Journal of Biotechnology Research
ISSN(e): 2413-3256, ISSN(p): 2413-8878
Vol. 9, Issue. 1, pp: 21-32, 2023
URL: https://arpgweb.com/journal/journal/16
DOI: https://doi.org/10.32861/jbr.91.21.32

Academic Research Publishing Group

Original Research **Open Access**

Screening and Validation of Rice OsAAP6 Interaction Protein

Bo Peng
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China

Jing Qiu
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China

Dong-Yan Kong
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China

Juan Peng
Xinyang Station of Plant Protection and Inspection, Henan Xinyang 464000, China

Yan Liu
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China

Gui-Ying Guo
Xinyang Academy of Agricultural Science, Henan Xinyang 464000, China

Xiao-Yu Sun
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China

Xiang-Jin Xu
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China

Yan-Fang Sun
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China

Rui-hua Pang
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China

Wei Zhou
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China

Jin-Hui Zhao
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China

Quan-Xiu Wang (Corresponding Author)
College of Life Sciences and Institute for Conservation and Utilization of Agro-bioresources in Dabie Mountains, Xinyang Normal University, Henan Xinyang 464000, China
Email: wwqqxx08@163.com

Article History
Received: 12 March, 2023
Revised: 25 May, 2023
Accepted: 20 June, 2023
Published: 28 June, 2023

Copyright © 2023 ARPG & Author
This work is licensed under the Creative Commons Attribution International

CC BY: Creative Commons Attribution License 4.0

Abstract

The protein content of rice seeds is an extremely important quality trait, but its genetic basis and molecular regulatory mechanism are still unclear. This study focuses on a positive regulatory gene *OsAAP6* of grain protein content in rice. Proteins that interact with *OsAAP6* were screened using yeast two hybrid experiments, and validated using in vivo point-to-point experiments and bimolecular fluorescence complementarity tests (BiFC). The main results are as follows: 98 positive colonies that may interact with OsAAP6 were screened from a rice cDNA library using yeast two hybrid technology. After sequencing and analysis, 40 proteins that may interact with *OsAAP6* were ultimately obtained. Through comparative analysis, three proteins (Pyruvate phosphate dikinase (PPDR), WRKY, and GreA) were selected from these 40 proteins that may interact with each other. In vivo point-to-point experiments in yeast and bimolecular fluorescence complementarity (BiFC) experiments in rice were used to further verify that PPDK, WRKY, and GreA can

interact with OsAAP6 protein, respectively. Therefore, the results of this study will provide important clues for further revealing the molecular mechanism by which the *OsAAP6* gene regulates grain protein content.

Keywords: Rice; OsAAP6; Interacting proteins; Screening and validation.

1. Introduction

Rice is one of the most important food crops in the world, with over half of the world's population relying on rice as their staple food [1]. Nearly two-thirds of the population in China relies on rice as their staple food. However, based on the current rate of global population growth, the global population will reach 10 billion by 2050 [2], which undoubtedly poses a great challenge to rice production. Breeders have attempted to utilize some high-yielding indica rice varieties to improve this yield requirement, but due to their poor taste and quality, they have not been favored by consumers and are difficult to promote commercially. Moreover, research has shown that improving rice quality often conflicts with increasing rice yield [3]. While improving the quality of rice, the yield of rice is often affected and decreases. Therefore, it is very important to improve rice quality without changing rice yield. The quality of rice mainly includes nutritional quality, cooking and eating quality, grinding and processing quality, and appearance quality [4]. In the past few decades, there has been a lot of research on rice quality, mainly including evaluation standards and measurement methods for rice quality, genetic research foundation and application of rice quality, influencing factors and improvement measures for rice quality, and localization of genes related to rice quality traits [5-10]. Although research on rice quality has been extensive, it can provide a theoretical basis for its causes and improvement measures. However, there are still many problems encountered in the practical breeding process of improving rice. Because the quality trait of rice is a complex quantitative trait that is influenced by both genes and environment [11], and in terms of environment, it is also susceptible to different environmental conditions [12-15]. Therefore, it is a very difficult and urgent problem to coordinate the relationship between rice yield, quality, and stress resistance, in order to obtain high-quality and high-yield rice varieties. Therefore, the cultivation and research of high-quality and high-yield new rice varieties is still urgent.

The main components of rice seed endosperm include starch and protein [16, 17], therefore, their relative content and composition are extremely important for the quality of rice. Protein, as the second major component in the nutritional quality of rice, is one of the main food sources for people to consume protein in their daily production activities. With the continuous improvement of people's living standards and increased awareness of nutrition and health, people will also have higher requirements for the nutritional quality of rice. Moreover, with the continuous development of the times, people's demand for high nutrient quality crops will continue to increase [18]. Therefore, in future research on crop breeding such as rice, improving the protein content and nutritional quality of crops is of great significance. At the same time, rice, as a model plant for crop molecular genetics and functional genomics research [19], studies the genetic regulation mechanism of its protein content, and improves rice breeding through scientific methods, so as to provide an important theoretical basis in the research of breeding new varieties of rice with high yield, high quality and strong resistance. Theoretical research on rice quality will provide guidance for the actual breeding process and provide global food production security.

Acid or dilute alkali gluten [20-22]. Glutenin is the protein that is most easily absorbed and digested by the human body among these four proteins, and it is also the protein with the highest content in rice seeds. Studies have shown that glutenin is encoded by 15 genes and can be roughly divided into four categories: A-type glutenin (including Glu A-1, Glu A-2, Glu A-3, Glu A-4), B-type glutenin (Glu B-1, Glu B-2, Glu B-3, Glu B-4, Glu B-5, Glu B-6), C-type glutenin, and D-type glutenin [11, 23-28]. Each type of glutenin is further divided into several subclasses, and the discovery of these different genes lays a solid foundation for further revealing the genetic research mechanism of rice proteins. The protein content of rice seeds is a typical quantitative trait controlled by multiple genes and is relatively protected by environmental influences.

More than 90% of the substances in rice seeds are starch and storage proteins [21, 29], which constitute the main nutritional components of rice. Moreover, studies have shown that the relative content ratio and composition structure of starch and storage protein in rice seeds are closely related to the formation of other quality traits of rice, such as appearance quality, cooking and eating quality, nutritional quality, and grinding processing quality [6, 30-33]. In addition, amino acids, as one of the important components of rice nutritional quality, not only play an important role in the growth, development, and metabolic processes of organisms, but also participate in numerous important physiological and biochemical reactions in plants. As an important substance in plant secondary metabolic reactions, they play an extremely crucial role [34-36]. With the continuous development of functional genomics research methods, there are more and more genetic research mechanisms of rice quality formation. Rice quality traits are a very complex genetic trait, controlled by multiple genes and easily influenced by external factors such as light, temperature, water, soil, etc [30]. At present, there have been many studies on the localization of QTL genes involved in regulating starch, protein, and amino acid content in rice. Therefore, elucidating its synthesis, regulation, and genetic basis on this basis has important theoretical significance and application value for genetic improvement of rice quality in the future.

