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RESEARCH ARTICLE

Salinity inhibits seed germination and embryo growth by reducing starch mobilization efficiency in barley

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Abstract

Barley is one of the world's earliest domesticated crops, which is widely used for beer production, animal feeding, and health care. Barley seed germination, particularly in increasingly saline soils, is key to ensure the safety of crop production. However, the mechanism of salt-affected seed germination in barley remains elusive. Here, two different colored barley varieties were used to independently study the regulation mechanism of salt tolerance during barley seed germination. High salinity delays barley seed germination by slowing down starch mobilization efficiency in seeds. The starch plate test revealed that salinity had a significant inhibitory effect on α-amylase activity in barley seeds. Further, NaCl treatment down-regulated the expression of *Amy1*, *Amy2* and *Amy3* genes in germinated seeds, thereby inhibiting α-amylase activity. In addition, the result of embryogenic culture system in vitro showed that the shoot elongation of barley was significantly inhibited by salt stress. These findings indicate that it is a feasible idea to study the regulation mechanism of salinity on barley seed germination and embryo growth from the aspect of starch-related source-sink communication.

KEYWORDS
barley, salinity, seed germination, starch mobilization, α-Amylase activity

1 | INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the most widely produced cereal crops worldwide. Besides being used for brewing beer and feeding animals, barley is also traditionally cooked along with rice in Asia. Since barley has similar nutritional value as corn and wheat, it is also processed into diverse foods, including pasta, breakfast cereals, baked products, and noodles (Islam et al., 2021). Therefore, whether it is used as a food crop or a cash crop, barley production is the key to ensuring human food security. Barley seeds with excellent performance during grain development, seed dormancy, and germination are critical for guaranteeing high yield and quality, which also is the key to meeting the high production and market demand.

Barley seeds contain the caryopsis, which can be divided into two types depending on whether the kernel has a hull or not. The structure of hulled barley seeds includes husk, pericarp, testa, aleurone layer, endosperm, and embryo. In cereal seeds, the embryo contains most of the genetic information, while the endosperm stores the nutrients and is the edible part (Diaz et al., 2019; Gomez et al., 2021). As the dominant component of the barley seed, starch comprises

amylose and amylopectin. Amylose is synthesized by the granule-bound starch synthase (GBSS) encoded by *Waxy*, while amylopectin is synthesized by various forms of the starch synthase (Huang, Sreenivasulu, & Liu, 2020; Källman et al., 2015; Li et al., 2018). Editing these starch synthesis genes in barley grains can affect their amylose content, resistant starch content, and starch fine structure, all of which are associated with their appearance quality, eating and cooking quality, nutritional quality, and germination traits (Huang, Li, et al., 2020).

As the planting of barley is mainly direct sowing, seed germination traits have always been the focus of the improvement and breeding of elite barley varieties. High-vigor seeds that can improve germination and seedling rates are suitable for direct seeding production with reduced labor cost and high work efficiency (Yu et al., 2021). Several genes, including *cathepsin B-like* (*HvPap-19*), *cathepsin F-like* (*HvPap-1*), and *proteinaceous inhibitors of cathepsin F-like* (*Icy-2*), which simultaneously regulate grain filling, composition, and germination, have been found in barley (Diaz et al., 2016; Gomez et al., 2021). Briefly, seed germination is a well-ordered series of physiological and morphological changes post imbibition and expansion of seeds, which usually begins with the rapid absorption of water and ends with radicle protrusion (Bewley, 1997; Li et al., 2016; Xiong, Feng, et al., 2022). In the early stage of germination, the activation and mobilization of the seed reserve provide most of the nutrients for the growth of heterotrophic embryos via the source-sink communication (Xiong et al., 2021). The classical model of starch degradation during barley germination postulates that the secretion of gibberellins (GAs) from the embryo to the endosperm cells via the scutellum, triggers the early seed reserve mobilization (Andriotis et al., 2016; Ritchie et al., 2000). GA in aleurone cells promotes amylase synthesis, which then is secreted to promote the degradation of starch to glucose (Gubler et al., 1995; Jacobsen & Beach, 1985). The more resistant amylose-only starch barley line shows a slower post-germination growth state than the wild-type control because of slower starch degradation efficiency based on weaker α-amylase activity during germination (Shaik et al., 2014). This mechanism by which amylase promotes the starch conversion into sugars that subsequently affects the germination and post-germination growth is common in cereal crops (Diaz et al., 2019; Wang et al., 2021; Xia et al., 2011; Xiong, Yu, et al., 2022).

