Development of an Efficient Preparation and Verifying System for Rice Gametophytic Male Sterility using RNAi on Candidate Gene and OsMYB86R as a Reporter

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Methodology

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Abstract

Background: Gametophytic male sterility plays an important role in the study of pollen development mechanism and the breeding of three-generation hybrid rice. Because of the inherent phenotypic and genetic characteristics of gametophytic male sterility, it is very hard to find and identify gametophytic male sterility, which makes the related research lay behind the sporophytic male sterility. On the other hand, with the abundance of gene transcription data, a large number of pollen-specific genes have been found, and most of them are possibly associated with gametophytic male sterility. In order to promote the research of these genes in pollen development and heterosis utilization, it is necessary to construct an efficient, economical and accurate method to test these genes and identify if they are gametophytic male sterility related genes.

Results: In this study, at first, the *OsC1/OsMYB86* gene involved in anthocyanin synthesis in rice was modified and identified, and the function of revised *OsMYB86R* gene was created as the same as *OsMYB86*, and *OsMYB86R* is more convenient to be used as a reporter gene because the restriction site is removed. Then, an ascorbic acid oxidase gene *OsPTD1* was downregulated using RNAi driven by its own promoter, which resulted in abnormal pollen tube growth; Lastly, the RNAi elements were linked with *OsMYB86R* and together-transferred into an *osmyb86* mutant, and the purple color segregation of T₁ and F₁ derived from positive plants were distorted, which showed that the *OsPTD1* related gametogenic male sterility was successfully prepared and determined.

Conclusion: In this study, an easy and efficient method to prepare and identify gametophyte male sterility was established by using the strategy of RNAi and *OsMYB86R* as reporter. Comparing with the current methods, the advantages of this method are: (1) in material preparation, save time, a generation less comparing with conventional method, also a lot of mutation screening maybe avoided; (2) in identification, less cost, no PCR, electrophoresis, and other processes needed, and no expensive chemicals and instruments required; (3) in the results, more accurate, much less background affection, no damage on the plant. As a result, it is an easy, efficient, low-cost, accurate method to prepare and identify gametophytic male sterility genes. It is very important in elucidating the mechanism of pollen development and promoting the utilization of heterosis.

Background

Plant male sterility plays an important role in the study of pollen development mechanism and heterosis utilization. Male sterility includes sporophytic male sterility (SMS) and gametophytic male sterility (GMS). These two types are quite different in heredity and phenotype. SMS is a type of male sterility caused by abnormal genes in the sporophytes. Also the SMS gene can be transmitted between generations either by female gametes or by male gametes. All the pollens in the SMS anther is sterile. In the field, the SMS anther is usually abnormal in shape, color, and size, and the sterile plant has few seeds setting. Therefore, it is easy to find and identify the SMS mutants. Because the accessibility of SMS mutants, a lot of studies have been carried out on the mechanism of pollen development and sporophytic male sterility by

using abundant SMS mutants.[1–4] By contrast, GMS is a type of male sterility caused by the abnormal genes of gametophytes or pollen itself. Also, the GMS gene can only be transmitted through the female gamete which leads to the very low probability of homozygous mutant. Half of the pollens in the anther is fertile and the other half is sterile. There is no significant difference between the anther of mutant and wild type in the field, and the seed setting ratio of GMS mutant is normal. So, it is very difficult to find and identify the GMS mutant directly[5–9]. Due to the above reasons, comparing with SMS, the finding and identifying of GMS are more difficulty than SMS. In officially published reports, GMS mutants were usually created by spontaneous mutation, induced mutation, and T-DNA insertion mutation and so on. After mutating, the GMS candidates were verified in their crossing or self-crossing generations by using PCR, or resistance on screening agents, or reporting traits to detecting the segregation distortion of mutated genes or selectable markers or reporters, respectively[7, 10–15]. These current strategies require special equipment, expensive chemicals, or invasive treatments, which results in concerns about the time-consuming, low-efficiency, low-accuracy, high-cost, damage to sample and so on. In fact, the difficulty causes the rare mutants of GMS, and the GMS study fall behind the SMS research.