Rice is the staple food for most people and also one of the main sources of protein intake. For developing countries and relatively poorer and underdeveloped countries, the protein content of rice is particularly important. The protein content in rice seeds varies greatly among different regions and varieties, ranging from 4.3% to 19.3% [34]. But overall, the average protein content of different rice varieties is 9.5% [34, 37]. According to the solubility of storage proteins in rice seeds and different extraction methods, their storage proteins can be divided into four categories: water-soluble albumin; Globulin soluble in dilute hydrochloric acid; Soluble in alcohol water mixture and

soluble in dilute solution. At present, research on rice protein content mainly focuses on QTL localization related to rice protein content [3], and there is still less research on its function. For example, the *OsAAP6* gene in rice is located on the long arm of rice chromosome 1 [6], Localization of several genes for type A glutenin revealed that both Glu A-1 and Glu A-4 genes were located on chromosome 1 Interestingly, localization studies have found that the Glu A-4 gene is a pseudogene, with Glu A-3 gene located on chromosome 3 and Glu A-2 gene located on chromosome 10, All other genes are distributed on chromosome 2, with only 2 genes identified for C-type glutenin and 1 gene identified for D-type glutenin [23-28]. Significant progress has been made in using specific biochemical methods for protein expression analysis and interaction protein screening of rice related genes [23, 38-41].

At present, some studies on the *OsAAP6* gene and *OsAAP6* gene family in rice have gradually deepened [42]. Many researchers use rice mutant materials to explore related genes that control rice protein content, and some related genes have been isolated and cloned [5, 6, 43]. However, the specific molecular regulation mechanism, as well as the synergistic regulation of these genes in protein synthesis and transport and other related factors are still unclear. Therefore, revealing the molecular regulatory network of *OsAAP6* gene and elucidating the regulatory mechanism of rice protein content is of great significance for deeply analyzing the molecular basis of rice protein synthesis and accumulation, and then using molecular breeding techniques to cultivate high-quality new rice varieties.

2. Test Materials

2.1. Test Strain

Yeast two hybrid test using strain *MaV203.*

2.2. Test Carrier

The yeast two hybrid experiment used vectors: pDONR222, pDEST32, pDEST22 (purchased from Invitrogen company).

The yeast two hybrid library pDEST22-cDNA was donated by Huazhong Agricultural University.

2.3. Main Instruments and Equipment

PCR instrument (5020); Visible spectrophotometer; Fluorescence microscope; Eppendorf centrifuge, etc.

3. Test Method

3.1. Yeast Two Hybrid Screening for Interacting Proteins

3.1.1. Preparation and Transformation of Yeast Receptive Cells

① Add 100 μ L of the prepared yeast receptive cells to a 1.5 mL centrifuge tube, followed by 10 μ L (5 μ g/μ L) of ssDNA, 1 μ L of carrier DNA, and 40% PEG-3350/1 of 500 μ L of vector DNA × LiAc/TE, operate on ice and mix gently and thoroughly.

② Place it in a 30 ℃ incubator for 30 minutes (gently mix every 10 minutes).

③ After taking it out, add 20 μ L DMSO and mix gently. Heat shock at 42 ℃ for 20 minutes (lightly flick every 5-10 minutes).

④ After heat shock, remove it and centrifuge at 12000 rpm for 30 seconds.

⑤ After removing the supernatant, add 100 μ L of sterile 0.9% (w/v) NaCl solution and gently resuspend the bacterial body.

⑥ Then apply it to the corresponding amino acid deficient plate and incubate it upside down at 30 ℃ for 3 d-5 days.

3.1.2. Toxicity Detection of Bait Expressed Proteins

Before screening for interacting proteins, it is necessary to first verify whether the expression of the *OsAAP6* gene in rice affects yeast *MaV203*, which means that the expression of OsAAP6 protein is non-toxic to yeast cells. Transfer the pDEST32 empty vector and the pDEST32-*OsAAP6* expression vector with the target gene into the yeast strain *MaV203*, apply them onto the SC Leu plate, and culture at 30 ℃ for 3 d-5 days. Observe and compare the growth of yeast cells on the two plates to identify whether the bait expression vector is non-toxic to yeast.

3.1.3. Bait Expression Protein Self-Activation Detection

If the Activation domain (AD) function of the decoy protein activates the expression of His reporter genes downstream, then yeast cells transformed with the decoy fusion plasmid can grow on the deficient medium lacking His. Therefore, this condition should be excluded before the screening test to inhibit its self-activating activity and reduce the false positive results during the screening test. His is known to encode an enzyme related to histamine biosynthesis, and its activity is specifically inhibited by 3-amino-1, 2, 4-triazole (3-Amino-1, 2, 4-triazole, 3AT), and it is econometric dependent. That is, under certain conditions, with the increase of 3AT concentration, the inhibition self-activation characteristics are enhanced. In this experiment, different gradients of 3AT concentration were set to screen out the best inhibition of self-activation of decoy protein.

2.1.4. Screening of Interacting Proteins

The decoy expression vector pDEST32-*OsAAP6* and rice cDNA library pDEST22-cDNA were transferred into yeast *MaV203* cells, coated in SC-Leu-Trp medium, and cultured at 30℃ for 3-5 days. Then, they were transferred to different defect types (SC-Leu-Trp-Ura, SC-Leu-Trp-His+3AT) and X-α-gal medium for growth, and the expression of reporter genes was observed.

3.2. Point to Point Validation in Yeast

The positive clones obtained through the above screening were cultured, sequenced, sorted out, and repeated sequences and vector sequences were removed, and 71 positive candidate clones were preliminarily obtained. After that, 40 potential interacting protein sequences were obtained. After gene annotation and statistical analysis, three candidate clones (Pyruvate phosphate dikinase, WRKY and FKBP, labeled as genes 1, 2 and 3, respectively) were selected. Then, the extracted plasmids were transferred into yeast cell *MaV203* together with pDEST32-*OsAAP6*, and the growth was observed on different amino acid defect medium. Finally, X-α-gal chromogenic reaction was performed. The interaction was verified again by rotary test in yeast.

3.3. BiFC Verification of Rice Protoplasts

3.3.1. Carrier Construction

Based on the restriction site characteristics of the selected BiFC test vectors (pSPYCE-35S and pSPYNE-35S), corresponding primers were designed to connect the *OsAAP6* gene to the pSPYCE-35S vector (the restriction site was *Bam* HI-*Xma*I), and the other three genes to be validated were connected to the pSPYNE-35S vector (the restriction site selection was divided into *PPDK*: *Xho* I-*Xma* I; *WRKY*: *Bam* HI-*Xm* I; *GreA*: *Bam* HI-*Xma* I). The final primers are as follows:

pSPYCE-35s::OsAAP6F: 5’-CGCGGATCCATGGACGTGGAGAAGGTGG-3’
pSPYCE-35s::OsAAP6R: 5’-CCCCCCGGGTCAGAGCTGCGTTTGGAAG-3’
pSPYNE-35S::1F: 5’-CCGCTCGAGATGGCGGTGATGGAGGAGC-3’
pSPYNE-35S::1R: 5’-CCCCCCGGGCTAGTTCGCTGTCAGGAGC-3’
pSPYNE-35S::2F: 5’-CGCGGATCCATGGCCGTGGACCTGATGG-3’
pSPYNE-35S::2R: 5’-CCCCCCGGGTTAGACATGTCCGTTGCCG-3’
pSPYNE-35S::3F: 5’-CGCGGATCCATGGGCTTCGAGAAGACGA-3’
pSPYNE-35S::3R: 5’-CCCCCCGGGTTACTGGGCGCTAAGAACC-3’

After the gene was amplified by PCR, the final expression vector was constructed by enzyme digestion and ligation between the gene and the corresponding empty vector. The plasmid was extracted from the correct carrier for final detection. Due to the high requirements of protoplast transformation test, the plasmids used must be sterile and endotoxin-free with high concentration requirements, so the extraction kit in QIAGEN kit was used to extract the plasmids.

