Scientific and Social Research, 2026, Volume 8, Issue 5
http://ojs.bbwpublisher.com/index.php/ssr
Online ISSN: 2981-9946
Print ISSN: 2661-4332

Analysis of Dynamic Changes of Endosperm Starch Content and Related Gene Expression at Different Stages of Rice Seed Germination

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Abstract: When seeds reach full maturity, they store large amounts of nutrients such as starch, which serve as the energy base for the subsequent germination process. This study took rice seeds as the research object, focusing on analyzing the changes in endosperm starch content at different germination stages and the expression of key genes involved in starch hydrolysis. Multiple technical methods were adopted, such as iodine staining microscopy, total RNA extraction, and reverse transcription-quantitative PCR (RT-qPCR), to detect and analyze the shape, quantity, and staining intensity of endosperm starch granules, and the relative expression of the *OsAmy1A* gene in rice seeds germinated for 1, 3, and 5 days. The results showed that during rice seed germination, the seeds presented obvious temporal developmental characteristics: no obvious germination phenomenon at 1 day, radicle protrusion at 3 days, and seedling emergence at 5 days. The endosperm starch content showed a continuous downward trend, which was consistent with the energy demand of seed development. As a key gene for starch hydrolysis, the relative expression of *OsAmy1A* increased gradually, with a significant increase between 3 and 5 days of germination. These two dynamic changes were closely related, which proved that the *OsAmy1A* gene accelerates the hydrolysis and transformation of endosperm starch by regulating the synthesis of α-amylase, thus providing the necessary energy and materials for seed germination and early seedling growth. The results of this study provide an experimental basis for further exploring the regulatory mechanism of material metabolism during rice seed germination, and also provide practical reference for optimizing rice seed storage, improving processing technology, and enhancing rice quality.

Keywords: Rice seeds; Seed germination; Endosperm; Starch content; *OsAmy1A* gene; Quantitative PCR; α-amylase; Metabolic regulation

Online publication: June 5, 2026

1. Background and objectives

Rice is one of the most important food crops in the world. The uniformity and vitality of its seed germination

will directly affect the growth of subsequent plants and then determine the final yield and quality. The endosperm is the main nutrient storage organ of rice seeds, in which starch accounts for 60%–80% of the dry weight of seeds and is the core energy source during seed germination. During seed germination, endosperm starch needs to be gradually decomposed into soluble sugars such as glucose and maltose under the joint action of a variety of hydrolases [1]. These soluble sugars are then transported to the growing parts, such as radicles and plumules, through the conductive tissues of endosperm and embryo, providing energy and carbon sources for cell division, differentiation, and organ formation.

Starch hydrolysis involves the participation of many enzymes. Among them, α-amylase is the key rate-limiting enzyme that controls starch degradation. Its coding genes mainly include *OsAmy1A*, *OsAmy3D*, and others [2]. Previous studies have proved that starch metabolic efficiency is closely related to seed germination rate and seedling stress resistance [3]. But up to now, there is still a lack of systematic quantitative analysis and detailed verification on the synergistic regulatory relationship between the dynamic changes of endosperm starch content and the expression of key hydrolase genes in different germination stages, including the imbibition stage, protrusion stage, and seedling stage.

Based on the above research background, this study took the 9522 rice variety as the experimental material. By accurately controlling the germination conditions, the study systematically observed the overall development status of seeds germinated for 1, 3, and 5 days, and detected the endosperm starch content and *OsAmy1A* gene expression level. The purpose of this study was to make clear four aspects: the phenotypic characteristics of rice seed growth and development at different germination stages; the dynamic changes of endosperm starch content with seed germination; the temporal expression characteristics of the *OsAmy1A* gene during seed germination; and the correlation between seed development status, starch content changes, and *OsAmy1A* gene expression. The results of this study can provide experimental support for in-depth analysis of the regulatory mechanism of material metabolism during rice seed germination, and also provide a theoretical basis for optimizing rice seed storage technology, improving processing technology, and breeding high-quality rice varieties, which is of great practical significance for improving the yield and quality of rice production [4].