Pollen development is not only regulated by sporophytic gene expression, but also by a large number of pollen genes[1]. The genes associated with GMS are usually expressed in microspore/pollen at the late stage of anther development. With the development of transcriptome and other gene expression detection methods, more and more pollen-specific genes are presented to us [1, 16, 17] It is speculated that most of these genes may be related to gametophyte development, which provides us with an opportunity to understand the mechanism of pollen development and GMS. In order to find out the GMS related genes from these pollen-specific genes, it is necessary to develop a simple, rapid, economical and accurate method for preparation and identification of GMS.

Anthocyanin pigmentation is obvious, and visible to the naked eye. The rice coleoptile purple line is a clear purple line that appears on each side of the coleoptile after the seed germination. Because of its early performance stage (budding stage) and stability, it is very suitable to use as reporter traits.[18, 19]. And *OsC1/OsMYB86* is a key gene encoding MYB transcription factor for anthocyanin biosynthesis in almost all organs including coleoptile in rice [18, 20–24]. Hence, *OsC1/OsMYB86* is an ideal reporter for transgenic events indicating.

In view of the low-efficiency and high-cost of preparation and identification of GMS in commonly used methods, a new method to create and verify GMS by using RNAi technology, and *OsMYB86R* (revised *OsMYB86*) as a reporter gene was designed and validated in this study. A GMS related gene *OsPTD1* was identified in rice using our new method. The purpose of this study is to provide an easy, efficient and low-cost method to create and identify GMS gene.

Results

OsMYB86R, revised from OsC1/OsMYB86, is workable when replacing GUS gene as a reporter in rice

OsC1/OsMYB86 gene is a functional gene for anthocyanin biosynthesis in rice. In our previous studies, the CDS sequence of *OsC1/OsMYB86* gene was used to replace *GUS* gene in vector pCAMBIA1301. The function of *OsC1/OsMYB86* gene was verified by complement test (data not shown). The transgenic positive plant showed purple color in many organs such as the stem base of seedling, leaf sheath, auricle, stigma apiculus, and coleoptile, which shows that it can be used as a reporter gene to indicate the transgenic positive events. While the CDS sequence of *OsMYB86* contains common restriction sites such as PstI, SacI, SalI and so on, if *OsMYB86* used as a reporter directly, it must influence the freedom of restriction site selection when the target gene is loaded. To remove these restriction sites, we optimized and modified the gene by artificial synthesis, and obtained *OsMYB86R*, and then replaced *GUS* gene in pCAMBIA1301 by homologous recombination, which resulted in an *OsMYB86R* functional verification vector pP35S-*OsMYB86R*-OE (Fig. 1A). The plastid was transformed into ZhongjiuB without purple color in all organs, the results showed that the stem base of seedling, leaf sheath, auricle, stigma and apiculus and coleoptile of the transgenic positive plants all showed purple color (Fig. 1B-E), which agrees with the complement test. And the purple color distribution of the coleoptile of the transgenic positive plants was wider and easier to observe than that of most wild type lines including R25 (Fig. 1E-F). The results showed that the revised *OsMYB86R* gene has the same function as *OsMYB86* in rice anthocyanin biosynthesis, and *OsMYB86R* was suitable for reporting transformation.

RNA interference of *OsPTD1* damages pollen tube development

Ascorbate acid oxidase (AAO) gene *NPT303* plays an important role in the growth of tobacco pollen tube, and the silencing of *NPT303* resulting in the abnormal growth of pollen tube[25]. There are more than 10 AAO genes in rice genome (<http://www.gramene.org/>), and most of them are function unclear. Among these AAO genes, *Os05g0485800* encodes a protein that is highly homologous to NPT303 and may be related to pollen tube growth, which was named as *pollen tube development gene 1*(*OsPTD1*). RT-qPCR showed that *OsPTD1* was expressed in anthers at the mid and late stages of anther development, but not in roots, stems, leaves, leaf sheaths and seeds (Fig. 2A). In order to find out whether it plays a role in pollen germination and growth, two vectors of *OsPTD1* RNA interference were constructed, which promoted by the constitutive promoter P35S and the specific promoter of *POsPDT1*, respectively (Fig. 2B-C), and transferred them into Zhonghua 11. The T₀ pollen germination assay showed that all the pollens of the RNAi promoted by P35S and the wild type could germinate and grow normally, only half of the pollens of the RNAi promoted by *POsPDT1* germinated and grew normally, and another half of the pollen germination was abnormal, most of the abnormally germinated pollen tube ruptures without growing (Fig. 2D-F). The above results shows that the RNAi under the *OsPDT1* itself promoter broke the growth of pollen tube, which may result in GMS.

