

A quantitative trait locus, *qSE3*, promotes seed germination and seedling establishment under salinity stress in rice

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SUMMARY

Seed germination is a complex trait determined by both quantitative trait loci (QTLs) and environmental factors and also their interactions. In this study, we mapped one major QTL *qSE3* for seed germination and seedling establishment under salinity stress in rice. To understand the molecular basis of this QTL, we isolated *qSE3* by map-based cloning and found that it encodes a K⁺ transporter gene, *OsHAK21*. The expression of *qSE3* was significantly upregulated by salinity stress in germinating seeds. Physiological analysis suggested that *qSE3* significantly increased K⁺ and Na⁺ uptake in germinating seeds under salinity stress, resulting in increased abscisic acid (ABA) biosynthesis and activated ABA signaling responses. Furthermore, *qSE3* significantly decreased the H₂O₂ level in germinating seeds under salinity stress. All of these seed physiological changes modulated by *qSE3* might contribute to seed germination and seedling establishment under salinity stress. Based on analysis of single-nucleotide polymorphism data of rice accessions, we identified a HAP3 haplotype of *qSE3* that was positively correlated with seed germination under salinity stress. This study provides important insights into the roles of *qSE3* in seed germination and seedling establishment under salinity stress and facilitates the practical use of *qSE3* in rice breeding.

Keywords: QTL, salinity stress, seed germination, HAK transporter, *Oryza sativa*.

INTRODUCTION

Soil salinity is a major factor affecting crop productivity worldwide, and rice (*Oryza sativa* L.), as the major staple food crop, is also affected by salinity stress (Wang *et al.*, 2012a,b). Approximately, 30% of the total rice-growing area in the world is affected by salinity stress (Prasad *et al.*, 2000; Takehisa *et al.*, 2004). Seedling establishment is a crucial determinant of crop yield in direct-seeding rice. High salinity inhibits seed germination and reduces seedling growth in rice (Wang *et al.*, 2011). Therefore, attaining a high capacity of seed germination and seedling establishment under salinity stress is an important objective of rice breeding.

Seed germination and seedling establishment are complex quantitative traits determined by multiple genetic factors. In rice, many quantitative trait loci (QTLs) for seed germination and seedling growth under salinity stress have been identified. Of these QTLs, QTLs conferring

salinity tolerance have been identified primarily at the seedling stage (Prasad *et al.*, 2000; Koyama *et al.*, 2001; Lin *et al.*, 2004; Ren *et al.*, 2005; Lee *et al.*, 2006; Cheng *et al.*, 2012; Wang *et al.*, 2012a,b; De Leon *et al.*, 2016, 2017; Puram *et al.*, 2017; Yu *et al.*, 2017), with a few reports of such QTLs at the seed germination stage (Wang *et al.*, 2011). At the seedling stage, the characteristics of Na⁺ and K⁺ concentrations and the Na⁺/K⁺ ratio are used to evaluate salinity tolerance. Several QTLs for Na⁺ uptake, K⁺ concentration and the Na⁺/K⁺ ratio have been reported (Koyama *et al.*, 2001; Lin *et al.*, 2004; Ren *et al.*, 2005; Wang *et al.*, 2012a). Only one QTL, *SKC1*, has been map-based cloned; this QTL encodes an HKT-type transporter, *OsHKT8*, and modulates the K⁺/Na⁺ homeostasis of seedlings under salinity stress (Ren *et al.*, 2005).

K⁺ has essential roles in the developmental and reproduction processes of plants (Gierth and Mäser, 2007) and

is important for salinity tolerance (Wu *et al.*, 1996; Shen *et al.*, 2015). Two kinds of transport systems, channels and transporters, are involved in K⁺ absorption and distribution in plants under salinity stress (Wang and Wu, 2013; Chen *et al.*, 2015; Shen *et al.*, 2015). Four multigene families of K⁺ transporters occur in plants: the *CHX*, *KEA*, *Trk/HKT* and *KT/KUP/HAK* families (Gupta *et al.*, 2008; Zhang *et al.*, 2012). Thirteen *KT/KUP/HAK* genes are found in Arabidopsis (Mäser *et al.*, 2001), and 27 occur in rice (Yang *et al.*, 2009). Several *KT/KUP/HAK* transporters have been found to be important for salinity tolerance in plants. *AtHAK5* plays a prominent role in K⁺ uptake under salinity stress in Arabidopsis; this uptake reduces the Na⁺/K⁺ ratio and increases salinity tolerance (Nieves-Cordones *et al.*, 2010; Jiang *et al.*, 2013). In rice, *OsHAK21* mediates K⁺ absorption by the plasma membrane and thereby plays crucial roles in the maintenance of Na⁺/K⁺ homeostasis under salinity stress (Shen *et al.*, 2015). However, the functions of *KT/HAK/KUP* transporters in response to salinity stress at the seed germination stage remain unclear.

Abscisic acid (ABA) is an important regulator of seed germination and abiotic stress responses (Zhu, 2002; Graeber *et al.*, 2012; Ryu and Cho, 2015). Under salinity stress, ABA biosynthesis-related genes, such as zeaxanthin oxidase, 9-*cis*-epoxycarotenoid dioxygenase, and ABA-aldehyde oxidase, are rapidly activated in plants, and ABA contents increase significantly (Xiong *et al.*, 2002; Zhu, 2002; Chinnusamy *et al.*, 2006; Ryu and Cho, 2015). The elevated levels of ABA help plants to stimulate stomatal closure, alter gene expression and accumulate osmo-compatible solutes, which increase stress tolerance (Cutler *et al.*, 2010; Kim *et al.*, 2010). In the presence of ABA, the formation of the ABA signaling pyrabactin resistance (PYR)/pyrabactin resistance-like (PYL)/regulatory component of ABA receptors (RCAR)/protein phosphatase 2C (PP2C) complex leads to the inhibition of PP2C activity, which allows the activation of SNF1-related protein kinase 2 (SnRK2). Activated SnRK2 then phosphorylates downstream substrate proteins such as transcription factors and thereby facilitates the transcription of ABA-responsive genes (Sah *et al.*, 2016). SnRKs are a class of Ser/Thr protein kinases that play key roles in plant stress responses (Chinnusamy *et al.*, 2004; Chae *et al.*, 2007; Lu *et al.*, 2007; Piao *et al.*, 2010).

Reactive oxygen species (ROS) are small molecules generated during plant development and stress responses, and they play a dual role in seed germination (Finkel, 1998; Bailly *et al.*, 2008). Low concentrations of ROS function as messengers in cellular signaling pathways that lead to seed germination, whereas high concentrations function as toxic products that lead to delayed or inhibited germination (Bailly *et al.*, 2008). Overaccumulation of ROS can cause DNA damage, protein oxidation and lipid peroxidation, which result in reduced seed germination (Mittler

et al., 2004; Bailly *et al.*, 2008; Parkhey *et al.*, 2012). Tight regulation is therefore required to balance ROS production and scavenging for successful germination. The associations between seed germination and the activities of ROS-scavenging systems such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) are well documented in Arabidopsis (Leymarie *et al.*, 2012), wheat (Ishibashi *et al.*, 2008), barley (Bahin *et al.*, 2011) and rice (Ye *et al.*, 2012). Additionally, SnRK3 proteins interact with ROS production under salinity stress in Arabidopsis (Verslues *et al.*, 2007). The potential connections between ABA and ROS levels in seed germination and seedling establishment under salinity stress deserve investigation in rice.

Previously, we have identified one salt-tolerant *japonica* landrace, Jiucaiqing, from Taihu Lake valley in Jiangsu Province, China (Wang *et al.*, 2011, 2012a,b). In the present study, one major QTL, *qSE3*, for seed germination and seedling establishment was identified from Jiucaiqing under salinity stress. The *qSE3* QTL was isolated using map-based cloning and found to encode a member of the *HAK* transporters, *OsHAK21*. Physiological analysis suggested that *qSE3* promoted seed germination and seedling establishment under salinity stress by modulating seed physiological states in rice, including increasing K⁺ and Na⁺ uptake and ABA levels and reducing the levels of ROS. In rice breeding, the application of *qSE3* will be useful to improve seed germination and seedling establishment under salinity stress.

RESULTS

Characterization of the *qSE3* locus and phenotype analysis

Germination rate and seedling establishment were significantly higher for IR26 than for Jiucaiqing under normal conditions (Figure 1a–c). By contrast, the values of these traits were significantly lower in IR26 than in Jiucaiqing under salinity stress (Figure 1d–f). To identify the elite genes in Jiucaiqing, we used 62 chromosome segment substitution lines (CSSLs) developed by introgressing chromosome segments of Jiucaiqing into IR26 for QTL mapping (Figure S1a–c). Three simple sequence repeats (SSR) markers, RM7642, RM7370 and RM6832, located on chromosome 3 were significantly associated with seed germination and seedling establishment under salinity stress (Figure S1d). The region encompassing these markers was designated a quantitative locus for seed germination and seedling establishment (*qSE3*).

