference was found in the expression levels of the nine salt-related genes between the mutant and wild-type seedlings. Meanwhile, the expression levels of the nine genes were upregulated in the mutants *pp2c68-1* and *pp2c68-2*, and the wild type; they were significantly higher in the mutant than in the wild type after salt stress treatment (Fig. 12). This result suggests that the nine genes may play roles in the salt stress response regulated by *OsPP2C68*, or *OsPP2C68* may regulate their expression to enhance rice resistance to salt stress.

Discussion

PP2CAs are important negative regulators in the ABA signaling pathway, and different PP2CA proteins participate in the transduction of ABA signals through distinct mechanisms[21,22]. The rice PP2CA gene families consists of 10 members. These rice *PP2CAs* have been reported to be sensitive to ABA. Some members of this family regulate various traits, such as seed germination, plant height, root length, fertility, and thousand-grain weight[23–25]. Research has also found functional redundancy among certain *PP2CAs*. For example, after knocking

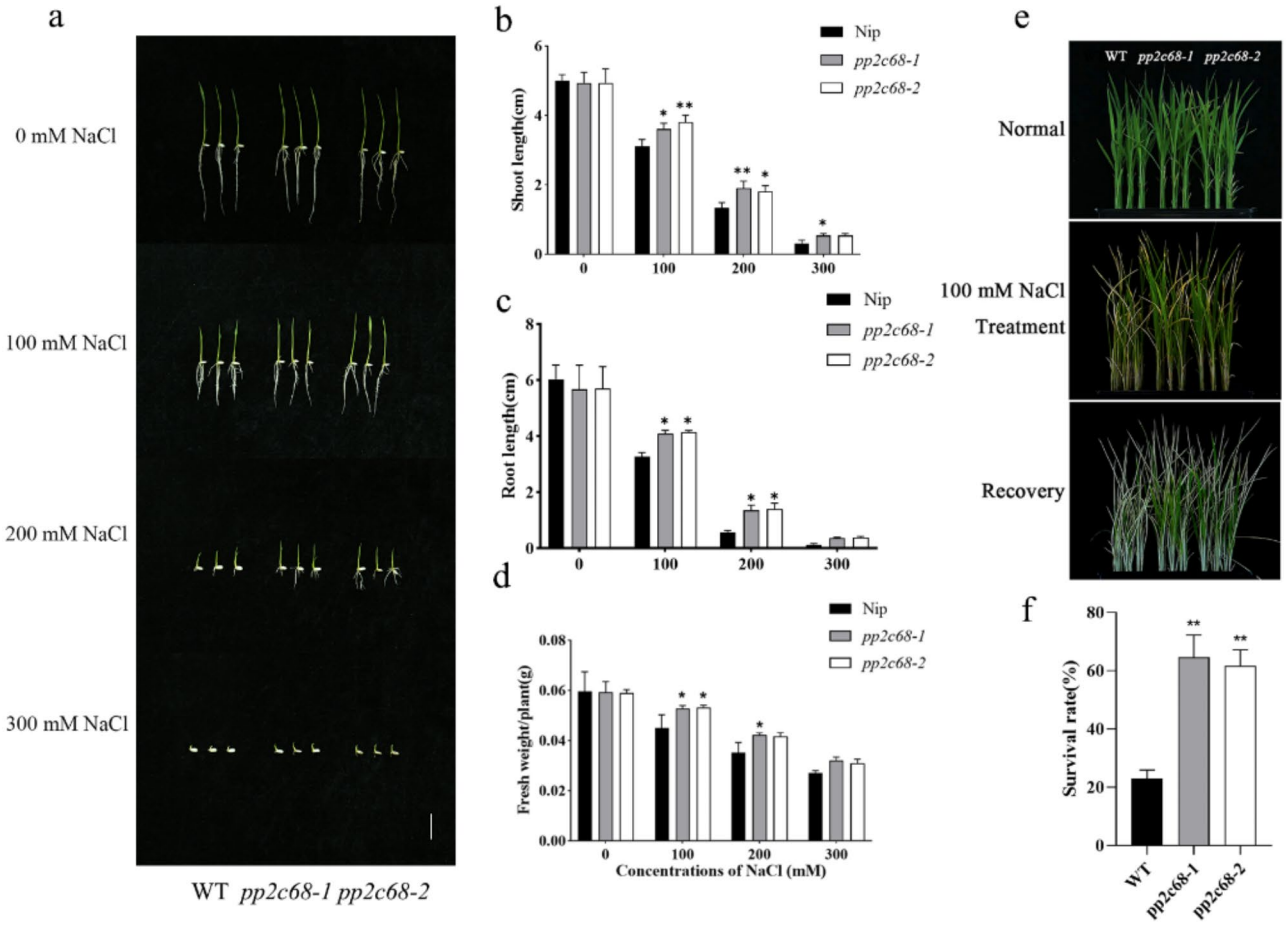

Fig. 10. Phenotype of wild type and *OsPP2C68* knockout mutant under NaCl treatment. (**a**) Growth of wild type and *OsPP2C68* knockout mutants under different concentration of NaCl treatment for 5 days. (**b-d**) Shoot length, root length and fresh weight statistics of *OsPP2C68* wild type and mutant under different concentration of NaCl treatment for 5 days. (**e**) Phenotype of wild type and *OsPP2C68* mutants before and after 100 mM NaCl treatment. (**f**) Survival rate of wild type and *OsPP2C68* mutants after recovery. *: Significant level, $0.01 < p < 0.05$.

out *OsPP2C49*, sensitivity to ABA did not change compared with the wild type[26]. This study demonstrates that the knockout mutant of *OsPP2C68* exhibits phenotypes related to seed germination, seedling growth, stomatal movement, and responses to abiotic stress, indicating the important role of this gene in regulating ABA signal transduction. In earlier phases of this research, overexpression plants of *OsPP2C68* were also generated. However, these materials exhibited slow growth during development and eventually died, preventing further investigation. The reasons behind these observations require further analysis.

