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The Effect of Salinity Concentration on Proline Dehydrogenase (ProDH) Gene Expression and Proline Accumulation in Black Rice and Red Rice

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ABSTRACT

The nutraceutical properties of the pigmented rice provide an opportunity to be widely cultivated. A tendency to grow rice on sub-optimal land such as saline soil is an alternative to support food security, but the adaptation under saline soil is still questionable. This study was performed to determine the stress resistance of black (var. Ketan Hitam), red (var. MS Pendek), and white rice (var. IR64) as a commercial variety through proline accumulation and *Proline dehydrogenase (ProDH)* expression under salinity stress and recovery. The results showed that salinity stress increased the proline content in rice plants, with var. IR64 rice and var. MS Pendek (red rice) accumulated the highest amounts of proline, and the expression of the *ProDH* in IR64 and MS Pendek was increased in the recovery phase. These results indicate the foundation for elucidating the mechanism response of black and red rice to salinity stress and recovery ability.

Keywords*:* salinity stress, proline, gene expressions, ProDH

INTRODUCTION

Salinity stress is caused by the excessive accumulation of dissolved salts, which can significantly impair plant growth and productivity. Salinity stress causes plants to experience osmotic stress, accumulating osmoregulatory compounds, such as proline, which mitigate the effects of reduced water potential within plant cells [1]. Osmotic adjustment is a critical mechanism that enables plants to optimize water uptake from the environment, serving as a primary defense strategy against dehydration [2]. The accumulation of proline under stress conditions is facilitated by activating enzymes involved in proline biosynthesis, while enzymes responsible for proline degradation are inhibited [3].

Proline dehydrogenase (ProDH) is an enzyme involved in proline degradation and is critical in maintaining cellular homeostasis [4]. Under dehydrated conditions, proline works as an

osmoregulatory compound, and in rehydrated conditions, it serves as an energy source through its catabolism [5]. The equilibrium between proline synthesis and proline degradation, referred to as proline homeostasis, is crucial for a plant's ability to withstand prolonged stress conditions [6]. This homeostasis is integral in preserving redox balance, enabling plants to resume growth and recover once the stress is alleviated [7].

Rice (*Oryza sativa* L.) is one of the most widely consumed cereal crops and a staple food for many global populations. It is classified as a salt-sensitive species, particularly vulnerable during the early vegetative and reproductive stages [8]. As reported by [9], the early vegetative phase occurs 15-25 days after planting. Salinity stress with an electrical conductivity (EC) level ≥ 8 dS/m is considered moderate salinity stress for rice plants, while an EC ≥ 14 dS/m is classified as high salinity stress [10]. According to [11], the salinity tolerance threshold for rice is approximately 3 dS/m, with yield reductions reaching up to 12% for each additional dS/m increase in salinity [12].

Black and red rice exhibit significant potential as functional foods due to their high nutraceutical content [13], which is beneficial for health [14]. Therefore, it has the potential for large-scale development. However, there is limited information regarding the resistance of black and red rice varieties to salinity stress [15]. Assessing their resilience to such stressors, including their capacity to recover once the stress is stopped, is essential. This study aims to investigate the effects of salinity stress and recovery on proline content and ProDH gene expression in three rice varieties: Ketan Hitam (black rice), MS Pendek (red rice), and IR64 (white rice, a commercial variety). By elucidating the recovery mechanisms of these plants following stress exposure, the findings of this research are expected to contribute valuable insights for the molecular development of salinity-resistant rice varieties, focusing on restoring metabolic homeostasis in plants before stress exposure.

MATERIALS AND METHODS

Plant Materials and Experimental Conditions

This study was conducted at the Agrotechno-Park Jubung, the Integrated Laboratory University of Jember, East Java, Indonesia. The rice seeds used in this experiment (IR64, MS Pendek, and Ketan Hitam) were sourced from the UPA Laboratory, University of Jember. Homogenous and healthy seeds were selected as plant material and cultivated under uniform conditions. Before planting, the seeds were soaked in water for 24 hours and sown for 20 days, representing the vegetative growth stage. Salinity treatment was simulated by applying NaCl to plants aged 21 – 28 days after planting (DAP). Three salinity levels were tested: 0 dS/m (control), 8 dS/m (moderate), and 14 dS/m (high). NaCl was applied over a one-week period, with plant samples collected at two distinct time points during the stress phase: 3 days after treatment (3 DAT) and 7 days after treatment (7 DAT). Following the stress period, the plants were irrigated with water to facilitate recovery. Recovery samples were then collected on day 1 and day 3 of the recovery phase (1 DAR and 3 DAR). The experimental design used was completely randomized.

