Postprint: Analysis of Physiological and Biochemical Indicators in Wild Rice Near-Isogenic Lines in Response to Low Temperature Stress

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Abstract

To understand the physiological mechanisms underlying cold tolerance in rice, this study employed the wild rice near-isogenic line cold-tolerant rice variety DC907 and its cold-sensitive recipient parent 9311 as experimental materials. Rice seedlings were subjected to cold stress treatment, and changes in physiological and biochemical indicators—including malondialdehyde (MDA), superoxide anion (O2-), soluble sugars, and antioxidant enzymes (superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX))—were detected and analyzed. The cold tolerance capacity of the two varieties was comprehensively evaluated using the membership function method. The results demonstrated that the O2- production rate, MDA content, and soluble sugar content all increased gradually with prolonged low-temperature stress. During the low-temperature stress period, both MDA content and O2- production rate were higher in 9311 than in DC907, while the soluble sugar content of DC907 was significantly higher than that of 9311 in the later stages of stress. Throughout the low-temperature stress process, the activities of antioxidant enzymes (CAT, POD, SOD, and APX) in DC907 were all higher than those in 9311. Specifically, the activities of CAT and POD increased with extended cold stress duration, whereas SOD and APX exhibited a trend of initial decrease followed by increase. The ranking of membership function values for these physiological indicators was consistent with the ranking of average membership function values, indicating that these indicators are closely associated with rice cold tolerance. Based on the comprehensive evaluation of membership function values, DC907 exhibited stronger cold tolerance capacity than 9311, which aligns with field observation results.

Full Text

Analysis of Physiological and Biochemical Indices in Response to Low Temperature Stress in Wild Rice Near-Isogenic Lines

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Abstract

To understand the physiological mechanisms underlying cold tolerance in rice, this study examined the cold-tolerant near-isogenic line DC907 and its cold-sensitive recurrent parent 9311. Rice seedlings were subjected to cold stress treatment at 8°C for 1, 3, and 5 days, and changes in physiological and biochemical indices were measured, including malondialdehyde (MDA), superoxide anion (O), soluble sugars, and antioxidant enzymes (superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX)). The cold tolerance of the two varieties was comprehensively evaluated using the membership function method. The results showed that O production rate, MDA content, and soluble sugar content all increased gradually with prolonged cold stress duration. Throughout the cold stress period, 9311 exhibited higher MDA content and O production rate compared to DC907. At the late stage of cold stress, DC907 showed significantly higher soluble sugar content than 9311. During cold stress, DC907 maintained higher activities of all antioxidant enzymes (CAT, POD, SOD, and APX) than 9311. CAT and POD activities increased with stress duration, while SOD and APX activities initially decreased then increased. The ranking of membership function values for these physiological indices was consistent with the ranking of average membership function values, indicating these parameters are closely correlated with rice cold tolerance. Comprehensive evaluation based on membership function values revealed that DC907 possessed stronger cold tolerance than 9311, which aligns with field observations.

Keywords: *Oryza sativa* L., low temperature stress, physiological index

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Wild rice represents an important crop resource in China, harboring rich genetic diversity and many favorable traits not found in cultivated rice, from which genes of significant practical value can be developed [1]. Guangxi is rich in common wild rice resources, and our key laboratory has previously identified several quantitative trait loci (QTLs) for seedling-stage cold tolerance from superior Guangxi common wild rice germplasm. By constructing a core collection of common wild rice and selecting two donors with maximum genetic difference and strong cold tolerance, we developed chromosome segment substitution line libraries covering the entire genome using the sequenced indica rice 9311 as the recurrent parent through continuous backcrossing and SSR marker-assisted selection. Using these cold tolerance QTL substitution lines for mapping, we have successfully localized multiple cold tolerance QTLs. Systematic observation of yield and botanical traits revealed no significant differences between the cold tolerance QTL substitution lines derived from wild rice and the recurrent parent 9311 [2]. The cold-tolerant variety DC907 is a near-isogenic line with clear and stable cold tolerance, developed by using the strongly cold-tolerant wild rice DP30 as the donor parent and indica rice 9311 as the recurrent parent, followed by continuous backcrossing with 9311 and screening for superior agronomic traits. Its genetic background is essentially identical to the recurrent parent 9311 [2-4]. The acquisition of this cold-tolerant near-isogenic line provides appropriate material for studying the physiological and molecular mechanisms of cold tolerance genes and offers a reliable foundation for breeding cold-tolerant rice varieties.