Salt stress is a key environmental factor limiting plant growth, which leads to osmotic stress, ionic stress and secondary stresses, particularly oxidative stress (Yang & Guo, 2018). Special attention should be paid to the harm of salinity to the morphogenesis of seed germination and seedling stage in crop, which will affect the subsequent development and reproduction stage. Liu et al. found that salinity inhibited rice seed germination by reducing the content of bioactive GAs, and exogenous bioactive GA could relieve this inhibition (Liu et al., 2018). In addition to decreasing the content of endogenous GA, increasing the content of endogenous ABA is also a feasible way to delay cotton seed germination (Chen et al., 2021). In addition, ABA also indirectly down-regulated the α-amylase activity during seed germination (Damaris et al., 2019).

In China's eastern coastal areas, barley is often grown as a pioneer crop for salt tolerance. In this present study, two local barley varieties (recently selected and cultivated by Jiangsu Coastal Area Institute of Agricultural Sciences) with good sensory and beer-making properties were used to study the mechanism of salinity affecting germination. Our study aims to investigate the important role of high salinity inhibition on starch mobilization efficiency in delaying barley seed germination and embryo growth.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

Two-rowed barley Yanmai7 (YM7) and Y12133 with good beer-making properties were used in this study. YM7 was obtained by crossing Supi4 and Shan2, while Y12133 was obtained from Yanhei1 × C2118. For grain quality analyses, two barley varieties were planted in the same field at the experimental farm of Jiangsu Coastal Area Institute of Agricultural Sciences in Yancheng, Jiangsu, China. During their growth period, the standard procedures for field management, disease treatment, and pest control were followed to prevent yield loss. All mature barley seeds were harvested and air-dried on the same day.

2.2 | Analysis of seed germination

Germination analysis of barley seeds was performed as described earlier (Gomez et al., 2021) with minor modifications. For each experiment, 30 barley seeds were sterilized with 70% (v/v) ethanol and washed twice with Milli-Q water. Sterilized seeds were placed in 10 cm × 10 cm culture plates and imbibed in solutions with 250 mM sodium chloride (NaCl) treatment or control treatment. The seeds were subsequently germinated in darkness in an artificial climate incubator (26 °C and 70% relative humidity). Seeds with a broken grain coat or testa were considered as successfully germinated (Diaz et al., 2016). The lengths of the shoots and roots of the germinated seeds were measured at the indicated time points using the ImageJ software. Each seed germination assay included at least three independent biological replicates.

2.3 | Qualitative analysis of α-amylase activity

The α-amylase activity was determined qualitatively using the starch board test method as described previously (Xie et al., 2007). The barley seeds were fully sterilized by soaking in 70% (v/v) ethanol for 20 minutes, and then washed twice with Milli-Q water. The embryoless half seeds were then transferred on 2% (w/v) agar in petri dishes and incubated in the dark at 28 °C for 3 days. The agar plate contained .2% potato starch, 20 mM calcium chloride, and 20 mM sodium succinate (pH 5.0). After incubation, the starch plate was soaked in iodine solution [.1% I_2 (w/v) and 1% KI (w/v)] for 5 minutes. The size of colorless haloes around the half seeds caused by starch hydrolysis is positively correlated with α-amylase activity. After being photographed, the diameter data of the haloes were measured using the ImageJ software.

2.4 | Analysis of total starch contents

The barley kernels were ground into powder using a coffee machine. The total starch content of the milled barley flour was determined using a K-TSTA total starch assay kit (Megazyme, Wicklow, Ireland). The detailed test steps follow the manufacturer's instructions.

2.5 | RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

At least 30 barley seeds were used for RNA extraction. Total RNA from germinated seeds was extracted with an RNAprep Pure Plant kit (Tiangen, Beijing, China). The first strand of cDNA was synthesized from 1.5 μg of total RNA in a 30 μl reaction volume with a FastQuant RT kit (Tiangen, Beijing, China). qRT-PCR was performed in 96-well blocks on a MyiQ real-time system (Bio-Rad, California, USA) using the 2 × SYBR Premix UrTaq II (Nobelab, Beijing, China). Three biological replicates were included for each sample. *α-Tubulin* was used as an internal control to normalize expression of the target genes. The primers for qRT-PCR were used in previous studies (Shen et al., 2020; Sheng et al., 2018), and their details are shown in Supplemental Table S1.

2.6 | In vitro shoot elongation assay

The in vitro embryo culture was performed following the published protocol in rice with minor modifications (Xiong et al., 2021). Briefly, the plumules were carefully isolated from barley seeds 4 h after imbibition (HAI) and cultured in 250 mM NaCl solution. After 72 hours of growth, the lengths of elongated shoots originating in the plumules were measured using the ImageJ software.

