Identification and fine mapping of a novel *qGR6.2* locus controlling rice salt tolerance during seed germination

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Abstract

Background Rice growth is frequently affected by salinity. When rice plants are exposed to high salinity, seed germination and seedling establishment are significantly inhibited. In particular, with the promotion of rice direct-seeding in Asia, improving rice salt tolerance during seed germination is of strong importance for rice breeding.

Results In this study, we found that the indica rice landrace Wujiaozhan (WJZ) showed a high capability of seed germination under both water (H 2 O) and salt (NaCl) conditions, particularly under high salt stress. The BC 1 F 2 population produced by crossing WJZ with japonica Nipponbare (Nip) was used to evaluate the germination traits under water (H 2 O) and salt (300 mM NaCl) conditions using germination rate (GR) and germination index (GI). A total of 13 quantitative trait loci (QTLs) were identified, including eight QTLs of GR, two QTLs of GI under H 2 O conditions, six QTLs of GR, and three QTLs of GI under 300 mM NaCl conditions. Six QTLs (qGR6.1 , qGR8.1 , qGR8.2 , qGR10.1 , qGR10.2 and qGI10.1) contributed to GR under both H 2 O and 300 mM NaCl conditions. Three QTLs (qGR6.2 , qGR10.1 and qGR10.2) under 300 mM NaCl conditions were identified at different time points of seed germination and shared the same region with qGI6 , qGI10.1 and qGI10.2 for GI. These QTLs could be used to improve seed germination ability via marker-assisted selection (MAS). One major effective salt-tolerance-specific QTL, qGR6.2, on chromosome 6 was further confirmed via the BC 2 F 2 population, which explained more than 20% of the phenotypic variation. Fine mapping results showed that qGR6.2 was narrowed to a 65.9-kb region between the Z654 and Z619 molecular markers, with eleven candidate genes being predicted. Based on the microarray database, there were high transcript abundances of six genes (LOC_Os06g10650 , LOC_Os06g10660 , LOC_Os06g10690 , LOC_Os06g10710 , LOC_Os06g10730 and LOC_Os06g10750) at all developmental stages, and only LOC_Os06g10750 was differentially expressed after salt incubation. RT-qPCR showed that two genes (LOC_Os06g10650 and LOC_Os06g10750) were significantly differentially expressed at 300 mM NaCl during seed germination. This result suggested that LOC_Os06g10650 and LOC_Os06g10750 might be the causal candidate genes for the major effective salt-tolerance-specific QTL qGR6.2 identified in WJZ, which may facilitate map-based cloning and help to elucidate the molecular mechanism underlying salt tolerance during seed germination.

Conclusions In our study, we identified 13 QTLs from indica landrace WJZ that confer seed germination traits under water and salt conditions. A major salt-tolerance-specific QTL qGR6.2 was confirmed and fine mapped to a 65.9-kb region flanked by the Z654 and Z619 markers. Our results provide information on the genetic basis of improving salt tolerance during seed germination by MAS.

Background

Soil salinity is the primary abiotic stress affecting crop growth and productivity worldwide (Zhu 2001). It is estimated that 6% of the Earth's landmass and 20% of irrigated land are affected by salinity (Munns and Tester 2008). Rice is the most important staple food, feeding more than half of the world's population. Compared to wheat and cotton, rice is more sensitive to salt stress, and approximately 30% of the rice-growing area in the world is affected by salinity (Takehisa et al. 2004). According to previous reports, high salinity inhibits seed germination and seedling establishment, reduces plant growth and diminishes rice yield (Wang et al. 2011; Kumar et al. 2013). Although saline soil could be improved by large-scale irrigation, drainage schemes, and chemical treatment, all these solutions are overly costly (Munns and Gilliham. 2015). Hence, genetic improvement of salt tolerance has been an important and feasible objective for rice breeding in coastal areas.

Salt tolerance is a polygenic trait highly influenced by the environment (Johnson et al. 1992; Wang et al. 2012b), which makes it difficult to identify the responsible QTLs and genes. To date, hundreds of salt-response QTLs have been reported at different developmental growth stages in rice (Ganie et al. 2019). Lin et al. (2004) detected two major QTLs (*qSNC-7* and *qSKC-1*) for Na^+/K^+ content in the seedling shoot on chromosomes 1 and 7. Based on the results of QTL mapping, this major QTL *qSKC-1* has been cloned, which encodes an HKT-type transporter protein regulating K^+ content in the shoot (Ren et al. 2005). Another major QTL*, Saltol,* was obtained and cultivated in salt-tolerant varieties by MAS, response to salt stress at the seedling stage (Thomson et al. 2010; Huyen et al. 2012).

Salt tolerance during seed germination is not consistently related to other stages (Johnson et al. 1992, Shi et al. 2017). To date, few studies aimed at genetically dissect salt tolerance during seed germination in rice. Wang et al. (2011) detected 16 QTLs of rice seed germination ability at 100 mM NaCl from the salt tolerance variety Jiucaiqing. Approximately 50 salt tolerance loci have been identified at the seed germination stage by genome-wide association analysis (GWAS) (Cheng et al. 2015; Shi et al. 2017; Yu et al. 2018). Fujino et al. (2008) reported that *qLTG3-1* responded to seed germination under salt stress because of tissue vacuolation and weakening. Recently, a QTL *qSE3* promoting seed germination and seedling establishment was identified from a *japonica* landrace Jiucaiqing at 300 mM NaCl, which encodes a potassium transport *OsHAK21* and mediates salt tolerance during seed germination through abscisic acid (ABA) metabolism (He et al. 2019). With the increasing promotion of rice direct-seeding methods in Asia, it is of considerable importance to explore more loci or genes for salt tolerance and develop salt-tolerant varieties by MAS during seed germination in rice.

In this study, an *indica* rice variety WJZ from 276 *indica* accessions (Cheng et al. 2015) was identified as high salt tolerance during seed germination. Seed germination evaluations were conducted by measuring the germination rate (GR) and germination index (GI) of a BC_1F_2 population derived from a cross between WJZ and the *japonica* variety Nipponbare (Nip) under H_2O and 300 mM NaCl conditions. A major salt-tolerance-specific QTL on the short arm of chromosome 6 for seed germination was identified by QTL mapping. Moreover, this major QTL *qGR6.2* was verified among the BC_2F_2 population and finally fine mapped to a 65.9-kb region between the Z654 and Z619 markers. Our work may help to elucidate the genetic and molecular basis of salt tolerance during seed germination.

Results

Characteristics of seed germination for two parents under salt stress

The germination rate (GR), seedling percentage (SP) and germination index (GI) for *indica* WJZ and *japonica* Nip seeds were evaluated after 10 days (d) of imbibition under H_2O and various salt concentration conditions (150, 200, 250, 300 and 350 mM NaCl). Both WJZ and Nip germinated readily, with approximately 100% of the GR and SP for WJZ and Nip under H_2O conditions (Table 1). However, WJZ had a significantly higher GI (13.93) than Nip (10.51), indicating that WJZ germinated faster than Nip under H_2O conditions. There was a significant decrease in GR, SP, or GI of both WJZ and Nip under various NaCl concentrations (Table 1), indicating that rice seed germination was inhibited and delayed by salt stress. When exposed to 350 mM NaCl, WJZ seeds displayed 80.03% of GR, in contrast to 12.22% of GR for Nip (Table 1), suggesting that WJZ was considerably more salt-tolerant than Nip during seed germination. Since the greatest variation in GR, SP and GI between the two rice parents was under 300 mM NaCl, seed germination was assessed with 300 mM NaCl in our later experiments.

To better understand the characteristics of the high seed germination ability and salt tolerance of WJZ, the dynamic traits of GR and SP between the two parents were further analyzed under H_2O and 300 mM NaCl conditions. Under H_2O conditions, although all seeds of both parents germinated and established seedlings after 6 d of imbibition (Fig. 1a, b), WJZ germinated faster and had higher values of GR and SP than Nip at the beginning of seed germination. Under 300 mM NaCl conditions, significant differences in GR and SP between WJZ and Nip were observed from 3 to 14 d during seed germination (Fig. 1c, d). The WJZ began to germinate after 3 d of imbibition, and its GR reached 90% after 7 d of imbibition (Fig. 1c), with a strong seedling establishment capacity being observed (Fig. 1d). However, Nip started to germinate after 7 d of imbibition and showed only 58.89% GR after 14 d of imbibition.

