ACADEMIA JOURNAL OF BIOLOGY 2025, 47(4): 103–111
DOI: 10.15625/2615-9023/22680

RESEARCH ON SALT TOLERANCE OF *OsProDH*-MUTANT BAC THOM 7 RICE VARIETY

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Received 8 April 2025; accepted 4 December 2025

ABSTRACT

Salt stress severely impacts rice productivity worldwide, with increasing soil salinization threatening food security in many regions. This study examined the physiological function of proline dehydrogenase (*OsProDH*) in salt stress responses using a Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9)-edited Bac Thom 7 (BT7) rice line carrying a 39-nucleotide deletion in the *OsProDH* gene. Seedlings of wild-type and mutant plants were subjected to 100−200 mM NaCl treatments to evaluate biochemical and physiological responses. Free proline quantification revealed distinct accumulation patterns, with the *OsProDH* mutant maintaining significantly higher proline levels before stress (1.8-fold) and during recovery (1.6-fold). Oxidative stress markers showed that the mutation significantly reduced salt-induced reactive oxygen species (ROS) accumulation, with H_2O_2 content decreased by 41% and malondialdehyde (MDA) levels reduced by 37% compared to wild-type plants under 100 mM NaCl. Antioxidant enzyme analysis revealed a selective enhancement pattern in the mutant, with peroxidase (POD) and ascorbate peroxidase (APX) activities increased by 42% and 38% respectively, during salt stress. Under severe salt stress (200 mM NaCl), physiological parameters, including survival rate and chlorophyll content, showed only minor improvements in the mutant despite substantial biochemical protection. These results demonstrate that *OsProDH* influences BT7 rice salt responses through multiple pathways, particularly in the regulation of proline metabolism and specific antioxidant enzyme activities. The findings contribute to our understanding of proline metabolism in cereal crops and provide insights for future development of salt-resilient rice varieties.

Keywords: Bac Thom 7, mutation, *OsProDH*, proline, salt tolerance.

Citation: Nguyen Duy Phuong, Ma Huyen Ngoc, Cao Le Quyen, 2025. Research on salt tolerance of *OsProDH*-mutant Bac Thom 7 rice variety. *Academia Journal of Biology*, 47(4): 103–111. https://doi.org/10.15625/2615-9023/22680

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INTRODUCTION

Due to climate change impacts, extreme environmental factors, including saline intrusion, are profoundly affecting rice production. The Bac Thom 7 (BT7) rice variety, known as one of the high-quality staple varieties in Northern Vietnam, is quite sensitive to salt stress (Ha et al., 2019). The emergence of advanced precision breeding technologies such as gene editing has opened significant prospects for improving salt tolerance in key rice varieties like BT7.

When encountering salt stress conditions, rice plants exhibit numerous metabolic responses to adapt to the environment. Proline is a common osmotic protectant that typically accumulates when rice plants experience salt stress (Maisura et al., 2014). Proline synthesis is controlled by P5CR (Δ1-Pyrroline-5-carboxylate reductase) and P5CS (Δ1-Pyrroline-5-carboxylate synthetase) enzymes, while proline degradation is catalyzed by ProDH (proline dehydrogenase) and P5CDH (Δ1-Pyrroline-5-carboxylate dehydrogenase) (Rizzi et al., 2015). In *Arabidopsis*, *AtProDH* has been found to be encoded by *AtProDH1* and *AtProDH2*. While *AtProDH2* expression is activated by salt and cold stress, inhibition of *AtProDH1* expression helps plants increase their resistance to cold and salt stress (Funck et al., 2010). Studies have shown that inactivation of NtProDH helps tobacco plants better withstand drought, salt, and cold stress conditions (Tateishi et al., 2005). Similarly, the Kongyu131 rice line with edited *OsProDH* gene demonstrated better heat tolerance compared to the wild-type control (Guo et al., 2020). Research on several Vietnamese rice varieties, such as TBR225, BC15, and Khang Dan 18 has also shown that drought and heat stress altered the expression levels of *OsProDH* (Phuong et al., 2024). However, to date, no studies have addressed the role of *OsProDH* in salt tolerance response in rice plants.