A residual heterozygous line (CSSL19) that contained *qSE3* was used to confirm QTL function. The allele of *qSE3* that came from Jiucaiqing significantly increased seed germination and seedling establishment in the IR26 background under salinity stress compared with those of IR26 (Figure S2). To minimize the effects of background

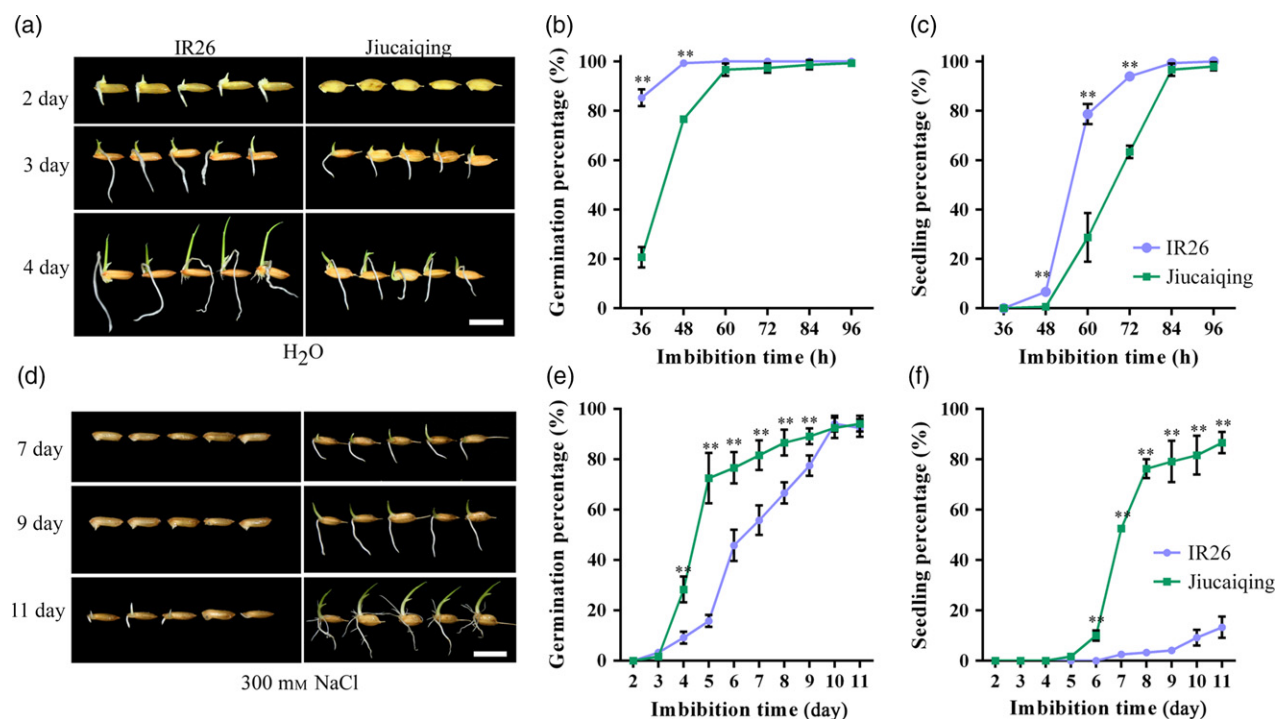


Figure 1. Comparison of seed germination and seedling establishment between Jiucaiqing and IR26 under normal conditions (a–c) or salinity stress (d–f). (b, e) Germination percentage; (c, f) seedling percentage. Bars = 10 mm. Each point represents the means \pm SD. ** Indicates the significant difference at 1% level according to Student's *t*-test.

variation, the near isogenic line (NIL) NIL(*qSE3*) was developed in which almost all of the genetic background was IR26 except for the introgressed segment (Figure 2c). No significant differences in seed germination and seedling establishment were observed between IR26 and NIL(*qSE3*) under normal conditions (Figure 2d,f–i), whereas NIL(*qSE3*) has significantly higher or lower values of seed germination and seedling establishment than did IR26 under salinity (Figure 2e, f–i), polyethylene glycol (PEG) (Figure S3a–e) and low temperature stresses (Figure S3f–j).

Map-based cloning of *qSE3*

To isolate the *qSE3* gene, 2368 individuals from the BC₄F₃ population were used to narrow down the *qSE3* locus to a genomic region between the markers YQ19 and RM6832 (Figure 3). High-resolution mapping was conducted using 939 BC₄F₄ families to further delimit the *qSE3* locus in a 46.57 kb region that contained eight predicted genes (Table S1). Of these genes, the most likely candidate gene of *qSE3* was K⁺ transporter *OsHAK21* (LOC_Os03 g37930) based on the following analyses. A previous study indicated that *OsHAK21* is involved in salinity tolerance in rice seedlings (Shen *et al.*, 2015). Sequence variation identified in an open reading frame (ORF) analysis indicated that Jiucaiqing and Nipponbare (Nip) have a similar sequence of *OsHAK21*. However, six single-base substitutions occurred in the coding region of *OsHAK21* between Jiucaiqing and IR26, of

which four substitutions, including single nucleotide polymorphism (SNP)1 (–6), SNP2 (–807), SNP4 (–3870) and SNP5 (–3889), led to amino acid substitutions (Figure 4a). The D2E mutation is located in the N-terminus region, the R676W and R681Q mutations are located in the C-terminus region of *OsHAK21*, and the S72P mutation occurs in the first transmembrane domain of *OsHAK21* (Figure 4b).

Eight single-base substitutions (at positions 143, 306, 308, 311, 493, 547, 551 and 568) and two indels (at positions 305 and 545) in the promoter region of *OsHAK21* were also observed between Jiucaiqing and IR26 (Figure 4c). Further quantitative real-time polymerase chain reaction (qRT-PCR) analysis showed that *OsHAK21* expression was significantly higher in the germinating seeds of NIL(*qSE3*) than in those of IR26 under salinity stress (Figure 4d). The *oshak21* mutant was used to confirm the candidate gene (Figure 5a–b). No significant differences in seed germination and seedling establishment were observed between Nip and *oshak21* under normal conditions, whereas *oshak21* had significantly lower and higher values of seed germination and seedling establishment, respectively, than did Nip under salinity stress (Figure 5c–g), suggesting the candidate gene is *OsHAK21*.

Expression pattern of *qSE3*

To expand our understanding of the physiological functions of *qSE3*, we determined the tissue distribution and

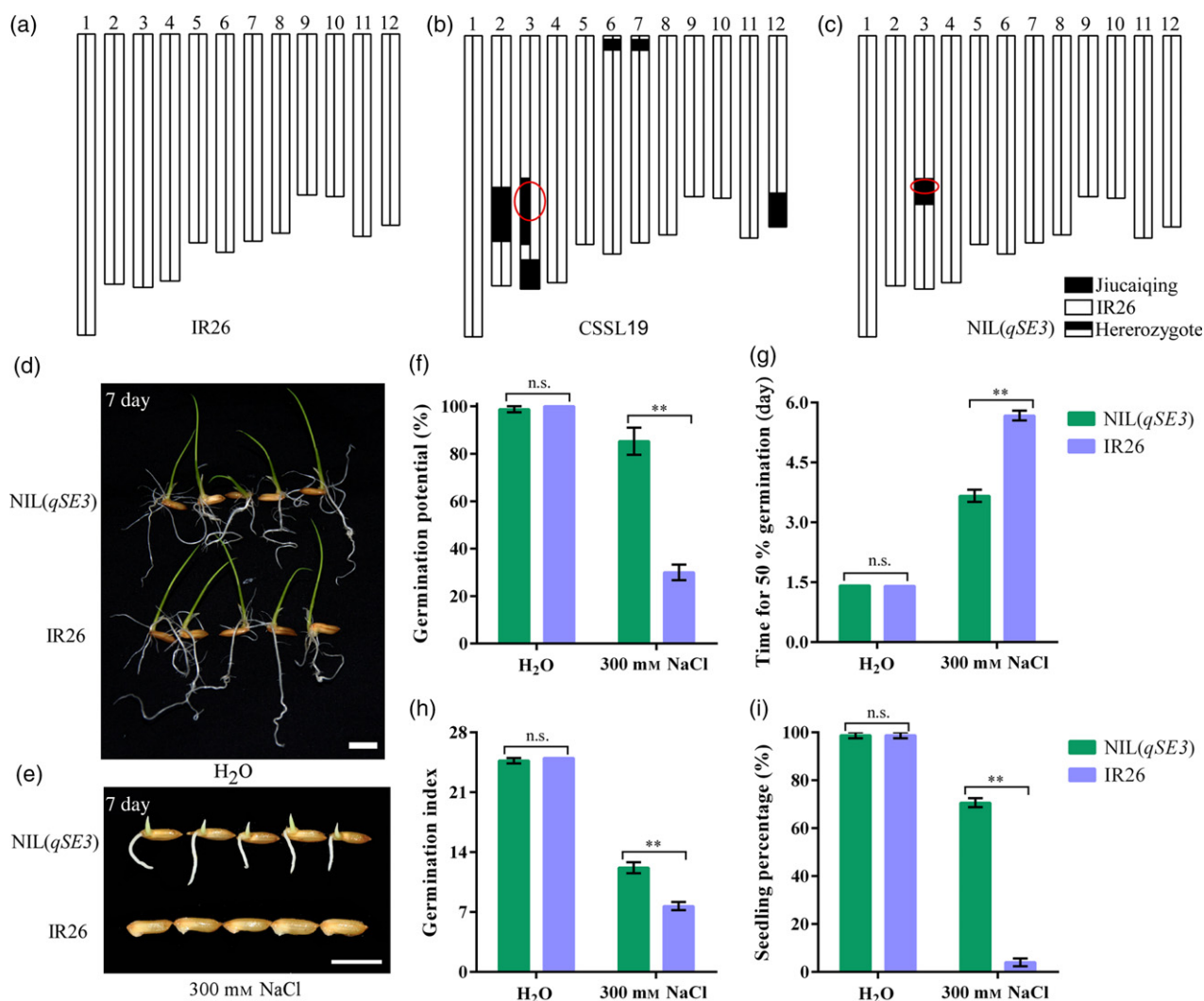


Figure 2. Comparison of seed germination and seedling establishment between NIL(*qSE3*) and IR26 under normal and salinity stress. Chromosomal location of the introgressed Jiucaiqing segments in CSSL19 (b) and NIL(*qSE3*) (c) in IR26 background (a). Seed germination under normal conditions (d) or salinity stress (e) at 7 days after imbibition. Bars = 10 mm. (f) Germination potential; (g) time for 50% of the germination percentage; (h) germination index; (i) seedling percentage. Each column represents the means \pm SD. ** Indicates the significant difference at 1% level according to Student's *t*-test. n.s. Results not significant.

expression pattern of *qSE3* during seed development and seed germination. qRT-PCR analysis demonstrated that *qSE3* was primarily expressed in the root followed by the stem in rice (Figure S4a). The transcript levels of *qSE3* increased significantly in the filling grain from 21 days after flowering (DAF) and sharply increased at the mature stage (42 DAF; Figure S4b). Additionally, *qSE3* expression was significantly induced by salinity stress during seed germination; the transcript levels of *qSE3* were increased significantly after 36 h of imbibition (Figures 4d, 5b). Similarly, the strongest GUS signals were detected in the root under both control and salinity stress conditions (Figure S4c); this finding is consistent with the results of the qRT-PCR assays.

qSE3 increases K⁺ and Na⁺ uptake in germinating seeds under salinity stress

Previous research has indicated that *OsHAK21* mediates K⁺ absorption in rice seedlings under salt stress (Shen *et al.*, 2015). To confirm and compare the transporter activity of *OsHAK21* encoded by the alleles from Jiucaiqing (*OsHAK21*^{JCQ}) and IR26 (*OsHAK21*^{IR26}), complementation tests were performed using a K⁺ uptake-deficient mutant of yeast strain CY162. The Arabidopsis *AtKAT1* gene was used as a positive control and increases the K⁺ uptake capability of CY162 cells at micromolar K⁺ concentrations (Anderson *et al.*, 1992). No significant differences were observed among