Variation in seed germination among the BC_1F_2 populations under salt stress

A BC_1F_2 population consisting of 181 individuals was derived from the selfing of one salt-tolerance BC_1F_1 single plant, which was produced by the backcross of one salt-tolerance F_3 individual (Nip × WJZ) with Nip (Fig. S1a). The variations in GR and GI among this BC_1F_2 population under H_2O and 300 mM NaCl conditions were analyzed. All the traits observed, including GR from 2 to 3 d and GI under H_2O conditions, GR from 5 to 13 d and GI under 300 mM NaCl conditions, showed a continuous

distribution and had a wide range of genetic variations (Fig. 2). Under H_2O conditions, there was a left-skewed distribution of GR after 2 d of imbibition, a right-skewed distribution at 3 d (Fig. 2a, b), and a symmetrical distribution of GI (Fig. 2c). Under 300 mM NaCl conditions, GR showed a left-skewed distribution at 5 d, a right-skewed distribution at 9, 11 and 13 d during seed germination (Fig. 2d, f-h), and a symmetrical distribution at 7 d (Fig. 2e). The GI under 300 mM NaCl conditions showed a right-skewed distribution (Fig. 2i). These results indicated that the traits of GR and GI are polygenic characteristics and might be regulated by various genes at the early and later stages of germination under either H_2O or NaCl conditions.

QTL mapping of seed germination traits under H_2O and salt conditions

A molecular linkage map consisting of 70 simple sequence repeat (SSR) or InDel (Insertion/Deletion) markers was constructed with the above BC_1F_2 population consisting of 181 individuals for QTL mapping of seed germination traits, GR and GI under H_2O and salt conditions. Under H_2O conditions, eight QTLs of GR during seed germination were identified on chromosomes 3, 6, 8 and 10, and two QTLs of GI were identified on chromosomes 6 and 10 (Table 2). GR for 2 d was regulated by three QTLs (*qGR8.1*, *qGR8.2* and *qGR10.1*), and GR for 3 d was regulated by six QTLs (*qGR3.1*, *qGR3.2*, *qGR3.3*, *qGR6.1*, *qGR10.1* and *qGR10.2*). The phenotypic variance of GR explained by a single QTL ranged from 7.32 to 23.99%. One major QTL, *qGR6.1*explained 23.99% of the phenotypic variation. The *qGI6.1* for the GI, identified within the interval of RM190~Z602 on chromosome 6, and *qGI10.1* within the interval of W13~W20 on chromosome 10, accounted for 10.39 and 8.86% of phenotypic variation, respectively. By comparison, *qGR6.1* and *qGI6.1* shared the same interval of RM190~Z602 on chromosome 6, and *qGR10.1* and *qGI0.1* shared the same interval of W13~W20 on chromosome 10 (Table 2). The additive effects of all these QTLs detected under H_2O conditions were negative, ranging from -0.36 to -9.95 (Table 2), suggesting that the positive alleles were derived from WJZ.

Under 300 mM NaCl conditions, six QTLs of GR and three of GI during seed germination were identified on chromosomes 6, 8, and 10, respectively (Table 2). All these QTLs showed a negative additive effect, indicating that the positive alleles originated from WJZ. Among the six QTLs of GR, *qGR6.2* and *qGR10.2* were continuously identified after 7, 9, 11, and 13 d of imbibition, *qGR10.1* at 5, 7,9,11, and 13 d (Fig. 3), and *qGR8.1* and *qGR8.2* only at 5 d and *qGR6.1* only at 13 d. It suggested that *qGR6.2*, *qGR10.2* and *qGR10.1* might be key QTLs of salt tolerance for seed germination(Table 2). The *qGR6.2* flanked by Z604 and RM276, explaining more than 20.0% of phenotypic variation, could be a major-effective QTL. Three QTLs of GI, *qGI6.2*, *qGI10.1*, and *qGI10.2,* accounted for 24.39, 17.41 and 13.18% of phenotypic variation, respectively (Table 2). By comparison, *qGR6.2* was co-localized with *qGI6.2* between Z604 and RM276 on chromosome 6, and *qGR10.1* shared the same region with *qGI10.1* in the interval of W13~W20 on chromosome 10, another chromosomal region (W20~RM6824) on chromosome 10 was identified to control GR and GI at the same time. Among those loci, a major QTL *qGR6.2* with a high LOD value (>8) could specifically enhance GR and GI under salt conditions (Table 2).

Validation and fine mapping of *qGR6.2*

To validate the major *qGR6.2* identified for GR under 300 mM NaCl conditions, we further structured a BC_2F_2 population consisting of 70 individuals. There was a significant peak between markers Z604 and Z605 based on GR at 13 d under salt stress, and its phenotypic variation and LOD values were 19.50% and 9.31, respectively (Fig. 4; Table S2). This result indicated that *qGR6.2* could obviously improve rice seed germination under salt stress.

A large BC_2F_3 population consisting of 1,205 individuals was developed to narrow the region of *qGR6.2*. Eighty-six recombinants were identified between Z604 and RM276 markers (Fig. 5a). Four new polymorphic markers (Z616, Z617, Z619 and Z605) between Z604 and RM276 were further developed. Based on the genotypes, the 86 recombinants were classified into four groups. Eighteen recombinant events were between Z604 and Z616, 57 recombinant events were between Z617 and Z619, and 11 recombinant events were between Z605 and RM276 (Fig. 5b). The progeny of these recombinants were tested to identify homozygous individuals in the heterozygous region of each group. Salt tolerance during seed germination was determined by the average values of these homozygous individuals derived due to segregation in the recombinant heterozygous region. In groups B or D, the average value of GR at 10 d for homozygous WJZ alleles was significantly higher

than that for Nip alleles, while there was no difference in groups A or C. *qGR6.2* was delimited between the Z617 and Z619 markers (Fig. 5b). Similarly, the larger BC_2F_4 population derived from heterozygous BC_2F_3 plants in markers Z617 and Z619 was developed, containing 2,318 individuals. Seventeen recombinants consisting of three types of recombination were obtained (E, F and G) (Fig. 5b), and the progeny assay of each homozygous individual from the recombinant group was conducted. Finally, the *qGR6.2* locus was narrowed down to a 65.9-kb region between markers Z654 and Z619 (Fig. 5b).

Prediction and expression analysis of candidate genes in the *qGR6.2* locus

According to the MSU Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu), eleven opening reading frames (ORFs) were annotated within the 65.9-kb region located in the *qGR6.2* locus, including five functional proteins, one transposon protein and five expressed proteins without annotation (Table 3). Five genes with functional annotation showed that *ORF1* (*LOC_Os06g10650*) encodes a tyrosine phosphatase (PTP) family protein, *ORF2* (*LOC_Os06g10660*) encodes a lysM domain-containing GPI-anchored protein 1 precursor, *ORF3* (*LOC_Os06g10670*) encodes an aspartic proteinase nepenthesin-1 precursor, *ORF5* (*LOC_Os06g10690*) encodes a PHD-finger domain-containing protein, and *ORF11* (*LOC_Os06g10750*) encodes an integral membrane protein DUF6-containing protein.

The expression profiles of 10 ORFs, including five functional proteins and five expressed proteins without annotation in various developmental stages and incubation of salt stress based on mRNASeq data and array database deposited in GENEVESTIGATOR (Fig. 6), were determined. The results showed that there were higher transcript abundances of seven genes, including *ORF1* (*LOC_Os06g10650*), *ORF2* (*LOC_Os06g10660*), *ORF3* (*LOC_Os06g10670*), *ORF5* (*LOC_Os06g10690*), *ORF7* (*LOC_Os06g10710*), *ORF9* (*LOC_Os06g10730*) and *ORF11* (*LOC_Os06g10750*), at all developmental stages, while almost no expression was observed for the other three genes, *ORF4* (*LOC_Os06g10680*), *ORF6* (*LOC_Os06g10700*) and *ORF8* (*LOC_Os06g10720*) (Fig. 6a). When the root and seedling samples were exposed to salt stress, *ORF11* (*LOC_Os06g10750*) showed significantly down-regulated expression patterns after salt incubation (Fig. 6b).