In our recent research using the CRISPR/Cas9 gene editing tool, we created a BT7 rice line carrying a 39-nucleotide deletion in *OsProDH* (Le et al., 2025). BT7 was selected as our model system because it represents a major commercial rice variety in Northern Vietnam production, yet shows significant salt sensitivity. This makes it an ideal candidate to demonstrate how *OsProDH* gene editing could effectively improve salt tolerance in economically important rice varieties. In this study, we continue to investigate the role of *OsProDH* in salt stress response in the BT7 rice variety by analyzing the physiological and biochemical reactions of the mutant rice line under stress conditions. This research will elucidate the function of *OsProDH* to serve future studies on breeding rice varieties resistant to adverse environmental factors using gene technology.

MATERIALS AND METHODS

Plant materials and growth conditions

Wild-type (WT) rice cultivar BT7 (*Oryza sativa* L. ssp. *indica*) was provided by Thaibinh Seed Corporation (Vietnam). The *OsProDH* mutant BT7 rice line was provided by the Department of Molecular Pathology, Vietnam Agricultural Genetics Institute (Vietnam). Germinated seeds were placed into 96-well plates and grown in 0.5X Murashige and Skoog (MS) medium under the conditions of 16 h light/8 h dark cycle at 26 ± 2 ^{o}C with 70−80% relative humidity. For salt stress treatment, two-week-old rice seedlings were transferred to 0.5X MS solution supplemented with 200 mM NaCl (for survival rate and chlorophyll content assessment experiments) (Guo et al., 2020) and 100 mM NaCl (for all other experiments) (Li et al., 2019; Rohman et al., 2016) and kept in the same conditions for 3 days. Plants were recovered from stress treatment by being transferred back to regular MS solution without NaCl and maintained under identical environmental conditions (16 h light/8 h dark cycle at 26 ± 2 ^{o}C with 70−80% relative humidity) for 7 days before final measurements were taken.

Total chlorophyll content

Leaves from 3-day salt-treated and non-treated plants were sliced into tiny pieces and incubated in 1 mL of 80% acetone: dimethyl sulfoxide (1:1, v/v) mixture overnight at room temperature in darkness. The supernatant from

each extraction sample was collected and the absorbance was measured at 663 and 645 nm using a spectrophotometer. Total chlorophyll content was calculated using the formula: $[(20.2 \times A_{645} + 8.02 \times A_{663}) \times V]/[1000 \times W]$, where V is the volume of the extract (mL) and W is the dry weight (g) of the sample. Results were expressed as mg chlorophyll per g dry weight (DW).

H_2O_2 and MDA content

Shoot samples from 3-day salt-treated and non-treated plants were homogenized in 1 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 × g for 15 min at 4 °C. For H_2O_2 determination, 0.5 mL of supernatant was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide. The reaction mixture was incubated in darkness at room temperature for 20 min. H_2O_2 content was determined by measuring absorbance at 390 nm against a calibration curve prepared with known concentrations of H_2O_2 (Li et al., 2019). Results were expressed as nmol H_2O_2 per g DW.

For malondialdehyde (MDA) determination, the extract was mixed with 0.5% thiobarbituric acid (TBA) prepared in 20% TCA. The mixture was incubated at 95 ^oC for 30 min, then quickly cooled in an ice bath and centrifuged at 10,000 × g for 10 min. The MDA content was calculated by measuring absorbance of the supernatant at 532 nm and 600 nm, using the formula: MDA (µM/g FW) = [(A532 – A600) × V]/(155 × W), where V is the volume of the extraction solution (mL), W is the dry weight of sample (g), and 155 $mM^{-1}.cm^{-1}$ is the molar extinction coefficient of MDA-TBA complex.

NBT staining

Superoxide (O_2^-) was visualized in leaves of 3-day salt-treated and non-treated plants according to the method described by Rohman et al. (2016) with minor modifications. Briefly, detached leaves were immersed in 0.1% nitroblue tetrazolium (NBT) solution prepared in 50 mM sodium phosphate buffer (pH 7.5) and incubated for 48 h in the dark at room temperature. After staining, leaves were decolorized by immersion in 100% ethanol for 24 h to remove chlorophyll. The treated leaves were then photographed to document the blue-purple formazan precipitates, which indicate sites of superoxide accumulation.