2.7 | Statistical analysis

All data are presented as means ± standard deviation (SD). The student's *t*-test was used to identify the level of significance for experiments with a single pairwise comparison (* $p < .05$, ** $p < .01$). Data from experiments with multiple comparisons were analyzed with Duncan's multiple range test at $p < .05$ (with different letters).

3 | RESULTS

3.1 | Two barley varieties have excellent yield components

As beer-producing varieties, YM7 and Y12133 seeds are converted into malt through the malting process when brewing, with the malt amount being closely related to the barley yield (Tomasi et al., 2019). The investigation results indicate that YM7 and Y12133 have the potential to achieve high yield when it comes to yield characters. There was no difference between the two barley varieties in terms of grain length, grain width, grain thickness, and 1,000-grain weight, with the number of grains per panicle being almost indistinguishable (Figure 1 and Table 1). The seed husks of the Y12133 and YM7 were black and yellow, respectively (Figure 1).

3.2 | High salinity delays barley seed germination

With the continuous water absorption of barley seeds, the dormancy-breaking seeds showed changes in their germination rate, shoot length, and radicle length, which are important indicators that reflect the germination process (Diaz et al., 2016). Under the treatment of

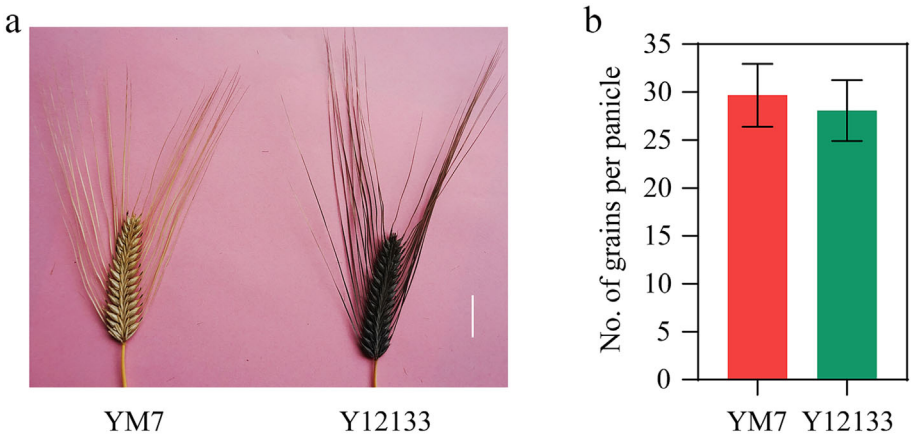

FIGURE 1 Panicle traits of the two barley varieties. (a) Shows the morphology of barley panicles. Scale bar = 20 mm. (b) Represents the number of grains per panicle of barleys. Error bars represent the SDs of three biological replicates (n = 3, each replicate contained 10 panicles).

TABLE 1 Grain traits of the two barley varieties.

	Grain length (mm)	Grain width (mm)	Grain thickness (mm)	1,000-grain weight (g)
YM7	8.86 ± .34	3.73 ± .04	2.78 ± .1	46.36 ± 1.61
Y12133	9.01 ± .39	3.7 ± .05	2.77 ± .12	47.1 ± .81

250 mM sodium chloride (NaCl), the salt tolerance phenotype of barley seeds during germination was investigated. After NaCl treatment, a noticeable delay in both germination and post-germination growth was observed, whether in YM7 or Y12133 (Figures 2 and 3). At each measured time point, the germination rate of the NaCl treatment was consistently lower than its corresponding control group, with a significant difference of approximately 50% (Figure 3a). Additionally, the shoot length of barley was observed to be significantly shorter under salt stress condition compared with the control group at 72 h after imbibition (HAI) (Figure 3b). The radicle length change was also consistent with that of shoot length at 72 HAI (Figure 3c). In conclusion, high salinity can inhibit barley seed germination, including shoot and radicle growth.