With the quantitative real-time PCR (RT-qPCR) approach, we subsequently detected the expression of all 11 ORFs in WJZ and Nip during seed germination under 300 mM NaCl conditions. The expression of *ORF1* (*LOC_Os06g10650*) was dramatically induced by salt stress after imbibition for 24 h with a nearly 20-fold change compared with 0 h in WJZ and 14-fold change compared with 0 h in Nip (Fig. 7a). *ORF2* (*LOC_Os06g10660*) and *ORF5* (*LOC_Os06g10690*) showed smooth expression (Fig. 7b, e). Eight genes, *ORF3* (*LOC_Os06g10670*), *ORF4* (*LOC_Os06g10680*), *ORF6* (*LOC_Os06g10700*), *ORF7* (*LOC_Os06g10710*), *ORF8* (*LOC_Os06g10720*), *ORF9* (*LOC_Os06g10730*), *ORF10* (*LOC_Os06g10740*) and *ORF11* (*LOC_Os06g10750*), showed down-regulated expression patterns during seed germination under salt stress (Fig. 7). Comparing the expression level between the two parents, we found that *ORF11* (*LOC_Os06g10750*) in WJZ was nearly 10-fold lower than that in Nip (Fig. 7k), indicating the different role of *ORF11* (*LOC_Os06g10750*) in the two parents. Taken together with gene function annotation and expression profiles, these results indicated that *ORF1* (*LOC_Os06g10650*), encoding a tyrosine phosphatase (PTP) family protein, and *ORF11* (*LOC_Os06g10750*), encoding an integral membrane protein DUF6-containing protein, might be the causal candidate gene for salt tolerance in the *qGR6.2* locus.

Discussion

Salinity seriously affects rice seed germination and seedling establishment, especially in the direct-seeding area, leading to rice reduction in yields (Wang et al. 2012b; Shi et al. 2017). In this study, the *indica* landrace WJZ from Yunnan Province in China showed a strong capability of seed germination and seedling establishment under high salinity. When exposed to 300 mM NaCl, the visible seed of WJZ could start to germinate after three days of imbibition and established normal seedlings after five days. This finding suggests that WJZ is an important germplasm resource with strong salt tolerance during seed germination, similar to N22-C-334-3 (Ashokkumar et al. 2013) and Jiucaiqing (He et al. 2019). WJZ was considerably higher than Nip with 167.62 *vs.* 84.18 cm of plant height (data not shown) and fell down easily in field planting. Hence, it is of great importance to explore elite genes controlling salt tolerance during seed germination from WJZ, which will be beneficial for improving rice salinity tolerance in direct-sowing areas. As reported in previous studies, rice suffered from salinity stress

during the whole growth stage, and salt tolerance at one developmental stage might be not correlated with that at other stages (Johnson et al. 1992, Chai et al. 2014, Shi et al. 2017). WJZ has a very strong tolerance to salt stress at the seed germination stage, and it is a subject that deserved to study the salt tolerance of WJZ at the seedling or reproductive stages. Through pyramiding various salt-tolerance loci, or those loci expressing at germination, seedling, tillering, or booting stages, it could be cultivated new rice varieties with salt tolerance during the whole growth stage, for production in rice direct-seeding areas or saline soils.

Evaluating the phenotype of salt tolerance comprehensively and accurately is the most crucial step for QTL mapping (Wang et al. 2012a). In this study, we evaluated the traits of seed germination under H_2O and NaCl conditions among the BC_1F_2 population, which was derived from a BC_1F_1 individual containing approximately 37.76% genetic region of Nip. The GR of this BC_1F_2 population showed a wide range of genetic variations from 2 to 3 d under H_2O conditions and from 5 to 13 d under NaCl conditions. There was a similar feature of GR under both H_2O and NaCl conditions, shifting from left-skewed distribution to right-skewed distribution during seed germination. This result suggested that GR was developmentally regulated by various genes and growth stage-specific under H_2O and NaCl conditions. Additionally, GR at 7 d under 300 mM NaCl conditions showed symmetrical distribution, suggesting that there was great genetic variation at this time point and might be a crucial period for breaking through the seed coat to germinate under salt stress (Fig. 2e).

In this study, a total of 13 QTLs controlling seed germination were identified via QTL mapping, including 10 QTLs under H_2O conditions and 9 QTLs under 300 mM NaCl conditions. Of these QTLs, six (*qGR6.1*, *qGR8.1*, *qGR8.2*, *qGR10.1*, *qGR10.2* and *qGI10.1*) were consistently effective under H_2O and 300 mM NaCl conditions, which might play key roles in seed germination. Under 300 mM NaCl, three QTLs (*qGR6.2*, *qGR10.1* and *qGR10.2*) of GR were identified at different time points of seed germination and they shared the same region with *qGI6.2*, *qGI10.1* and *qGI10.2* for GI. It was also found that some QTLs, such as *qGR8.1,* only contributed to GR at the early stage (5 d) of seed germination, and others, such as *qGR6.1,* only contributed at the late stage (13 d) of seed germination. At least three QTLs (*qGR6.1*, *qGR6.*2 and *qGI6.2*) were identified as major QTLs, as they explained more than 20.0% of phenotypic variation. All these QTLs could improve rice seed germination and salt tolerance via gene pyramiding in future research. By comparing chromosomal locations of reported QTLs, five QTLs (*qGR3.3*, *qGR6.2*, *qGR10.1*, *qGI6.2* and *qGI10.1*) in the BC_1F_2 population were located in the same or adjacent regions as previously reported QTLs. *qGR3.3* was near the region of *qLTG-3-2* for low-temperature germination ability (Fujino et al. 2004) and *qGR3-1* for germination rate (Cui et al. 2002). *qGR6.2* and *qGI6.2* were close to the *qIR-6* position for seed germination under salt stress (Wang et al. 2011), and one gene, *OsRR22* was located within this region and involved salt tolerance at the seedling stage (Takagi et al. 2015). The regions of *qGR10.1* and *qGI10.1* were similar to *qSKC10* and *qRKC10* identified for the shoot and root potassium content under salt stress at the seedling stage (Wang et al. 2012a). This result indicated that these co-localized QTLs at the different developmental stages were the weightily genomic regions for salt tolerance in rice.

The major QTL *qGR6.2* with a high LOD value (>8), enhancing GR and GI under salt conditions, was further validated in the BC_2F_2 population. *qGR6.2* was finally mapped in a region of 65.9 kb between markers Z654 and Z619. A total of 11 candidate genes were predicted in the Nipponbare genome. Of these genes, we found that *ORF1* (*LOC_Os06g10650*) encoded tyrosine phosphatase (PTP) family protein and *ORF11* (*LOC_Os06g10750*) encoded integral membrane protein DUF6 containing protein with differential expression by exposure to salt stress based on mRNA-Seq data. According to previous studies, PTP family proteins have been reported to regulate signal transduction and control plant growth and development (Stoker, 2005). *AtPTP1*, the first PTP family gene in the plant, was upregulated by high salt stress (Xu et al. 1998). An integral membrane protein DUF6 containing protein *At01g09380* was involved in salt stress and drought stress (Sun et al. 2009). Furthermore, mRNA-Seq data showed high transcript abundances of *ORF1* (*LOC_Os06g10650*) and *ORF11* (*LOC_Os06g10750*) at all developmental stages. The different expression patterns of these two genes from RT-qPCR were also observed in WJZ and Nip after seed imbibition at 300 mM NaCl, suggesting different roles in the two parents. These results indicated that *ORF1* (*LOC_Os06g10650*) and *ORF11* (*LOC_Os06g10750*) might be the causal candidate genes of *qGR6.2* in response to salt tolerance during seed germination. Genetic transformation by CRISPR/Cas9 will be performed to validate the functions of these two causal genes.

In summary, we identified 13 QTLs for seed germination traits under H_2O and salt conditions, which provides information on the genetic basis of improving salt tolerance during seed germination by MAS. Of these loci, the major QTL *qGR6.2*, specifically for salt tolerance during seed germination, was fine mapped within a region of 65.9 kb with two more likely causal genes, *ORF1* (*LOC_Os06g10650*) and *ORF11* (*LOC_Os06g10750*). Sequence analysis and genetic transformation will be carried out in the future to validate the function of the candidate genes and elucidate the molecular mechanism underlying salt tolerance at the seed germination stage. The major QTL *qGR6.2* could be highly useful for improving the salt tolerance of seed germination by the MAS strategy.

Materials And Methods

Plant materials

The high salt-tolerant *indica* WJZ was crossed with the salt-sensitive *japonica* Nip and the progeny were continuously self-crossed to produce the F_3 population. One salt-tolerance F_3 individual was backcrossed with Nip twice to generate the BC_2F_2 population, then followed self-cross in this study. The BC_1F_2 and BC_2F_2 populations were used for QTLs mapping, and BC_2F_3, BC_2F_4 and BC_2F_5 for fine mapping of target QTL (Fig. S1). All plants were grown in a paddy field at the Jiangpu Experimental Station of Nanjing Agricultural University (Jiangsu Province, China) in the summer of 2014, 2015, 2016, 2017 and 2018 with 17 cm between plants within a row and 33 cm between rows. The seeds of each line or individual were harvested at maturity and dried at 42°C for 7 d to break seed dormancy and then stored at -20°C.