ProDH Expression Analysis

Total RNA was isolated using AccuZol™ a total RNA isolation reagent and approximately 1 µg of RNA was used for first-strand cDNA synthesis with the AccuPower® CycleScript™ RT Premix

(dT20) solution reagent kit, following the manufacturer's instructions. The cDNA product was then amplified using PCR under the following conditions: 95°C for 5 min, 95°C for 30 s, 61°C for 30 s, 72°C for 1 min, and 72°C for 5 min as the final elongation. Primers used to amplify antioxidant gene expression are listed in Table 1.

Table 1: Primer sequences for gene expression analysis

Gene	Primer Sequence	Molecular Weight (bp)	References
Os-ProDH	F: GGTTGGTCTTCCTTCAGGTGTGC R: CATCAACATCATCAAACACCACTAT	106	[16]
Actin	F: TCCATCTTGGCATCTCTCAG R: GTACCCGCATCAGGCATCTG	335	[17]

Proline Content Analysis

Proline content was quantified using the method described by [18], and the results were referenced against a pure proline standard curve, with units expressed as µg/mg. To prepare the sample, a reactant mix comprised 1% ninhydrin, 60% acetic acid, 20% ethanol, and 19% distilled water (aquadest). Approximately 50 mg of the sample was ground using a mortar, then 1 mL of a solvent mixture of 40% ethanol and 60% aquadest was added. The sample was then transferred to an Eppendorf tube and incubated at 4°C for 24 hours. After incubation, the sample was centrifuged at 10.000 rpm for 10 minutes. An aliquot of 200 µL of the supernatant was combined with 800 µl of the reactant mix in an Eppendorf tube and heated at 95°C for 60 minutes. After cooling to room temperature, the sample was centrifuged at 2.500 rpm for 5 minutes. The final supernatant was transferred to a cuvette and analyzed using a spectrophotometer at a wavelength of 520 nm.

Total Soluble Protein Content Analysis

Total soluble protein content was determined using the Bradford method [19], with Bovine Serum Albumin (BSA) as the standard protein. A total of 0.3 g of sample was ground with a mortar, then 900 µl of phosphate buffer was added to create a homogenate. The homogenate was then transferred to an eppendorf and centrifuged at 10.000 rpm for 10 minutes to separate the soluble protein in the supernatant from insoluble material. Subsequently, 5 µL of supernatant was mixed with 45 µL of distilled water and 950 µL of Bradford reagent. The mixture was vortexed thoroughly to ensure complete protein and dye interaction. The absorbance value was measured at 595 nm using a spectrophotometer. The protein concentration was determined by comparing the absorbance values to a standard curve generated from known concentrations of BSA.

Total Chlorophyll Content Analysis

Total chlorophyll content was quantified using the spectrophotometric method [20]. A 0.5 g rice leaf sample was collected and weighed. The leaf was then ground using a mortar until a smooth homogenate was obtained. The ground leaf tissue was extracted with 1.5 mL of 100% methanol and stirred to facilitate the release of chlorophyll from the tissue. The resulting homogenate was transferred to an Eppendorf tube and centrifuged at 10.000 rpm for 10 minutes to separate the supernatant from the pellet. The supernatant was carefully collected