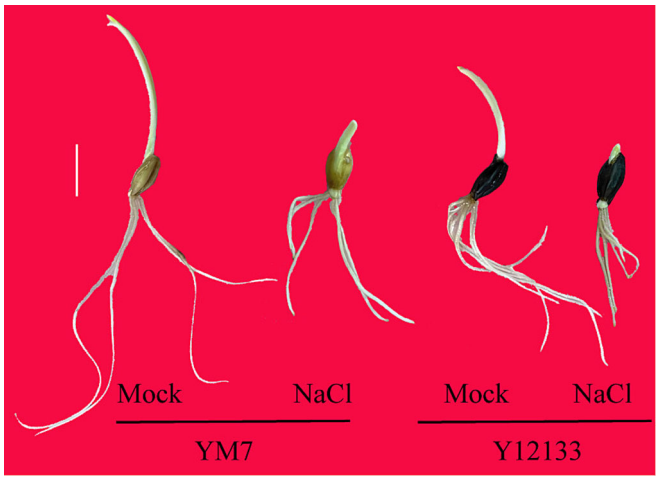

FIGURE 2 Morphology of the germinating seeds at 72 h after imbibition of YM7 and Y12133 with or without 250 mM NaCl. Scale bar = 10 mm.

3.3 | Starch mobilization efficiency in germinated barley seeds weakened by NaCl treatment

In barley, starch mobilization determines the process of seed germination. To investigate the correlation between NaCl treatment and germination delay, we analyzed the starch content of seeds both at the initial stage (0 HAI) and 72 HAI. The study found that the starch content in YM7 seeds was 56.30% at 0 HAI (Figure 4a). At 72 HAI, the starch content of YM7 treated with NaCl and the control group decreased to 52.89% and 50.48%, respectively (Figure 4b). Subsequent analysis revealed that the seeds treated with NaCl experienced

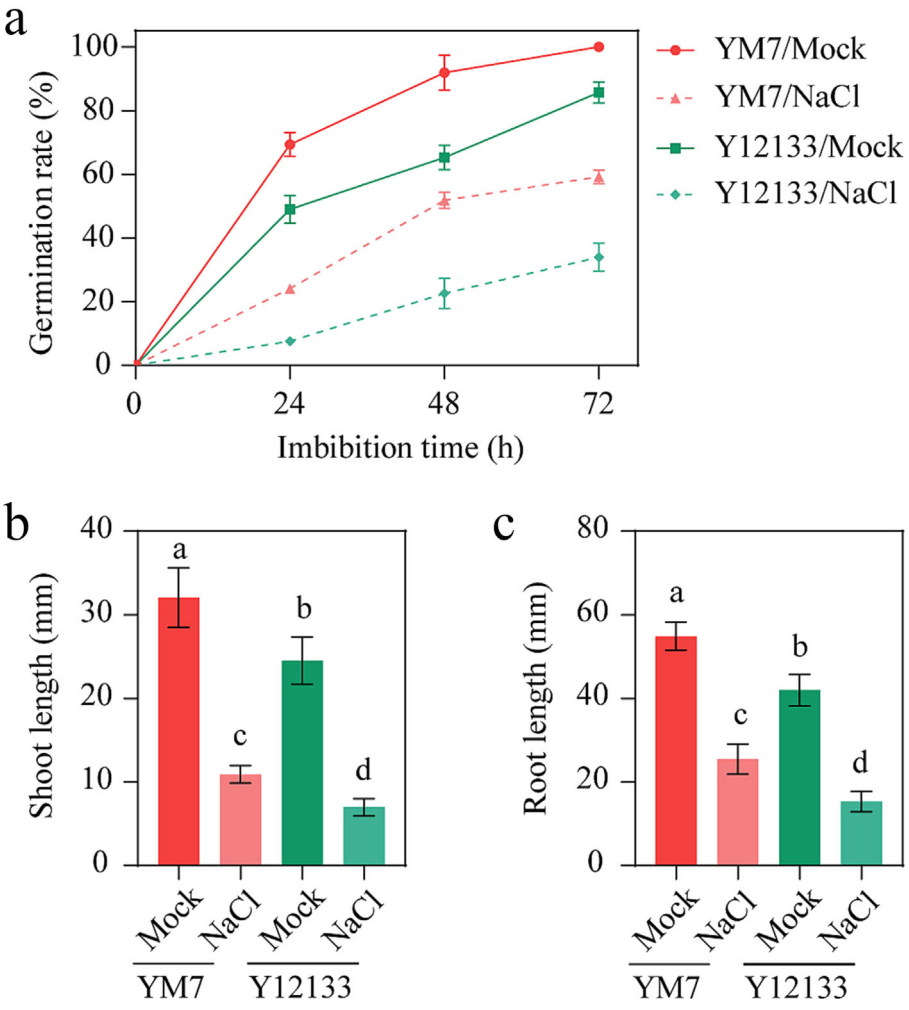

FIGURE 3 Salt stress inhibited the seed germination of barley. (a) the germination rate from 0 to 72 h after imbibition (HAI). (b) the shoot length at 72 HAI with or without 250 mM NaCl. (c) the root length at 72 HAI with or without 250 mM NaCl. Error bars represent the SD (n = 3, each replicate contained 30 seeds). Different letters indicate the significant differences at $p < .05$ by Duncan's multiple range test.