Construction and verification of a GMS preparation and identification system

In order to confirm *OsPTD1* to be a gene related to GMS, two vectors for preparation and identification of GMS was designed and constructed based on *OsPTD1* gene. The P35S and P*OsPTD1* promoted *OsPTD1*-RNAi elements were linked with *OsMYB86R*, which generated RNAi vectors of P35S-*OsPDT1*_P35S-*OsMYB86R* and P*OsPTD1*-*OsPDT1*_P35S-*OsMYB86R*, respectively (Fig. 3A, and E). Also, they were transferred into Zhongjiu B separately (Fig. 3B, and F). The two type RNAi positive plants (purple color), as male parents, was crossed with a male-sterile Zhongjiu B-*osabcg15* mutant with no purple color. At the same time, the male parents were allowed to self-crossing. Purple segregations in coleoptile of F₁ and T₁ were investigated 3 days after seed germination in wet soil in a disk (Fig. 3C, D, G and H). The results showed that the T₁ and F₁ of P35S promoted RNAi exhibited 3:1 and 1:1 segregation (purple color: no purple color), respectively, which did not match with the genetic characteristics of GMS. However, the color segregation ratio of the T₁ of P*OsPTD1* promoted RNAi did not show 3:1 ratio, it showed 1:1 ratio (Table 1). And only a few seeds, F₁ produced by P*OsPTD1* promoted RNAi male plants, displayed purple coleoptile, which indicated a distorted segregation ratio to the expected 1:1 ratio (Table 1). The distorted segregation ratio of the P*OsPTD1* promoted RNAi generations is just the genetic characteristic of GMS, which indicates that P*OsPTD1* driven RNAi produced a gametophytic defect. The above results showed the *OsPTD1* related GMS materials by using the newly designed method was successfully prepared. The results indicate the identifying of GMS could be carried out easily, rapidly, and economically in three days after seed germination.

Table 1
The segregation ratio of coleoptile purple color in generations of *OsPDT1* RNAi

Parents or Combinations	generation	purple color : purple colorless		
		Obs ratio	Exp ratio	$\chi^2_{0.05,1}$
P35S- <i>OsPTD1</i> RNAi_ P35S- <i>OsMYB86R</i> -1(A)	T ₁	143:57	3:1	1.13
P35S- <i>OsPTD1</i> RNAi_ P35S- <i>OsMYB86R</i> -2(B)	T ₁	152:48	3:1	0.06
ZhongjiuB- <i>osabcg15</i> ×A	F ₁	98:87	1:1	0.54
ZhongjiuB- <i>osabcg15</i> ×B	F ₁	82:72	1:1	0.53
P <i>OsPTD1</i> - <i>OsPTD1</i> RNAi _ P35S- <i>OsMYB86R</i> -1(C)	T ₁	112:88	1:1	2.65
P <i>OsPTD1</i> - <i>OsPTD1</i> RNAi _ P35S- <i>OsMYB86R</i> -2(D)	T ₁	103:88	1:1	1.03
ZhongjiuB- <i>osabcg15</i> ×C	F ₁	29:227	0:256	—
ZhongjiuB- <i>osabcg15</i> ×D	F ₁	18:170	0:188	—

Discussion

OsMYB86R is a favorable reporter gene

After transformation, it is better to identify the positive transformants as soon as possible. Reporter gene is a visible indicator of whether the target gene is transferred or not, also it can be widely used in identifying positive transgenic plants. To satisfy various research purposes, more and more requirements were raised to reporter genes. An ideal reporting gene should have the following characteristics: stable phenotype; easy detection; less background affect; low-cost; no damage on plants, environment and health [26–28].