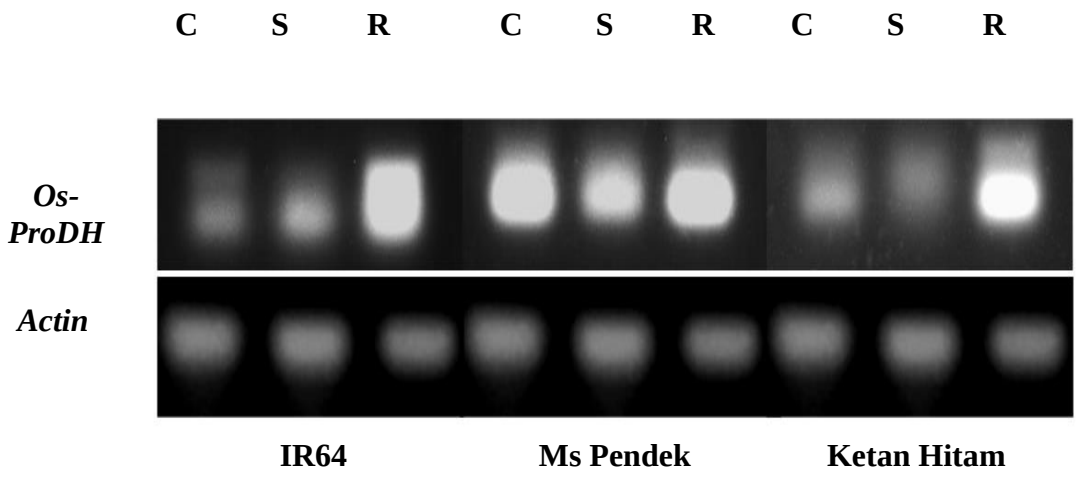
and placed in a cuvette for spectrophotometric analysis. Absorbance was measured at three wavelengths: 470 nm, 652 nm, and 665 nm to determine the total chlorophyll content.

RESULTS

Relative Expressions of *ProDH*

Molecular analysis was conducted to investigate the regulation of the *ProDH* in plants exposed to salinity stress during the subsequent recovery phase. *ProDH* encodes the ProDH enzyme, which plays a crucial role in proline degradation and is essential for maintaining cellular homeostasis. The PCR products, shown as DNA bands in Figure 2, were generated using cDNA as the template. The cDNA was synthesized through reverse transcription (RT) of messenger RNA (mRNA) extracted from rice leaf samples. The expression of the *ProDH* in plants exposed to salinity stress and recovery conditions is depicted in Figure 2.

The results indicated that *ProDH* expression was significantly elevated during the recovery phase in all three rice varieties (IR64, MS Pendek, and Ketan Hitam), with similar intensities in the DNA bands, as quantified using ImageJ. An interesting observation was found in MS Pendek, where *ProDH* expression remained relatively constant under normal and recovery conditions. Furthermore, MS Pendek exhibited a notably higher relative expression of ProDH during the stress phase compared to the other varieties, IR64 and Ketan Hitam. The lowest expression of *ProDH* in IR64 and Ketan Hitam differed. In IR64, the lowest ProDH expression was observed under normal conditions, whereas in Ketan Hitam, it was observed during the stress phase.



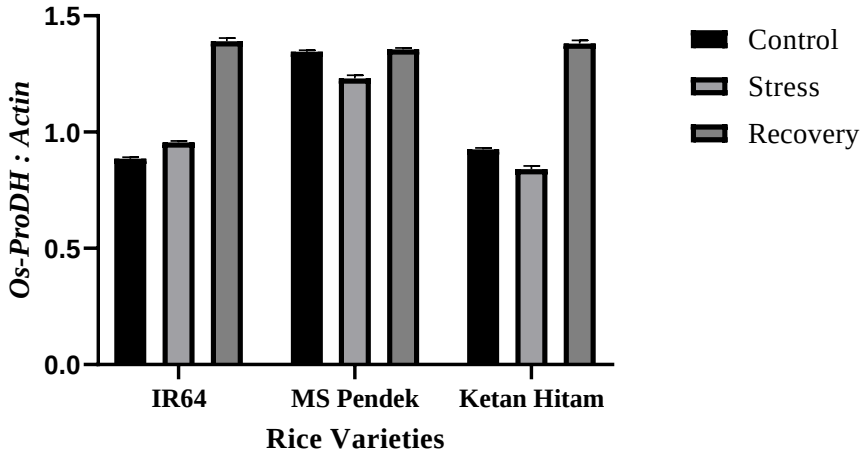


Figure 2: Relative Expressions of *ProDH*

Proline Content

Proline content was analyzed under three levels of salinity: 0 dS/m (control), 8 dS/m (medium), and 14 dS/m (high). Plants were exposed to these salinity treatments for 7 days, after which the stress was terminated, initiating the recovery phase on the 8th day (1 DAR). Observations continued until the 10th day (3 DAR). As shown in Figure 3, proline content increased in response to salinity stress in all rice varieties. A decline in proline content was observed upon cessation of salinity stress (recovery phase).