3.3.2. Preparation and Transformation of Rice Protoplasts

3.3.2.1. Preparation of Rice Protoplasts

① Boil a water bath at 55℃ and divide 10 mL of 0.6 M mannitol into a 50mL conical flask.

② Take out 20-30 yellowing rice seedlings, cut off the roots, and then cut 7-8 cm of young rice stems upwards from the incision. Then cut them into 0.5 mm long and quickly balance them in 0.6 M mannitol for 10 minutes.

③ Use a 1mL gun to suck out 0.6 M mannitol, slowly add the enzymatic hydrolysate, wrap it in tin foil, and vacuum for 30 minutes.

④ Horizontal shaking table less than 40 rpm, enzymatic hydrolysis for 4-5 hours.

⑤ When the enzymatic hydrolysis is about to be completed, prepare a W5 solution, rinse 350 with a sieve, and place a 50mL round low centrifuge tube on an ultra clean workbench to blow dry.

⑥ Gently shake the triangular flask containing the enzymatic hydrolysate for 30 seconds, add 10 mL of W5 solution, shake the bed horizontally at 80 rpm, release for 10 minutes, and release the protoplasts twice with W5 solution, gently shake for 10 minutes each time. Filter the solution into a 50mL round bottom centrifuge tube, gently and avoiding light.

⑦ 100 g, set acceleration and deceleration to 0, centrifuge at room temperature for 5 minutes.

⑧ Carefully use the cut gun tip to absorb the supernatant and discard it. Leave 1 mL of liquid at the bottom of the tube, add 1 mL of W5 solution, gently shake the protoplasts, and place them in a dark room for 60-90 minutes.

⑨ Centrifuge 100 g at room temperature for 5 minutes and discard the supernatant. Suspend the protoplast weight in 1 mL of MMG solution for the next transformation step.

3.3.2.2. Transformation of Rice Protoplasts

① Take 10 separately μ Add L plasmid into a sterile clean 1.5 mL centrifuge tube, then slowly and gently add 100 μ L protoplast suspension, gently rotate to shield and mix well.

② Slowly add 120 along the centrifuge tube wall μL of PEG-CaCl2 solution, mix gently and let stand at room temperature for 10-15 minutes.

③ Add 480 μ Gently invert the W5 solution of L to stop the conversion.

④ 200 g room temperature for 5 minutes, and the supernatant was discarded.

⑤ Add 0.5 mL of W1 solution, resuspend the protoplasts, and gently transfer them to the culture plate. 300 pre added to the culture board μ W1 solution of L.

⑥ Incubate at room temperature in dark for 12-16 hours.

3.3.3. Fluorescence Observation

Centrifuge 100 g of the overnight cultured cells and collect them for 5 minutes. Use a pipette to gently draw an appropriate amount of bottom cells onto a slide, cover the slide gently, and observe under a fluorescence microscope. Pay attention to avoiding light as much as possible during the entire process

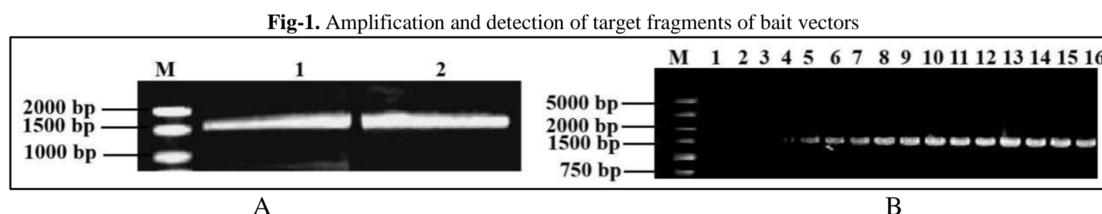
4. Results and Analysis

4.1. Yeast Two Hybrid Screening for Interacting Proteins

4.1.1. Construction of Bait Expression Vector

Using the constructed pMD19-T-*OsAAP6* vector as a template, the target fragment was amplified using the designed primers with attB connectors. As shown in Figure 1 A Agarose gel results below, the bands are within the expected correct band size range. After gel cutting and recycling, the final yeast bait expression vector pDEST32-*OsAAP6* was constructed through a two-step recombination reaction of BP and LR. Convert it to E. coli *DH5α* After growth, PCR detection of bacterial fluid was performed in cells. As shown in Figure 1, the size of the B band is correct and sequencing is required. The final sequencing correct sequence obtained is the final yeast bait expression vector pDEST32-*OsAAP6*.

Fig-1. Amplification and detection of target fragments of bait vectors

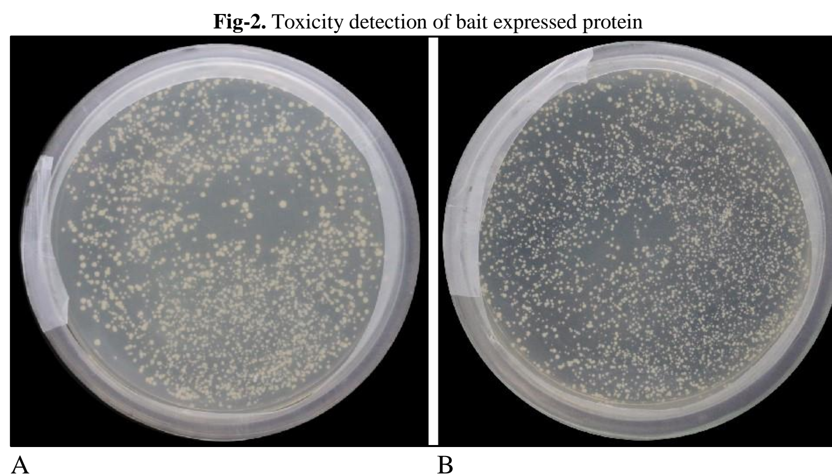


Note：A：Amplification of *OsAAP6*；B：detect the expression vector of pDEST32-*OsAAP6* by PCR with bacteria solution

4.1.2. Toxicity Detection of Bait Expression Vectors

Before conducting yeast two hybrid screening experiments, it is necessary to first verify whether the expression of rice *OsAAP6* gene will affect yeast *MaV203*, that is, to prove that the expression of OsAAP6 protein is non-toxic to yeast cells. Transfer the pDEST32 empty vector and the pDEST32-*OsAAP6* expression vector with the target gene into the yeast strain MaV203, apply them onto the SC Leu plate, and culture at 30 ℃ for 3 d-5 days. Observe and compare the growth of yeast cells on the two plates to identify whether the bait expression vector is toxic to yeast. As shown in Figure 2, the growth status of yeast cells transformed with rice *OsAAP6* gene is similar to that of yeast cells not transformed with *OsAAP6* gene. At the same time, monoclonal clones were selected and grown overnight on YPAD liquid culture medium at 30 ℃, and their OD600 values were all above 0.8, indicating that the rice OsAAP6 protein can be expressed normally in yeast and has no toxic effect on yeast cells.

Fig-2. Toxicity detection of bait expressed protein



Note：A：pDEST32 was transfered into yeast MaV203 cells；B：pDEST32-OsAAP6 was transfered into yeast MaV203 cells

4.1.3. Self-Activation Detection of Decoy Expression Vector

If the decoy protein has self-activation, that is, it has the function of transcriptional activation domain, then the expression of downstream His reporter gene can be activated. If yeast cells transformed with bait-fusion plasmids can grow on the deficient medium lacking His, it is not possible to determine whether the expression of downstream

reporter genes is caused by the bait-caught interacting proteins in the library or by the auto-activation of the bait-protein. It dramatically increases the probability of screening for a false positive. Therefore, for decoy expressed proteins with self-activating activity, the self-activating activity should be inhibited to reduce the false positive results in the screening process.