2. Materials

The rice variety 9522 was chosen for this experiment, with seeds plump, evenly sized, undamaged, and free of pathogens and insect pests.

2.1. Experimental instruments

The instruments used in the experiment included a constant temperature water bath, a low-temperature high-speed centrifuge, a UV-visible spectrophotometer, adjustable pipettes (0.5–10 μL, 10–100 μL, 100–1000 μL, Thermo), a real-time fluorescent quantitative PCR instrument, an optical microscope, a constant temperature incubator, and a liquid nitrogen tank.

2.2. Experimental reagents

The reagents included 1% sodium hypochlorite solution (analytical grade), DEPC-treated water (RNase-free, Tiangen Biotech (Beijing) Co., Ltd.), Trizol reagent (Invitrogen), isopropanol (analytical grade), chloroform

(analytical grade), reverse transcription kit (FastQuant RT Kit, Tiangen Biotech (Beijing) Co., Ltd.), SYBR Green real-time fluorescent quantitative PCR kit (SuperReal PreMix Plus, Tiangen Biotech (Beijing) Co., Ltd.), iodine staining solution (1 g I_2 and 2 g KI dissolved in 100 mL distilled water, prepared and used immediately), and *Actin* reference gene primers and *OsAmy1A* gene-specific primers (synthesized by Sangon Biotech (Shanghai) Co., Ltd.).

2.3. Experimental consumables

The consumables included RNase-free 2 mL EP tubes, RNase-free 1.5 mL EP tubes, RNase-free pipette tips (10 μL, 200 μL, 1000 μL, Axygen), sterile Petri dishes (9 cm in diameter, Corning), sterile filter paper (9 cm in diameter, Whatman), glass slides, coverslips (Fisher Scientific), centrifuge tube racks, and pipette tip boxes.

3. Experimental methods

3.1. Seed disinfection and cultivation

First, the screened rice seeds were rinsed with sterile water to remove surface impurities. Then, they were soaked in 1% sodium hypochlorite solution for 10 minutes, during which they were gently shaken every 2 minutes to ensure that the disinfection was thorough. After disinfection, the seeds were rinsed with sterile water for 3 times, and each rinsing time was not less than 1 minute to completely remove the residual sodium hypochlorite solution, so as to avoid inhibiting seed germination.

The disinfected seeds were evenly spread on Petri dishes covered with three layers of moist sterile filter paper. Each Petri dish was placed with 50 seeds, and an appropriate amount of sterile water was added to make the filter paper fully moist without standing water. The Petri dishes were placed in a constant temperature incubator at 28 ℃ for 3 days in the dark, so as to simulate the imbibition and protrusion stages of seed germination. After that, the seedlings were transferred to a light incubator with 16 hours of light (light intensity 3000 lux) and 8 hours of darkness for 2 days, so as to simulate the seedling growth stage. Seed samples were collected at 1 day (1 DAG), 3 days (3 DAG), and 5 days (5 DAG) after seed soaking and cultivation. Each group was set with three biological replicates, and 5 seeds were randomly selected from each replicate for subsequent experiments.

3.2. Observation of the overall development status of seeds

The seed samples of each stage were collected, and the seed coat was removed. The development characteristics of the seeds were observed and recorded under a stereomicroscope, including the water absorption of the seeds and the protrusion of the radicles. At the same time, the seeds were photographed and recorded to intuitively show the development status of different germination stages.

3.3. Observation of starch content by iodine staining

3.3.1. Slide preparation

The seeds were cut along the midline with a dissecting knife, and the embryo was removed. The middle part of the endosperm tissue was cut into 50 μm thick slices with a blade, which were placed on glass slides. One drop of iodine staining solution was added, stained at room temperature for 30 seconds, and the excess

staining solution was blotted with absorbent paper. Finally, one drop of sterile water was added to mount the slide.