Evaluation of seed germination under H_2O and NaCl conditions

A total of 30 healthy grains from each line were surface-sterilized with 0.5% sodium hypochlorite solution for 15 min and then rinsed three times with sterile distilled water. Seeds were imbibed in a Petri dish (diameter 9 cm) with 40 mL quartz (diameter 1~2 mm) and 20 mL solution for 10 d under H_2O condition and 14 d under NaCl condition, respectively. The different NaCl solutions (0 mM, 150 mM, 200 mM, 250 mM, 300 mM, and 350 mM) were applied for two parents to determine the fitting salt concentration of treatment. 0 mM and 300 mM NaCl solutions were used for the BC_1F_2 population to detect QTLs responsible for seed germination under H_2O and NaCl conditions. The evaluation of seed germination under 300 mM NaCl was conducted for fine mapping of target QTL among the BC_2F_2, BC_2F_4 and BC_2F_5 population. All seeds were grown at 25±1°C in a growth chamber under 12 h light/12 h day conditions. Seed germination was defined as the emergence of the radicle (2 mm) through the surrounding tissue, and the seedling establishment was considered when the root length reached the seed length and the shoot length reached half of the seed length (Cheng et al. 2015). The germination rate (GR) was calculated daily. The germination index (GI) was calculated as GI = $\sum$ (*Gt*/t), where *Gt* is the number of germinated seeds on day *t* (Wang et al. 2011). Three replications of each line were performed.

DNA extraction and PCR analysis

Total genomic DNA was extracted from young leaves of each plant using the cetyltrimethylammonium bromide (CTAB) method. According to the International Rice Microsatellite Initiative (IRMI, http://www.gramene.org) (MoCouch et al. 2002), a total of 167 SSR markers were polymorphic between WJZ and Nip and scattered on 12 chromosomes (Table S1). PCR was performed as described by Chen et al. (1997). The PCR products were separated by electrophoresis through 8% nondenaturing polyacrylamide gels and visualized by silver staining.

QTL mapping

Among 167 SSR markers with polymorphism between WJZ and Nip, 70 heterozygous molecular markers in the BC_1F_1 individual, were used to construct a primary genetic map with 181 individuals from the BC_1F_2 population by Mapmaker/Exp 3.0 (Lander et al. 1987). Since the salt-tolerance BC_1F_1 individual was derived from backcrossing of one salt-tolerance F_3 individual with Nip, its genetic region was approximately 37.76% of Nip (Fig. S1 b). The total genetic distances of 498.24 cM

were obtained with an average genetic distance of 7.22 cM among the BC_1F_2 population. GR at 2 and 3 d under H_2O conditions and GI, GR at 5, 7, 9, 11 and 13 d and GI under 300 mM NaCl conditions were used for QTL mapping. QTL analysis was carried out by Inclusive Composite Interval Mapping (ICIM) (Li et al. 2008) with a threshold of LOD >3 operating 1000 permutations. The phenotypic variance and additive and dominance effects of each QTL were estimated.

Validation and fine mapping of *qGR6.2*

A linkage map of 70 individuals from the BC_2F_2 population was analyzed with 11 SSR markers (Table S3) on chromosome 6 to ensure the presence of the major QTL *qGR6.2*. To fine-map the target QTL *qGR6.2*, another ten polymorphic InDel markers were developed and used for fine mapping. A total of 1,205 BC_2F_3 individuals were used to screen recombinants between the Z604 and RM276 markers, and 2,318 BC_2F_4 individuals were used to screen recombinants between the Z617 and Z619 markers. In total, seven types of recombinants were identified. Twenty progenies of each recombinant were planted and screened for homozygous plants from each group. These homozygous plants (BC_2F_4, BC_2F_5) were tested for seed germination under 300 mM NaCl conditions. The average GR value of seed each group after 10 d of imbibition was used for fine mapping.

Prediction and expression analysis of candidate genes

Open reading frames (ORFs) in the region of markers Z654 and Z619 were predicted by the Rice Annotation Project Database (http://rice.plantbiology.msu.edu/). GENEVESTIGATOR (https://genevestigator.com/gv/) was employed to analyze the expression patterns of eleven candidate genes based on 2836 Affymetrix microarray datasets and 421 mRNASeq data with a significance level of $P < 0.05$ in salt incubation.

Seeds of two parents were sampled after 0 h, 6 h, 12 h, 24 h, 36 h, and 48 h imbibition at 300 mM NaCl, frozen quickly in liquid nitrogen and stored at -80℃ for RNA extraction. Total RNA was isolated from approximately 80～100 mg powder with a total RNA Kit (BioTeke, http://www.bioteke.com). The first-strand cDNA was synthesized with random oligonucleotides using the HiScript II Reverse kit (Vazyme Biotech, http://www.vazyme.com/) according to the manufacturer's protocol. To measure the mRNA levels of genes, RT-qPCR was conducted using a CFX96 Real-time System (Bio-Rad, USA) with SYBR Green Mix (Vazyme). The rice housekeeping gene *OsActin* (*LOC_Os03g50885*) was used as an internal control (Zhou et al. 2008). The PCR conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. A final melt curve stage of 65-95°C was performed to confirm the specificity of the primers. Relative quantification of transcript levels was obtained based on the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The amount of the transcripts in the WJZ after imbibing for 0 h was set at 1.0. All of the primers used for RT-qPCR were designed according to http://quantprime.mpimp-golm.mpg.de/ (Table S4). Three biological replicates were conducted.

Data analysis

The experimental data were analyzed using Statistical Analysis System (SAS) software (Cary, NC, USA) and compared with Student's *t*-test at the 5% and 1% levels of probability.

Abbreviations

WJZ: Wujiaozhan; Nip: Nipponbare; GR: Germination rate; SP: Seedling percentage; GI: Germination index; QTLs: Quantitative trait loci; MAS: Marker-assisted selection; GWAS: Genome-wide association analysis; SSR: Simple sequence repeats; InDel: Insertion/Deletion; ORF: Opening reading frame; PTP: Protein tyrosine phosphatase; CTAB: Cetyltrimethylammonium bromide; ABA: abscisic acid; RT-qPCR: quantitative real-time PCR.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of supporting data

The datasets supporting the conclusions of this article are provided within the article and its additional files.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

JP Cheng, HS Zhang and P Zeng conceived the project and designed the research. P Zeng performed most of the experiments and analyzed the data; PW Zhu performed salt tolerance identification for fine mapping; P Zeng and LF Qian constructed the genetic map; and XM Qian, YX Mi, Zefeng Lin and SN Dong participated in developing plant populations. P Zeng and JP Cheng wrote the manuscript. H Aronsson provided technical assistance with English writing. HS Zhang and JP Cheng supervised and complemented the writing. All authors read and approved the final manuscript.

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Not applicable.

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Supporting Information

Figure S1 Overview of the mapping populations and the genetic basis of BC_1F_1. (a) A flow chart that describes the construction of the mapping population in this study. (b) The genetic basis of a BC_1F_1 single plant that derived from backcrossing between one salt-tolerance individual from F_3 and Nip. The light blue, light green, and gray regions represent segments of the 12 chromosomes (listed as 1 to 12) derived from Nip, heterozygous, and the centromere, respectively. The mapped markers are positioned by chromosome assignment from the high-density restriction fragment length polymorphism linkage map and described in Table S1.

Table S1 Summary of 167 pairs of polymorphic SSR or InDel markers between WJZ and Nip.

Table S2 Validation of *qGR6.2* controlling salt tolerance at the seed germination stage using the BC_2F_2 population. Chr: chromosome; LOD: the likelihood of odds; PVE: variation explained by each putative QTL; ADD: the additive effect is the effect of substituting WJZ allele for Nip allele; its negative value indicates that WJZ contains the positive allele; DOM: dominance effect.

Table S3 Simple sequence repeats (SSR) and InDel markers for fine mapping of *qGR6.2*

Table S4 Primers for expression pattern analysis of candidate genes by RT-qPCR.