Because of the possibility to meet the above requirements, some plant endogenous genes have been screened and used as reporter gene. Among this type genes, anthocyanin-related gene is one of the most widely used endogenous reporter genes in plants, such as maize, wheat, Arabidopsis, tobacco and tomato [27, 28]. The anthocyanin biosynthesis pathway involves many genes, and it is assumed that at least 16 genes are involved in anthocyanin biosynthesis in rice[29, 30]. Due to the limited carrying capacity of the vector, it is impossible to load all genes controlling anthocyanin synthesis into a same vector as the reporter genes. Scientists often use a compromise strategy to solve the problem: selecting the appropriate genetic background material, in which most anthocyanin synthesis genes are functional, as the transgenic host; using one to several anthocyanin genes as the reporter [28, 29, 31, 32].

OsC1/OsMYB86 is a homologous gene of the anthocyanin biosynthesis gene *C1* in maize, and it is a functional gene for anthocyanin biosynthesis in rice. And the purple color phenotype controlled by *OsC1/OsMYB86* are visible and stable, which indicates it is suitable to be used as a reporter gene. [18, 19, 21, 23, 24]. For better understanding and using this gene, *OsMYB86R* which was optimized from *OsMYB86* was created, and in which the usual restriction sites in *OsMYB86* CDS were removed. The revised *OsMYB86R* had normal function like *OsMYB86*, and could indicate the positive transformants quickly, directly, accurately and harmlessly in the early T_0 generation. It is also easy to separate the positive plants from the next generations of transgenic plants out. Because the sequence of *OsMYB86R* is only 819 bp, which saves a lot of space for the loading of more and longer target gene. Moreover, the usual restriction sites in *OsMYB86R* have been removed, it also does not affect the freedom of restriction sites selection when the target gene is loaded. Therefore, *OsMYB86R* is a desirable reporter gene.

In addition, Zhongjiu B is a stable maintainer line of *Indica* rice with a shorter growth period than Zhonghua 11 (a commonly used *Japonica* variety in rice transgene). The transgenic efficiency of Zhongjiu B is higher than the two popular *Indica* rice Minghui 63 and 9311. And Zhongjiu B, transformed by only one reporter gene *OsMYB86R*, will display purple color in multi organs, which indicates that other anthocyanin pigment genes in Zhongjiu B are normal. Therefore, Zhongjiu B is a good *Indica* rice host for the transformation when *OsMYB86R* gene as a reporter.

RNAi promoted by candidate gene own promoter can be used to prepare gametophytic male sterility (GMS) efficiently

GMS is a type of male sterility caused by abnormal genes in pollen itself. The discovery or preparation of GMS mutants will be helpful to the GMS gene cloning, function analyzing, and mechanism understanding about GMS. It is helpful to elucidate the mechanism of pollen development. Gametophytic male sterility plays an important role in the male sterility seed production system of three-generation hybrid rice, in which the pollen lethal elements are gametophytic male sterility [33–36]. Therefore, the preparation of GMS also has key value in providing the new pollen lethal element.

The inherent phenotypic and genetic characteristics of GMS make it very difficult to find and breed its mutants. Only a few GMS genes were identified in rice, including *RIP1*[6], *mga36*[8], *GT1*[15], *OsImpβ1*[5], *RUPO*[14]. It is far behind the study of sporophytic male sterility, which is obstructive not only for elucidating the mechanism of pollen development, but also for developing new system of nuclear sterility production. Spontaneous mutation, induced mutation, T-DNA insertion mutation, gene editing and down-regulation expression, any one of them may result in GMS. The first three methods, due to the uncertainty of the target gene, will inevitably lead to a large number of mutants unrelated to GMS, and the efficiency of obtaining GMS is low; And gene editing, focusing its clear target gene, can ensure greatly to improve the efficiency of GMS mutation, but it is necessary to eliminate the transgenic positive plants in the next generation and detect the mutants again, and also to overcome the problems such as the continuous mutation by gene editing and the influence on subsequent analysis.