Antioxidant enzyme assays

Fresh leaf samples of 3-day salt-treated and non-treated plants were ground in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and 1% PVP. After centrifugation at 12,000 × g for 20 min at 4 ^oC, the supernatant was used for enzyme assays as described by Li et al. (2019). Blank controls containing all reagents except the enzyme extract were included for each assay to account for non-enzymatic reactions. CAT activity was determined by monitoring H_2O_2 degradation at 240 nm (extinction coefficient of 39.4 $M^{-1}.cm^{-1}$). POD activity was measured by guaiacol oxidation at 470 nm (extinction coefficient of 26.6 $mM^{-1}.cm^{-1}$). APX activity was determined by ascorbate oxidation at 290 nm (extinction coefficient of 2.8 $mM^{-1}.cm^{-1}$). All enzyme activities were expressed as U/mg DW.

Proline content

Leaves were collected from plants at three time points: before salt treatment, 3 days after salt treatment, and 7 days after recovery. The samples were ground with 3% sulfosalicylic acid (w/v) and filtered through filter paper. The extract was mixed with an equal volume of 1% ninhydrin acid and glacial acetic acid (1:1, v/v). After incubation at 100 ^oC for 1 h, the mixture was cooled and toluene was added. The mixture was vigorously vortexed and allowed to separate into phases. The absorbance of the toluene phase was measured at 520 nm. The L-proline content was calculated using a standard curve prepared with known concentrations of L-proline.

Salt stress tolerance

To evaluate salt tolerance, both WT and mutant Bac Thom 7 plants were subjected to 200 mM NaCl treatment for 3 days. Subsequently, all plants were transferred back to normal growth conditions. The survival rate was recorded after 7 days of recovery.

Experimental design and statistical analysis

Each experiment was conducted with at least 30 plants, from which 5 plants were randomly selected for analyses. All samples collected from each treatment were divided into two parts: one part was dried to determine dry weight (DW), while the remaining part was used for analyses. For each biological replicate, three technical replicates were measured and averaged to obtain a single value. Mean values ± standard deviation (SD) are presented in graphs from at least three independent experiments. The data from each treatment were subjected to ANOVA analysis with a significance level at $P < 0.05$ using MS Excel 2016.

Place and time of experiments

All laboratory experiments were conducted at the Department of Molecular Pathology, Vietnam Agricultural Genetics Institute (Ha Noi, Vietnam). Rice cultivation was performed at Thai Nguyen University of Agriculture and Forestry (Thai Nguyen, Vietnam). The experiments were conducted from January to December 2024.

RESULTS

***OsProDH* is involved in mitigating oxidative damage under salt stress conditions**

As shown in Figure 1, the *OsProDH* mutant (MU) exhibited different proline accumulation patterns compared to wild-type (WT) plants across the three examined stages. Under control conditions (CT), the basal proline content in mutant plants was significantly higher than in wild-type plants. When exposed to salt stress (100 mM NaCl for 3 days), both genotypes responded with substantial increases in proline accumulation, reaching similar levels with no statistically significant differences between mutant and wild-type plants. After the 7-day recovery period under normal conditions, proline levels decreased in both genotypes compared to their salt-stressed state. However, the *OsProDH* mutant maintained significantly higher proline levels than wild-type plants during this recovery phase. These results demonstrate that the *OsProDH* mutation alters proline accumulation patterns in Bac Thom 7 rice, particularly under normal conditions and during recovery from salt stress.