Cold damage is a major limiting factor in rice production. Reports indicate that over 15 million hectares of rice cultivation worldwide are affected by low temperature stress, with 24 countries experiencing serious rice cold injury problems. Although rice is widely cultivated in China from 53°27 N to 18°90 N, almost all rice-producing regions suffer from varying degrees of low temperature damage, with annual losses of 5-10 billion kg of grain in disaster years [5]. Current molecular research on rice cold tolerance remains primarily at the gene mapping stage [6-11], while studies on physiological and biochemical changes under low temperature stress are relatively limited [12]. Therefore, investigating the physiological and biochemical mechanisms of cold tolerance in wild rice near-isogenic lines can lay the foundation for further molecular mechanism studies and provide theoretical support for improving cold tolerance in rice varieties, holding significant practical importance. Single physiological indices have limitations in reflecting plant stress resistance, whereas average membership function values enable multi-index comprehensive evaluation, offering a more reasonable and accurate assessment of plant stress tolerance [13-17]. This study compared differences in various physiological indices under cold stress and analyzed correlations between individual index membership function values and average membership function values to rationally evaluate physiological indices related to rice cold tolerance, aiming to elucidate the physiological basis of cold tolerance in rice seedlings.

1.1 Experimental Materials

The rice varieties used were the cold-tolerant wild rice near-isogenic line DC907 and its cold-sensitive parent 9311, provided by the State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources at Guangxi University.

1.2 Experimental Methods

Rice seeds were soaked in 1‰ carbendazim for 12 hours, rinsed repeatedly with distilled water, and then placed in a 28°C constant temperature incubator for germination. Seedlings were cultivated hydroponically in a greenhouse. Each variety was subjected to three cold treatment groups (1, 3, and 5 days of cold treatment) and one control group (normal temperature cultivation), with three replicates per group. At the three-leaf stage, the three treatment groups for each variety were placed in an artificial climate chamber at (8±0.5)°C with 3,000 lx illumination (12 h/day) at specific time points (1, 3, and 5 days before the end of the experiment). After 1, 3, and 5 days of cold treatment, the uppermost fully expanded functional leaves were collected from both treatment and control groups to measure physiological indices including MDA content, O production rate, soluble sugar content, and activities of SOD, POD, CAT, and APX.

1.3 Detection Methods for Physiological Indices

MDA content was determined using the thiobarbituric acid method, soluble sugar content by the anthrone-sulfuric acid method, SOD activity by the nitroblue tetrazolium photoreduction method, and POD activity by the guaiacol method. CAT activity was measured using the hydrogen peroxide method. APX activity assay was modified from Nakano and Asada [18], O determination was modified from Wang et al. [19], and other assays were modified from Li [20].

1.4 Data Processing Methods

Data were processed and tabulated using Excel, graphed using Origin 2019, and analyzed using SPSS 19. The membership function formulas were as follows:

For indices positively correlated with cold tolerance (formula a):

$$U_i = \frac{X_{ij} - X_{min}}{X_{max} - X_{min}}$$

For indices negatively correlated with cold tolerance (formula b):

$$U_i = 1 - \frac{X_{ij} - X_{min}}{X_{max} - X_{min}}$$

Where U_i is the membership function value, X_{ij} is the mean of three replicates for a given physiological index at different treatment times, X_{min} is the minimum value among the three replicates, and X_{max} is the maximum value.

2.1 Changes in Superoxide Anion Production Rate Under Low Temperature Stress

Superoxide anions can cause lipid peroxidation, destroy plant cell membrane structures, and directly react with proteins and nucleic acids, causing protein cross-linking and nucleic acid structure damage, thereby altering cell function. Under normal conditions, O production and scavenging remain balanced, with imbalance occurring only when plants encounter external stress [21,22]. Therefore, O production rate can reflect the degree of stress experienced by plants. As shown in Figure 1 [Figure 1: see original paper], the timing and magnitude of O production rate changes differed between DC907 and 9311 as low temperature treatment duration increased. In DC907, O production rate peaked after 1 day of cold stress then declined, whereas in 9311, it continued to rise, peaking at 3 days before decreasing slightly. Although ANOVA showed no significant intraspecific or interspecific differences in O production rate between the two varieties at various treatment times (Figure 1, Table 1), the higher O production rate in 9311 during the late cold stress stage indicates that DC907 suffered less O -mediated damage than 9311.