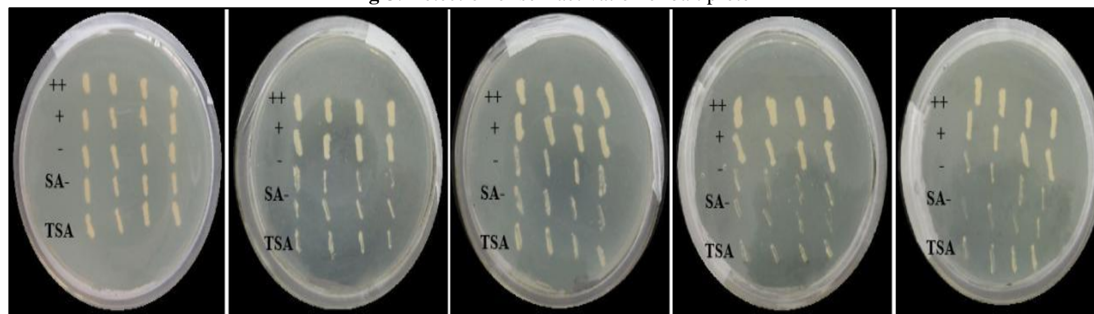
His is known to encode an enzyme related to histamine biosynthesis, the activity of which can be specifically inhibited by 3AT, and it is econometric dependent at certain concentrations. Under certain 3AT metering control, with the increase of 3AT concentration, the inhibition of self-activation will be enhanced. In this experiment, different gradients of 3AT concentration were set to screen out the best inhibition of self-activation of decoy protein. As shown in Figure 3, strong interaction control, weak interaction control, non-interaction control and self-activated negative control were set up at the same time. In the absence of 3AT, compared with the negative control, the growth of the decoy protein was better, that is, the decoy protein may have weaker expression. With the increase of 3AT concentration, the expression of decoy protein showed an obvious trend of decline, which preliminatively proved that 3AT could well inhibit the self-activation activity of OsAAP6 decoy protein. When the concentration of 3AT was 75 mM, it could be seen from the figure that yeast cells basically no longer grew, similar to the negative control, which indicated that the expression of decoy proteins had been basically inhibited. Therefore, we finally determined that the optimal concentration of 3AT to inhibit the expression of the decoy protein was 75 mM, which could better inhibit the self-activation activity of the decoy protein, thus greatly reducing the false positive results in the screening process of yeast two-hybrid.

4.1.4 Screening of Interacting Proteins

Transfer the bait plasmid pDEST32-*OsAAP6* and the rice cDNA library plasmid pDEST22-cDNA into yeast *MaV203* cells, coat them in SC Leu Trp medium, and incubate at 30 ℃ for 3 d-5 days. As shown in Figure 3 A, the yeast growth status is good, indicating that the bait plasmid and library plasmid have been successfully transferred into yeast cells and can be used for the next screening library test. Gently pick yeast cells grown on SC Leu Trp deficient medium with a toothpick onto SC Leu Trp Hi+75 mM medium and grow for 3 d-5 days in a 30 ℃ constant temperature incubator. As shown in Figure 3 B, there has been a significant difference in yeast growth. Some yeast colonies grow very large, some are very small, and some even do not grow. Observe and take photos. At this point, yeast cells grown on SC Leu Trp Hi+75 mM medium are positive yeast cells that successfully expressed the His reporter gene. Then, yeast cells grown on SC Leu Trp Hiss+75 mM deficient medium were gently picked with a toothpick onto SC Leu Trp Ura medium and grown for 3 d-5 days in a 30 ℃ constant temperature incubator. As shown in Figure 4 C, the yeast cells growing at this time are positive yeast cells expressing the Ura reporter gene. From Figure 4 C, it can be seen that there are significant differences in the growth of yeast cells on SC Leu Trp Ura medium, with some cells growing significantly and others very small. The yeast cells with minimal growth may indicate a weak interaction between the screened protein and OsAAP6 protein.

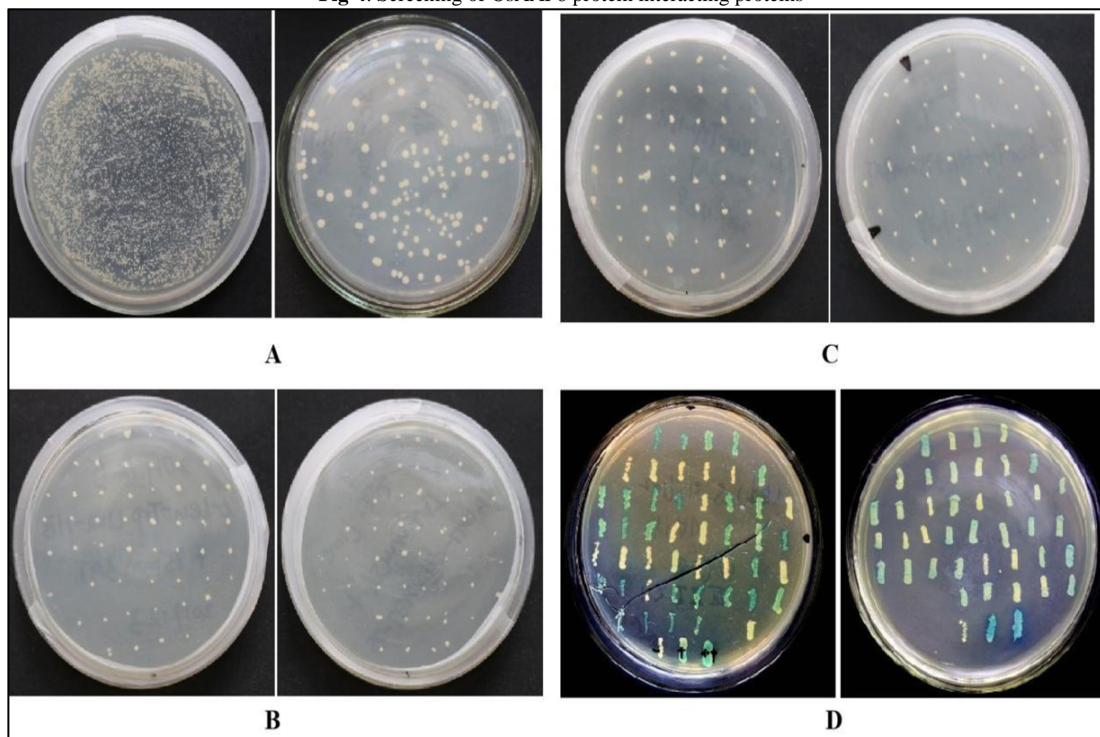
5-bromo-4-chloro-3-indole-α-D-galactoside has been widely used in the detection of yeast two-hybrid screening interaction protein assay for GAL4 system. It is widely used to identify positive blue clones quickly and easily by simple blue-white spot screening principle on culture medium, and there is no time consuming detection of β-galactosidase reporter gene in the process. In this experiment, in order to ensure the reliability of screening and reduce the probability of false positives, the positive clones grown on SD-Leu-Trp-Ura were transferred to YPDA containing X-α-gal for chromogenic reaction. As shown in Figure 4 D below, the white colony is the result of no interaction, while the blue colony is the positive clone screened, and the darker the blue, the stronger the interaction.