ABA is an important regulator of stomatal movement. Research has identified *OsPP2C09*, *OsPP2C50*, and *OsPP2C53* in rice as major negative regulators of ABA signaling during stomatal closure. In addition, the *OsPP2C09* mutant exhibits significantly reduced stomatal length and increased stomatal density[25,27]. This study found that *OsPP2C68* also negatively regulates stomatal movement, but it does not affect stomatal density or length. This result indicates that *OsPP2C68* and *OsPP2C09* have distinct regulatory mechanisms for stomata.

This study discovered that the expression of *OsPP2C68* is induced by drought and salt stress. Following drought and salt treatments, the survival rates of the *OsPP2C68* knockout mutants were significantly higher than that of the wild type. The mutants exhibited increased Pro content, enhanced activities of SOD and CAT, and reduced levels of MDA and electrolyte leakage. These findings indicate that the knockout mutants promote the synthesis of osmolytes and antioxidant enzymes under drought and salt stress conditions, enhancing the plant's ability to scavenge reactive oxygen species and reduce cellular membrane damage, mitigating the detrimental effects of drought and salt stress. Furthermore, under drought and salt stress, the knockout mutants of *OsPP2C68* exhibited upregulation in the expression of drought- and salt-related genes. These results indicate that the knockout of *OsPP2C68* affects the synthesis of osmolytes and antioxidant enzymes within the plant, and the expression of genes related to drought and salt stress response. Consequently, this condition enhances the drought and salt tolerance of rice plants.

At present, many experimental studies have utilized members of the PP2C family as bait to screen for proteins that interact with PP2Cs in the ABA signaling pathway. This approach aims to identify ABA receptors or