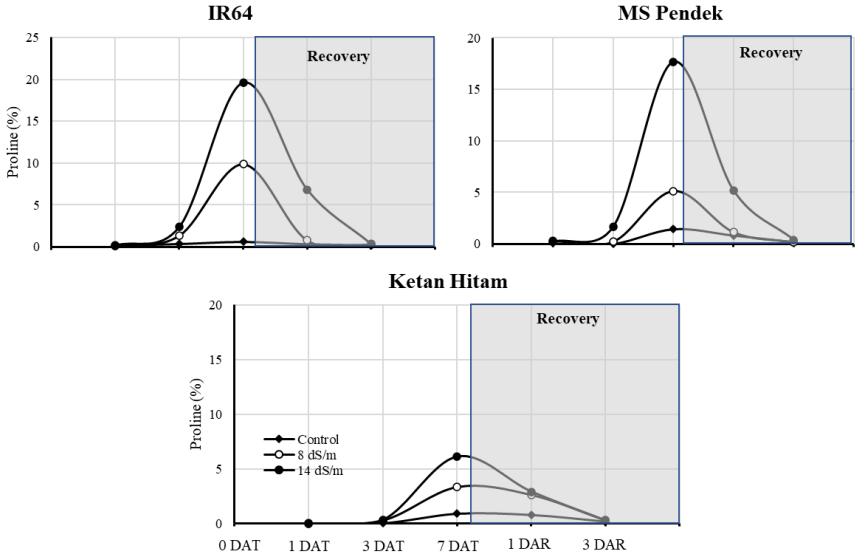


Figure 3: Proline Content in IR64, MS Pendek and Ketan Hitam under Salinity Stress and Recovery

The highest proline content was observed on the seventh day of salinity stress, with the highest salinity level was 14 dS/m. A similar peak in proline accumulation was recorded on the same day at a salinity level of 8 dS/m; the levels were lower than those at 14 dS/m. During the

recovery phase, a significant reduction in proline content was noted at both salinity (8 dS/m and 14 dS/m). The decrease in proline content progresses, reaching the lowest levels on the third recovery day. These findings suggest that proline accumulation is a key response to salinity stress, while its subsequent decline during recovery indicates a return to normal metabolic processes.

Total Soluble Protein Content

The results presented in Figure 3. indicate a decrease in total soluble protein content under salinity stress conditions across all varieties, with both increasing salinity levels and prolonged stress duration. Notably, the most significant reduction in total soluble protein content occurred at a salinity level of 14 dS/m after 7 days of stress, showing a drastic percentage decrease.

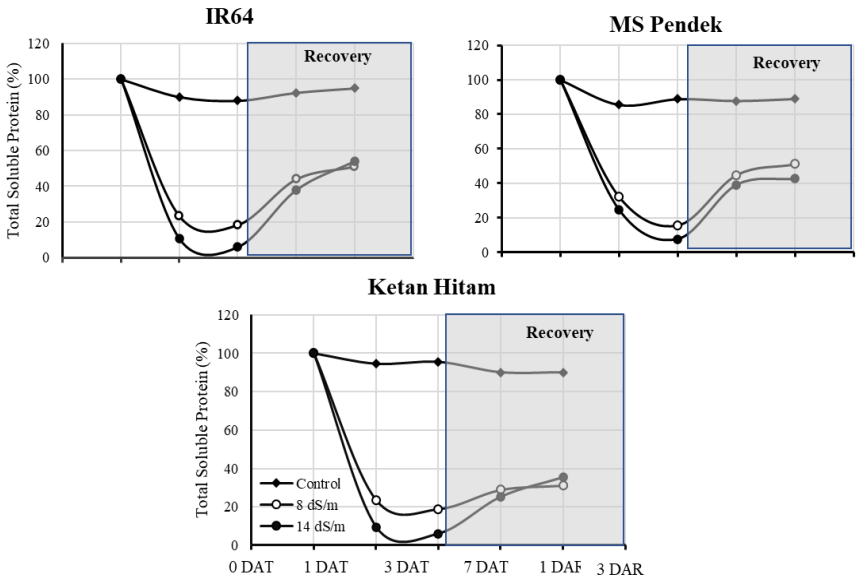


Figure 4: Total Soluble Protein Content in IR64, MS Pendek, and Ketan Hitam under Salinity Stress and Recovery

During the recovery phase, an increase in the total soluble protein content was observed in each variety. The highest percentage of total soluble protein occurred sequentially in the IR64, MS Pendek, and Ketan Hitam varieties, with values of 54.06, 42.61, and 35.33%, respectively. These results suggest that IR64 and MS Pendek exhibit faster recovery abilities than Ketan Hitam rice, as indicated by the greater re-formation of total soluble proteins.