Fig-3. Detection of self-activation of bait protein



Note : ++ : Strong positive interaction control ; + : Weak positive interaction control; -: Negative interaction control; SA- : Negative self-activation control; TSA: Test of self-activation; A : 0 mM 3AT; B: 25 mM 3AT; C: 50 mM 3AT; D: 75 mM 3AT; E: 100 mM 3AT.

Fig-4. Screening of OsAAP6 protein interacting proteins



Note：A: The growth of yeast cells on SC-Leu-Trp, the left was diluted 10 × and the right was diluted 50 ×; B: The growth of yeast cells on SC-Leu-Trp-Ura; C: The growth of yeast cells on SC-Leu-Trp-His+75 3AT; D: The growth of yeast cells on YPAD.

4.1.5 Determination of Candidate Genes

98 positive colonies were screened through yeast double hybridization, and yeast plasmids were extracted after cultivation. Due to the difficulty in extracting yeast plasmids, the concentration is low. Therefore, first transform it into E. coli *DH5α* Culture, and then send the Escherichia coli solution for sequencing. Statistical analysis was conducted on the sequencing results, with 27 sequencing failures or no alignment results. The remaining 71 were compared, eliminating duplicate and vector sequences, and ultimately obtaining 40 positive clones that may have interacted. The 40 positive clones obtained were statistically analyzed, and their gene numbers and basic functional descriptions are shown in Table 1.

Through database comparison and literature analysis of the 40 positive clones obtained, we initially identified three candidate genes: Pyruvate phosphate dikinase 1 (PPDK). There are reports that PPDK plays an important role in seed development and may participate in the conversion of amino acids and starch synthesis during seed development; The transcription factors WRKY 7 and GreA 19 play an important role in regulating gene expression.

Table-1. Comparison results of positive clones by sequencing

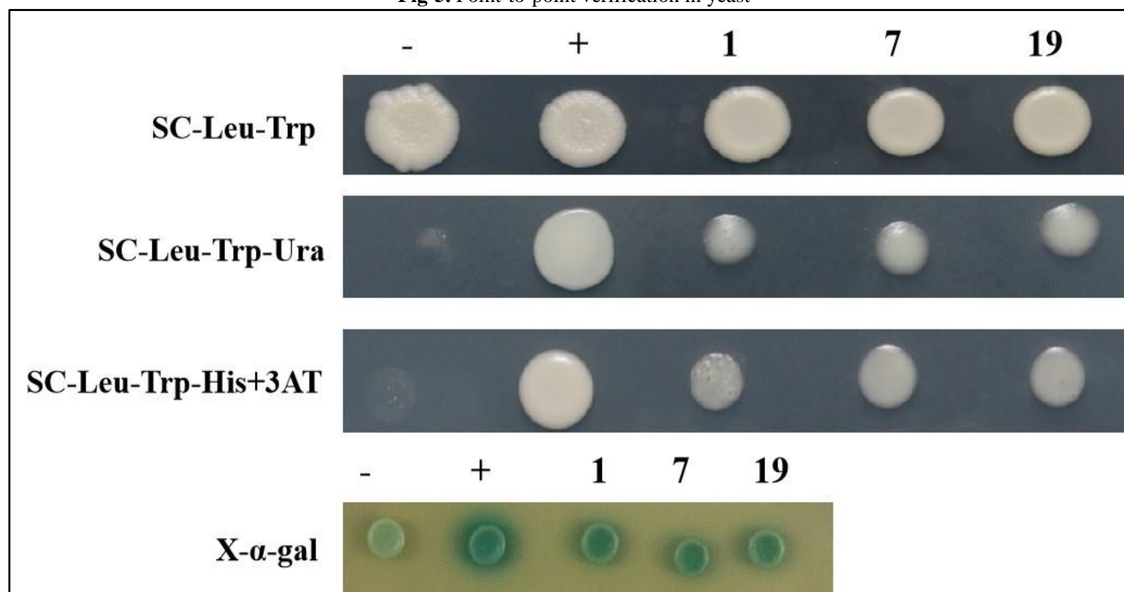
编号	基因功能描述	基因ID	相似度
Number	Gene description of function	Gene ID	Identity
1	Pyruvate phosphate dikinase, C-terminal domain	Loc_Os01g16960	100%
2	FAD-linked reductases, N-terminal domain	Loc_Os02g56850	99.349%
3	Cysteine rich domain	Loc_Os03g21040	99.713%
4	Actin-fragmin kinase, catalytic domain	Loc_Os02g07790	99.796%
5	Esterase domain of haemagglutinin-esterase-fusion glycoprotein HEF1	Loc_Os06g05630	91.736%
6	RNA polymerase subunit RPB10	Loc_Os12g07980	100%
7	WRKY DNA-binding domain	Loc_Os04g51560	99.444%
8	UEV domain	Loc_Os02g42314	100%
9	Ribosomal protein L3	Loc_Os11g37080	100%
10	Sedlin (SEDL)	Loc_Os02g21250	100%
11	ERF1/Dom34 middle domain-like	Loc_Os01g67134	99.837%
12	Osmotin, thaumatin-like protein	Loc_Os01g62260	100%
13	Expressed protein At2g23090/F21P24.15	Loc_Os06g49490	100%
14	Phosphorelay protein-like	Loc_Os08g44350	99.209%
15	Mago nashi protein	Loc_Os12g18880	100%
16	Actin-fragmin kinase, catalytic domain	Loc_Os06g47530	100%
17	Pyruvate phosphate dikinase, C-terminal domain	Loc_Os08g27840	100%
18	lambda integrase N-terminal domain	Loc_Os01g04020	100%

19	GreA transcript cleavage factor, C-terminal domain	Loc_Os02g52290	100%
20	Rna1p (RanGAP1), N-terminal domain	Loc_Os02g42412	100%
21	Formate dehydrogenase/DMSO reductase, C-terminal domain	Loc_Os03g05730	100%
22	Osmotin, thaumatin-like protein	Loc_Os09g36580	99.871%
23	LexA repressor, N-terminal DNA-binding domain	Loc_Os04g18090	100%
24	Actin-fragmin kinase, catalytic domain	Loc_Os02g41580	92.152%
25	PHD domain	Loc_Os12g40190	96.935%
26	Aquaporin-like	Loc_Os03g05290	98.805%
27	Cellulases catalytic domain	Loc_Os02g34560	98.288%
28	Catalase-peroxidase KatG	Loc_Os01g22230	98.391%
29	Capz beta-1 subunit	Loc_Os07g23200	97.572%
30	PHD domain	Loc_Os07g41740	96.19%
31	L15e	Loc_Os05g19370	99.504%
32	Heat shock protein 70kD (HSP70), peptide-binding domain	Loc_Os11g47760	97.035%
33	Leucine rich effector protein YopM	Loc_Os07g41694	95.181%
34	S100 proteins	Loc_Os06g46950	98.408%
35	Clathrin adaptor core protein	Loc_Os01g74610	97.409%
36	Glycerol-3-phosphate (1)-acyltransferase	Loc_Os01g57360	95.25%
37	HAD-related	Loc_Os05g43770	98.095%
38	ADC synthase	Loc_Os09g19734	97.287%
39	Archaeal histone	Loc_Os05g36280	98.054%
40	Ribosomal protein L37e	Loc_Os05g48320	97.849%

4.2. Point-to-Point Verification in Yeast

The three candidate gene plasmids (pDEST22-*PPDK*, pDEST22-*WRKY*, pDEST22-*GreA*) and decoy plasmids (pDEST22-*OsAAP6*) were co-transferred into yeast *MaV203* cells, respectively. Then the colonies were grown on SC-Leu-Trp medium. After monoclonal growth, single colonies were selected and diluted with sterilization ddH2O and then inoculated with 10 μL on SC-Leu-Trp-ura, SC-Leu-Trp-his +75 mM 3AT, YPAD+X-α-gal medium for 3-5 days. As shown in Figure 5, the interaction between OsAAP6 protein and PPDK, WRKY and GreA was confirmed by the growth of different defective medium and the positive control and negative pairs.