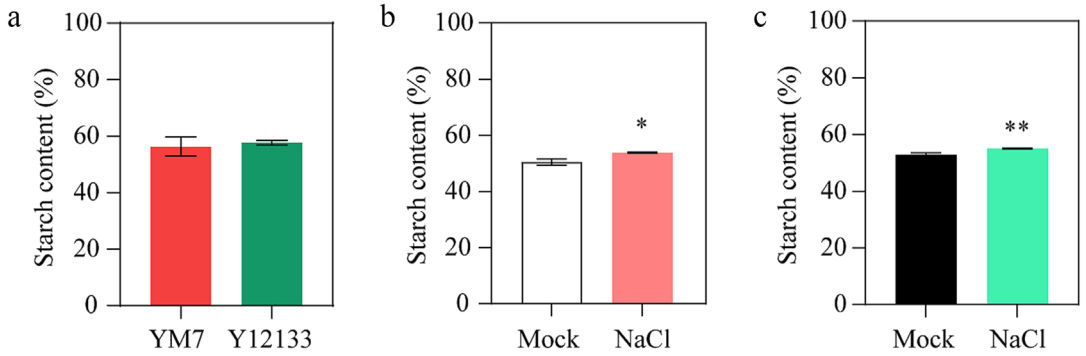

FIGURE 4 Determination of starch content in barley seeds. (a) Seeds of YM7 and Y12133 germinated for 0 hours. (b) Seeds of YM7 germinated for 72 hours under NaCl treatment. (c) Seeds of Y12133 germinated for 72 hours under NaCl treatment. Error bars represent the SDs of the three biological replicates (n = 3, each replicate contained 10 seeds). *, $p < .05$; **, $p < .01$ (Student's *t*-test).

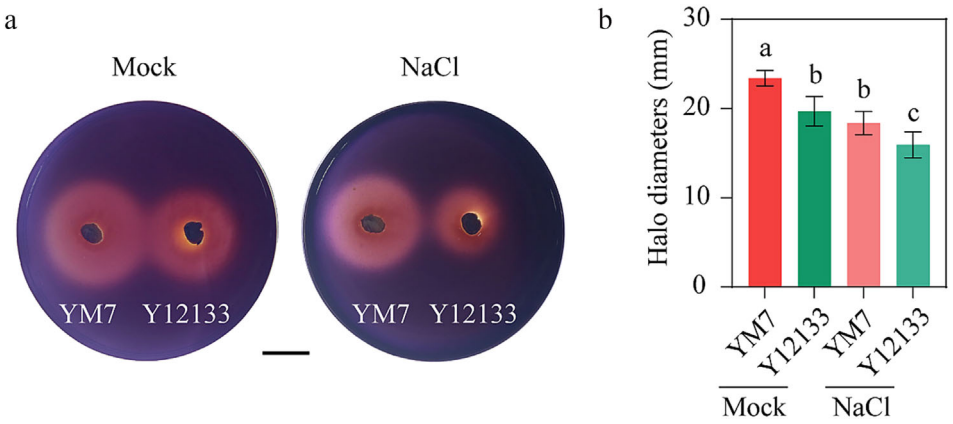

FIGURE 5 Analysis of α-amylase activity after NaCl treatment. (a) the qualitative comparison of α-amylase activity with or without NaCl treatment at 72 HAI. Scale bar = 10 mm. (b) Quantitative analysis of the halo diameters in the starch plate test. Error bars represent the SDs of the three biological replicates (n = 3, each replicate contained 10 seeds). Different letters indicate the significant differences at $p < .05$ by Duncan's multiple range test.

a 3.41% reduction in starch content, whereas the control group experienced a 5.82% reduction. Similar starch results were also observed in Y12133 seeds treated and untreated by NaCl (Figure 4c). These findings suggest that NaCl treatment may have hindered barley seed germination by slowing down the reduction of starch content.

3.4 | High salinity inhibits α-amylase activity in barley seeds

α-amylase mobilizes the degraded storage starch, which positively regulates the barley seed germination (Andriotis et al., 2016). Therefore, we study whether NaCl also regulates seed germination by the starch plate test, a method for qualitative comparison of α-amylase activity. Briefly, the embryo-less half-seeds can secrete α-amylases to degrade starch in plate, thus producing the colorless halo around the seed. There is a positive correlation between halo diameter and α-amylase activity of seed. Our results showed that the halo diameters around YM7 and Y12133 in the salt-treated plate were smaller than those in the control plate (Figure 5a). In more detail, high salinity reduced the halo diameter of YM77 by 32.02% and Y12133 by 28.85%, suggesting that reduced α-amylase activity could be one important reason for the delayed seed germination with NaCl treatment (Figure 5b).