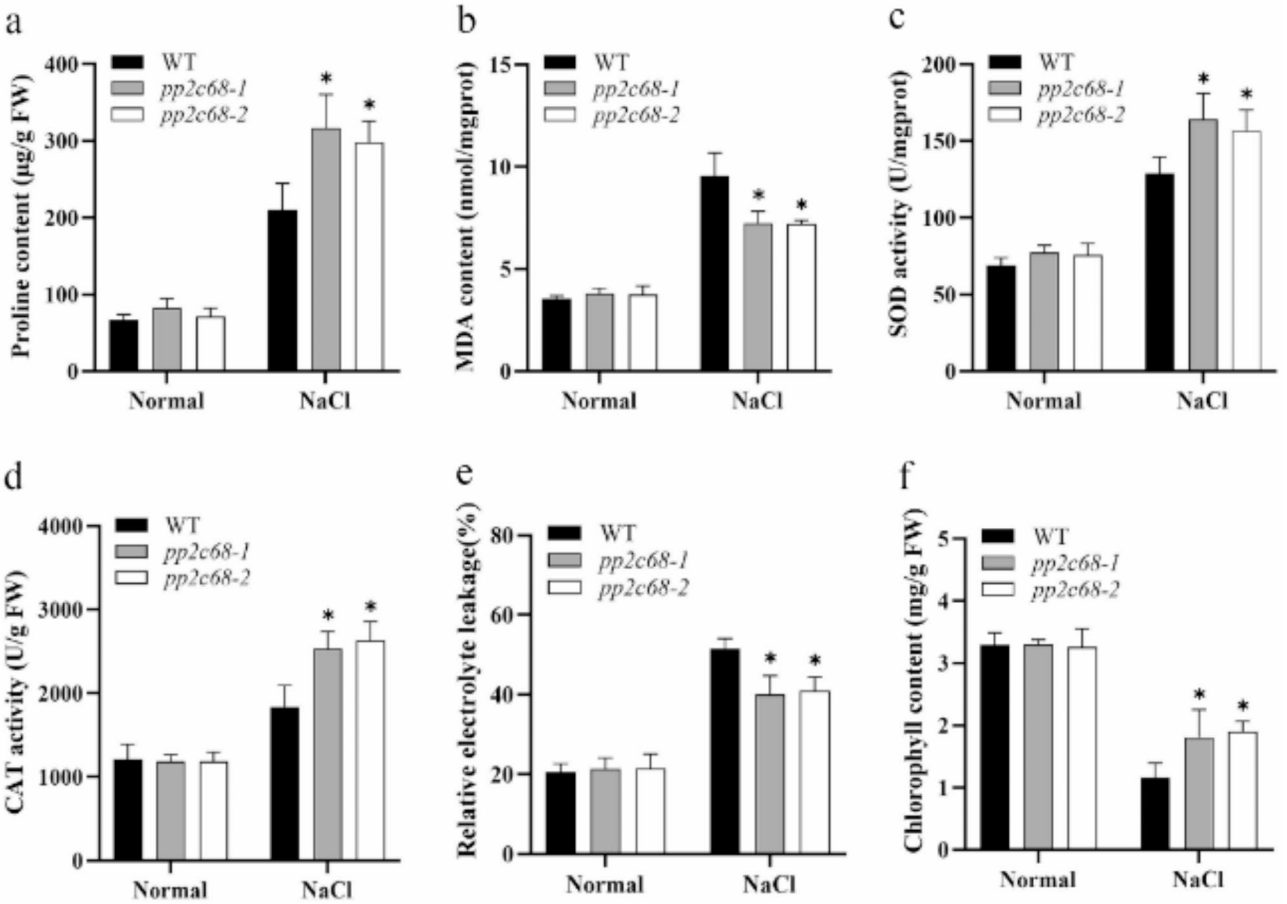

Fig. 11. Analysis of changes in physiological indexes of wild type and *OsPP2C68* mutant under NaCl tolerance. (**a**) Pro content. (**b**) MDA content. (**c**) SOD activity. (**d**) CAT activity. (**e**) Relative electrolyte leakage. (**f**) Chlorophyll content. *Significant level, $0.01 < p < 0.05$.

downstream transcription factors involved in the ABA signaling pathway. Such effort has significantly advanced the understanding of the functions of *PP2CAs* and laid the foundation for deciphering the intricate regulatory mechanisms of the ABA signaling pathway. However, further research is necessary to investigate the interacting proteins of *OsPP2C68* in the ABA signaling pathway.