Total Chlorophyll Content

The effect of salinity stress on the percentage of relative changes in total chlorophyll content (%) in rice plants is illustrated in Figure 5 below:

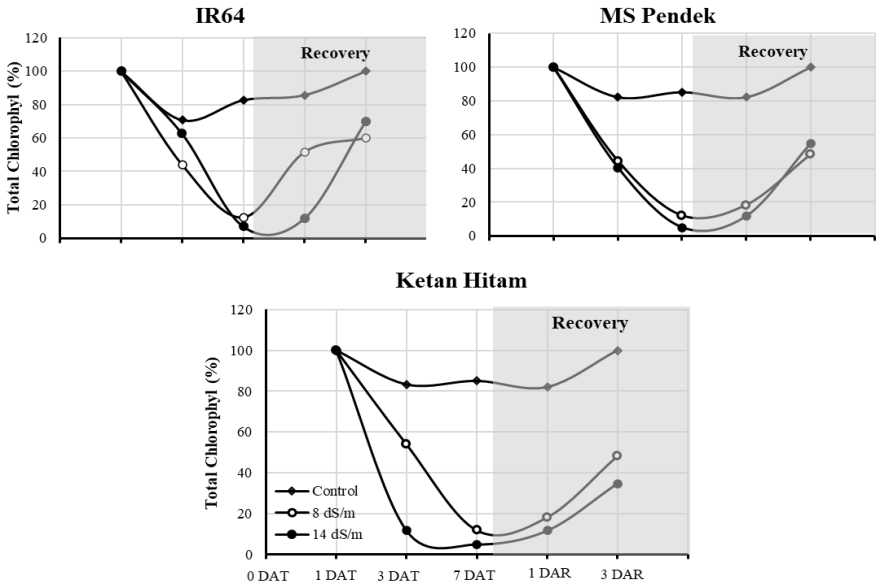


Figure 5: Total Chlorophyll Protein Content in IR64, MS Pendek, and Ketan Hitam under Salinity Stress and Recovery

Based on the research results in Figure 5, salinity stress significantly reduced the relative percentage of changes in total chlorophyll content in rice plants. The most considerable decrease in chlorophyll content occurred at a salinity level of 14 dS/m with a stress duration of 7 days across the IR64, MS Pendek, and Ketan Hitam varieties. However, all varieties showed increased total chlorophyll content during the recovery phase. The increase in total chlorophyll content from the highest stress level to the recovery phase was 66.81% for IR64, 49.75% for MS Pendek, and 29.75% for Ketan Hitam.

DISCUSSION

The expression of a gene is indicated by the formation of mRNA [21], as reflected in the thickness of the DNA band. A thicker DNA band signifies a higher quantity of mRNA produced, which correlates with increased gene expression. As shown in Figure 2, the relative expression of *ProDH* was up-regulated during the recovery phase, aligning with the role of *ProDH* in proline catabolism once the stress is alleviated [22]. The breakdown of proline contributes to ATP production through F_1F_0-ATPase [23], providing an essential energy source for plant regrowth after the stress is removed [24]. These findings are consistent with the observed increase in gene expression during recovery, highlighting ProDH's role in facilitating energy production for recovery processes.

The relative expression of *ProDH* was decreased and closely associated with proline accumulation. However, high relative expression of *ProDH* was also observed under normal conditions, particularly in MS Pendek. Under normal conditions, *ProDH* may be expressed as part of the plant's preparedness for potential stress [25]. This upregulation enables the plant to respond more rapidly to stress before proline levels accumulate to harmful concentrations, facilitating quicker adaptation to environmental challenges.