Tables

Table 1 Phenotypic values of seed germination for the two parents under different NaCl concentration conditions

NaCl concentration (mM)	GR (%)		SP (%)		GI	
	WJZ	Nip	WJZ	Nip	WJZ	Nip
0	100.00±0.00	99.33±0.82	100.00±0.00	99.33±0.82	13.93±0.43**	10.51±0.44
150	99.33±0.82**	87.77±2.74	99.33±0.82*	85.57±3.60	12.00±0.19**	6.62±0.30
200	98.67±0.82*	86.63±4.08	96.67±2.37**	68.90±1.35	10.47±0.11**	4.96±0.13
250	95.53±2.74*	77.77±4.90	88.90±7.20**	51.13±3.60	8.24±0.79**	3.97±0.11
300	95.53±2.74**	34.43±3.60	65.53±7.58**	1.11±0.82	6.92±0.25**	1.79±0.10
350	80.03±10.80**	12.22±3.61	26.67±8.17*	0.00±0.00	5.09±0.71**	0.76±0.15

GR: Germination rate, SP: Seedling percentage, GI: Germination index, WJZ: Wujiaozhan, Nip: Nipponbare; * and ** indicate the significant differences compared to WJZ at 5% and 1% levels, respectively.

Table 2 QTLs analysis of seed germination traits among BC1F2 population under H_2O and 300 mM NaCl conditions

Treatment	Traits	QTLs	Days after imbibition	Chr.	Left Marker	Right Marker	LOD	PVE (%)	Add	Dom
H_2O (control)	GR	*qGR3.1*	3	3	Y25	W33	3.22	7.32	-4.92	5.87
		qGR3.2	3	3	RM6832	RM3513	4.00	9.36	-5.52	6.08
		qGR3.3	3	3	RM3513	RM8277	3.57	9.75	-5.35	4.77
		qGR6.1	3	6	RM190	Z602	6.11	23.99	-8.51	3.73
		qGR8.1	2	8	RM3572	RM6208	3.32	15.75	-6.69	-14.58
		qGR8.2	2	8	RM6208	Y61	3.42	14.97	-8.81	-11.51
		qGR10.1	2	10	W13	W20	3.33	9.25	-9.95	-1.11
			3	10	W13	W20	3.33	8.95	-3.93	2.89
		qGR10.2	3	10	W20	RM6824	3.36	8.66	-4.01	3.10
	GI	*qGI6.1*		6	RM190	Z602	3.69	10.39	-0.44	0.04
		qGI10.1		10	W13	W20	3.28	8.86	-0.36	0.06
300 mM NaCl	GR	*qGR6.1*	13	6	RM190	Z602	3.14	7.07	-5.40	4.54
		qGR6.2	7	6	Z604	RM276	8.80	20.14	-1.86	-19.02
			9	6	Z604	RM276	10.56	23.82	-1.30	-18.69
			11	6	Z604	RM276	9.93	22.18	-1.24	-15.48
			13	6	Z604	RM276	10.58	23.54	-1.19	-12.69
		qGR8.1	5	8	RM3572	RM6208	4.00	18.80	-7.76	-8.00
		qGR8.2	5	8	RM6208	Y61	3.84	14.48	-7.17	-6.24
		qGR10.1	5	10	W13	W20	3.89	11.74	-7.65	-3.37
			7	10	W13	W20	5.69	14.07	-11.00	2.90
			9	10	W13	W20	6.19	14.90	-9.81	3.75
			11	10	W13	W20	6.59	16.22	-8.63	4.33
			13	10	W13	W20	5.94	14.35	-6.54	3.15
		qGR10.2	7	10	W20	RM6824	4.40	9.49	-9.66	0.59
			9	10	W20	RM6824	5.35	11.20	-9.30	0.92
			11	10	W20	RM6824	5.78	12.16	-8.36	1.08
			13	10	W20	RM6824	5.53	11.52	-6.52	1.19
		qGI6.2		6	Z604	RM276	11.20	24.39	-0.05	-0.72
	GI	*qGI10.1*		10	W13	W20	7.50	17.41	-0.41	0.14
		qGI10.2		10	W20	RM6824	6.50	13.18	-0.38	0.05

GR: Germination rate, GI: Germination index, Chr: chromosome, LOD: the likelihood of odds, PVE: Variation explained by each putative QTL, ADD: the additive effect is the effect of substituting WJZ allele for Nip allele; its negative value indicates that WJZ contains the positive allele, DOM: Dominance effect.

Table 3 Predicted candidate genes of *qGR6.2*

Number	Candidate genes	Putative protein function
ORF1	*LOC_Os06g10650*	Tyrosine phosphatase family protein, putative, expressed
ORF2	*LOC_Os06g10660*	LysM domain-containing GPI-anchored protein 1 precursor, putative, expressed
ORF3	*LOC_Os06g10670*	Aspartic proteinase nepenthesin-1 precursor, putative, expressed
ORF4	*LOC_Os06g10680*	Expressed protein
ORF5	*LOC_Os06g10690*	PHD-finger domain containing protein, putative, expressed
ORF6	*LOC_Os06g10700*	Expressed protein
ORF7	*LOC_Os06g10710*	Expressed protein
ORF8	*LOC_Os06g10720*	Expressed protein
ORF9	*LOC_Os06g10730*	Expressed protein
ORF10	*LOC_Os06g10740*	Transposon protein, putative, unclassified, expressed
ORF11	*LOC_Os06g10750*	Integral membrane protein DUF6-containing protein, expressed

Figures

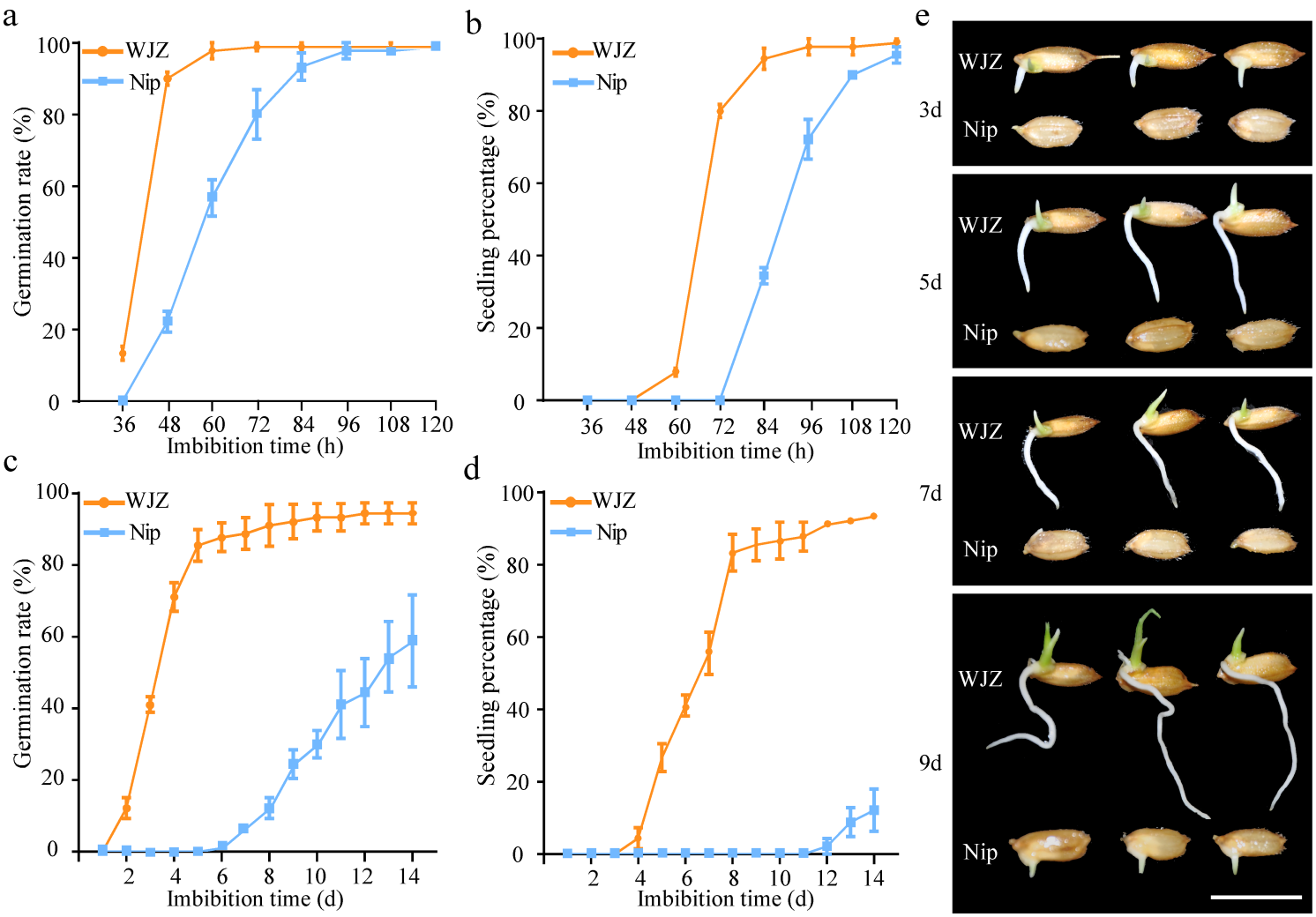

Figure 1

Evaluation of seed germination and seedling establishment between WJZ and Nip under H2O and 300 mM NaCl conditions. (a-b) Quantification and statistical analysis of germination rate (a) and seedling percentage (b) of two-parent varieties from 36 h to 120 h under H2O condition. (c-d) Quantification of germination rate (c) and seedling percentage (d) of two-parent varieties from 1 to 14 d at 300 mM NaCl. Each point represents the mean ± standard deviation. (e) Morphology of two varieties during seed germination after 3, 5, 7 and 9 d at 300 mM NaCl. Scale bar = 1 cm.