Materials and methods

Plant growth conditions and stress treatments

To examine the transcript levels of the *OsPP2C68* gene under various abiotic stresses and hormone treatments, the transcript levels of the *OsPP2C68* gene under different stress conditions were studied using the *japonica* rice variety, Nipponbare, as the research material. The seeds were sterilized, allowed to germinate for 2 days at 28 °C, and subsequently grown hydroponically in a growth chamber The hydroponic solution followed the method of the International Rice Research Institute[28]. During the third-leaf stage of seedling development, treatments with 100 μM ABA, 20% polyethylene glycol (PEG), 100 mM NaCl, and cold stress(4℃) were applied at varying durations. Leaf samples were collected at specified time points, and total RNA was extracted from the leaves for further analysis.

RNA isolation and qRT-PCR analysis

Total RNA was extracted from Nipponbare by using TRIzol reagent, and complementary DNA (cDNA) was synthesized using a Thermo Fisher reverse transcription kit. RT-qPCR was performed using an ABI PRISM 7500 qPCR instrument and ChamQ Universal SYBR qPCR Master Mix. Triple quantification was performed for each cDNA sample. The experiment employed *OsActin* as an internal reference control for RT-qPCR, and relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method[29].

Plasmid construction and plant transformation

To construct the gene knockout strain, we utilized the CRISPR/Cas9 system for targeted genome modification in rice[30,31], A 20 bp single guide RNA sequence (GGTGTGCTGTGATTCTGCGG) was cloned into the *pOs-sgRNA* vector and then subcloned into the Cas9 vector *pYLCRISPR/Cas9Pubi-H*. The CRISPR/Cas9 construct was transformed into Nipponbare.