RNA interference (RNAi) is a technique which can effectively affect gene function by down-regulating target gene expression. The choice of promoter and target can affect RNAi specificity and efficiency. In this study, the strategy of RNAi driven by target gene own promoter was used, the *OsPTD1* down-regulated GMS was obtained successfully. Except for the abnormal pollen fertility, there were no significant changes in other characters of the interfering plants. At the same time, the constitutive 35S promoter to drive RNAi of *OsPTD1* gene was used. The results showed that the pollen fertility was not affected and no GMS materials were obtained.

The reason for the difference in specificity and efficiency of RNAi is as follows: interference driven by target gene own promoter not only ensures that the resulting double-stranded MicroRNAs degrade the target mRNA in a specific tissue (pollen) on time, moreover, the newly transferred own promoter may compete with the original promoter for the transcription factor and reduce the expression of the target gene. which together may specifically and efficiently decrease the expression of the target gene. While other promoters, such as 35S, may not be expressed in pollen and may not have the chance or ability scrambling for transcription factors. Therefore, the recommendation from this study is to use the own promoter to drive the RNAi of the candidate gene.

The combination of candidate gene RNAi and OsMYB86R makes the preparation and identification of GMS with high efficiency, accuracy and low cost

GMS mutants were obtained traditionally by natural mutation, induced mutation, T-DNA insertion mutation or gene editing. After mutagenesis, it is necessary to determine whether they were GMSs or not according to the genetic characteristics of their offspring. The identify strategy is to carry out genotype

analysis by PCR or chemical resistance analysis by using screening marker genes in self crossing generation and hybridizing F_1 generation (mutant as male parent). Thereafter, segregation distortion is used to determine the mutant is GMS. In other words, if the segregation ratio of self-bred offspring is 1:1 instead of 3:1, and the mutated loci is not transfer through pollen learned from F_1 , it is GMS [7, 10, 11]. For example, *TMS1*[12], *OsGT1*[15], and *gaMS-1*[13] were prepared and determined as GMS by using above methods. These current methods are cumbersome and involve the use of multi reagents and equipment, which inevitably leads to problems of low-efficiency, high-cost, low-accuracy and sample damage. For gene editing strategy, scientists have to spend another generation remove the editing component for overcoming continuous editing, and screen out the mutant with no transgenic elements, which undoubtedly lengthens the identification time for at least one generation.

In this study, using the strategy combining RNAi technology and *OsMYB86R* reporting technology, a method for GMS preparation and identification at the same time was designed. by transferring the linked *OsPTD1*-RNAi element and *OsMYB86R* into Zhongjiu B with no purple color in any organ, the GMS genetic resources of *OsPTD1* were prepared successfully. And the genetic identification of GMS was determined only three days after the seed germination, by using the purple color segregation distortion in the T_1 and F_1 progenies derived from T_0 positive plants of RNAi.

There are three strategies to prepare GMS including random mutation (like spontaneous mutation, induced mutation and T-DNA insertion mutation and so on), gene editing (ZFNs, TALENs and CRISPR-Cas, etc.), and down regulation gene expression (such as RNAi and anti-RNA). The third strategy RNAi has been used in this study. Comparing with random mutation strategy, RNAi is much more efficient, less work and less cost for GMS preparation, because the burdensome mutant screening work is avoided in RNAi, while it is necessary in random mutation just because there is no definite target gene. Furthermore, RNAi strategy saves time compared to gene editing, because gene editing requires removing editing elements in generation T_1 to avoid influence on flowing study by persistent editing. So, if gene editing strategy is used to produce GMS, the genetic population for segregation distortion analysis could only be constructed in T_1 not in T_0 , while it would be finished in T_0 when RNAi strategy is adopted. It means one generation time will be saved. At last, in identification, the first two strategies require PCR, resistance detection, or fluorescence testing to analyze inheritance behavior, which needs specialized equipment and expensive chemicals, and invasive sampling procedures. However, in the RNAi linked *OsMYB86R* strategy, we use anthocyanin-related genes as reporter genes to analyze their genetic characteristics, which is more direct, simple, timeless, harmless, low-cost and high-accuracy than commonly used methods based on PCR detection, resistance testing, or widely used report genes such as *GUS*, *FPs*, and *Luc* and so on.