Proline catabolism via ProDH is also linked to nitrogen metabolism in plants. Under normal growth conditions, plants utilize various nitrogen sources, and proline degradation plays a crucial role in nitrogen recycling [26]. ProDH activity in this context ensures the efficient use of proline, a nitrogen-containing compound essential for plant growth and development. The upregulation of *ProDH* under normal conditions reflects the need for a balanced proline metabolism in plants [6]. ProDH regulates proline homeostasis, contributes to energy production, and maintains proper nitrogen metabolism [27]. These functions underscore the importance of ProDH in both stress responses and general plant growth and development.

Proline is an amino acid that plants synthesize as a protective mechanism against osmotic stress. Proline acts as an osmoregulator, helping maintain the cells' osmotic balance. According to [28], plants generally increase proline content in response to rising salinity levels, and this content decreases once salinity stress is alleviated. Based on the graph in Figure 3, proline content increased in each variety when exposed to salinity stress. Subsequently, proline levels decreased when the salinity stress was removed during recovery.

The research results indicated that the highest proline content was observed at a salinity level of 14 dS/m with a salinity application period of 7 days for the IR64, MS Pendek, and Ketan Hitam varieties. This condition represents a state of severe stress for the plants, leading to a significant accumulation of proline, which was higher compared to the salinity level of 8 dS/m and the stress duration of 3 days. As stated by [29], plants typically increase proline content with higher salinity levels and longer stress durations as a survival mechanism under abiotic stress conditions. Proline content in IR64 and Ketan Hitam also increases under control conditions, consistent with the findings of [30], who noted that plants can synthesize proline under stress and non-stress conditions. Proline plays a crucial role in cell growth and differentiation, as it is part of the protein cell wall component that functions in cell wall differentiation, plant development, and stress tolerance [31].

Apart from being an osmoregulatory, proline is an important energy source, supporting various metabolic processes necessary to continue growth and recovery in plants under stress [32]. According to [29], proline is no longer required as an osmoregulatory molecule under recovery conditions. Excessive proline accumulation under optimal conditions can harm plants, as it may lead to reactive oxygen species (ROS) formation. Therefore, proline must be rapidly catabolized to prevent toxicity and allow the plant to resume its normal physiological processes and complete its life cycle.

Proteins are composed of amino acids that play crucial roles in plant metabolism. Variations in the total soluble protein content indicate amino acid synthesis in response to salinity stress [33]. Under stressful conditions, plants break down proteins into free amino acids to help maintain cellular osmotic pressure. According to [34], dehydration causes the cytoplasmic fluid in the plant to become more viscous, leading to protein aggregation and denaturation. The reduction in total soluble protein content in this study suggests that protein degradation during the stress phase results in the formation of amino acids, particularly proline, which acts as an osmoregulatory compound. This aligns with [35], who note that protein degradation yields free amino acids, essential for maintaining cellular osmotic balance.

Chlorophyll content, a key pigment component in plant leaves, is susceptible to salinity stress. Under salinity stress conditions, the chlorophyll content in plant leaves is typically lower than that of those growing in optimal environments. This decline in chlorophyll content under salinity stress is commonly reported in various studies [36]. The study by [37] suggests that excessive proline accumulation can have toxic effects, primarily by generating ROS, such as hydrogen peroxide (H2O2), leading to lipid peroxidation and subsequent chlorophyll degradation. This degradation is often manifested by a shift in leaf colour from green to yellowish. Additionally, elevated salinity concentrations significantly reduce the rates of net carbon fixation and adversely affect photosynthetic pigments, including carotenoids and chlorophyll[38].

CONCLUSIONS

The relative expression of *ProDH* generally decreases under salinity stress. However, it is upregulated during recovery, indicating its involvement in proline metabolism, specifically in proline accumulation during stress and subsequent catabolism during recovery. Elevated proline levels do not necessarily correlate with enhanced salinity tolerance; tolerance is primarily determined by the plant's ability to recover after stress alleviation. This recovery capacity is largely contingent upon the plant's ability to degrade proline and restore normal physiological functions during recovery. In this regard, the rice varieties IR64 and MS Pendek exhibited the highest levels of proline accumulation during the stress phase; however, they also demonstrated a marked reduction in proline content during recovery, suggesting that these varieties possess robust adaptive mechanisms in response to environmental stress. In contrast, Ketan Hitam exhibits a higher salinity tolerance, as evidenced by its elevated *ProDH* expression during the recovery phase and relatively low proline accumulation under stress conditions.

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