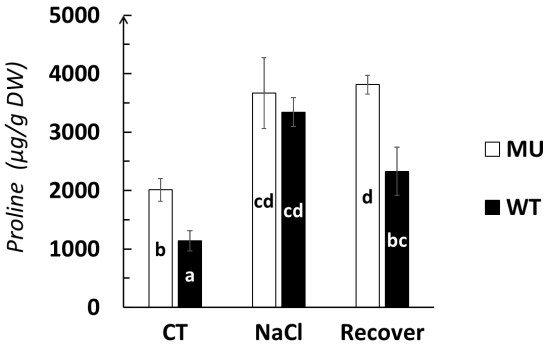

Figure 1. Proline accumulation in *OsProDH* mutant (MU) and wild-type (WT) Bac Thom 7 plants. Proline content was evaluated before salt treatment (CT), after 3 days of 100 mM NaCl treatment (NaCl), and after 7 days of recovery from salt stress under normal conditions. Values represent means ± SD (n = 3). Different letters indicate significant differences between means (ANOVA, $P < 0.05$)

***OsProDH* mutation reduces oxidative damage under salt stress**

Reactive oxygen species (ROS) accumulation serves as a primary indicator of salt-induced cellular damage in plants. To assess how *OsProDH* affects this critical stress response, we compared oxidative markers between mutant and wild-type BT7 rice.

The NBT staining and H_2O_2 quantification (Figs. 2A, 2B) revealed distinct ROS patterns between genotypes. Under control conditions, both lines showed minimal superoxide presence and equivalent H_2O_2 levels. However, salt exposure triggered significant ROS production, with wild-type plants accumulating substantially more superoxide (indicated by darker blue formazan deposits) and hydrogen peroxide compared to the mutant. These results suggest the *OsProDH*-mutant plants possess enhanced ROS scavenging capacity under salt stress.

As a direct consequence of elevated ROS levels, lipid peroxidation (measured as MDA

content, Fig. 2C) increased significantly in both genotypes during salt stress. However, consistent with the lower ROS accumulation, the *OsProDH* mutant maintained significantly reduced MDA levels compared to wild-type plants, demonstrating that lower reactive oxygen species generation translated to decreased membrane damage under saline conditions.

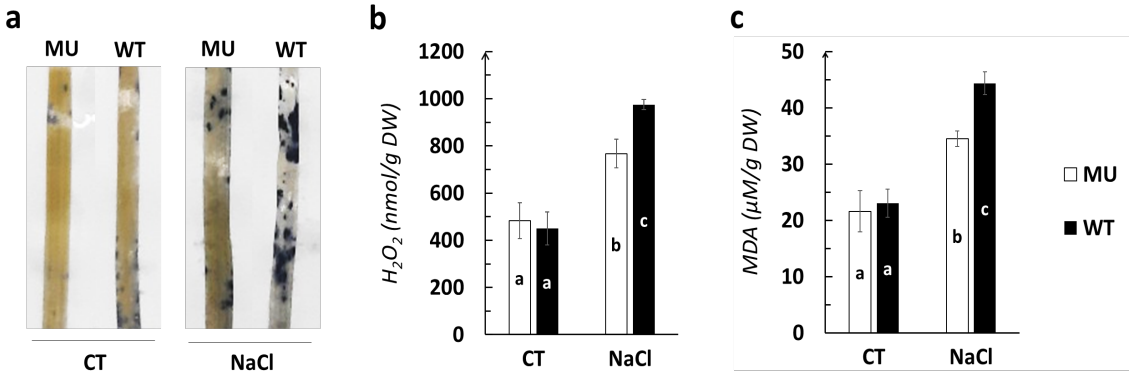

Figure 2. Salt-induced oxidative stress in wild-type (WT) and *OsProDH* mutant (MU) Bac Thom 7 seedlings. Measurements in leaves before salt treatment (CT), after 3 days of 100 mM NaCl treatment (NaCl): (a) NBT staining of leaves (blue spots indicate O_2^- accumulation); (b) H_2O_2 content; (c) MDA content. Values represent means ± SD (n = 3). Different letters indicate significant differences between means (ANOVA, $P < 0.05$)

The data collectively indicate that the *OsProDH* mutation confers enhanced protection against salt-induced oxidative damage through more effective ROS management and subsequent reduction in cellular membrane deterioration.

Indirect effects of *OsProDH* expression on antioxidant enzyme protection under salt conditions

Antioxidant enzymes constitute a critical defense mechanism against ROS-induced cellular damage. Analysis of key detoxifying enzymes revealed distinctive patterns between wild-type and *OsProDH* mutant plants across different conditions.