In conclusion, the strategy for preparation and identification of GMS has the advantages of harmless, short-time, high-efficiency, low-cost, high-accuracy and reliability. In addition, pollen lethal factor and fluorescent protein are two key components in present technology of nuclear sterility seed production. If the GMS produced by this method aborts completely (no transgenic element transfer through pollen), the RNAi elements and linked *OsMYB86R* can be used to replace the pollen lethal factor and the fluorescent

protein gene in the current system, which must be useful in developing new nuclear sterility seed preparation system. Obviously, this strategy can also be used for the preparation and identification of GMS in other crops. A simple sketch map for the reference to facilitate the understanding and application was shown in Fig. 4.

Methods

Plant materials and growth conditions

The *Japonica* rice line Zhonghua 11 were kindly provided by Dr. Li Ping, Shanghai University. The *indica* line R25, multi organs displaying purple color, is a self-breeding restorer, and YR25 without purple color, is an *osmyb86* mutant from R25. Zhongjiu B, another *osmyb86* mutant without purple color in all organs, is a maintainer line with a shorter growth period than Zhonghua 11, were kindly provided by China National Rice Research Institute. And Zhongjiu B-*osabcbg15* is a nuclear male sterility material, an *osabcbg15* near-isogenic line of Zhongjiu B, which was derived from the crosses and backcrosses between Zhongjiu B (the recurrent parent) and *osabcbg15*[37]. All rice plants used in this study were grown in fields at Jinghong, Yunnan.

Vector construction

For functional verification of *OsMYB86R*, the *OsMYB86R* was synthesized by Shanghai GenerayBiotech Co., Ltd after *OsMYB86*'s restriction sites being eliminated, and code being optimized according rice preference. And the target fragment was amplified using primers 86F and 86R and the synthesized *OsMYB86R* as a template. The *GUS* gene in the pCAMBIA1301 vector was then substituted with the PCR product of *OsMYB86R* by using a homologous recombination strategy described as user manual of In-Fusion HD Cloning Kit (Takara Biotech), which generated the pP35S-*OsMYB86R*-OE vector. This vector was transformed into the Zhongjiu B calli using an Agrobacterium-mediated method. The sequence of *OsMYB86R* and primers 86F/R are showed in Supplementary data.

For RNAi of *OsPDT1* gene, all of the RNAi elements, including promoter of *POsPDT1* or P35S, target sequence (left arm), stem-loop structure (first intron of *OsMYB86*), complementary sequence of target (right arm), and Tnos, was synthesized as one fusion sequence by Shanghai Generay Biotech Co., Ltd. The whole RNAi sequence was loaded between the restriction sites *SalI* and *SbfI* of pCAMBIA1301, which generated the vectors of pP35S-*OsPDT1*-RNAi and pP35S-*OsPDT1*-RNAi35S-*OsC1*. And these two vectors were transformed into Zhonghua 11, respectively.

For preparation and identification of *OsPDT1* GMS, the synthesized RNAi sequences was inserted in pP35S-*OsMYB86R*-OE by using *SalI* and *SbfI*, which generated pP35S-*OsPDT1*-*OsPDT1*-P35S-*OsMYB86R* and pP35S-*OsPDT1*-P35S-*OsMYB86R*. Thereafter, they were transformed into Zhongjiu B, respectively.