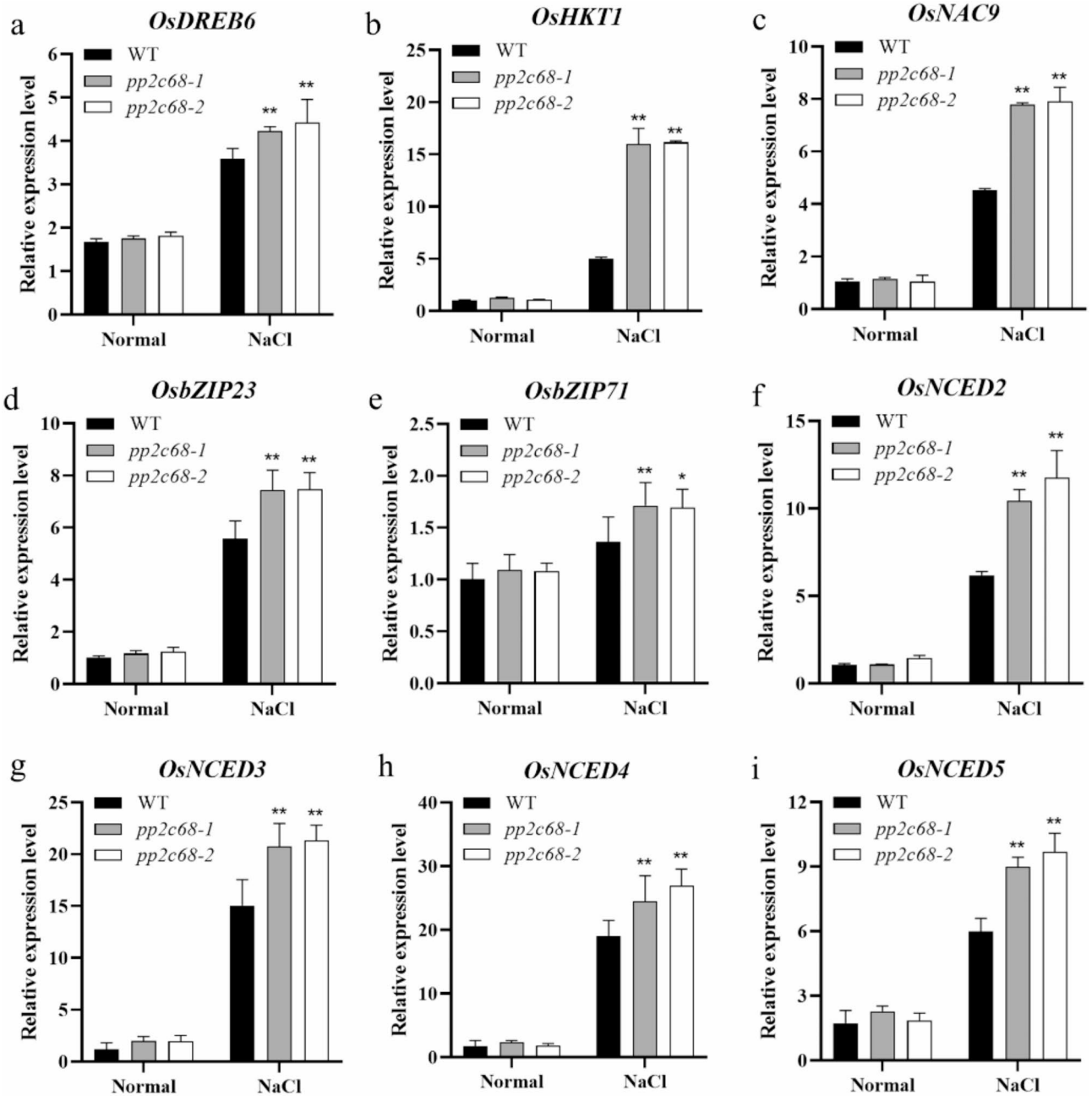

Fig. 12. Analysis of relative expression of NaCl relative gene in wild type and *OsPP2C68* mutants. (**a**) Relative expression of *OsDREB6*, (**b**) Relative expression of *OsHKT1*, (**c**) Relative expression of *OsNAC9*, (**d**) Relative expression of *OsbZIP23*, (**e**) Relative expression of *OsbZIP71*, (**f**) Relative expression of *OsNCED2*, (**g**) Relative expression of *OsNCED3*, (**h**) Relative expression of *OsNCED4*, (**i**) Relative expression of *OsNCED5*. Data represent mean ± SD ($n = 3$). Asterisks indicate statistically significant differences (Student's t-test; * $p < 0.05$, ** $p < 0.01$).

A 2000 bp fragment upstream of the *OsPP2C68* start codon was amplified from rice genomic DNA. The amplified promoter fragment was digested by *Eco*RI and *Hind*III enzymes and cloned into the pCAMBIA1301-GUS vector. The *OsPP2C68* promoter-GUS constructs were transformed into Nipponbare, and *OsPP2C68* knockout mutants of rice were obtained. All transgenic rice plants were obtained using agrobacterium-mediated transformation[32].

GUS staining and subcellular localization

During the rice maturation stage, various tissues from *OsPP2C68* promoter-GUS transgenic plants were collected for GUS staining analysis by following the previously described experimental procedure[33,34]. The *OsPP2C68*-GFP vector plasmid was constructed by ligating the coding sequence (CDS) of the *OsPP2C68* gene to the green

fluorescent protein (GFP) expression vector pCAMBIA1390 with a ubiquitin promoter driven. Transfection of the nuclear red fluorescent reference vector plasmid *OsLC2*-RFP and *OsPP2C68*-GFP into rice protoplasts was conducted, followed by incubation in darkness at 28 °C for 16–24 h. Observations were made using a laser scanning confocal microscope LSM 880 to identify the site where fluorescence was expressed[35,36].

ABA sensitivity assay

The sensitivity of seed germination to ABA was determined by inoculating seeds onto 1/2 MS medium that contained ABA. Fully filled and uniform seeds of the wild type and *OsPP2C68* knockout mutants were selected for seed germination assay. The hulled mutant and wild-type seeds were sterilized, dried, and inoculated into 1/2 MS medium without ABA and with 1, 2, 5, and 10 μM ABA for germination, with 20 seeds per dish in three replicates. Seed germination was counted once at 12 h. The seed germination rate was determined by the number of seeds in each dish.

Response of Transgenic plants to abiotic stress

To analyze the gene for stress tolerance, seeds of the wild-type and *OsPP2C68* knockout mutants were inoculated and grown on 1/2 MS medium for 3 days at 28 °C in an incubator under 14 h light/10 h dark cycles. Seeds with uniform growth were transferred to 1/2 MS medium that contained different concentrations of ABA, mannitol, and NaCl for 5 days. Then, all the seedlings were counted for germination percentage, shoot length, root length and fresh weight.