3.3.2. Observation and recording

The slides were observed under an optical microscope, and three different fields of view were selected for each sample to take photos. The starch content was judged according to the staining intensity: the darker the color, the higher the starch content; the lighter the color, the lower the starch content. The staining results were intuitively described as dark blue, blue, and light blue.

3.4. Total RNA extraction and cDNA synthesis

3.4.1. Total RNA extraction

Endosperm tissues were collected from seeds at different developmental stages and immediately put into pre-chilled RNase-free 2 mL EP tubes. 500 μL Trizol reagent was added, and the samples were fully ground with a tissue grinding rod until a uniform slurry was formed. The mixture was placed at room temperature for 5 minutes to ensure that nucleic acids and proteins were completely separated. Then, 100 μL chloroform was added to the tube, shaken vigorously for 15 seconds, and placed at room temperature for 3 minutes, then centrifuged at 4 ℃ and 12,000 r/min for 15 minutes.

About 200 μL of the upper aqueous phase was carefully sucked into a new RNase-free 1.5 mL EP tube, and an equal volume of isopropanol was added. The tube was gently inverted to mix well, and placed at −20 ℃ for 30 minutes to precipitate RNA. Then, it was centrifuged at 4 ℃ and 12,000 r/min for 10 minutes, the supernatant was discarded, 500 μL 75% ethanol (prepared with RNase-free water) was added, and the RNA precipitate was rinsed by gently inverting the tube. It was then centrifuged at 4 ℃ and 7,500 r/min for 5 minutes, and the supernatant was discarded. The RNA precipitate was air-dried at room temperature for 5–10 minutes, and excessive drying should be avoided. The dried precipitate was dissolved in 30 μL DEPC-treated water, and the prepared RNA sample was stored at −80 ℃ for later use.

3.4.2. Detection of RNA purity and integrity

The OD_{260}/OD_{280} ratio of RNA samples was measured by a UV-visible spectrophotometer. The ratio between 1.8 and 2.0 indicated that the RNA purity was qualified, and there was no protein or phenolic contamination.

3.4.3. cDNA synthesis

With qualified total RNA as the template, cDNA was synthesized according to the instructions of the reverse transcription kit. The total reaction volume was 20 μL, including 4 μL 5× FastQuant RT Buffer, 1 μL RT Enzyme Mix, 1 μL FQ-RT Primer Mix, 2 μg RNA template, and RNase-free water was added to make up to 20 μL. The reaction was carried out at 42 ℃ for 15 minutes for reverse transcription, and then heated at 95 ℃ for 3 minutes to inactivate the reverse transcriptase. The product was stored at −20 ℃ for later use.

3.5. Quantitative PCR detection of *OsAmy1A* gene expression

3.5.1. Primer design and synthesis

According to the coding region sequences of the rice *OsAmy1A* gene (accession number: LOC_Os08g36910) and the *Actin* reference gene (accession number: LOC_Os03g50885) published in GenBank, specific primers were designed by using Primer Premier 5.0 software. The primer sequences were as follows.

OsAmy1A upstream primer: 5′-GCTACGCATACATCCTCACC-3′,
downstream primer: 5′-GCTAAACTGAGAGTCTCGTCATC-3′;
Actin upstream primer: 5′-GAGATCACTGCCTTGGCTCC-3′,
downstream primer: 5′-CGATAACAGCTCCTCTTGGC-3′.

The specificity of the primers was verified by BLAST alignment, which was synthesized by Sangon Biotech (Shanghai) Co., Ltd., with HPLC grade purity.