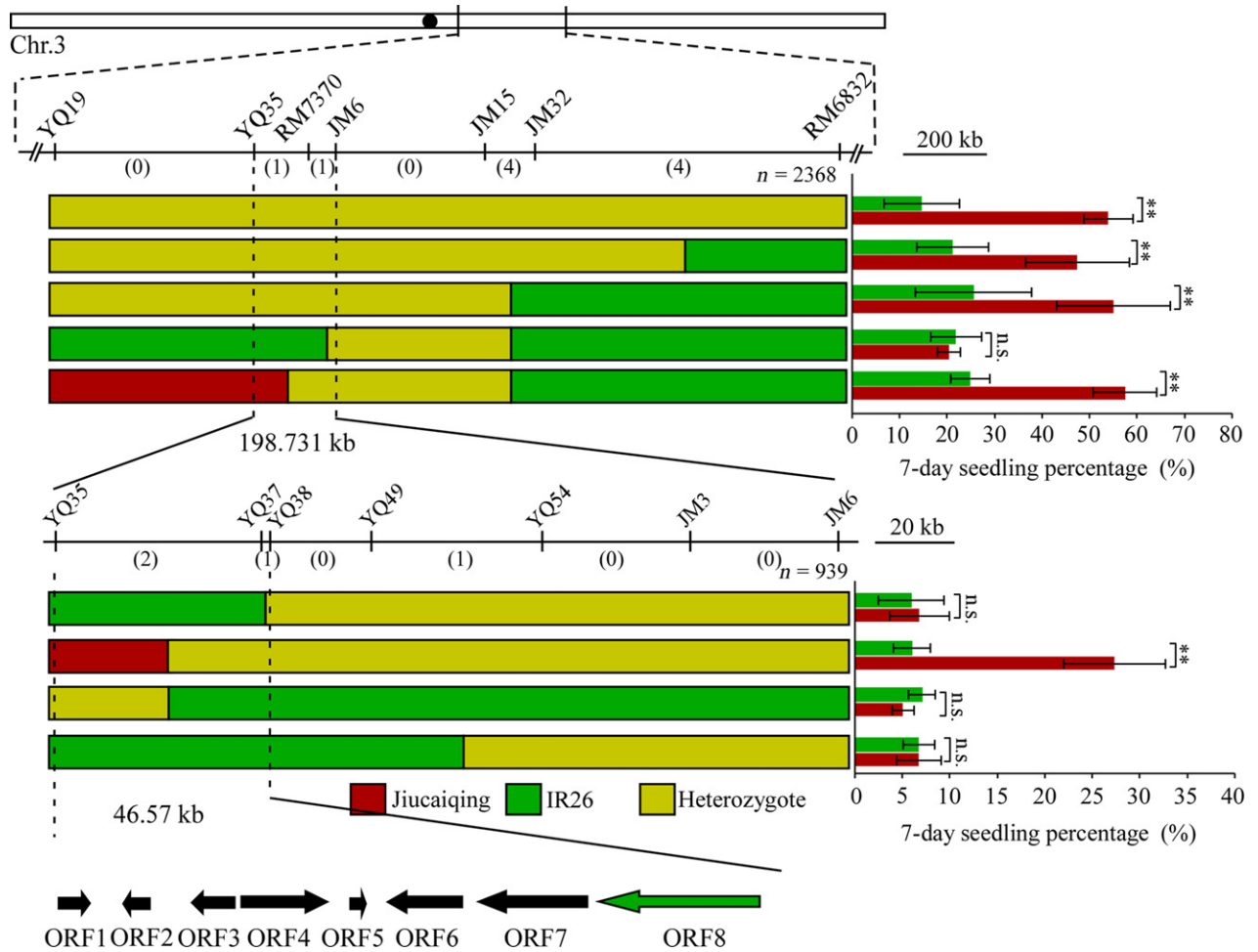


Figure 3. Fine mapping of *qSE3*. The *qSE3* locus was narrowed down to a 46.57 kb region between markers YQ35 and YQ38 on chromosome 3. Numbers below the horizontal line are the number of recombinants. Red bars represent the Jiucaiqing genotype region; green and yellow bars represent IR26 genotype and heterozygous genotype, respectively. Right: the seedling percentage at 7 days after salinity stress. Bars indicate the mean values \pm SD. **Indicates significant difference 1% level according to Student's *t*-test. n.s. Results not significant. Among the eight genes in this region, ORF 8 corresponds to *OshAK21* (Shen *et al.*, 2015).

transformants when they were cultured under 100 mM KCl conditions (Figure 6a). The strain CY162 transformed with the empty pYES2 vector and *OshAK21*^{IR26} were unable to grow under 10 mM and 5 mM KCl conditions, however *AtKAT1* and *OshAK21*^{JCQ} expression rescued its growth. This result indicated that the *OshAK21* allele from Jiucaiqing conferred significant K⁺ uptake and growth in yeasts at moderate external K⁺ concentrations.

To further determine whether *qSE3* affected K⁺ uptake in germinating seeds under salinity stress, the K⁺ content and net K⁺ uptake rate were compared between NIL(*qSE3*) and IR26 and between Nip and *oshak21*. K⁺ accumulation and K⁺ net uptake rates were significantly higher in NIL(*qSE3*) and Nip than in IR26 and *oshak21* respectively (Figure 6b, d). As described above, a similar ORF sequence in *OshAK21* was observed between Jiucaiqing and Nip. Therefore, we confirmed that the *OshAK21* allele from Jiucaiqing (*qSE3*)

could improve the capacity for K⁺ transport in germinating seeds under salinity stress. Previous research has found that *OshAK21* cloned from Nip may not be permeable to Na⁺ in yeast (Shen *et al.*, 2015). This finding suggests that *OshAK21* encoded by the allele from Jiucaiqing may not have the capacity for Na⁺ transport. However, the Na⁺ content and Na⁺ net uptake rates were significantly higher in NIL(*qSE3*) and Nip than in IR26 and *oshak21* respectively (Figure 6c, e). As a result, there was no significant reduction of Na⁺/K⁺ ratios in germinating seeds in NIL(*qSE3*) and Nip than in IR26 and *oshak21* respectively (Figure 6f).

qSE3 elevates ABA level in germinating seeds under salinity stress

ABA is involved in mediating seed germination and stress responses in plants. We predicted that *qSE3* would induce additional ABA biosynthesis in germinating seeds with

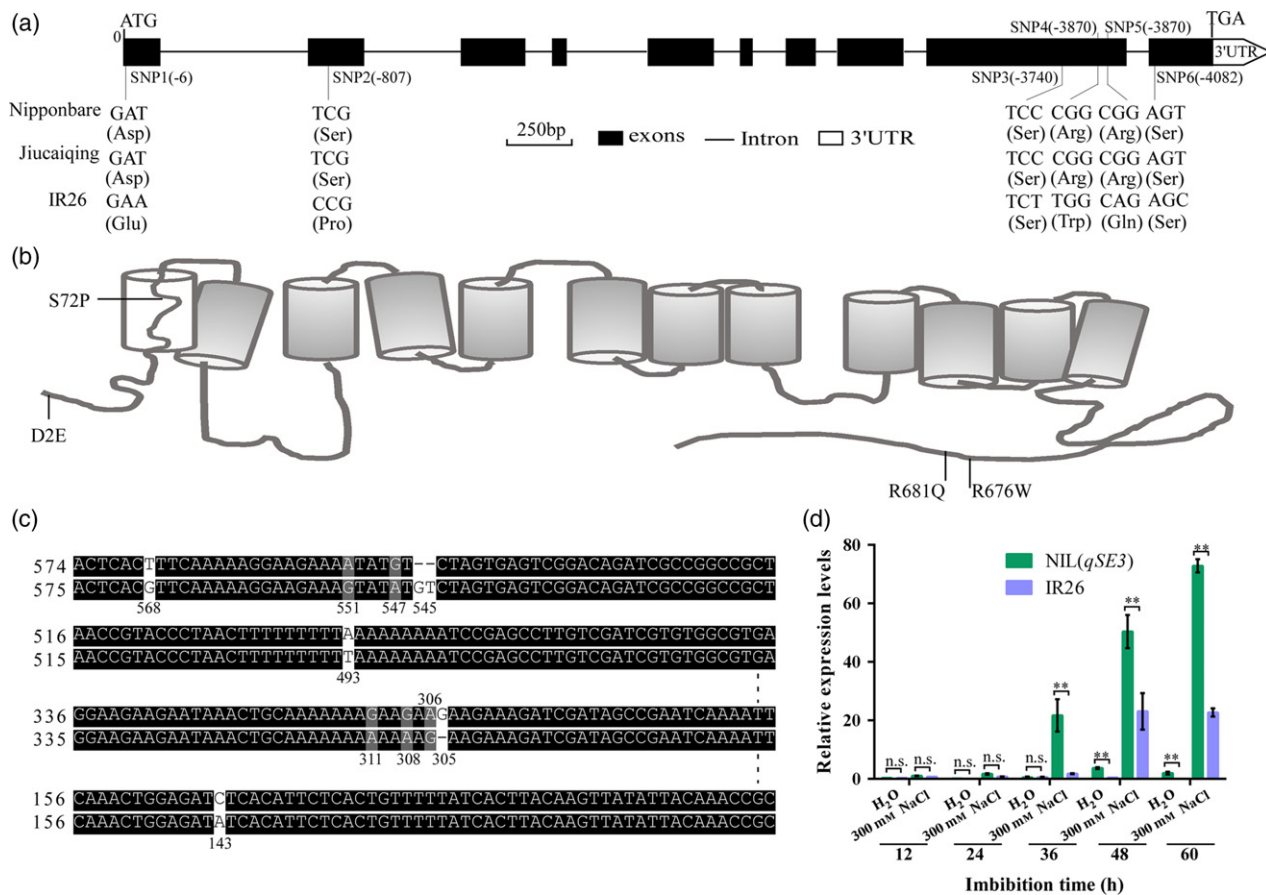


Figure 4. Confirmation of *qSE3* candidate gene. (a) Comparison of the nucleotide sequence in the open reading frames (ORFs) of *OsHAK21* among Nipponbare, Jiuciqing and IR26. Black boxes indicate exons, and white box shows the 3' untranslated region. Solid lines represent introns. Nucleotide variation presented below the horizontal line, and amino acid listed in brackets. (b) Model of *OsHAK21* topology and location of mutations. The proposed model for *OsHAK21* protein is shown with 12 transmembrane domains and along the cytoplasmic C-terminus. (c) Comparison of the nucleotide sequence in the promoter region of *OsHAK21* between Jiuciqing and IR26. (d) Comparison of the *OsHAK21* expression between NIL(*qSE3*) and IR26 in germinating seeds under normal conditions or salinity stress using a qRT-PCR approach. Gene expression was normalized to that of *OsActin* gene control. The relative expression levels were represented by fold change relative to the expression level of NIL(*qSE3*) in aqueous conditions. Each column represents the means \pm SD. ** Indicates the significant difference at 1% levels according to Student's *t*-test. n.s. Results not significant.