To understand the difference of Na/K accumulation in germinated barley seeds under salt stress, we detected the expression of some salt-responsive genes, including three salt overly sensitive genes (*SOS1*, *SOS2* and *SOS3*), three Na^+/H^+ antiport genes (*NHX1*, *NHX3*, *NHX5*) and one high affinity potassium transporter gene (*HKT1;5*) (Fu et al., 2018). The results showed that the expression of these genes was up-regulated in seeds treated with NaCl at 36 HAI and 72HAI (Figure 6). To further study which α-amylase family member directly connects the salt pathway and seed starch degradation, we also examined the expression of α-amylase genes in barley. The qRT-PCR revealed that the expression of *Amy1*, *Amy2* and *Amy3* genes decreased significantly in YM7 after NaCl treatment at 36 HAI and 72 HAI (Figure 6a and c). Similar results were found in Y12133 (Figure 6b and d). These results are consistent with our starch mobilization and amylase qualitative data, indicating that high salinity can inhibit the activity of α-amylase in germinated barley seeds.

3.5 | High salinity hinders shoot elongation

Salt affects the degradation efficiency of starch in germinated seeds, which leads to different sugar supply required for embryo growth. To eliminate the potential interference from the endosperm, we used the in vitro embryogenic culture system to study whether embryo growth