Fig-5. Point-to-point verification in yeast



Note:-: negative control; +: positive control; 1 : pDEST32-*OsAAP6* and pDEST22-*PPDK* were transformed into *MaV203*; 7: pDEST32-*OsAAP6* and pDEST22-*WRKY* were transformed into *MaV203*; 19: pDEST32-*OsAAP6* and pDEST22-*GreA* were transformed into *MaV203.*

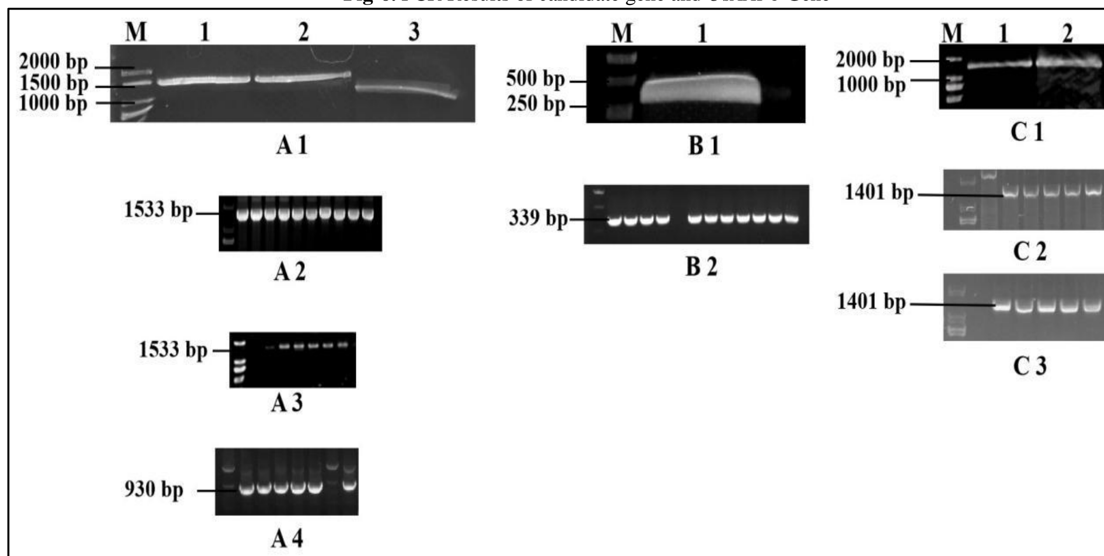
4.3. BiFC Verification of Rice Protoplasts

4.3.1. Carrier Construction

Three selected candidate genes (*PPDK*, *WRKY*, *GreA*) and rice *OsAAP6* gene were cloned and constructed into fluorescent expression vectors pSPYNE-35S and pSPYCE-35S, respectively. Lane 2 in Figure 6-A1 below shows the full-length CDS fragment of *PPDK* gene (1533 bp). Figure 6-A1 Lane 3 shows the full-length CDS fragment of *WRKY* gene (930 bp). Figure 6-B1 Lane 1 shows the full-length CDS fragment of *GreA* gene (339 bp). In Figure 6-C1, lane 2 shows the full-length CDS fragment of *OsAAP6* gene (339 bp). The fragments were recovered and

connected to the T vector, transformed into Escherichia coli, and then sent to the bacterial solution for sequencing. After extracting the plasmid with the correct sequencing bacterial solution, double enzyme digestion was performed, and finally the fragments were connected to the final fluorescent expression vectors pSPYNE-35S and pSPYCE-35S. The PCR detection results of the final carrier bacterial solution after transformation are shown in Figure 6-A3, A4, B2 and C3. The corresponding results with correct sequencing were selected to extract the plasmid.

Fig-6. PCR Results of candidate gene and *OsAAP6* Gene



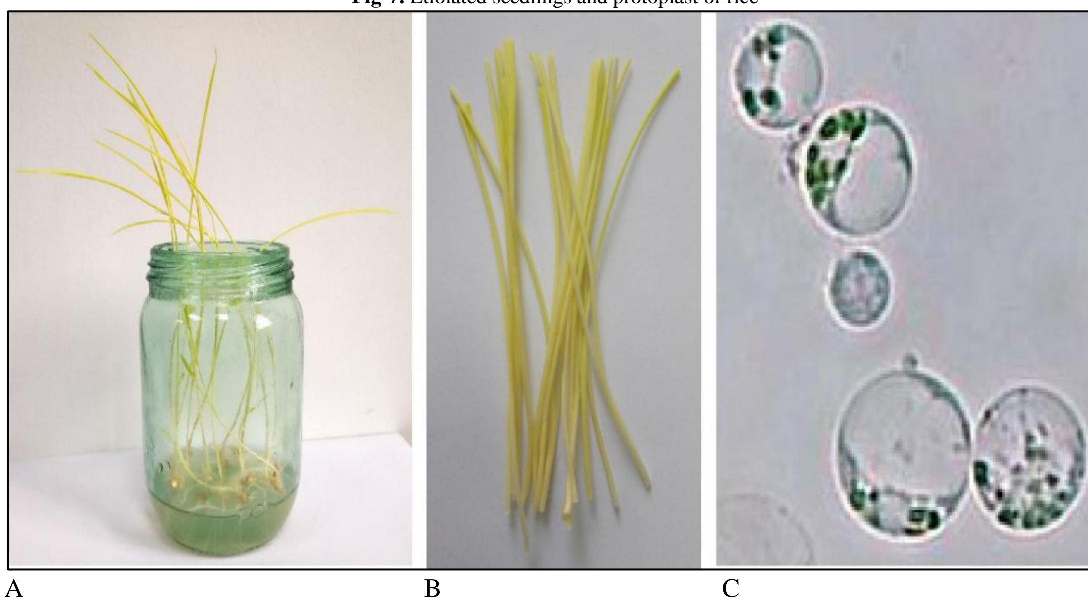
Note: The line 1 is *PPDK* (*Eco*R I-*Xma*I, pull-down test), The line 2 is *PPDK* (*Xho*I-*Xma*I, BiFC test), The line 3 is *WRKY*（*Bam* HI-*Xma*I); A2、A3、A4 are the bacterial liquid PCR results of the final vector respectively; B1: The line 1 is *GreA* (*Bam* HI-*Xma*I), B2 is the bacterial liquid PCR results of the final vector; C1: The line 1 is *OsAAP6* (*Hind*III-*Bam* HI), The line 2 *OsAAP6* (*Bam* HI-*Xma* I), C2、C3 are the bacterial liquid PCR results of the final vector respectively.

4.3.2. Rice Protoplast Transformation and Fluorescence Observation

Planting yellow rice seedlings, after growing for 9 days to 11 days (as shown in Figure 7-A), take 7-8 cm of the young stem from the root upwards to prepare rice protoplasts (as shown in Figure 7-B). The morphology of the protoplasts under a microscope is shown in Figure 7-C.