increasing Na⁺ accumulation under salinity stress. As expected, the ABA levels were significantly higher in germinating seeds of NIL(*qSE3*) and Nip than in those of IR26 and *oshak21* respectively under salinity stress (Figure 7b). ABA content is modulated by a balance between biosynthesis and catabolism, which are involved in the genes for 9-*cis*-epoxycarotenoid dioxygenase (NCED) and ABA 8'-hydroxylase (ABA8ox) (Figure 7a). Significantly higher transcript levels of *OsNCED3* and *OsNCED4*, but lower transcript levels of *OsABA8ox3*, were observed in NIL(*qSE3*) than in IR26 (Figure 7c, d). No significant difference in *OsNCED* transcript levels was observed between Nip and *oshak21*, but the transcript levels of *OsABA8ox2* and *OsABA8ox3* were significantly lower in Nip than in *oshak21*. *OsSAPKs* are the key components of the ABA signaling pathway (Figure 7a). The expression levels of *OsSAPK4*, *OsSAPK9* and *OsSAPK10* were significantly higher in NIL(*qSE3*) and Nip

than in IR26 and *oshak21* respectively (Figure 7e). These observations suggested that *qSE3* affected ABA biosynthesis and the signaling process in germinating seeds under salinity stress in rice.

qSE3 decreases ROS level in germinating seeds under salinity stress

ROS production is known to be increased under stress conditions. H₂O₂ is the most stable ROS. To determine whether *qSE3* reduces ROS accumulation through ABA regulation in germinating seeds under salinity stress, the levels of H₂O₂ were determined and compared between NIL(*qSE3*) and IR26 and between Nip and *oshak21*. Generally, the levels of H₂O₂ were significantly lower in NIL(*qSE3*) and Nip than in IR26 and *oshak21* respectively (Figure 8a). Antioxidant enzymes, including CAT, APX and peroxidase (POD), are among the primary defenses

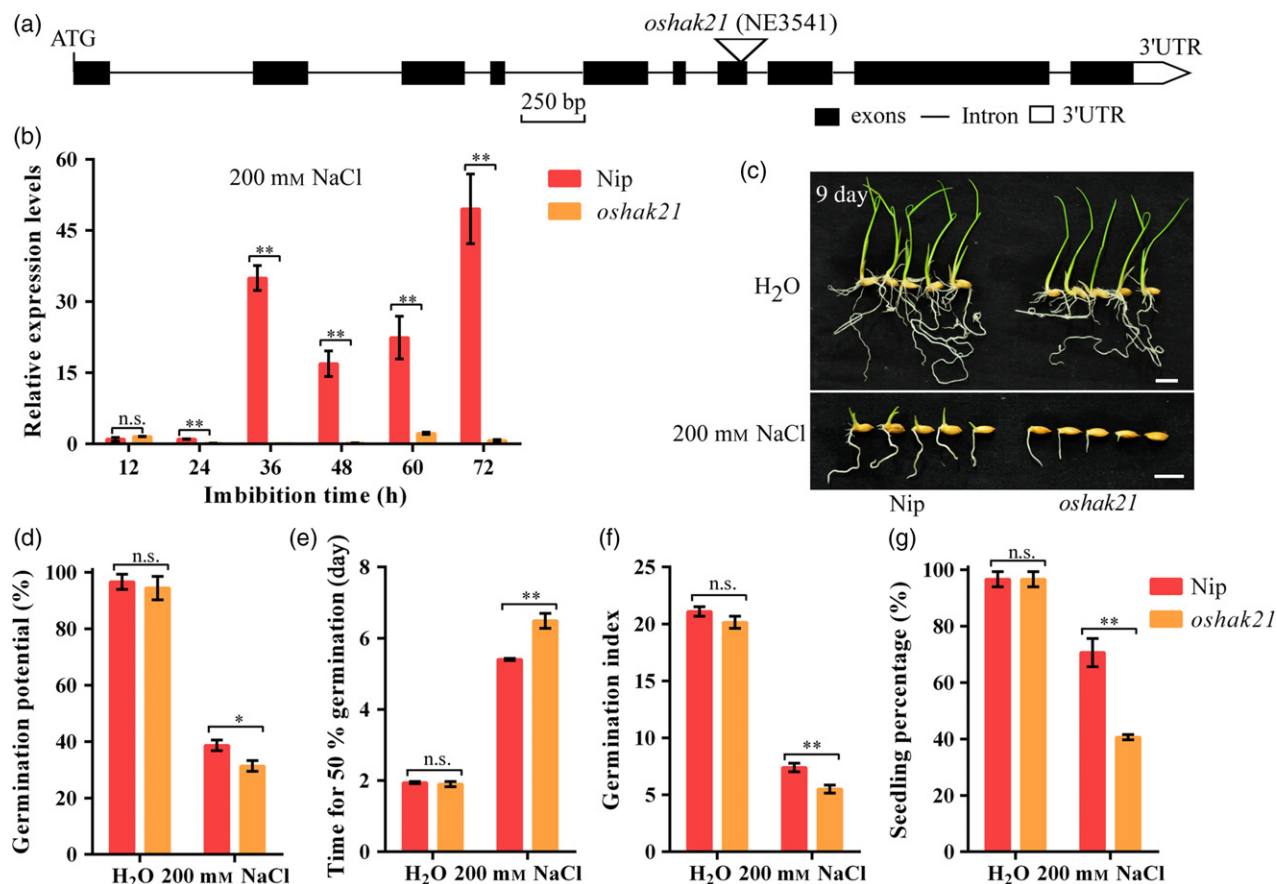


Figure 5. Comparison of seed germination between Nip and *oshak21* mutants under normal conditions or salinity stress. (a) Gene structure of *OsHAK21* with a T-DNA insertion. Triangle represents the T-DNA. Black and white boxes represent exons and the untranslated region (UTR) of *OsHAK21*, respectively. Solid lines represent introns. (b) qRT-PCR analysis of *OsHAK21* in germinating seeds of Nip and *oshak21* under salinity stress. Gene expression was normalized to that of *OsActin* gene control. The relative expression levels were represented by fold change relative to the expression level of Nip at the 12 h imbibition stage. (c) Seed germination after 9 days under normal conditions or salinity stress. Bars = 10 mm. (d) Germination potential; (e) time for 50% of the germination percentage; (f) germination index; (g) seedling percentage. Each column represents the means \pm SD. * and ** Indicate significant difference at 5% and 1% levels according to Student's *t*-test, respectively. n.s. Results not significant.

against ROS. The activities of CAT, APX and POD were significantly higher in NIL(*qSE3*) and Nip than in IR26 and *oshak21* respectively (Figure 8b–d), which coincided with lower levels of H₂O₂ in NIL(*qSE3*) and Nip than in IR26 and *oshak21* respectively. These results suggested that *qSE3* improved seed germination and seedling establishment under salinity stress by reducing oxidative damage.

Association of a haplotype of *qSE3* with seed germination under salinity stress

To investigate whether allelic constitution at the *qSE3* locus affected seed germination under salinity stress, SNPs in the region ~2 kb upstream and ~2 kb downstream of *qSE3* were analyzed using the SNP data of rice accessions (McCouch *et al.*, 2016). Five haplotypes of *qSE3* were identified among these accessions (Figure 9a). Eight accessions with HAP3 showed increased seed

germination under salinity stress (Figure 9b–d). The 2036T SNP of HAP3 was the SNP most significantly associated with seed germination in those eight accessions. To confirm this observation, the association between seed germination and 2036T SNP was further analyzed using 222 rice accessions (Table S2). We observed that 14 rice accessions harboring 2036T SNP exhibited higher seed germination than did 188 rice accessions with 2036C SNP under salinity stress (Figure 9e–g). These results indicated that HAP3 and 2036T SNP of *qSE3* were significantly and positively correlated with seed germination under salinity stress.

Application of *qSE3* for direct seeding

To investigate the application of *qSE3* for direct seeding, seedling establishment and seedling growth was compared between NIL(*qSE3*) and IR26 when seeds were directly sown in soil. No significant differences were