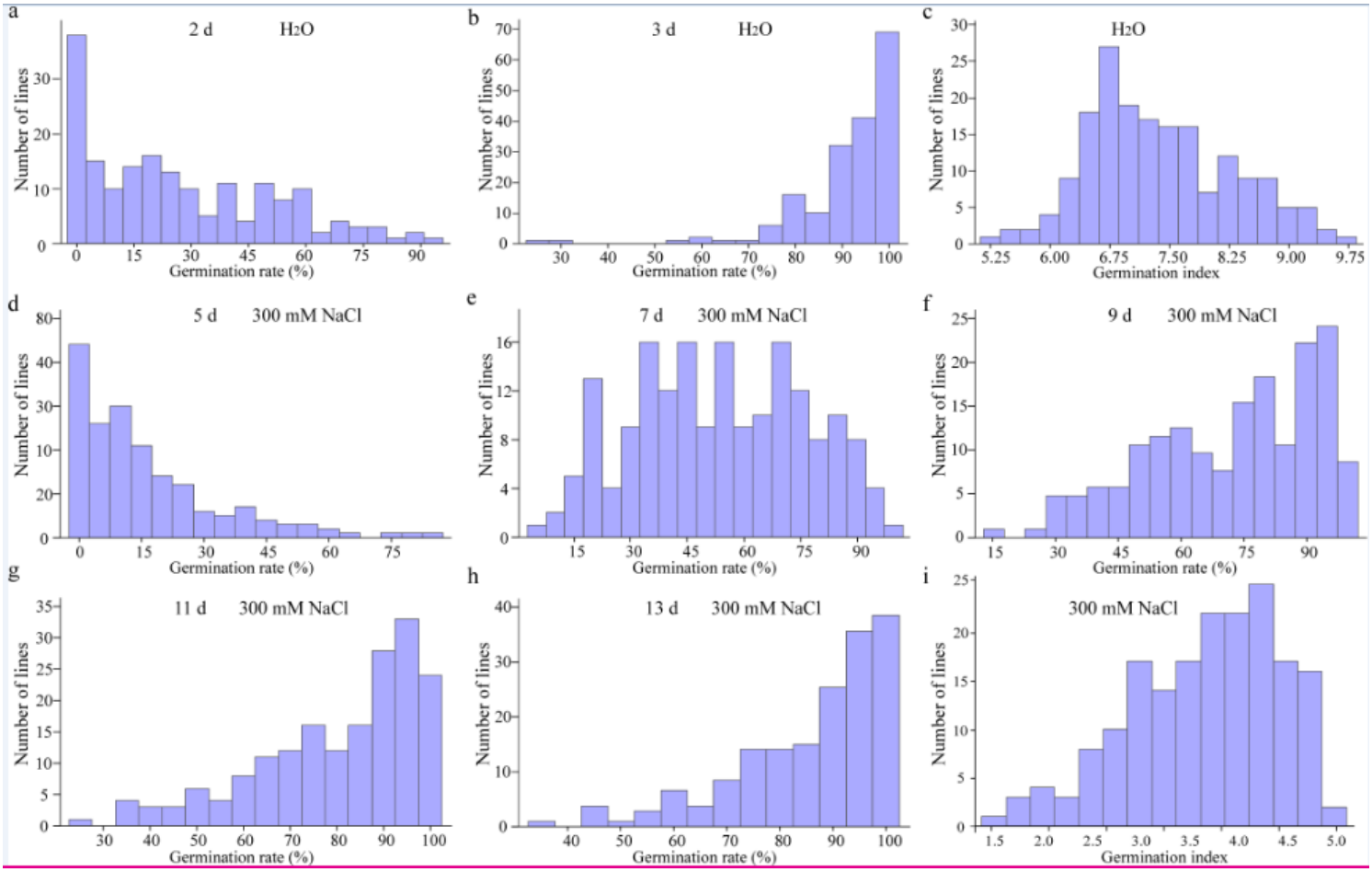

Figure 2

Performance of seed germination traits in the BC1F2 population on different days under H2O and 300 mM NaCl conditions. Performance of GR after 2 d (a) and 3 d (b) imbibition, and GI (c) under H2O condition, GR after 5 d (d), 7 d (e), 9 d (f), 11 d (g), 13 d (h) imbibition and GI (i) under 300 mM NaCl condition.

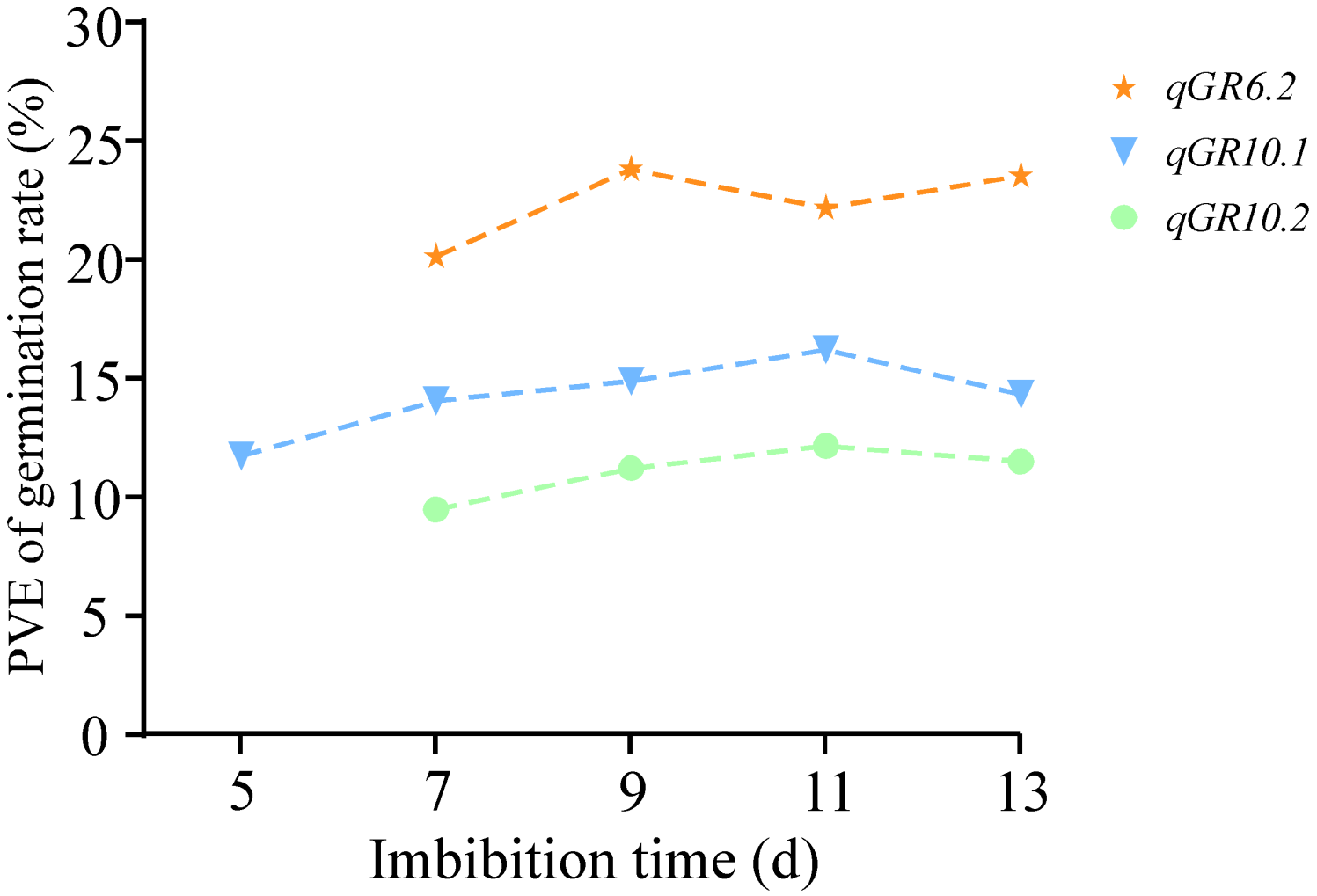

Figure 3

Putative continuously identified QTLs controlling GR in the BC1F2 population under 300 mM NaCl conditions from 5 to 13 d