2.2 Changes in MDA Content Under Low Temperature Stress

MDA is the final product of membrane lipid peroxidation and can bind to proteins, causing intra- and inter-molecular cross-linking that alters protein spatial structure [23-25]. Changes in MDA content reflect not only the degree of biomembrane damage but also cellular functional impairment. Studies have shown that cold stress leads to MDA accumulation, making it a commonly used stress marker [26]. As shown in Table 1 and Figure 2 [Figure 2: see original paper], MDA content in 9311 remained higher than in DC907 throughout the cold stress period. Both varieties showed synchronized significant increases in MDA content with prolonged stress, though the magnitude and absolute levels differed. From day 3 of cold stress onward, MDA content in 9311 was significantly higher than in DC907. These results indicate that oxidative damage in rice increased with cold stress duration, leading to elevated MDA content, with 9311 experiencing greater damage than DC907.

2.3 Changes in Soluble Sugar Content Under Low Temperature Stress

Soluble sugars function as osmotic regulators in cells, and their increased content can enhance water retention capacity while protecting cold-sensitive proteins [13,27,28]. They may participate in damage repair and play important roles in maintaining cell integrity [29]. Therefore, soluble sugar content can reflect plant resistance to low temperature stress. As shown in Figure 3 [Figure 3: see original paper], soluble sugar content in both rice varieties increased with cold stress duration, reaching maximum levels after 5 days. Notably, 9311 showed a decline from day 3 to day 5, whereas DC907 demonstrated continuous significant elevation throughout the stress period. No significant difference in soluble sugar content was observed between the two varieties under control conditions (with 9311 slightly higher than DC907), but significant differences emerged after 5 days of cold stress, with DC907 showing substantially higher content. This suggests that increased soluble sugar content is strongly associated with DC907's cold tolerance and plays an important role in its cold resistance during the late stress stage.

2.4 Changes in Antioxidant Enzyme Activities Under Low Temperature Stress

Reactive oxygen species (ROS) are important signaling molecules in plants that accumulate extensively during low temperature stress, ultimately causing cellular oxidative damage and plant death [30]. Plants possess numerous ROS-scavenging enzymes, including SOD, CAT, POD, and APX, which convert O and H O into less reactive substances, reducing or eliminating their attack on membrane lipids and preventing peroxidation. These enzymes work synergistically to maintain ROS at low levels, protecting cells from oxidative damage [19,31]. The activity levels of these antioxidant enzymes reflect the cell's capacity to scavenge ROS and resist external stress—higher activity indicates stronger ROS scavenging ability and greater protection for rice seedlings [12].

2.4.1 Changes in SOD Activity Under Low Temperature Stress

SOD is a superoxide radical scavenger and the only antioxidant enzyme capable of reducing O to H O while oxidizing another O to O [32]. Its activity increases under stress conditions to enhance plant resistance. As shown in Figure 4A, SOD activity in both 9311 and DC907 exhibited a decreasing-then-increasing trend with prolonged cold stress, reaching maximum activity after 5 days. Both varieties showed significantly elevated SOD activity compared to controls after 5 days of cold stress. Throughout the entire cold stress period, DC907 maintained significantly higher SOD activity than 9311, indicating superior cold tolerance in DC907.

2.4.2 Changes in CAT Activity Under Low Temperature Stress

Significance analysis in Table 1 and Figure 4B revealed that CAT activity in both DC907 and 9311 increased significantly with cold stress duration, both within and between varieties. As shown in Figure 4B, DC907 exhibited a substantial increase in CAT activity after 5 days of cold stress, significantly higher than 9311. This suggests a correlation between CAT activity and DC907's cold tolerance following low temperature stress.

2.4.3 Changes in POD Activity Under Low Temperature Stress

POD is an iron-containing proteinase that plays an important role in plant cold tolerance. Studies have shown POD induction in bananas during cold stress reduces lipid peroxidation and maintains leaf cell water potential, thereby increasing cold tolerance [33]. As shown in Figure 4C, POD activity in both DC907 and 9311 increased slowly during the first 3 days of cold stress. However, after 5 days, POD activity in DC907 increased dramatically and was significantly higher than in 9311, where no obvious change occurred. This suggests that the substantial increase in POD activity plays an important role in DC907' s cold tolerance.

