

Article

Molecular Breeding of Zheyongyou810, an *Indica*–*Japonica* Hybrid Rice Variety with Superior Quality and High Yield

Jian Song, Yongtao Cui, Honghuan Fan, Liqun Tang and Jianjun Wang *

Zhejiang Academy of Agricultural Sciences, Hangzhou 310022, China; songj@zaas.ac.cn (J.S.); cuiyt@zaas.ac.cn (Y.C.); xixi615@163.com (H.F.); tanglq@zaas.ac.cn (L.T.)

* Correspondence: wangjj@zaas.ac.cn; Tel.: +86-057186404370

Abstract: Rice heterosis has played a significant role in improving rice yields in China since the 1970s. The utilization of heterosis between the *indica* and *japonica* subspecies of rice is especially important in this regard. However, inter-subspecies hybridization between *indica* and *japonica* rice is hampered by the inadequate seed-setting rate and inferior rice quality of hybrid offspring, which has limited its development. The discovery and cloning of wide-compatibility gene *S5-n* and the granular starch synthase gene *Wx* have provided a solution to this problem, paving the way for the breeding of inter-subspecies hybrid rice. Here we demonstrated the breeding process of Zheyongyou810 and its performances in regional trials. Employing marker-assisted selection through the utilization of KASP (Kompetitive Allele-Specific PCR), a high-yielding and superior-quality *indica*–*japonica* hybrid rice variety named Zheyongyou810 was developed. Zheyongyou810 achieved an average yield of 10,737.75 kg/ha (12.58% higher than control Jiayou5) in the national regional trials across 2019 and 2020, and 11,777.25 kg/ha (13.36% higher than Jiayou5) in the production trial in 2020, respectively. The quality of rice has also been improved, reaching the standards set by the Ministry of Agriculture of the People's Republic of China for Grade 3 premium rice (NY/T593). In 2021, Zheyongyou810 was approved by the national examination showing broad application prospects.

Keywords: *indica*–*japonica* hybrid rice; quality; yield; marker-assisted selection; molecular breeding; KASP marker



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1. Introduction

Over the past four decades, substantial advancements have been made in harnessing heterosis among rice varieties. However, the practical application of heterosis among rice subspecies in production has been impeded by hybrid incompatibility. The primary challenge stems from the limited cloning and utilization of genes controlling the fertility of *indica*–*japonica* hybrids.

In the 1980s, Japanese scholars identified a phenomenon known as “wide-compatibility” rice, which exhibited a significantly higher seed setting rate when crossbred with both *indica* and *japonica* rice [1]. Despite this discovery, the specific gene responsible for this trait remained elusive, with speculation centered on the *S5* gene region of the wide-compatibility rice variety. A breakthrough occurred in 2008 when Academician Zhang’s team identified the *S5* gene, later featured as the cover story in PNAS [2]. Their pioneering research, titled “A killer-protector system regulates both hybrid sterility and segregation distortion in rice,” was successfully published in the journal “Science” in 2012. This publication successfully unveiled the mechanism behind the reproductive isolation between the rice *indica* and *japonica* subspecies [3].

The *S5-n* gene encodes an aspartyl protease that influences the fertility of female gametes and the seed setting rate in rice [2]. Further investigations revealed that the *S5* locus encompasses three alleles: *S5-i*, an *indica* allele; *S5-j*, a *japonica* allele; and *S5-n*, a neutral allele. In *indica* and *japonica* rice, the *S5-i* and *S5-j* proteins exhibit two amino acid variations, contributing to hybrid sterility and the establishment of reproductive isolation,