Under normal conditions, all three antioxidant enzymes (CAT, POD, and APX) exhibited comparable baseline activities between wild-type and *OsProDH* mutant plants, with no statistically significant differences observed (Fig. 3). This pattern indicates that in the absence of environmental stress, *OsProDH* mutation does not substantially alter the constitutive antioxidant enzyme profile in rice, suggesting that both genotypes maintain similar ROS management capacities under optimal growing conditions.

Salt stress affected the antioxidant enzyme activities differently. For catalase (CAT), neither the wild-type nor the mutant plants showed significant changes in activity under salt stress compared to control conditions, and no significant differences were observed between genotypes (Fig. 3A). In contrast, both POD and APX activities increased significantly in both genotypes under salt stress compared to their respective controls. Importantly, the *OsProDH* mutant exhibited significantly higher POD and APX activities compared to wild-type plants under salt conditions (Figs. 3B, 3C).

These findings demonstrate that in salt stress conditions, BT7 rice selectively enhances peroxide-scavenging enzymes (POD and APX) rather than CAT. Moreover, the *OsProDH* mutation, which promotes proline accumulation, appears to provide additional protection to these key antioxidant enzymes, enabling their enhanced activity during salt stress and contributing to improved ROS detoxification capacity.

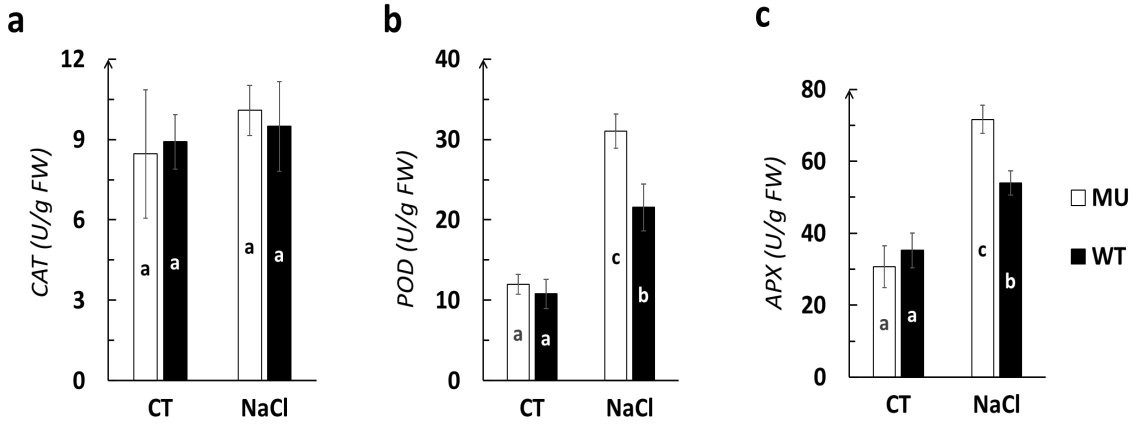

Figure 3. Activities of antioxidant enzymes in wild-type (WT) and *OsProDH* mutant (MU) Bac Thom 7 plants. (a) CAT, (b) POD, and (c) APX activities were measured under normal conditions (CT) and after 3 days of 100 mM NaCl treatment (NaCl). Values represent means ± SD (n = 3). Different letters indicate significant differences between means (ANOVA, $P < 0.05$)

Assessment of salt tolerance in *OsProDH*-mutant BT7 rice

To evaluate whether the *OsProDH* mutation affects overall salt tolerance, we examined the phenotypic responses of both genotypes under severe salt stress conditions.

When subjected to severe salt stress (200 mM NaCl), both mutant and wild-type plants displayed significant injury symptoms, including leaf rolling, chlorosis, and growth inhibition (Fig. 4A). After the recovery period, the *OsProDH* mutant showed a marginally higher survival rate (15.0%) compared to wild-type plants (7.2%) following salt treatment (Fig. 4B). However, despite this visible numerical difference, statistical analysis indicated no significant difference in survival rates between genotypes under salt stress ($P < 0.05$).