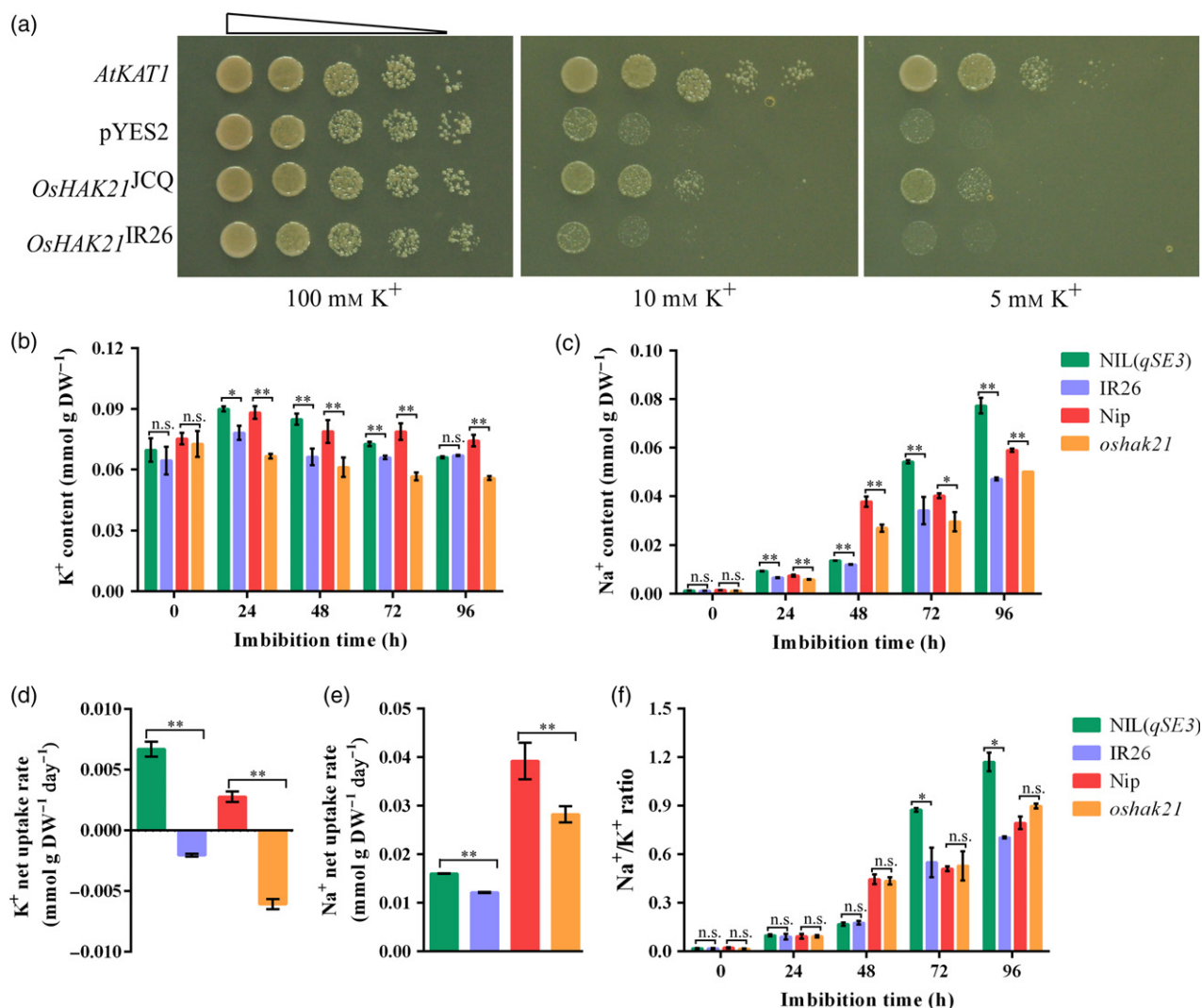


Figure 6. Comparison of K⁺ and Na⁺ uptake between NIL(*qSE3*) and IR26 and between Nip and *oshak21* in germinating seeds under salinity stress. (a) Functional analysis of OSHAK21 in the K⁺ uptake-deficient mutant strain CY162 of *Saccharomyces cerevisiae*. Growth status of CY162 cells expressing *AtKAT1*, empty vector (pYES2) and *OsHAK21* encoded by the allele from Jiucaiqing (*OsHAK21^{JCQ}*) and IR26 (*OsHAK21^{IR26}*) on SD-URA medium supplemented with 100 mM, 10 mM and 5 mM KCl. The white triangle indicates 1:10 serial dilutions of yeast cells placed on the media. Three independent clones were tested for each condition and outcomes were similar. (b) K⁺ content; (c) Na⁺ content; (d) K⁺ net uptake rate; (e) Na⁺ net uptake rate; (f) Na⁺/K⁺ ratio. Each column represents the means \pm SD. * and ** indicate the significant difference at 5% and 1% levels according to Student's *t*-test, respectively. n.s. Results not significant.

observed between NIL(*qSE3*) and IR26 under normal conditions at 7 days after sowing (Figure 10a, b, f–h). However, seedling establishment and seedling growth were significantly higher in NIL(*qSE3*) than in IR26 under salinity stress (Figure 10c–h). Under salinity stress, the seedling survival of NIL(*qSE3*) was significantly higher than that of IR26 at 14 days after sowing (Figure 10e). Agronomic traits were similar between NIL(*qSE3*) and IR26, including heading date, plant height, effective tillers, 1000-grain weight and panicle length, among others (Figure S5). These results suggested that *qSE3* can improve the performance of plants directly seeded

under salinity stress while not influencing agronomic traits.

DISCUSSION

Salinity has a strong effect on seed germination, causing low germination rates and low seedling establishment in rice (Wang *et al.*, 2011). In this study, we observed that *japonica* landrace Jiucaiqing was an elite germplasm that had high capacities of seed germination and seedling establishment under salinity stress. Identification and utilization of elite genes from Jiucaiqing is important for the improvement of salinity tolerance in rice. Here, the elite

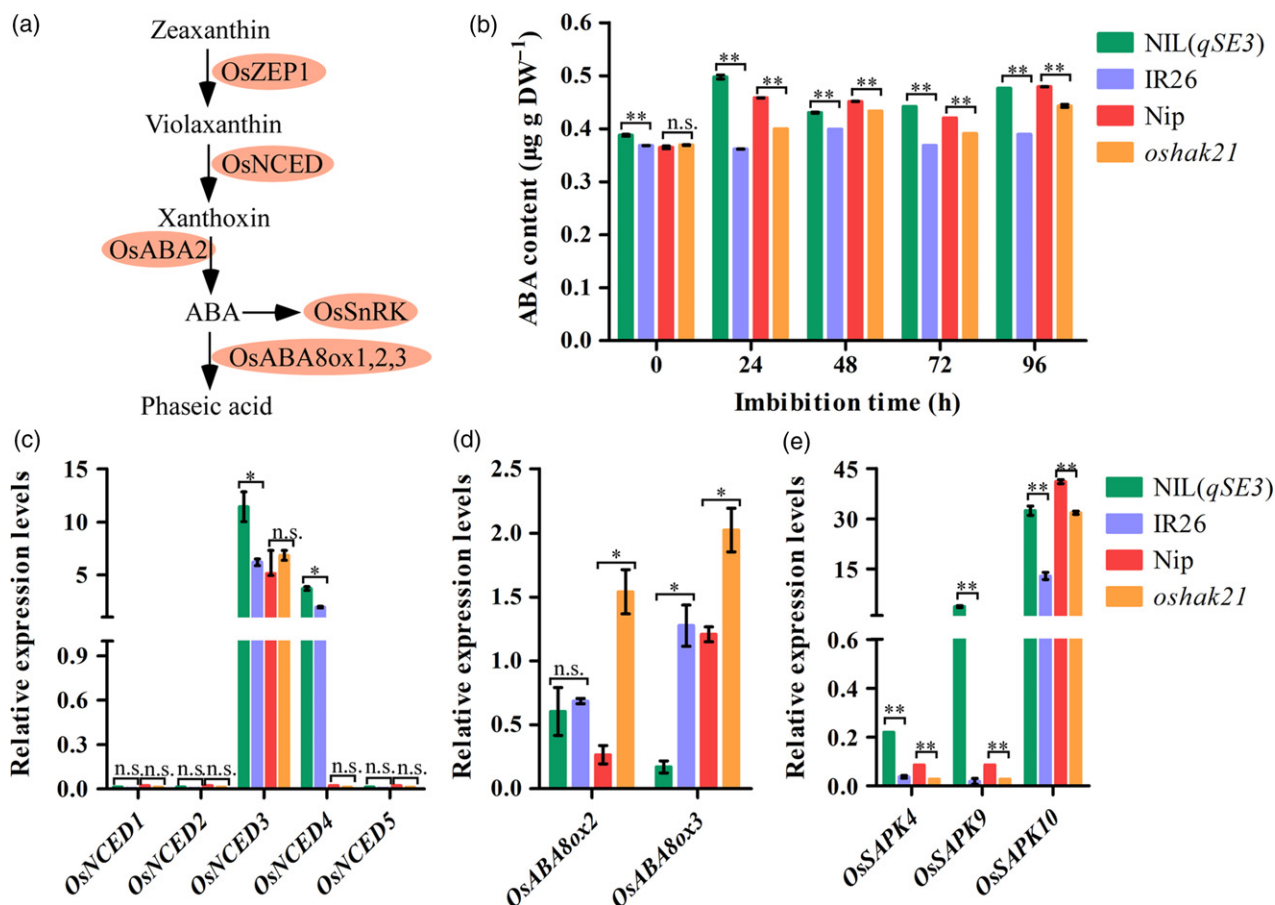


Figure 7. Comparison of ABA levels between NIL(*qSE3*) and IR26 and between Nip and *oshak21* in germinating seeds under salinity stress. (a) General overview of ABA metabolism pathway in rice according to previous reports. (b) ABA content in germination seeds. Relative expression levels of ABA biosynthesis-related genes (c), catabolism-related genes (d) and signaling-related genes (e) in 24 h-imbibed seeds. Gene expression was normalized to that of the *OsActin* gene control. The relative expression levels were represented by fold change relative to the expression level of *OsNCED1* in NIL(*qSE3*). Each column represents the means \pm SD. * and ** Indicate the significant difference at 5% and 1% levels according to Student's *t*-test, respectively. n.s. Results not significant.

allele for *qSE3* in Jiucaiqing was successfully identified; this allele significantly increased seed germination and seedling establishment under salinity stress. We also observed that *qSE3* significantly increased seed germination and seedling establishment under PEG and low temperature conditions. Previous results indicated that *qLTG3-1* modulates seed germination under various conditions that are tightly associated with the vacuolation of tissues covering the embryo (Fujino *et al.*, 2008). To reveal whether *qSE3* and *qLTG3-1* have similar functions, we investigated the possible mechanisms of *qSE3* in seed germination under salinity stress in this study.