3.5.2. Quantitative PCR reaction

The synthesized cDNA was used as a template, and the SYBR Green real-time fluorescent quantitative PCR kit was used for the reaction. The total reaction volume was 10 μL, including 4 μL 2× SuperReal PreMix Plus, 0.3 μL upstream primer (10 μmol/L), 0.3 μL downstream primer (10 μmol/L), 1 μL cDNA template, and 4.4 μL RNase-free Water. The reaction procedure was: pre-denaturation at 95 ℃ for 15 minutes, then 40 cycles of denaturation at 95 ℃ for 10 seconds, annealing at 58 ℃ for 20 seconds, and extension at 72 ℃ for 20 seconds. Each sample was set with three technical replicates.

3.5.3. Data processing

The relative expression level of the *OsAmy1A* gene was calculated by the 2^-ΔΔCt method, where ΔCt=Ct(*OsAmy1A*)-Ct(Actin), and ΔΔCt=ΔCt(treatment)-ΔCt(control) (1 DAG sample served as the control group) [5].

4. Conclusion and discussion

4.1. Phenotypic characteristics of seed growth at different germination stages

The germination process of rice seeds presents obvious sequential development characteristics, as shown in **Figure 1**. At 1 day of germination (1DAG), the seeds were in the imbibition stage, without obvious germination signs, and the radicles did not protrude. During this period, the seeds mainly absorbed water through imbibition, which laid the foundation for the subsequent metabolic activities. At 3 days of germination (3DAG), the seeds entered the protrusion stage, the radicles broke through the seed coat and began to elongate, and the metabolic activity of the seeds was fully activated, entering the preparation stage for rapid growth. At 5 days of germination (5DAG), the seeds developed into seedlings, the radicles were significantly elongated, the plumules continued to grow, some seedlings unfolded their first true leaves, and the initial roots were formed, which meant that the seeds completed the transformation from heterotrophic growth to autotrophic growth.

Figure 1. Temporal development characteristics of rice seed germination

4.2. The change of starch content was shown by iodine staining

The color change of iodine staining clearly reflected the slow decrease in starch content, as shown in **Figure 2**. At 1DAG, the endosperm tissue showed dark blue after staining, and the color was uniform, indicating that the starch content in the seeds was the highest at this time, and the energy warehouse was full. At 3DAG, the staining color was much lighter than that at 1DAG, which indicated that the starch had been decomposed to provide energy for the growth of radicles. At 5DAG, the staining color was lighter than that at 3DAG, showing that the starch continued to decompose to provide sufficient energy for the rapid growth of seedlings.

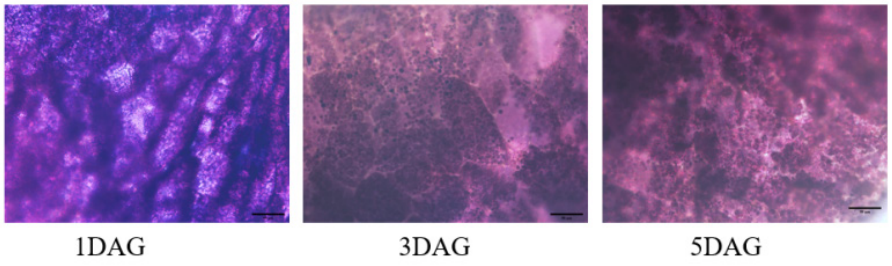

Figure 2. Changes in starch content during germination of rice seeds

4.3. The expression changes of the *OsAmy1A* gene

The results of quantitative PCR showed that the expression level of the *OsAmy1A* gene increased continuously, as shown in **Figure 3**. Taking the expression level of 1DAG as the standard (set to 1), the expression level increased by about 40 times at 3DAG and about 380 times at 5DAG. This showed that the gene was continuously activated during seed germination, and its expression intensity was getting stronger and stronger. The increase in gene expression was consistent with the fading trend of iodine staining: the stronger the gene expression, the more starch is decomposed, and the lighter the staining color.