In this study, the wild-type and *OsPP2C68* knockout mutants were grown in normal hydroponic solution until the three-leaf stage at 28 °C in an incubator under 14 h light/10 h dark cycles. Thereafter, they were transferred to hydroponic solutions that contained 100 μM ABA, 25% PEG, and 100 mM NaCl for stress treatments[37–40]. After the appearance of phenotypic differences, the plants were returned to normal hydroponic solution for a recovery period of 7–10 days. Subsequently, the survival rates of rice seedlings were calculated. Survival rate was determined as the ratio of the number of surviving plants in each hydroponic container to the total number of plants subjected to treatment. Each experiment was repeated three times.

Conducting reproductive stage soil drought stress analysis on the wild-type and *OsPP2C68* knockout mutants involved growing seedlings under soil conditions until the reproductive stage. The seedlings were subjected to water drought stress until leaf wilting, curling, and yellowing occurred. Subsequently, they were rewatered for recovery growth. Finally, phenotypic data were measured and statistically analyzed to assess the effects of drought stress on both genotypes.

Observation of rice leaf stomata

We selected rice flag leaves of roughly the same size, washed the middle part of the back of each leaf with distilled water, dried the leaves, and then evenly applied a 2–3 cm long layer of transparent nail polish. After the nail polish had dried to form a film, we slowly peeled it off by using tweezers and placed it on a microscope slide for observation. The types of rice stomata could be classified into three categories: fully open, partially open, and fully closed stomata. We observed the types of rice stomata under a microscope, evaluating stomatal density (number/mm^2) by using scanning electron microscopy images of the wild-type and *OsPP2C68* knockout mutant leaf samples. Each measurement was repeated using three random fields of view (each with an area of 0.24 mm^2). The length and width of the stomata were measured using the same stomatal apparatus, conducting three independent repetitions.

Enzyme activity assay

The activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were determined in accordance with their respective assay kit instructions (Jiancheng Bioengineering Institute, Nanjing, China). Plant tissue was ground in ice-cold phosphate buffer (pH 7.4) at nine times the volume (1 g tissue: 9 mL buffer), followed by centrifugation of the sample at 3500 rpm for 10 min. Absorbance of the supernatant was measured at 420, 550, and 595 nm.

Determination of free proline, malondialdehyde and chlorophyll content

Free Pro content was measured in accordance with the method used by Song et al.[41]. MDA content was measured following the method described by Duan et al.[42]. The chlorophyll content of rice plants was measured by grinding 0.1 g of fresh leaves, and the absorbance of each sample was tested via colorimetry at 652 nm by using a UV 2400 ultraviolet/visible spectrophotometer[43]。.

Relative conductivity measurement

The procedure involved weighing 0.1 g of clean rice leaf samples by using an electronic balance. The leaves were then cut into approximately uniform-sized fragments by using scissors and placed in 15 mL centrifuge tubes that contained 10 mL of double-distilled water. The tubes were left to soak overnight for 12 h. After soaking, the electrical conductivity (EC) of the solution was measured using a conductivity meter and recorded as R1. A boiling water bath was boiled for 0.5 h. After the water had cooled down, conductivity value was measured and recorded as R2 (relative conductivity = R2/R1 × 100%).

Data analyses

All data were expressed as mean standard deviation and represented by error bars. Data analysis was completed by comparing raw data from all individuals by using the Microsoft Excel program. Graphs were created using GraphPad Prism 8.0 and Adobe Photoshop.

Primers

The sequences of the primers used in the study are listed in Supplementary Table S2.

Data availability

All data were available with in this article and its supplementary files. All constructs and transgenic plants are available upon request. The *OsPP2C68* gene sequences are available from the China Rice Data Center (http://www.ricedata.cn/index.htm) using the accession numbers LOC_Os09g15670. Source data are provided with this paper.

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Author contributions

L.C. participated in the selection of articles. B.W. and Y.L. conceived and conducted the experiments; B.Z. and H.X. participated in analyzing the results; J.C., F.W., W.L., M.L. provided assistance during the experiments. B.W. and Y.L. wrote the paper. All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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