Reducing Na⁺ uptake, increasing K⁺ accumulation and thereby decreasing the Na⁺/K⁺ ratio is an important strategy for salinity tolerance in seedlings (Munns and Tester, 2008; Yang *et al.*, 2014). Germinating seeds cannot exclude Na⁺ or Cl⁻ (Tester and Davenport, 2003) or accumulate these in vacuoles as can older seedlings (Greenway and Munns, 1980). We predicted that a different mechanism of

salinity tolerance might occur in germinating seeds than that in seedlings of rice. In this study, the major QTL *qSE3* was map-based cloned and found to encode the potassium transporter *OsHAK21*. *OsHAK21* mediates K⁺ absorption by the plasma membrane and plays crucial roles in the maintenance of Na⁺/K⁺ homeostasis in seedlings under salinity stress (Shen *et al.*, 2015). It is known that Na⁺ will translate from roots to shoots under salinity stress in plants. However, the seedling including shoots and roots has not been established after 72 h and 96 h imbibition under salinity stress in this study. Therefore, the Na⁺ and K⁺ concentrations were conducted only in the germinating seeds in this study. We observed that K⁺ and Na⁺ uptake increased simultaneously in germinating seeds modulated by *qSE3* under salinity stress, resulting in no significant reduction of Na⁺/K⁺ ratios. A different capacity of K⁺ transport was observed between *OsHAK21*^{JCQ} and *OsHAK21*^{IR26} in this study. The C-terminus of HAK transporters is considered to be critical in determining the capacity of K⁺ transport (Kim

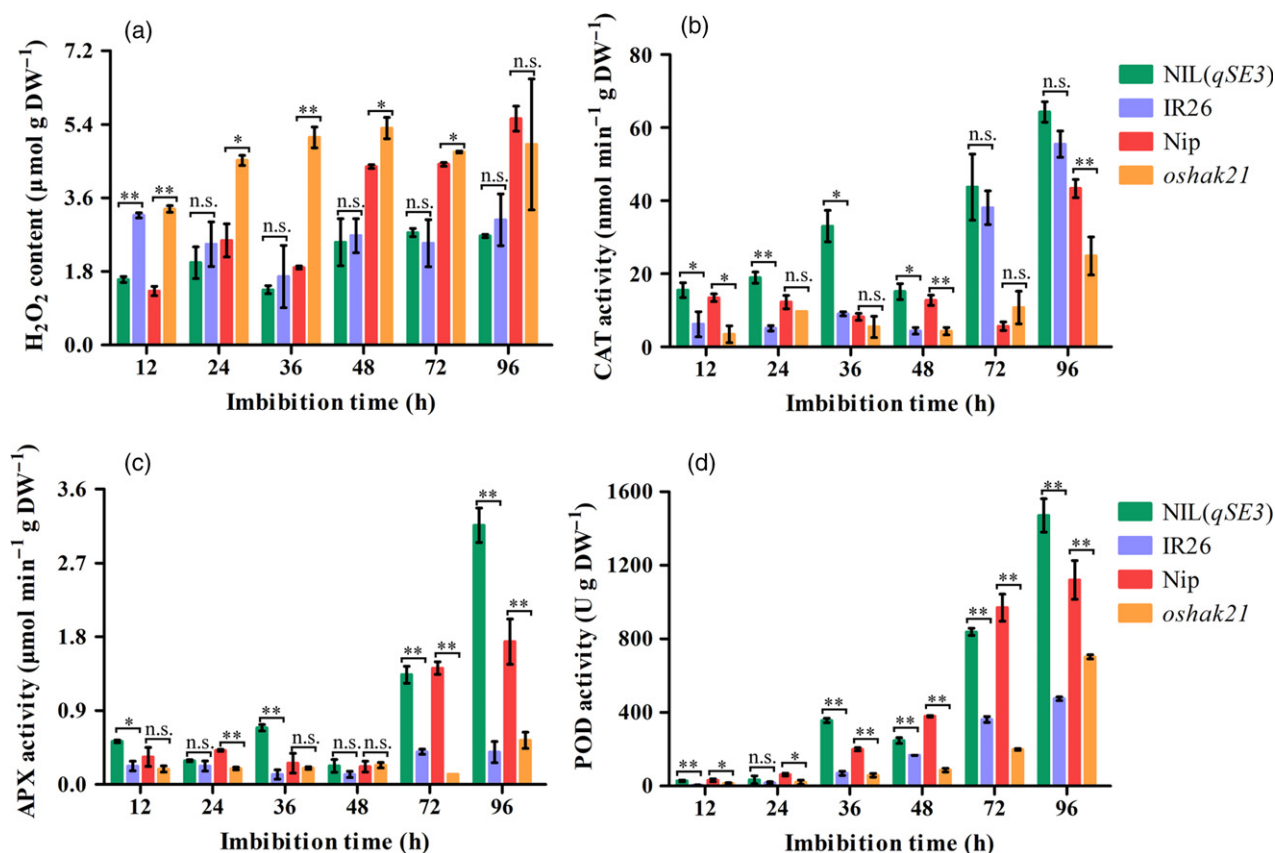


Figure 8. Comparison of H₂O₂ levels and antioxidant enzymes activities between NIL(*qSE3*) and IR26 and between Nip and *oshak21* in germinating seeds under salinity stress. (a) H₂O₂ content; (b) CAT activity; (c) APX activity; (d) POD activity. Each column represents the means ± SD. * and ** Indicate the significant difference at 5% and 1% levels according to Student's *t*-test, respectively. n.s. Results not significant.

et al., 1998; Rubio *et al.*, 2000; Mangano *et al.*, 2008). We observed that the R676W and R681Q mutations locate in the C-terminus region of OsHAK21^{JCQ} and OsHAK21^{IR26}, suggesting this protein region might be critical in determining the capacity for K⁺ transport.

Some members of the KT/HAK/KUP family are characterized as Na⁺-permeable transporters. However, none of HAK transporters from higher plants has been shown to mediate high-affinity Na⁺ uptake (Benito *et al.*, 2012). Furthermore, Shen *et al.* (2015) indicated that OsHAK21 may not be permeable to Na⁺ in yeast. Therefore, we speculated in this study that OsHAK21 may not mediate Na⁺ transport in germinating seeds under salinity stress. However, a significant increase in Na⁺ uptake was observed in germinating seeds by regulation of *qSE3* under salinity stress. Concerning the low-affinity Na⁺ uptake of OsHAK21, it is generally accepted that Na⁺ can enter the plant through ion channels (Maathuis, 2014), including glutamate-like receptors, cyclic nucleotide gated channels, or possibly other, non-identified, non-selective cation channels (Nieves-Cordones *et al.*, 2016). Whether *qSE3* contributes to Na⁺ uptake through the activation of ion channels under salinity stress requires

further investigation. How did *qSE3* promote seed germination under salinity stress with the accumulation of Na⁺ in this study? The regulation of *qSE3* in salinity tolerance at the seed germination stage might include other regulatory mechanisms.

Rapid accumulation of ABA is a characteristic of plants exposed to abiotic stress (Xiong *et al.*, 2002; Zhu, 2002; Ryu and Cho, 2015). In this study, *qSE3* regulation elevated ABA biosynthesis and ABA signaling responses in germinating seeds under salinity stress, which might be due to its promotion of Na⁺ accumulation. ABA is well known to mediate the inhibition of seed germination. How did the accumulation of ABA promote seed germination and seedling establishment under salinity stress in this study? ABA functions through a PYR/RCARs-PP2Cs-SnRKs signaling module to regulate multiple physiological processes (Hubbard *et al.*, 2010). Previous studies have indicated that the ABA signaling genes *OsRK1*, *SnRK1A*, *SnRK2* and *OsCIPK31* play key roles in regulating seed germination and stress responses (Xiong and Yang, 2003; Chae *et al.*, 2007; Lu *et al.*, 2007; Piao *et al.*, 2010). Accordingly, we observed that the expression of *OsSAPK4*, *OsSAPK9* and

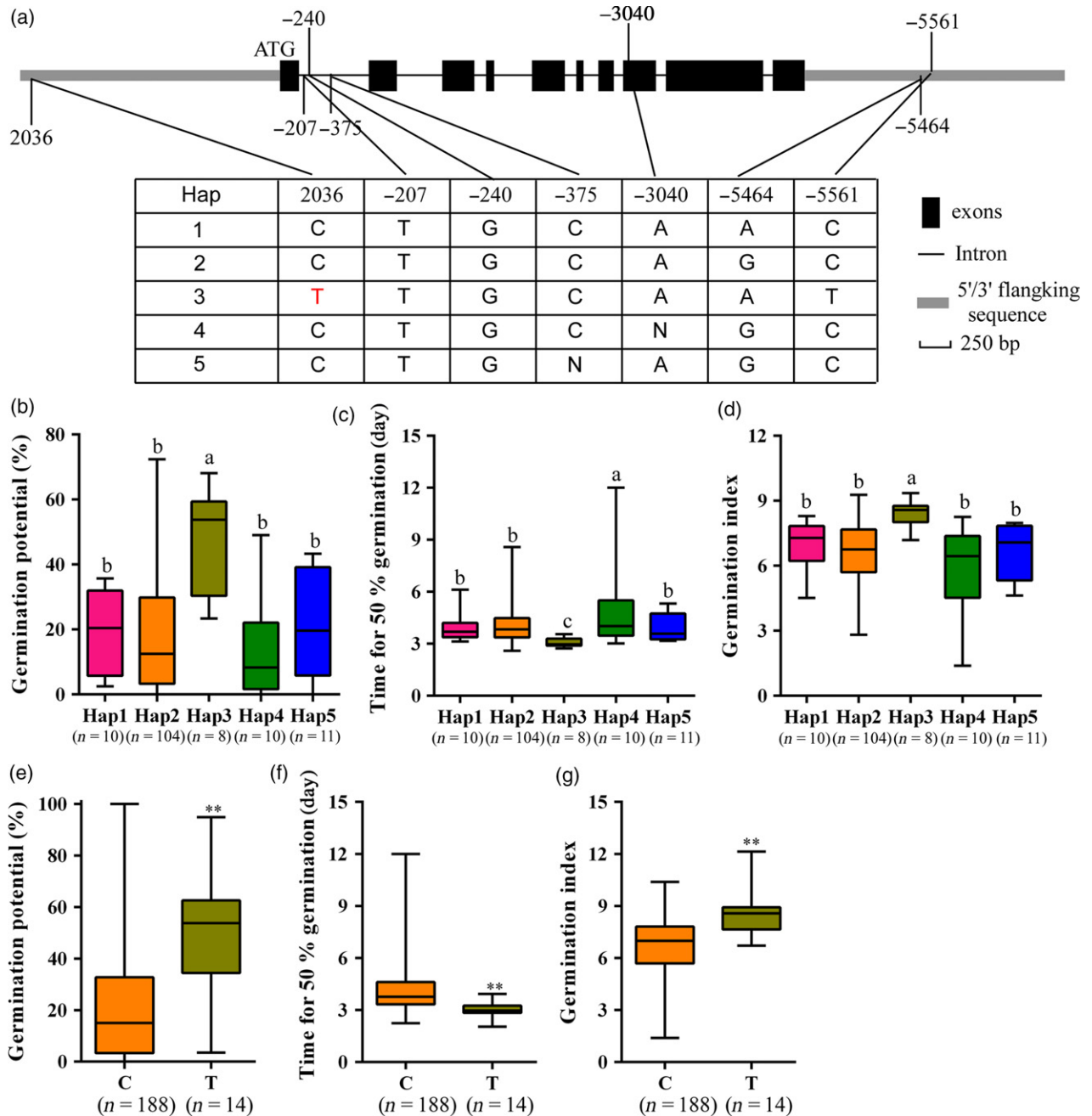


Figure 9. Haplotype of *qSE3* associated with seed germination under salinity stress. (a) Haplotypes of *qSE3* identified in the region ~2 kb upstream and ~2 kb downstream of the gene. Black boxes and solid lines represent exons and introns, respectively. The 2036T SNP is the most significantly associated with seed germination under salinity stress. (b–d) Germination phenotype of accessions harboring different haplotypes under salinity stress. (e–g) Germination phenotype of accessions harboring 2036T or 2036C SNP under salinity stress. (b, e) Germination potential; (c, f) time for 50% germination; (d, g) germination index. Number of rice accessions listed in brackets. Each column represents the means \pm SD. Different lowercase letters represent significant difference at 5% level according to least significant difference (LSD) test. * and ** indicate the significant difference at 5% and 1% levels according to Student's *t*-test, respectively.