distinguishing the *indica* and *japonica* subspecies. However, the presence of the *S5-n* allele acts as a bridge, facilitating gene flow between these two subspecies. Generally, hybrids with the genotype *S5-i/S5-j* tend to be a predominantly sterile, whereas hybrids with genotypes containing *S5-n* along with either *S5-i* or *S5-j* tend to be predominantly fertile [3]. In 2023, the research team led by Jianmin Wan successfully identified the main locus *RSH12*, responsible for the occurrence of hybrid pollen sterility in the context of inter-subspecies hybridization between *indica* and *japonica* rice in the journal “Cell”. This locus comprises two closely linked genes informally named “destroyers” and “guardians”. The “destroyers” negatively impact the fertility of all pollen, resulting in sterility. They were found to interact with a vital protein within the mitochondria, the cell’s energy factory, disrupting its energy generation capacity, leading to pollen sterility. Conversely, the “guardians” interact with the “destroyers”, preventing their entry into the mitochondria and neutralizing their detrimental effects. Additionally, the “guardians” transport the “destroyers” to the autophagosome, a cellular organelle responsible for degradation, thus eliminating their negative impact and ensuring the normal pollen development. Interestingly, the functional types of this gene are inherited only by some *indica* rice farmers, while *japonica* rice farmers may not have inherited this functional type due to geographical differences. As these genes are unevenly distributed among rice species or subspecies, the phenomenon of pollen sterility after their hybridization is common [4].

With the continuous improvement in living standards, the demand for high-quality rice, especially in terms of taste and cooking quality, has been on the rise. Rice taste quality has become a pivotal factor for consumers when purchasing rice. This quality of rice is primarily determined by the expression of the wax gene (*Wx*), which regulates amylose content. Japanese scientist Yoshio Sano’s discovery of the *Wx* gene in 1984, marked a significant milestone [5], leading to the identification of multiple alleles, including *Wx^a*, *Wx^b*, *Wxⁱⁿ*, *Wx^{op}*, *Wx^{lp}*, *Wx^{mq}*, and *Wx^{mp}* [6–11]. In non-glutinous varieties, the *Wx* gene exhibits two allelic variants, *Wx^a* and *Wx^b*. In *indica* rice, *Wx^a* is the predominant variant, contributing to higher amylose content, which yields firmer and less sticky cooked rice. In contrast, in *japonica* rice, *Wx^b* is the prevalent variant, leading to lower amylose content, and resulting in stickier and softer cooked rice. Sequence analysis reveals that compared to *Wx^a*, *Wx^b* undergoes a specific genetic mutation, where a G nucleotide is replaced with a T nucleotide at the 5’ splice site of the first intron, causing a reduction in intron 1 splicing efficiency and abnormal splicing, ultimately leading to decreased amylose content [6,11,12]. Even within the F₂ generation of *indica-japonica* hybrid rice, which displays the same F₁ phenotype, there can be significant variability in amylose content, potentially impacting a significant impact in the taste and texture of cooked rice [11,13]. Therefore, the selection and breeding of parents with the desired *Wx* gene isotype based on molecular markers play a pivotal role in enhancing the hybrid rice’s quality.

To generate an *indica-japonica* hybrid rice variety that exhibits high yield and superior quality, our initial focus was on developing a restorer line which carries the *S5-n* and *Wx^b* alleles using KASP marker-assisted selection. Subsequently, the new *indica-japonica* hybrid rice Zheyong810 was developed through breeding with the new *indica* restorer Zhehuizhi810 and the *japonica* CMS line Zhe08A. Finally, Zheyong810 participated in the official trials of “single-season late japonica rice in the Yangtze River middle and lower reaches” in 2019 and 2020. The variety underwent official evaluations for yield, grain quality, and other agronomic characteristics.

2. Materials and Methods

2.1. Plant Materials

The new hybrid rice variety Zheyong810 was developed through a cross between the cytoplasmic male sterile japonica line Zhe08A and the indica restorer line Zhehuizhi810. Both parent lines carried the favorable allele *Wx^b* at the *Wx* locus for low amylose content but contained different alleles at *S5* locus. Zhehuizhi810 was created from an indica cross of F1317 and H702. F1317 is a high-yield restorer line with the *S5-n* allele, while H702 is a

superior-quality restorer line with the Wx^b allele. The allelic information at the *S5* and *Wx* loci of the aforementioned parents and hybrid variety are listed in Table 1.

Table 1. Rice materials used in this study.