2.4.4 Changes in APX Activity Under Low Temperature Stress

Plants accumulate large amounts of oxidizing substances including H O under stress conditions. Ascorbate peroxidase (APX) can decompose H O and is considered to play an important role in plant stress resistance. As shown in Figure 4D, APX activity in 9311 decreased initially then recovered, remaining below control levels throughout the cold stress period. In contrast, DC907 showed decreased APX activity after 1 day of stress, but activity increased significantly with prolonged stress, exceeding control levels at both 3 and 5 days. During the late cold stress stage (3 and 5 days), APX activity in DC907 was significantly higher than in 9311. Since APX is primarily located in the chloroplast stroma and thylakoid membranes, its high activity in DC907 would help protect chloroplasts from cold damage throughout the stress period, which is crucial for maintaining physiological function. This indicates that DC907 has stronger capacity for ROS scavenging and chloroplast damage resistance than 9311 under low temperature stress.

2.5 Membership Function Values

Since MDA and O reflect biomembrane damage and are negatively correlated with cold tolerance, inverse membership functions were used for analysis. The remaining five indices (SOD, POD, CAT, APX, and soluble sugar content) reflect cellular damage resistance and are positively correlated with cold tolerance, thus employing standard membership functions. Previous studies have noted that single physiological indices have limitations in expressing plant stress resistance [13,27,34], while membership function analysis provides more accurate evaluation [35,36]. Therefore, using average membership function values for comprehensive assessment of rice cold tolerance is more reasonable. DC907 exhibited higher average membership function values than 9311, and the comprehensive evaluation ranking of cold tolerance (DC907 > 9311) matched phenotypic observations (Figure 5 [Figure 5: see original paper]). The ranking of membership function values for O production rate, MDA content, POD activity, and APX activity was consistent with the average membership function value ranking, indicating these indices are closely related to rice cold tolerance (Table 2).

3. Conclusion and Discussion

Plants face various abiotic stresses during their life cycles, including low temperature, salinity, drought, and heavy metal ion stress [37]. These stresses typically cause osmotic and oxidative damage, affecting normal growth and development while reducing yield [38]. Low temperature stress adversely affects rice during seed germination, morphogenesis, seedling growth, and reproductive stages, generally causing metabolic disorders, growth retardation, and reduced reproductive capacity, with prolonged stress leading to plant death. Soluble sugars are important osmotic regulators whose accumulation in rice can mitigate low temperature damage. In this study, soluble sugar content in rice leaves increased after cold stress, with DC907 showing significantly higher content than 9311 after 5 days, suggesting that rice maintains osmotic balance by increasing soluble sugar content under cold stress. The accumulation of MDA and O can reflect the degree of plant damage during stress. In this study, 9311 showed higher O production rates than DC907 as cold stress duration increased, indicating greater damage to 9311. MDA content also increased significantly under cold stress, causing membrane damage, with 9311 showing significantly higher MDA content than DC907 throughout the stress period, further reflecting DC907' s superior cold tolerance.

Antioxidant enzyme systems in plants are typically located in different cellular compartments and work together to scavenge ROS. SOD acts first, reducing superoxide radicals to hydrogen peroxide. Low temperature disrupts CAT dynamic balance, increasing its activity to scavenge excess H O [32]. POD, as an important ROS-scavenging enzyme, catalyzes reactions between H O and phenolic compounds to defend against cold damage. POD activity continued to increase during cold stress, contributing to low temperature resistance. APX in DC907 maintained high activity throughout the cold stress period, scavenging H O to reduce membrane lipid peroxidation and decrease MDA content. As an osmotic regulator, soluble sugars may also play important roles in maintaining protein stability. The increase in soluble sugar content during cold stress appears strongly associated with DC907' s cold tolerance. In this study, as cold stress duration increased, O production rate and antioxidant enzyme activities showed opposite trends—when antioxidant enzyme activities were low, O production rate increased, and vice versa. This likely reflects the action of SOD, which defends against superoxide radical damage through the dismutation reaction: O + O + 2H → H O + O . Since H O is less reactive, it can be further decomposed by CAT, POD, and APX. Therefore, under cold stress, antioxidant enzyme systems in rice seedlings reduce oxidative damage by increasing their activities.

The changes in these physiological indices in the two near-isogenic rice varieties under low temperature stress elucidate the physiological basis of cold tolerance in rice seedlings. Comprehensive evaluation using membership functions confirmed that DC907 possesses greater cold tolerance than 9311.

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Note: Figure translations are in progress. See original paper for figures.