RNA preparation and quantitative real-time PCR analysis

Total rice RNAs were extracted from roots, stems, leaves, leaf sheaths, and flowers of the wild type plants at heading stage, and seeds at the filling stage using a RNAprep Pure Plant Kit (Tiangen Biotech). First-strand cDNA was synthesized from 1 µg total RNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time, TaKaRa). Quantitative real-time PCR was conducted using a TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa) on a Bio-Rad CFX96 system according to the manufacturer's instructions. *OsActin1* was used as internal control, and relative transcript levels were analyzed using the $2^{-\Delta\Delta CT}$ method[38]. Three biological replicates were performed. Microsoft Office Excel 2016 was used to analyze data, and Student's two-tailed paired t-test was used to determine the statistical significance of data. RT-qPCR primer sequences are showed in Supplementary data (Talbe S1).

pollen germination in vitro

The germination medium was composed of 18% sucrose, 5% potato starch and 0.005% H₃BO₃. And the solid medium is prepared in advance according to the following steps: evenly mixing the ingredients, heating and melting in a microwave oven, cooling and evenly spreading on the slide. Immediately after floret opening, pollen grains were shed onto the solid germination medium. After 30-min incubation at 28~30°C, the slides were observed by a Nikon SMZ1500 stereoscope and photographed with a Nikon DS-5Mc digital camera.

Coleoptile purple color observation and inheritance analysis

The transgenic positive plants with anthocyanin color were self-crossed to obtain T₁ generations. And the same positive plants were used as male parent to pollinate male sterility of Zhongjiu B-*osabcg15* to obtain F₁ generations. After being soaked and pregerminated, the seeds were evenly planted in a mud tray, covered with fresh-keeping film to keep wet, and under natural light. The purple color of coleoptile was investigated after 2 to 3 days growth. The segregation of purple: no-purple was counted and calculated, and chi-square test was conducted to verify whether the observed values were consistent with the theoretical values.

Abbreviations

GMS: gametophytic male sterility; SMS: sporophytic male sterility; MYB: myeloblastosis; AAO: ascorbic acid oxidase

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting this article are included within the article and its additional files.

Competing interests

The authors declare no potential competing interests.

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Authors' contributions

Y, WP, and SD designed and performed the experiments. K, WJ, and JX performed the experiments analyzed the data. LN and SL designed the experiments. LB, ZW, and Y designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Phenotype of OsMYB86R gene a) refers to the structure of pP35S-OsMYB86R-OE vector. The left sides of Pictures b), c), d), and e) refer to the color of different organs in Zhongjiu B, the right sides of Pictures b), c), d), and e) refer to the color of different organs in OsMYB86R overexpression positive plant. The traits in pictures b), c), and d) are from T0 plants, Trait in picture e) is from T1 plant. The left side of picture f) is purple wild type R25, the right side of picture f) is osmyb86 mutant YR25. The Arrows points to the purple colored tissue. Scale bars, 0.2 cm

Figure 2

The expression pattern of OsPTD1gene by RT-qPCR and the RNAi pollen germination in vitro a) refers to the RT-qPCR analysis on OsPTD1 expression pattern. b) refers to the structure of pP35S-OsPDT1-RNAi. c) The structure of pPOsPTD1-OsPDT1-RNAi. Picture d) refers to the germination of wild pollen. Picture e) The germination of the P35S promoted RNAi pollen. Picture F refers to the germination of the POsPTD1 promoted RNAi pollen, and arrows indicating rupture of RNAi pollen. 30 minutes after germinating, all photos were taken. Scale bars, 20μm

Figure 3

The inheritance characteristics of OsPDT1 RNAi a) and e) refer to the structure of RNAi vector of pP35S-OsPDT1_P35S-OsMYB86R and pP OsPDT1-OsPDT1_P35S-OsMYB86R, respectively. Pictures b), c), and d) refer to the T0 positive plant, T1 and F1(positive plant as male parent) of P35S-OsPDT1_P35S-OsMYB86R. Pictures f), g), and h) refer to the T0 positive plant, T1 and F1(positive plant as male parent) of POsPDT1-OsPDT1_P35S-OsMYB86R. Scale bars, 0.8 cm

Figure 4

A strategy for gametophytic male sterility preparation and identification a) refers to schematic diagram of GMS preparation and identification. b) refers to the vector structure of this strategy. When this strategy used, the green highlighted should be replaced according candidate GMS related gene. X, GMS candidate; PX, promoter of X; LA, left arm of RNAi-X; IC, the first intron of *OsC1*; RA, right arm of RNAi-X.

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