Line/Variety	Subspecies	S5 Locus	Wx Locus
F1317	<i>indica</i>	<i>S5-n</i>	Wx^a
H702	<i>indica</i>	<i>S5-i</i>	Wx^b
Zhehuizhi810	<i>indica</i>	<i>S5-n</i>	Wx^b
Zhe08A	<i>japonica</i>	<i>S5-j</i>	Wx^b
Zheyu810	<i>indica-japonica</i>	<i>S5-n/S5-j</i>	Wx^b

2.2. Development of KASP Markers

In order to identify any genetic alterations at the *S5* and *Wx* loci, two KASP markers were devised. Based on the analysis of approximately 200 bp of DNA sequences flanked both upstream and downstream the SNP locus, KASP primers were crafted in accordance with established protocols using Primer 3 [14]. The conventional fluorescence probes, including FAM (5'-GAAGGTGACCAAGTTCATGCT-3') and HEX (5'-GAAGGTCGGAGTCAACGGATT-3'), were used alongside an allele-specific primer targeting the SNP in the 3' end (Table 2).

Table 2. Primers for KASP assay used in this study.

Gene	Primer	Primer Sequence (5'-3') *
<i>Wx</i>	Kwxa-F1	GAAGGTGACCAAGTTCATGCTAGGAAGAACATCTGCAAGG
	Kwxb-F2	GAAGGTCGGAGTCAACGGATTAGGAAGAACATCTGCAAGT
	Kwx-R	TTAATTAATTAATTTCCAGCCC
<i>S5</i>	KS5i-F1	GAAGGTGACCAAGTTCATGCTAAGAAGGGATTAAATTTGCTCGCT
	KS5n-F2	GAAGGTCGGAGTCAACGGATTAAAGAAGGGATTAAATTTGCTCGCG
	KS5-R	CATACTGTGCTTTGCTACTTACCG

* FAM-probe: GAAGGTGACCAAGTTCATGCT, HEX-probe: GAAGGTCGGAGTCAACGGATT; The italic letter in the 3' end refers to SNP.

2.3. DNA Isolation and KASP Genotyping

Rice penultimate leaves (1–2 cm in size) were collected during the heading stage and placed into a 96-well plate. The plate was freeze-dried for 24 h. Two steel beads were added to each well, and a 2 min grinding step was performed. Next, 400 µL of DNA buffer was added, followed by incubation in a 55 °C water bath for 20 min. After the incubation period, 400 µL of isopropanol was added and carefully mixed to ensure thorough integration. The sample was then centrifuged at 3700 rpm for 20 min, followed by supernatant discarding and 1000 µL of 75% ethanol adding. Another 10 min centrifugation step at 3700 rpm followed, and the supernatant was discarded. The sample was air-dried and rehydrated with 200 µL of molecular-grade water. The DNA concentration was calibrated to a final concentration of 50 ng/µL using the precise measurements obtained from Nanodrop One C-1000 spectrophotometer (Thermo Fisher, Wilmington, NC, USA).

For KASP genotyping, a PCR instrument was employed using 96-well plates. The included reaction mix was prepared by combining 2 µL DNA, which was adjusted to a concentration of 50 ng/µL, with 5 µL of 2× KASP Master Mix. Additionally, 0.14 µL of each KASP primer was included in the mixture. Fluorescence signals post-PCR were measured using a plate reader. *Wx* and *S5* SNPs were genotyped using the KASP assay, with distinct color codes for homozygous genotypes. The KASP assays were conducted by Shanghai Bay Gene Bio technologies Limited (Shanghai, China). The genotyping data obtained were visualized utilizing SNP viewer2 (LGC Limited, Cambridge, UK).

2.4. Experiment Field Management and Investigation of Agronomic Characteristics and Rice Quality

The parent materials, namely F1317, H702, and Zhehuizhi810, were cultivated in a research field situated at Zhejiang Academy of Agricultural Sciences (30°27' N, 120°14' E, Zhejiang) during both summer and winter seasons, as well as in Lingshui (18°50' N, 109°94' E, Hainan). In the years 2019 and 2020, hybrid Zheyongyou810 seeds participated in the single-season late japonica rice trials organized by the National Joint Organization. The control variety used for comparison was Jiayou 5. According to the requirements of the national regional trials, Zheyongyou810 was tested at 10 locations across provinces (please refer to Table S1 for site information). Each trial site featured 3 randomly arranged plots, with 240 plants per plot. The agronomic characteristics such as growing period, effective panicles, plant height, panicle length, number of filled grains per panicle, seed setting rate, thousand grain weight, and yield data were recorded and compiled into reports by each trial site according to national standards.