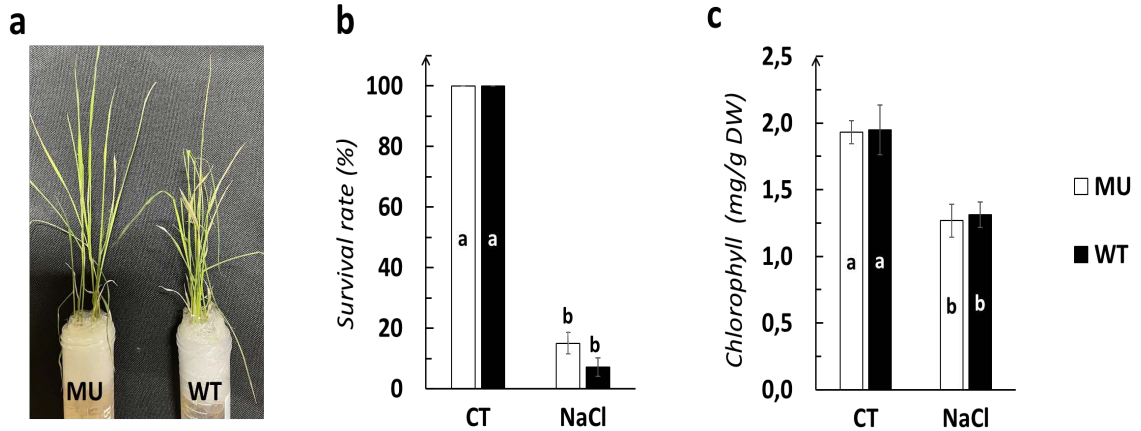

Figure 4. Salt-resistant phenotype of *OsProDH* mutant (MU) and wild-type (WT) Bac Thom 7 rice seedlings. (a) Representative images of 2-week-old plants after 3 days of 200 mM NaCl treatment. (b) Survival rates of plants under normal conditions (CT) and after 7 days of recovery from 3-day treatment with 200 mM NaCl (NaCl). (c) Chlorophyll contents of plants under normal conditions (CT) and after 3 days of 200 mM NaCl treatment (NaCl). Values represent means ± SD (n = 3). Different letters indicate significant differences between means (ANOVA, $P < 0.05$)

The limited improvement in survival rates corresponded with salt-induced damage to the photosynthetic apparatus. Chlorophyll content decreased substantially in both genotypes to approximately 65% of their respective controls (Fig. 4C), with no significant differences between mutant and wild-type plants. This parallel reduction in chlorophyll content likely contributed to the poor recovery observed in both genotypes, as plants with severely compromised photosynthetic capacity following stress would have limited energy resources for recovery and repair processes.

These results indicate that while the *OsProDH* mutation provides some biochemical protection against salt-induced oxidative damage (Figs. 1–4), it confers only a modest improvement in overall physiological salt tolerance under severe stress conditions in the Bac Thom 7 rice variety, insufficient to maintain photosynthetic activity or significantly enhance survival rates.

DISCUSSION

Role of *OsProDH* in proline regulation under salt stress

The differential proline accumulation pattern in our mutant reveals complex molecular regulation of proline metabolism during salt stress cycles. While both genotypes reached similar proline peaks during salt exposure, the distinctive accumulation before and after stress suggests *OsProDH* activity is subject to sophisticated temporal regulation.

This pattern can be explained by stress-responsive transcriptional control of proline metabolism genes. Under salt stress, strong activation of biosynthetic genes likely masks the effects of impaired catabolism, creating a "ceiling effect" where maximum proline accumulation is determined primarily by biosynthesis rate (Batista-Silva et al., 2019). The regulatory balance likely shifts dramatically post-stress, when biosynthesis pathways are downregulated but catabolism becomes essential for metabolic recovery.

The maintained elevated proline in the BT& rice during recovery represents "metabolic memory" that could prime plants for subsequent stress episodes. This suggests potential crosstalk between *OsProDH* activity and stress signalling pathways, possibly involving abscisic acid (ABA) or reactive oxygen species as mediators. This was stated in a recent research which indicates proline metabolism enzymes serve as stress-signalling hubs beyond their catalytic functions (Bhaskara et al., 2015).