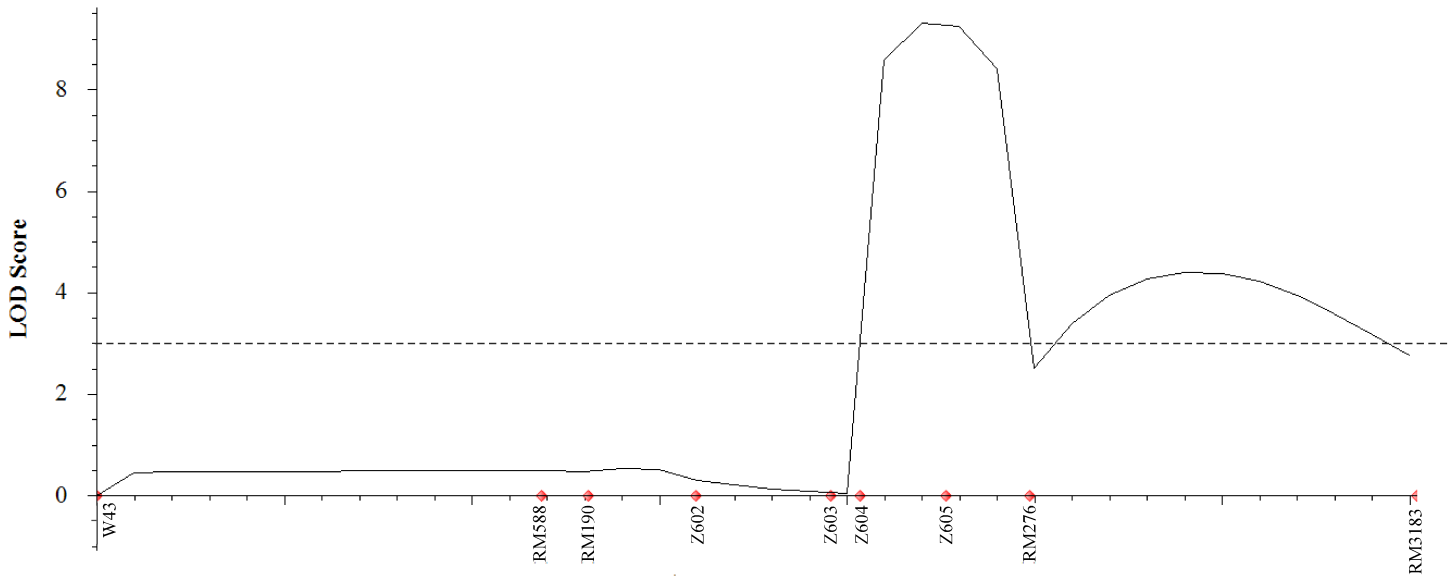

Figure 4

LOD score curves of qGR6.2 controlling the GR of the BC2F2 population at 13 d in 300 mM NaCl conditions. The LOD curve indicates the strength of evidence for the presence of qGR6.2 within Z604~Z605 on chromosome 6. Dashed lines show LOD thresholds of 3. 0. Marker names and genetic distances are shown below the chromosome.

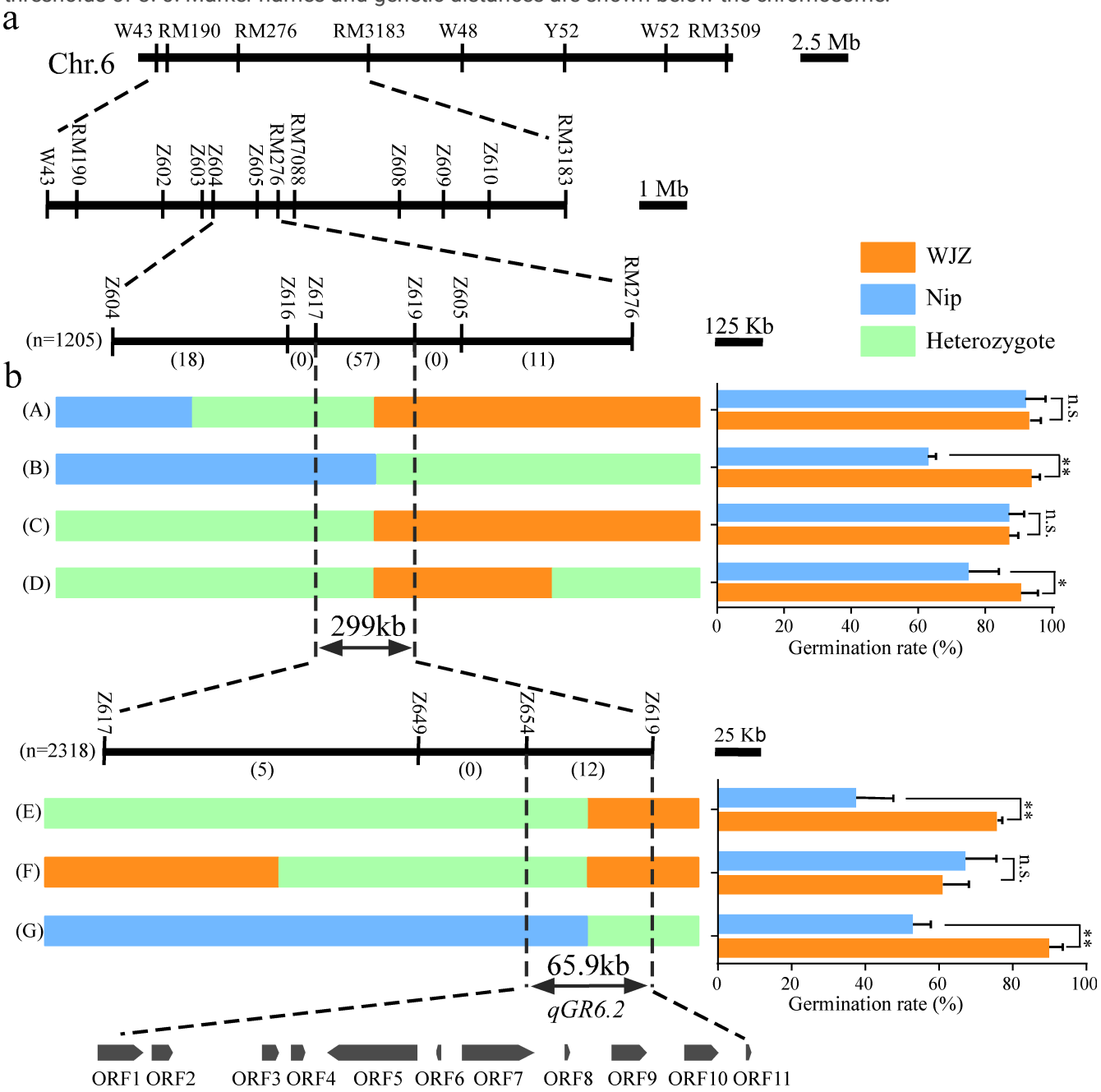

Figure 5

Fine mapping of qGR6.2. (a) A high-resolution physical map of qGR6.2 linked two markers, Z604 and RM276, on the short arm of chromosome 6. Numbers between two markers represent recombinant events. (b) Progeny tests of homozygous recombinants delimited qGR6.2 to an approximately 65.9-kb region flanked by the markers Z654 and Z619. Orange bars, light blue bars and light green bars on the left represent the WJZ, Nip and heterozygous genotypes, respectively. On the right, the bar diagram refers to the mean GR of progeny separated from the heterozygous region in each recombinant group after imbibition for 10 d, orange and light blue represent the WJZ and Nip genotypes of the heterozygous region in each recombinant group respectively. The physical map is based on RAP-DB (http://rapdb.lab.nig.ac.jp/index.html). Arrows indicate eleven ORFs in this region according to the Rice Genome Annotation Project (RGAP).