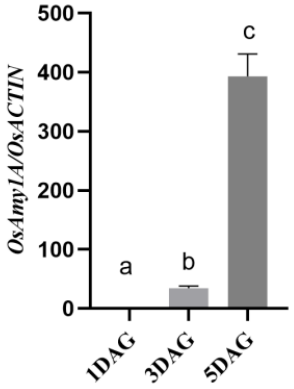

Figure 3. The relative expression level of the *OsAmy1A* gene during seed germination

5. Conclusion and prospect

This study revealed the stage characteristics and molecular regulatory mechanism of rice seed germination, and clarified the precise cooperative relationship between gene expression, material metabolism, and

morphogenesis. Under suitable environmental conditions, including adequate water, temperature (25–30 °C), and humidity (70%–80%), the germination of rice seeds showed obvious stage changes. On the first day, the seeds completed the imbibition process, the volume expanded significantly, and the seed coat softened. On the third day, the radicles broke through the seed coat and protruded. On the fifth day, the coleoptiles and primary roots continued to grow and develop into seedlings with complete morphology, which had preliminary photosynthetic capacity and nutrient absorption structure.

During this dynamic process, the metabolic activity inside the seeds changed significantly. Through iodine staining observation, it was found that with the progress of germination, the staining color of the endosperm gradually faded, from the initial dark blue to light brown or yellowish brown on the fifth day, indicating that the starch content stored in the seeds was continuously decreasing. These decomposed starches were converted into soluble sugars under the action of hydrolases such as α-amylase, providing the necessary energy for seed germination and seedling growth.

At the same time, the results of real-time fluorescent quantitative PCR showed that the expression level of the *OsAmy1A* gene increased continuously with the progress of germination. The gene expression increased significantly from the third day of germination, and the expression level on the fifth day was more than 300 times that in the early stage of germination. Further analysis found that there was a significant positive correlation between the expression activity of the *OsAmy1A* gene and the starch decomposition rate: the stronger the gene expression, the more α-amylase is synthesized, and the higher the starch decomposition efficiency.

These findings form a complete metabolic regulatory network. The *OsAmy1A* gene promotes the formation of carbohydrates by regulating the starch decomposition process, and provides sufficient energy for morphogenesis processes such as radicle elongation and coleoptile protrusion, thus supporting the smooth growth of seedlings. This process reflects the multi-level coupling mechanism of gene regulation, material metabolism, and morphogenesis in plant physiological processes.

From the practical application point of view, the results of this study have important agricultural value. First, the storage conditions of rice seeds can be optimized according to the temporal expression characteristics of the *OsAmy1A* gene. By accurately controlling the temperature, humidity, and gas composition of the storage environment, the early expression of the *OsAmy1A* gene in a non-germinating state can be effectively inhibited, the unnecessary consumption of endosperm starch can be reduced, and the storage life and use value of seeds can be prolonged. Second, molecular breeding technologies such as CRISPR/Cas9 can be used to directly edit the regulatory elements of the *OsAmy1A* gene, optimize its expression pattern and time, so as to cultivate new rice lines with uniform germination, strong seedlings, and high cold and drought resistance. These new lines can improve the stability of rice seedling emergence uniformity, seedling rate, and final yield under adverse conditions. In addition, the phenotype-gene expression correlation analysis model established in this study can also provide a method and theoretical reference for the study of seed germination of important gramineous crops such as wheat and maize.

Nevertheless, the present study still has several limitations. First, only 9522 rice varieties were used as the research object. Different rice varieties may have differences in seed size, starch composition, germination rate, and other aspects. Future research should expand the variety scope, including indica rice, japonica rice, hybrid rice, and other types of rice. Second, this study was carried out under controlled conditions and did not involve the influence of environmental factors such as temperature, water, and salt-

alkaline stress. Subsequent studies can set up different environmental treatments to explore the expression dynamics of *OsAmy1A* under different stress conditions. In addition, seed germination is a complex biological process involving multiple genes and multi-level regulation. Future research should integrate multi-omics technologies such as transcriptomics, proteomics, and metabolomics, so as to deeply understand the overall regulatory mechanism of rice seed germination from the perspective of systems biology.

Disclosure statement

The author declares no conflict of interest.

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