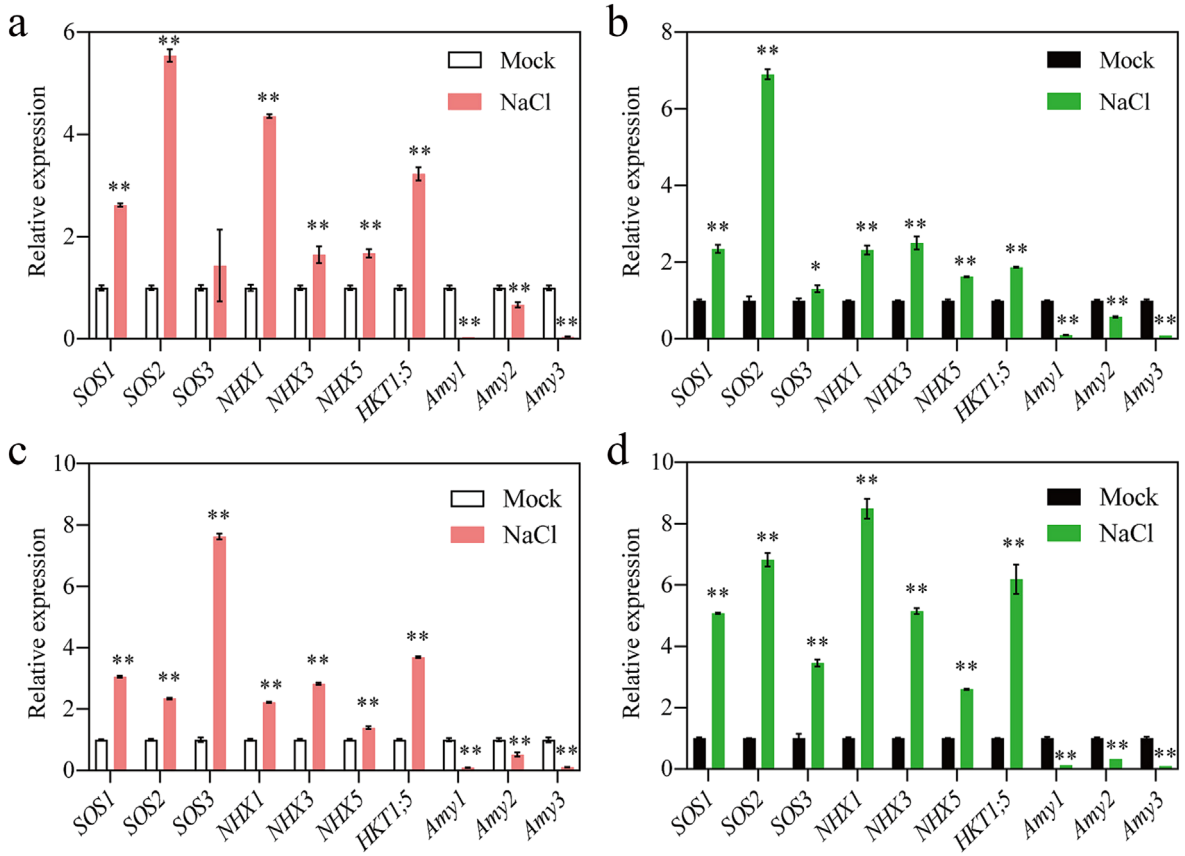

FIGURE 6 Expression of salt-responsive genes and α-amylase genes is regulated by NaCl treatment in barley seeds. (a) qRT-PCR analysis in YM7 at 36 HAI. (b) qRT-PCR analysis in Y12133 at 36 HAI. (c) qRT-PCR analysis in YM7 at 72 HAI. (d) qRT-PCR analysis in Y12133 at 72 HAI. Error bars represent the SDs of the three biological replicates (n = 3, each replicate contained 20 seeds). *, $p < .05$; **, $p < .01$ (Student's *t*-test).

was affected by salinity under equal sugar supply. In this assay, isolated plumules at 2 HAI were cultured in vitro. After 72 hours of cultivation, the shoot length under NaCl treatment was significantly shorter than that of the control, which was observed in both two barley varieties (Figure 7).

In summary, all data show that NaCl treatment can delay barley seed germination both by inhibiting starch mobilization and by preventing shoot elongation.

4 | DISCUSSION

Barley, as one of the most salt-tolerant cereals, may be a pioneer in shifting crop production to saline lands. Therefore, much attention has been paid to breeding and deciphering the underlying mechanism of salt-tolerant barley varieties (Gupta et al., 2022; Hura, 2020; Jadidi et al., 2022; Jiang et al., 2022; Munns & Tester, 2008). After growing in the solution or sand culture containing NaCl for 3 weeks, barley showed a higher increase of the shoot dry matter than other cereals, including rice (*Oryza sativa*), durum wheat (*Triticum turgidum ssp durum*), and bread wheat (*Triticum aestivum*) (Munns & Tester, 2008). However, at 250 mM NaCl, the shoot dry matter of barley hardly increased. Interestingly, low salinity (1–20 mM NaCl) promoted seedling growth as salt stimulates the synthesis of phenolic components by promoting related gene and protein expression and key enzyme activity (Wang et al., 2020). Considering that most barley seeds are planted in coastal areas by direct seeding production, it is particularly important to study the mechanism of high salt tolerance during seed germination. In this study, two barley varieties with good yield characters and different seed husk colors were used to test the salt tolerance during seed germination. High salinity could delay the germination process by more than 48 h, along with the shortening of shoot length and root length (Figures 2 and 3). Ion transporters, including *SOS*, *HKT* and *NHX* family members, play key roles in Na and K transporting and K/Na homeostasis under salt stress (Munns & Tester, 2008; Shen et al., 2020). We also detected the expression of *SOS1*, *SOS2*, *SOS3*, *NHX1*, *NHX3*, *NHX5* and *HKT1;5* in barley seeds, and the results showed that they were up-regulated in response to salt signal (Figure 6).

In cereal, source-sink communication and its regulatory mechanism during seed germination is a classical research direction of plant science. Once the germination starts, there will be a supply chain of carbon flowing from the endosperm (source) to the heterotrophic embryo (sink) (Lee et al., 2014; Yu et al., 2015). For source, the conversion of starch to glucose in endosperm is catalyzed by various hydrolytic enzymes including α-amylase. In sink, the turned-