OsSAPK10 increased significantly in germinating seeds modulated by *qSE3* under salinity stress, suggesting that *qSE3* regulation was involved in the ABA signaling pathway. In *Arabidopsis*, SnRK3s are involved in ROS regulation through the interaction of CAT under salinity stress

(Verslues *et al.*, 2007). Recently, rice *SAPK1* and *SAPK2* have been reported to improve ROS detoxification following salt stress by promoting ascorbic acid contents and SOD and CAT expression levels in plants (Lou *et al.*, 2018). We hypothesized that induced *OsSAPKs* expression might

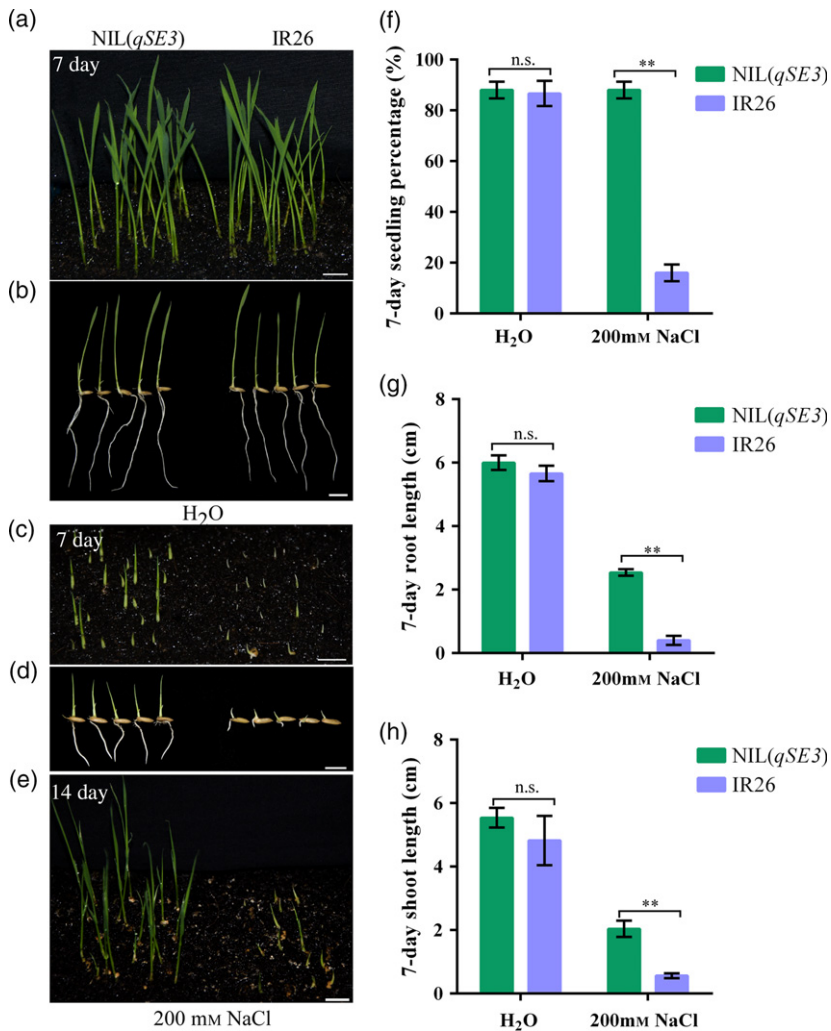


Figure 10. Comparison of seedling establishment and seedling growth between NIL(*qSE3*) and IR26 when seeds are directly sown in soil. Seedling establishment and seedling growth under normal conditions (a, b) or salinity stress (c, d) at 7 days after sowing. (e) Seedling survival under salinity stress at 14 days after sowing. Bars = 10 mm. (f) Seedling percentage; (g) root length; (h) shoot length. Each column represents the means \pm SD. **Indicates the significant difference at 1% level according to Student's *t*-test. n.s. Results not significant.

be involved in the reduction of ROS levels under the action of ROS-detoxification enzymes in this study. Maintaining a low level of ROS is important for seed germination and seedling establishment under salinity stress in rice.

Previous studies have shown that salinity tolerance is developmentally regulated and growth stage-specific in rice (Cheng *et al.*, 2015). Therefore, the development of rice varieties with vigorous germination and seedling growth simultaneously under salinity stress is difficult. According to our results and a previous study (Shen *et al.*, 2015), *OsHAK21* improves salinity tolerance at both the seed germination and seedling stages in rice. The application of *qSE3* might be useful for breeding varieties suitable for direct seeding. Determination of the allelic diversity of *qSE3* with a focus on newly identified tolerant rice accessions is of interest. After analyzing the SNP data of rice accessions, the HAP3 haplotype was identified as positively correlated with seed germination under salinity stress. Eight accessions that harbor the HAP3 haplotype, i.e. Sabharaj, RTS14, Kiang-Chou-Chiu, Ai-Chiao-Hong,

Peh-Kuh-Tsao-Tu, Taichung Native 1, Zhenshan 2 and Peh-kuh, were identified as salinity tolerant accessions in this study. The identified haplotype and accessions might be useful for improving salinity tolerance in rice.

In conclusion, the major QTL *qSE3* for rice seed germination and seedling establishment under salinity stress was identified and cloned in this study and encodes the potassium transporter *OsHAK21*. *qSE3* contributed to seed germination and seedling establishment under salinity stress and might be involved in altering K⁺ and Na⁺ uptake and ABA and ROS levels in germinating seeds (Figure 11). K⁺ uptake increased significantly in germinating seeds under salinity stress because of *OsHAK21* mediation of K⁺ absorption. However, the mechanism underlying the regulatory effect of *qSE3* on Na⁺ uptake remains to be elucidated. Increased movement of Na⁺ into the cell, together with the protection against Na⁺-induced cell damage mediated by K⁺ accumulation, will cause increases in the ABA biosynthesis and ABA signaling responses in germinating seeds under salinity stress, and will contribute to reducing

ROS levels and promoting seed germination and seedling establishment. The function of ABA on reducing ROS level to improve seed germination under salinity stress warrants investigation in the future.

EXPERIMENTAL PROCEDURES

Plant materials

Japonica Jiucaiqing, *indica* IR26 and 62 CSSLs, developed by introgressing chromosome segments of Jiucaiqing into IR26 (Cheng *et al.*, 2016), were used for QTL mapping. A residual heterozygous line (CSSL19) that contained the QTL was used to produce BC₄F₃ and BC₄F₄ populations with one or two generations of selfing for fine mapping. NILs were screened for fine mapping and functional analysis. *japonica* Nipponbare was used as the wild-type control and *oshak21* mutant line on the Nipponbare background was used in functional analysis (Shen *et al.*, 2015). In total 222 accessions from the rice diversity panel were used haplotype analyses (Table S2; Eizenga *et al.*, 2014). All plants were grown in an experimental field at Nanjing Agricultural University. All seeds were harvested at their maturity stage and dried at 42°C for 7 days to break seed dormancy (Wang *et al.*, 2011).

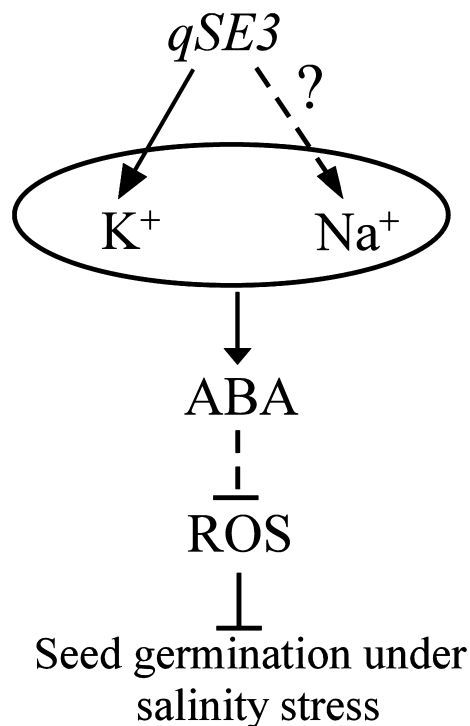


Figure 11. Hypothetical model of the role of *qSE3* on seed germination and seedling establishment under salinity stress in rice. *qSE3* expression in germinating seeds improves the uptake of K⁺ and Na⁺ under salinity stress. Increased K⁺ and Na⁺ accumulation in germinating seeds enhances ABA biosynthesis and ABA signaling responses. ROS levels will be reduced in germinating seeds under ABA regulation, which contributes to seed germination and seedling establishment under salinity stress. Arrows and lines with an slanted dashes indicate positive and negative effects, respectively. Solid lines indicate direct regulation, while dashed lines indicate indirect regulation.

Evaluation of seed germination

Seed germination was conducted as previously described by Wang *et al.* (2011). Fifty seeds of Jiucaiqing, IR26, CSSLs and NIL per replicate were imbibed in Petri dishes (diameter 9 cm) with 20 ml of 300 mM NaCl at 30 ± 1°C for 11 days, whereas 50 seeds each of Nip and *oshak21* were treated with 200 mM NaCl because of the lower salinity tolerance on the Nip background. IR26 and NIL seeds were also treated with 10 ml of 20% PEG6000 (polyethylene glycol with an average molecular weight of 6000 Da) at 30 ± 1°C for 7 days and with 10 ml of distilled water at 15 ± 1°C for 14 days and were sowed 1 cm deep in soil and treated with 200 mM NaCl for 14 days.