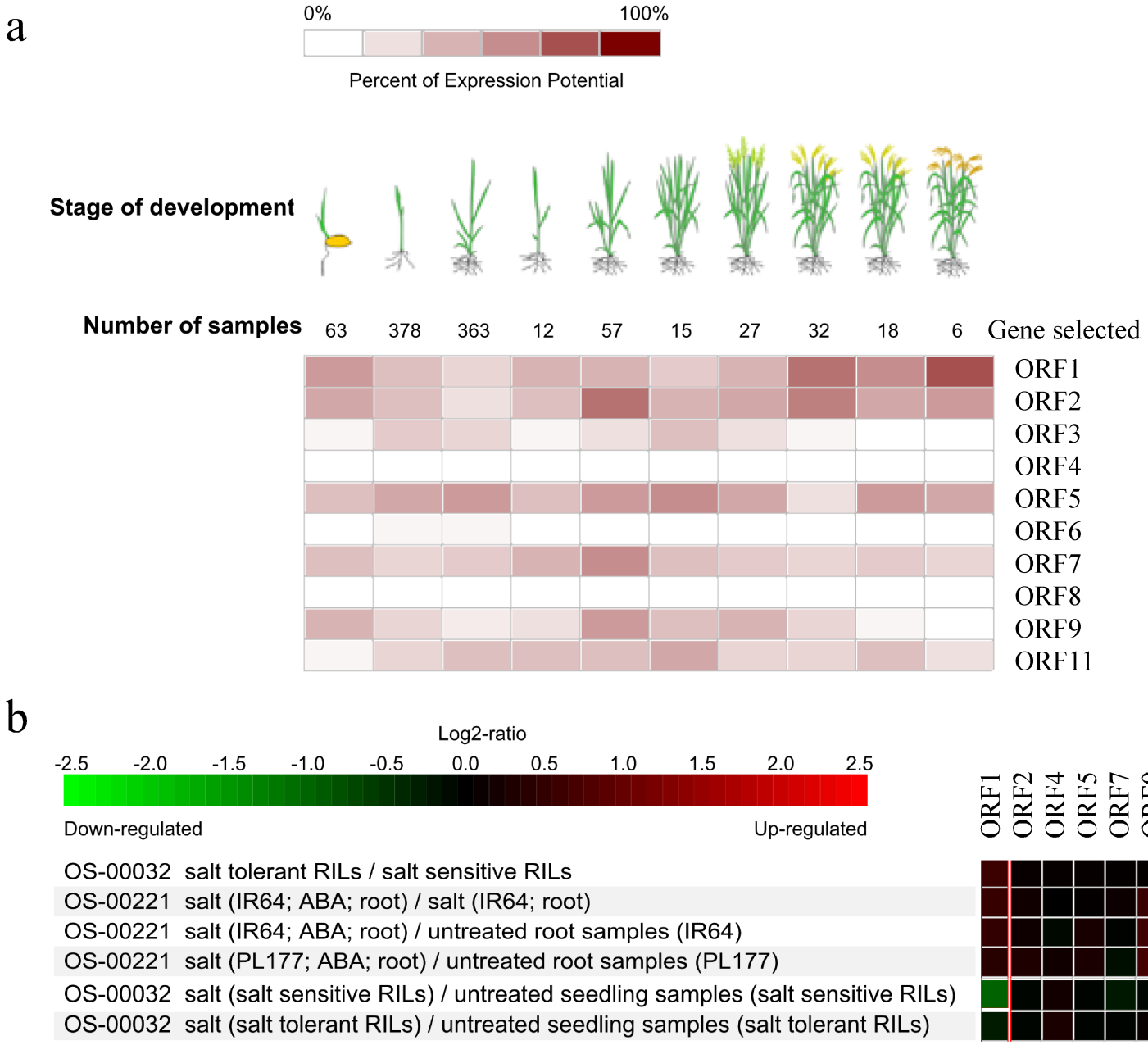

Figure 6

Expression patterns of candidate genes in various developmental stages (a) and response to salt stress (b) based on mRNA-Seq data and Affymetrix microarray datasets from GENEVESTIGATOR (http://www.genevestigator.com).

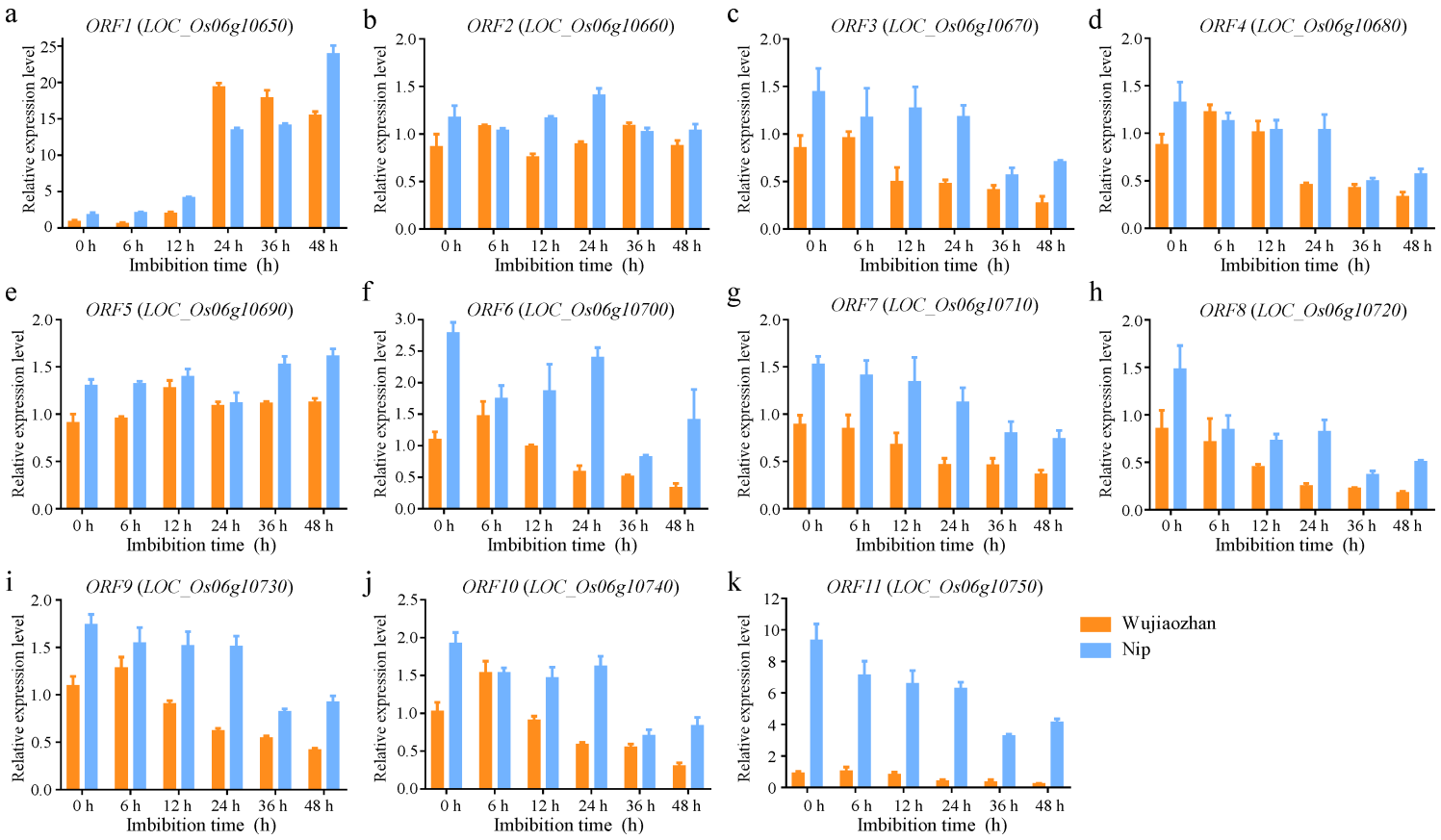

Figure 7

Expression levels of candidate genes between WJZ and Nip during seed germination at 300 mM NaCl. Expression analysis of 11 ORFs (a-k) after seed imbibition for 0 h, 6 h, 12 h, 24 h, 36 h and 48 h at 300 mM NaCl between WJZ and Nip. Data represent the mean ± SD (n=3).

Supplementary Files

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