The vectors pSPYNE-35S:: *PPDK*, pSPYNE-35S:: *WRKY*, pSPYNE-35S:: *GreA*, and pSPYCE-35S:: *OsAAP6* were co transformed into rice protoplasts. If the two proteins interact, yellow fluorescence can be emitted. As shown in Figure 8-A, obvious yellow fluorescence was found on the membrane of protoplast somatic cell cells transfected with pSPYNE-35S:: *GreA* and pSPYCE-35S:: *OsAAP6* vectors, which proves that GreA may interact with OsAAP6. As shown in Figure 8-B, weak yellow fluorescence was observed in the cell membrane of the lower right corner of the protoplasts transferred with pSPYNE-35S:: *WRKY* and pSPYCE-35S:: *OsAAP6* vectors. This result suggests that WRKY may also have weak interactions with OsAAP6; However, a relatively obvious yellow fluorescence was found in the rice protoplast somatic cell cells transfected with pSPYNE-35S:: *PPDK* and pSPYCE-35S:: *OsAAP6* vectors, which indicates that the protein PPDK and OsAAP6 may have a relatively obvious interaction.

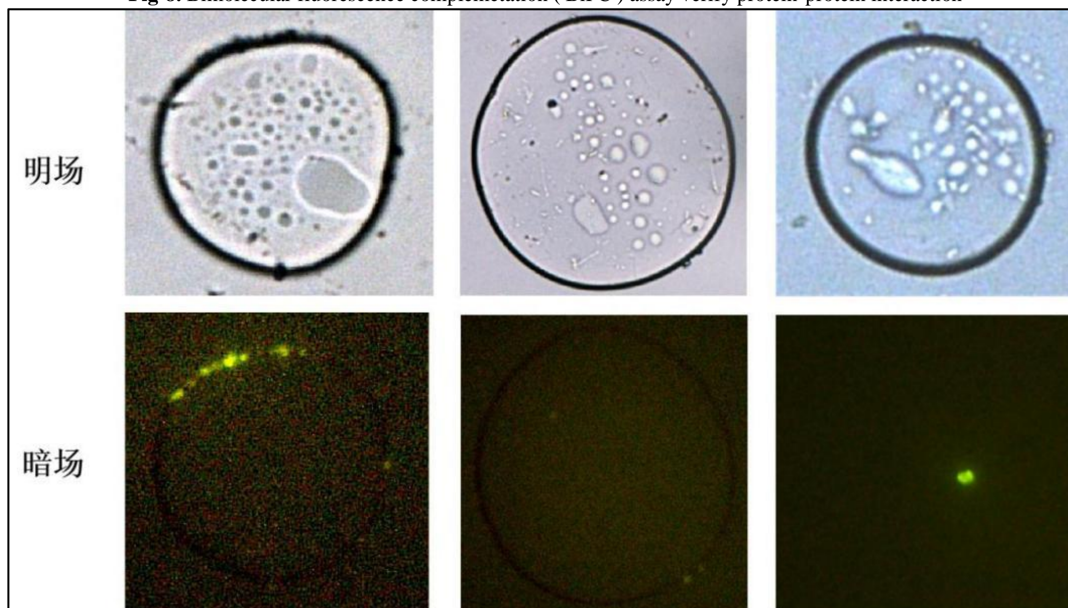
Fig-7. Etiolated seedlings and protoplast of rice



A B C

Note：A：etiolated seedlings of rice; B: young stem of etiolated seedlings; C: protoplast of rice

Fig-8. Bimolecular fluorescence complemetation (BiFC) assay verify protein-protein interaction



A B C

Note: A: pSPYNE-35S::*GreA* and pSPYCE-35S::*OsAAP6* aretransformed into protoplast; B: pSPYNE-35S::*WRKY* and pSPYCE-35S::*OsAAP6* aretransformed into protoplast; C: pSPYNE-35S::*PPDK* and pSPYCE-35S::*OsAAP6* are transformed into protoplast.

5. Conclusion

A total of 98 positive colonies that may interact with *OsAAP6* were screened from a rice cDNA library using yeast two hybrid technology. After sequencing and analysis, 40 proteins that may interact with *OsAAP6* were ultimately obtained. The in vivo point-to-point and BiFC experiments were used to further validate the interaction between PPDK, WRKY, GreA, and OsAAP6 proteins. Therefore, our results will provide important clues for further revealing the molecular mechanism by which the *OsAAP6* gene regulates grain protein content.

Acknowledgments

This work was financially supported by National Natural Science Foundation of China (U2004141, 31801332), Key Project of Science and Technology in Henan Province (222102110141; 222102110115), Key Scientific Research Projects of Universities in Henan Province (21A180001), Xinyang Innovation Project (20210007), Graduate Research Innovation Fund Project of XYNU (2022KYJJ066, 2022KYJJ068), Scientific Research Fund Project of XYNU (2022-DXS-131).

References

[1] Peng, B., Kong, D. Y., and Pang, R. H., 2017. "Detection and application of functional markers of *badh2* gene in south henan japonica rice variety." *Southwest Agricultural Journal,* vol. 30, pp. 1693-1699.

[2] Shewry, P. R., 2007. "Improving the protein content and composition of cereal grain." *Journal of Cereal Science,* vol. 46, pp. 239-250.

[3] Pandey, M. K., Rani, N. S., and Madhav, M. S., 2012. "Different isoforms of starch-synthesizing enzymes controlling amylose and amylopectin content in rice (*Oryza sativa* L.)." *Biotechnology Advances,* vol. 30, pp. 1697-1706.

[4] Chen, C., Huang, J. J., and Zhu, L. Y., 2013. "Varietal difference in the response of rice chalkiness to temperature during ripening phase across different sowing dates." *Field Crops Research,* vol. 151, pp. 85-91.

[5] Chen, Y., Wang, M., and Ouwerkerk, P., 2012. "Molecular and environmental factors determining grain quality in rice." *Food and Energy Security,* vol. 1, pp. 111-132.

[6] Kong, D. Y., Peng, B., and Peng, Y., 2018. "Effect of sowing date on grain endosperm chalkiness of different rice varieties." *Journal of Plant Sciences,* vol. 6, pp. 41-51.

[7] Peng, B., Kong, H. L., and Li, Y. B., 2014. "*OsAAP6* function as a an important regulator of grain protein content and nutritional quality in rice." *Nature Communications,* vol. 5, p. 4847.

[8] Wang, S. K., Li, S., and Liu, Q., 2015. "The OsSPL16-GW7 regulatory module determines grain shape and simultaneously improves rice yield and grain quality." *Nature Genetics,* vol. 47, pp. 949-954.

[9] Wang, Y. X., Xiong, G. S., and Hu, J., 2015. "Copy number variation at the *GL7* locus contributes to grain size diversity in rice." *Nature Genetics,* vol. 47, pp. 944-948.

[10] Zhou, W., Wang, X., and Zhou, D., 2017. "Overexpression of the 16-kDa amylase/trypsin inhibitor RAG2 improves grain yield and quality of rice." *Plant Biotechnology Journal,* vol. 15, pp. 568-580.

[11] Zhang, Q. F., 2007. "Strategies for developing green super rice." *Proceedings of the National Academy of Sciences of the United States of America,* vol. 104, pp. 16402-16409.

[12] Cheng, X. J. and Luo, C. P., 2023. "The effect of different nitrogen application rates on the quality of conventional rice varieties." *Modern Agricultural Technology,* vol. 834, pp. 12-17.