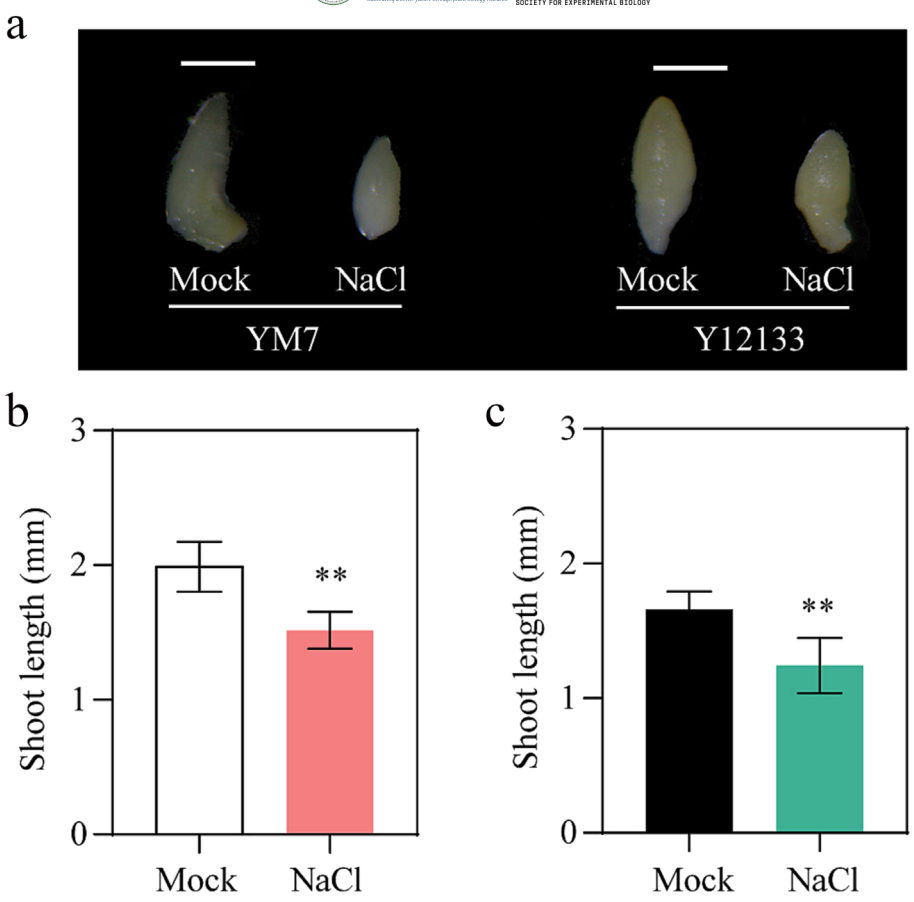

FIGURE 7 In vitro shoot elongation assay in response to NaCl treatment. (a) Physiological morphology of shoot length following treatment with salinity for 72 h. (b) Quantitative data of shoot length in YM7. (c) Quantitative data of shoot length in Y12133. Error bars represent the SDs of the three biological replicates (n = 3, each replicate contained 8 plumules). **, $p < .01$ (Student's *t*-test).

over glucose is used directly for growth or for the synthesis of other polysaccharides (Xiong et al., 2021). Interfering with any other link can affect the original process of seed germination. The best example in barley is that gibberellin (GA) accelerates starch mobilization by increasing the secretion of α-amylase to promote seed germination (Gubler & Jacobsen, 1992; Rajjou et al., 2012; Washio, 2003). NaCl treatment reduced starch mobilization in barley (Figure 4), indicating that the gene expression of α-amylase was inhibited by salt signal. Therefore, we used the starch plate test to prove that high salinity inhibited the α-amylase activity in YM7 and Y12133 (Figure 5). Further, qRT-PCR test also confirmed that the expression of α-amylase genes *Amy1*, *Amy2* and *Amy3* was down-regulated by salt signal (Figure 6). It has been found in rice that NaCl treatment reduces bioactive GA content to inhibit seed germination by decreasing α-amylase activity via down-regulation of α-amylase gene expression (Liu et al., 2018). Exogenous salicylic acid (SA) can reduce the damage of salinity to rice seed germination by positively regulating GAs and ABA homeostasis (Liu et al., 2022). Further, Li et al. found that ABA-mediated salt stress tolerance in rice depends on the short-term brassinosteroid (BR) signal activation (Li et al., 2021). As to whether these regulatory mechanism exists in barley, more evidence of the interaction between salt stress and plant hormone is still needed.

In addition to starch mobilization in barley, we had also studied whether salt stress can interfere with the embryo growth. The result of embryogenic culture system showed the barley shoot growth in vitro was significantly inhibited by salt stress (Figure 7). Therefore, it is necessary to pay attention to the direct inhibition of salinity on embryo growth to reduce the delayed barley germination.

AUTHOR CONTRIBUTIONS

Conceptualization, Min Xiong and Gongneng Feng; methodology, Bin Peng; software, Zhou Zhou; validation, Huiquan Shen and Xiao Xu; formal analysis, Gongneng Feng; investigation, Jian Xu and Min Xiong; resources, Yuxiang Shen; data curation, Changya Li and Lina Deng; writing—original draft preparation, Min Xiong; writing—review and editing, Jian Xu and Gongneng Feng; supervision, Yuxiang Shen; project administration, Min Xiong; funding acquisition, Min Xiong, Gongneng Feng and Bin Peng. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

PEER REVIEW

The peer review history for this article is available in the Supporting Information for this article.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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SUPPORTING INFORMATION

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