These findings suggest salt tolerance strategies should consider the entire stress-recovery cycle rather than focusing solely on acute stress responses. Temporary inhibition of *OsProDH* during recovery phases might be more advantageous than constitutive suppression throughout the plant's lifecycle, similar to findings in Arabidopsis, where temporal control of *AtProDH* expression affected stress adaptation (Funck et al., 2010).

Proline accumulation enhances antioxidant defense through selective enzyme protection

The selective enhancement of POD and APX activities in our *OsProDH* mutant under salt stress reveals a previously unrecognized connection between proline metabolism and specific antioxidant pathways in salt-stressed rice. This enzyme-specific protection pattern differs from the generalized antioxidant responses typically observed during salt stress.

Proline likely provides targeted protection in these organelles, explaining why peroxide-scavenging enzymes show enhanced protection while CAT, primarily localized in peroxisomes, remains unaffected (Foyer & Noctor, 2016). Additionally, proline's amphipathic properties may allow it to accumulate at membrane interfaces where it can specifically protect membrane-associated POD isoforms from salt-induced denaturation (Mansour & Ali, 2017).

These findings suggest that engineering proline metabolism for enhanced salt tolerance should focus on compartment-specific accumulation rather than total cellular proline levels, as the protective effects appear pathway-specific rather than universal.

Bridging biochemical protection and physiological tolerance

The notable disconnect between enhanced biochemical protection and limited physiological improvement in our *OsProDH*-mutant rice highlights fundamental constraints in engineering salt tolerance through single-pathway modifications. This discrepancy highlights the complex nature of salt tolerance mechanisms. While proline accumulation effectively mitigated oxidative damage, other salt-induced physiological disruptions remained unaddressed. The severe stress imposed (200 mM NaCl) likely overwhelmed the protective capacity conferred by the single-gene mutation. Under such conditions, ion toxicity becomes the predominant damaging factor, with Na^+ directly inhibiting photosynthetic machinery and metabolic enzymes regardless of ROS levels (Munns & Tester, 2008). The similar chlorophyll degradation in both genotypes suggests that proline-mediated protection couldn't prevent Na^+-induced damage to photosystems.

Another possible explanation involves energy constraints. Maintaining elevated proline levels and antioxidant enzyme activities requires significant metabolic resources. During severe stress, this enhanced protection may compete with essential recovery processes for limited energy resources, potentially explaining why biochemical improvements didn't translate to significant physiological tolerance (Chen et al., 2016). Yang et al. (2018) observed similar limitations in other single-gene modifications that enhanced specific protective mechanisms but failed to confer comprehensive stress tolerance.

These findings suggest that future research should focus on combining *OsProDH* modification with complementary salt tolerance mechanisms, particularly those targeting ionic homeostasis and membrane transport. Additionally, evaluating the *OsProDH* mutation under moderate salt stress conditions more relevant to agricultural settings might better reveal its potential contribution to practical salt tolerance improvement.

CONCLUSION

Our study demonstrates that *OsProDH* mutation in BT7 rice alters proline accumulation patterns, reduces oxidative damage under salt stress, and selectively enhances specific antioxidant enzyme activities (POD and APX) without affecting CAT. While these biochemical improvements translated to only modest physiological salt tolerance, they reveal proline's role beyond osmotic adjustment as a selective protective molecule in antioxidant defense. This research highlights both the potential and limitations of single-gene modifications for salt tolerance, suggesting that future breeding strategies should combine *OsProDH* manipulation with complementary mechanisms addressing the multiple aspects of salt stress for more effective enhancement of salt tolerance in rice varieties. Although the *OsProDH*-mutant showed modest physiological improvements under severe salt stress, its enhanced biochemical protection mechanisms suggest potential utility in moderately saline agricultural environments where stress levels do not exceed the protective capacity of the single-gene modification. This fundamental understanding of *OsProDH* function provides a foundation for future breeding strategies.

Acknowledgements: This research was funded by the Ministry of Science and Technology under the project "Research on the application of precise mutation methods to enhance drought/heat tolerance in Vietnamese rice varieties using CRISPR/Cas9 technology" (Grant No. ĐTĐL.CN-52/22). We would like to express our sincere gratitude!

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