[13] Peng, B., He, L. L., and Tian, X. Y., 2019. "Expression analysis of *OsAAP6* gene under different nitrogen fertilizer conditions and its impact on the nutritional quality of rice grains." *Southwest Agricultural Journal,* vol. 32, pp. 1973-1979.

[14] Peng, B., Song, X. H., and Duan, B., 2018. "The effect of different sowing dates on the quality traits of Japanese "Jinjinqing" rice." *Southwest Agricultural Journal,* vol. 31, pp. 1772-177.

[15] Peng, B., Yang, N. N., and He, L. L., 2019. "The effect of sowing date on the chalkiness characteristics of endosperm in different fragrant rice varieties in southern Henan." *North China Agricultural Journal,* vol. 34, pp. 122-129.

[16] Tian, Z. X., Qian, Q., and Liu, Q. Q., 2009. "Allelic diversities in rice starch biosynthesis lead to a diverse array of rice eating and cooking qualities." *Proceedings of the National Academy of Sciences of the United States of America,* vol. 106, pp. 21760-21765.

[17] Zeng, D. L., Tian, Z. X., and Rao, Y. C., 2017. "Rational design of high-yield and superior-quality rice." *Nature Plants,* vol. 59, pp. 1-6.

[18] He, Y., Wang, S., and Ding, Y., 2013. "Identification of novel glutelin subunits anda comparison of glutelin composition between japonica and indica rice(*Oryza sativa* L.)." *Journal of Cereal Science,* vol. 57, pp. 362-371.

[19] Duan, M. and Sun, S. S. M., 2005. "Profiling the expression of genes controlling rice grain quality." *Plant Molecular Biology,* vol. 59, pp. 165-178.

[20] Kim, C. S., Gibbon, B. C., and Gillikin, J. W., 2006. "The maize mucronate mutation is a deletion in the 16-kDa gamma-zein gene that induces the unfolded protein response." *Plant Journal,* vol. 48, pp. 440-451.

[21] Peng, B., Pang, R. H., and Sun, Y. F., 2016. "Research progress on starch synthesis and regulation in rice seeds." *Jiangxi Agricultural Journal,* vol. 28, pp. 15-21.

[22] Shewry, P. R. and Halford, N. G., 2002. "Cereal seed storage proteins: structures, properties and role in grain utilization." *Journal of Experimental Botany,* vol. 53, pp. 947-958.

[23] He, W., Qu, M. Y., and Wei, Y. D., 2023. "Analysis of stress-induced expression and screening of interacting proteins of rice ribokinase gene." *Journal of Agricultural Biotechnology,* vol. 31, pp. 1-14.

[24] Katsube-Tanaka, T., Duldulao, J. B., and Kimura, Y., 2004. "The two subfamilies of rice glutelin differ in both primary and higher-order structures." *Biochimica et Biophysica Acta-Proteins and Proteomics,* vol. 1699, pp. 95-102.

[25] Kawakatsu, T., Yamamoto, M. P., and Hirose, S., 2008. "Characterization of a new rice glutelin gene GluD-1 expressed in the starchy endosperm." *Journal of Experimental Botany,* vol. 59, pp. 4233-4245.

[26] Kusaba, M., Miyahara, K., and Iida, S., 2003. "Low glutelin content1: a dominant mutation that suppresses the glutelin multigene family via RNA silencing in rice." *Plant Cell,* vol. 15, pp. 1455-1467.

[27] Satoh, H., Shibahara, K., and Tokunaga, T., 2008. "Mutation of the plastidial alpha-glucan phosphorylase gene in rice affects the synthesis and structure of starch in the endosperm." *Plant Cell,* vol. 20, pp. 1833-1849.

[28] Wang, Y., Ren, Y., and Liu, X., 2010. "*OsRab5a* regulates endomembrane organization and storage protein trafficking in rice endosperm cells." *Plant Journal,* vol. 64, pp. 812-824.

[29] Jiang, L., Yu, X., and Qi, X., 2013. "Multigene engineering of starchbiosynthesis in maize endosperm increases the total starch content andthe proportion of amylose." *Transgenic Research,* vol. 22, pp. 1133-1142.

[30] Cao, Z. Z., Zhang, Q. F., and Wei, K. S., 2012. "Response of some key enzymeactivities involved in nitrogen metabolism to high temperature atfilling stage and its relation to storage protein accu-mulation in ricegrain." *Acta Agronomica Sinica,* vol. 38, pp. 99-106.

[31] Monirujjaman, M. and Ferdouse, A., 2014. "Metabolic and physiological roles of branched-chain amino acids." *Molecular Biology,* vol. 2014, pp. 1-6.

[32] Peng, B., Kong, D. Y., and Peng, Y., 2019. "Genetic improvement of grain quality promoted by high and new technology in rice." *Journal of Agricultural Science,* vol. 11, pp. 81-94.

[33] Yang, F., Chen, Y., and Tong, C., 2014. "Association mapping of starchphysicochemical properties with starch synthesis-related genemarkers in non-waxy rice (*Oryza sativa* L.)." *Molecular Breeding,* vol. 34, pp. 1747-1763.

[34] Lu, K. Y., Li, L. Z., and Zheng, X. F., 2009. "Genetic dissection of amino acid content in rice grain." *Journal of the Science of Food and Agriculture,* vol. 89, pp. 2377-2382.

[35] Nguyen, H. C., Hoefgen, R., and Hesse, H., 2012. "Improving the nutritive valueof rice seeds: Elevation of cysteine and methionine contents in riceplants by ectopic expression of a bacterial serine acetyltransferase." *Journal of Experimental Botany,* vol. 63, pp. 5991-6001.

[36] Shen, Y., Jia, Q. L., and Liu, M. Z., 2016. "Genome-wide characterizationand phylogenetic and expression analyses of the caleosin genefamily in soybean, common bean and barrel medic." *Archives of Biological Sciences,* vol. 68, pp. 575-585.

[37] Ye, G., Liang, S., and Wan, J., 2010. "QTL mapping of protein content in rice using single chromosome segment substitution lines." *Theoretical and Applied Genetics,* vol. 121, pp. 741-750.

[38] Fang, J., Li, J., and Zhu, J. M., 2022. "Screening, identification, and expression analysis of rice osbhlh156 interacting proteins." *Acta Physiologica Sinica,* vol. 58, pp. 1485-1495.

[39] Huang, L. P., Liu, L. N., and Jiang, M. Y., 2022. "Screening and gene expression of interaction protein zfp36 in rice zinc finger protein." *Genomics and Applied Biology,* vol. 41, pp. 1316-1328.

[40] Peng, B., Tian, X. Y., and Liu, Z. Y., 2021. "The application of modern biotechnology in protein interaction research-a review." *Journal of Agriculture and Crops,* vol. 7, pp. 39-47.

[41] Sun, Y. D., Yang, Y., and Liu, C. Y., 2022. "Construction of a rice yeast two hybrid library and screening of PID2 intracellular domain interaction proteins." *Molecular Plant Breeding,* vol. 20, pp. 3931-3937.

[42] Peng, B., Kong, D. Y., and Huang, Y. J., 2018. "Bioinformatics analysis of the structure and function of OsAAP6 gene in rice." *Southwest Agricultural Journal,* vol. 31, pp. 429-436.

[43] Takaiwa, F., Oono, K., and Wing, D., 1991. "Sequence of three members and expression of a new major subfamily of glutelin genes from rice." *Plant Molecular Biology,* vol. 17, pp. 875-885.