Seeds were considered germinated when the radicle protruded through the seed coat. Seedlings were considered established when the root length reached the seed length and the shoot turned green. Percentage germination at 3 days (normal condition) or 5 days (stress condition) was called the germination potential (GP). T_{50} was the time at which 50% germination was achieved and was calculated using GERMINATOR software (Joosen *et al.*, 2010). The germination index (GI) was calculated as follows: $GI = \sum (Gt/t)$, where Gt is the number of the germinated seeds on day t (Wang *et al.*, 2010). The seedling percentage (SP) was calculated each day. Following 0, 24, 48, 72 and 96 h of imbibition under salinity stress, approximately 1 g of each sample was harvested to detect the levels of Na⁺, K⁺, ABA, H₂O₂ and antioxidant enzyme activities. Three biological replicates were made.

Map-based cloning

The mean values of GI and SP in the individual CSSLs were compared with those of the recurrent parent IR26 for QTL mapping. QTLs were assigned to the introgressed region when significant differences in GI and SP were observed in CSSLs (Cheng *et al.*, 2016). Genomic DNA of seedlings from the BC₄F₃ and BC₄F₄ populations was extracted using the cetyltrimethylammonium bromide (CTAB) method to screen recombinants for fine mapping (Murray and Thompson, 1980). Markers used for fine mapping are listed in Table S3. The candidate gene was predicted using the data at <http://rice.plantbiology.msu.edu/cgi-bin/gbrowse>. The genome sequence and region ~1 kb upstream of the candidate gene were amplified from the genomic DNA of Jiucaiqing and IR26 using Phanta[®] Super-Fidelity DNA polymerase (Vazyme Biotech Co., Ltd, Nanjing, Jiangsu, China).

Expression analysis

Total RNA was extracted from developing grains (at 0, 7, 14, 21, 28, 35 or 42 DAF) and germinating seeds (at 12, 24, 36, 48, 60 and 72 h after imbibition) in Nip using the TransZol Plant kit (Transgen, www.transgen.com) according to the manufacturer's protocol. First-strand cDNA was synthesized with random oligonucleotides using the HiScript[®] II Reverse Transcriptase system (Vazyme Biotech Co., Ltd.). qRT-PCR was carried out in a total volume of 20 µl containing 2 µl of cDNA, 0.4 µl of gene-specific primers (10 µM), 10 µl of SYBR Green Mix and 7.2 µl of RNase-free ddH₂O using the Roche LightCycler480 Real-time System (Roche, Swiss Confederation). PCR conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. Rice *OsActin* and *18S rRNA* genes were used as internal controls. Primers used for qRT-PCR are listed in Table S4. Normalized transcript levels of gene expression were calculated using the comparative C_T method (Livak and Schmittgen, 2001). Transgenic plants carrying the *OsHAK21* promoter–GUS fusion construct in Nip were used for a

GUS staining assay. Histochemical staining of GUS activity was performed as described by Lagarde *et al.* (1996). Three biological replicates were made.

Yeast strains and culture conditions

AtKAT1, *OsHAK21^{JCQ}* (cloned from Jiucaiqing) and *OsHAK21^{IR26}* (cloned from IR26) coding sequences were cloned into the expression vector pYES2. The K⁺ uptake-deficient CY162 (*MAT α* , *ura3-52*, *leu2*, *trk1 Δ* , *his3 Δ 200*, *his4-15* and *trk2 Δ 1::pCK64*) strain of the yeast was used (Shanghai Weidi Biotechnology Co., Ltd. Shanghai, China). The yeast transformations were performed using the LiAC/ssDNA/PEG method as described previously (Horie *et al.*, 2011), and the growth assays were performed on SD-URA (Synthetic Dextrose Minimal Medium without Uracil) medium. Three biological replicates were made.

Evaluation of K⁺ and Na⁺ levels

Approximately 1 g of each sample was dried in an oven at 85°C for 3 days, weighed and then extracted with nitric acid using a microwave digestion system. K⁺ and Na⁺ contents were measured using inductively coupled plasma mass spectrometry (ICP-MS; Perkin Elmer, Nexion 300X, USA). The net uptake rates of K⁺ and Na⁺ were calculated as described by Nieves-Cordones *et al.* (2010): net uptake rate = $(C_2 - C_1)/(t_2 - t_1) \times (R_1 + R_2)/2$, where C is the total K⁺ or Na⁺ content, R is the dry weight of the seeds, and t is the time at harvest; the subscript numbers 1 and 2 indicate the start and end of the period for which the uptake rate was calculated: $t_2 - t_1 = 2$ days. $(R_1 + R_2)/2$ = the mean seed dry weight. Na⁺ or K⁺ content was expressed as $\mu\text{mol g DW}^{-1}$ (dry weight) d^{-1} . Three biological replicates were made.

Hormone quantification

Approximately 1 g of each sample was rapidly frozen in liquid nitrogen and homogenized into a powder. Endogenous ABA was extracted from each sample using 5 ml of 80% (v/v) pre-cooling methanol at 4°C for 12 h, followed by centrifugation at 8000 *g* at 4°C for 10 min. The supernatant was collected, and 0.1 g of poly(vinylpyrrolidone) was added to adsorb the phenolic compounds and pigments at 4°C for 1 h. The mixture was then centrifuged at 12 000 *g* at 4°C for 10 min. Next, the supernatant was washed with 5 ml of 100% (w/v) and 5 ml of 80% (v/v) methanol and passed through C18 columns (C18 Sep-Park[®] Cartridges; Waters Corp., Milford, MA, USA). The extract was freeze dried, dissolved in 2 ml of 75% aqueous methanol, and then filtered through 0.22- μm membrane filters. The final 2 μl of filtrate solution was used for ABA quantification using a high-performance liquid chromatography (HPLC) system (Waters Instruments Inc., USA). ABA content was determined using the external standard method and is expressed as $\mu\text{g g DW}^{-1}$. Three biological replicates were made.

Evaluation of H₂O₂ level

H₂O₂ levels were assayed based on the Huang and Song method (2013) with minor modifications. Approximately 1 g of each sample was rapidly frozen in 5 ml of cold acetone (−20°C) and homogenized into a powder. A titanium reagent (20% titanic tetrachloride in concentrated HCl, v/v), NH₄OH and H₂SO₄ were added to the homogenate. Then, the mixture was centrifuged at 12 000 *g* at 4°C for 10 min, and the absorbance of the supernatant was determined immediately at 415 nm. The H₂O₂ content was expressed as $\mu\text{mol g DW}^{-1}$. Three biological replicates were made.

Evaluation of antioxidant enzyme activities

CAT, APX and POD activities were measured using commercial assay kits following the manufacturer's instructions (Suzhou Keming Bioengineering Company, Suzhou, Jiangsu, China). Approximately 0.1 g of powder of each sample was added to 1 ml of sodium phosphate buffer (50 mM, pH 7.0). After centrifugation at 8 000 *g* at 4°C for 10 min, the supernatant was collected to determine CAT, APX and POD activities. Three biological replicates were made.

CAT activity was determined based on monitoring absorbance at 240 nm. The reaction mixture contained 10 μl of enzyme extract and 190 μl of a solution of 50 mM phosphate buffer (pH 7.0) and 10 mM H₂O₂ in a final volume of 200 μl at 25°C. CAT activity was calculated using the following formula: CAT activity ($\text{nmol min}^{-1} \text{g DW}^{-1}$) = $918 \times \Delta A_{240}/\text{DW}$.

APX activity was determined by monitoring absorbance at 290 nm. The reaction mixture contained 140 μl of 50 mM phosphate buffer (pH 7.0), 20 μl of 7.5 mM AsA, 20 μl of 300 mM H₂O₂ and 20 μl of enzyme extract in a final volume of 200 μl at 25°C. APX activity was calculated using the following formula: APX activity ($\mu\text{mol min}^{-1} \text{g DW}^{-1}$) = $1.79 \times \Delta A_{290}/\text{DW}$.

POD activity was determined based on monitoring absorbance at 470 nm. The reaction mixture contained 10 μl of enzyme extract, 60 μl of ddH₂O, 120 μl of 0.1 mM acetic acid buffer (pH 5.4), 30 μl of 20 mM guaiacol and 30 μl of 22 mM H₂O₂ in a final volume of 250 μl at 25°C. One unit of POD activity was defined as an absorbance change of 0.005 units per minute at 470 nm. POD activity was calculated using the following formula: POD activity (U g DW^{-1}) = $5000 \times \Delta A_{470}/\text{DW}$.

Haplotype analyses

To determine the haplotypes of the target gene, 700 000 SNP markers of rice accessions listed at <https://ricediversity.org/data/index.cfm> (McCouch *et al.*, 2016) were used. SNPs in the region ~2 kb upstream and ~2 kb downstream of the target gene were located according to the Rice Genome Annotation Project MSU7 database (Rice Genome Browser: <http://rice.plantbiology.msu.edu>). Seed germination was conducted using 222 accessions under 200 mM NaCl conditions for 10 days (Table S2). Haplotypes represented at least eight investigated accessions that were previously used for a comparative analysis of phenotypes (Dong *et al.*, 2016).

Data analysis

Experimental data were analyzed using SAS software (Cary, NC, USA), and significant differences among samples were compared using Student's *t*-test or using the least significant difference (LSD) test at the 5% and 1% levels of probability.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. QTL mapping for seed germination and seedling establishment under salinity stress.

Figure S2. Comparison of seed germination and seedling establishment between CSSL19(*qSE3*) and CSSL19(*qse3*) under salinity stress.

Figure S3. Comparison of seed germination and seedling establishment between NIL(*qSE3*) and IR26 under PEG and low temperature stresses.

Figure S4. Expression patterns of *OsHAK21* in rice.

Figure S5. Comparison of agronomic traits between NIL(*qSE3*) and IR26.

Table S1. Candidate genes located between YQ35 and YQ38 for *qSE3*

Table S2. Information of 222 accessions used for haplotype analyses

Table S3. Primer pairs used for fine mapping in this study

Table S4. Primer pairs used for gene cloning and qRT-PCR analyses in this study

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