The rice samples for rice quality were obtained by cultivating and harvesting seeds from experimental plots managed by Wuhu Qingyi River Seed Industry Co., Ltd. (Wuhan, China), the Crop Institute of Shanghai Academy of Agricultural Sciences, and Zhejiang Academy of Agricultural Sciences in both 2019 and 2020. Rice quality analysis was outsourced to the Center for Food Quality Supervision and Testing (Wuhan) Ministry of Agriculture and Rural Affairs PRC.

2.5. Data Statistical Analysis

Experimental data were statistically analyzed using Excel (Microsoft, Albuquerque, NM, USA) and SPSS11.5 software (IBM, Armonk, NY, USA). One-way ANOVA was conducted to analyze the variance, and the *t*-test was utilized at significance levels of $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Molecular Marker-Assisted Selection of Restorer Line Zhehuizhi810

The breeding process of the restorer line Zhehuizhi810 is depicted in Figure 1 and described in detail below.

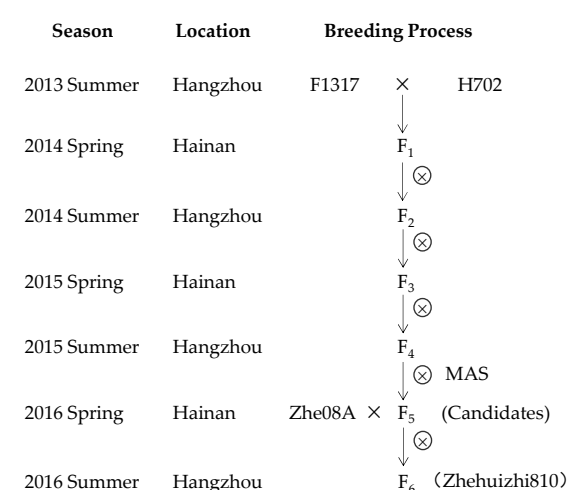


Figure 1. Marker-assisted selection breeding of the restore line Zhehuizhi810.

In the year 2013, the female progenitor, a highly productive restorer line named F1317 was employed as the female progenitor, while the *indica* restorer line H702 served as the male counterpart, culminating in a hybrid creation. The resulting first-generation (F₁) progeny was meticulously nurtured as a single plant in Hainan during the spring season of 2014. Subsequently, for the second-generation (F₂), seeds were obtained through a combination of direct seeding and mixed harvesting in Hangzhou during the summer of 2014. The third-generation (F₃) seeds were reaped in Hainan during the spring of 2015,

leading to the cultivation of a fourth-generation (F_4) single-plant population in Hangzhou during the summer of 2015. Among these, a total of 93 plants exhibiting superior agronomic traits were selected for genotyping using *S5* and *Wx*.

The *S5* KASP marker classified the 93 plants into three groups, with 8 plants having the T/T genotype (*S5-i*) with a red signal as H702; 82 plants having the G/G genotype (*S5-n*) with a blue signal as F1317; one plant having the G/T genotype (*S5-i/S5-n*) with a purple signal; and two plants which could not be genotyped. Similarly, the *Wx* KASP marker divided the 93 individual plants into three groups: 9 plants had the G/G genotype (*Wx^a*) with a red signal as F1317; 69 plants had the T/T genotype (*Wx^b*) with a blue signal as H702; and 15 plants had the G/T genotype (*Wx^b/Wx^a*) with a purple signal. Subsequently, 62 individual plants were found to carry both *Wx^b* and *S5-n* alleles, making them suitable as intermediate materials for breeding a new variety in the next generation. In the winter of 2015, the selected 62 individual plants were re-verified using *S5* KASP and *Wx* KASP, and the results showed that both *S5-n* and *Wx^b* were present (Figure 2).

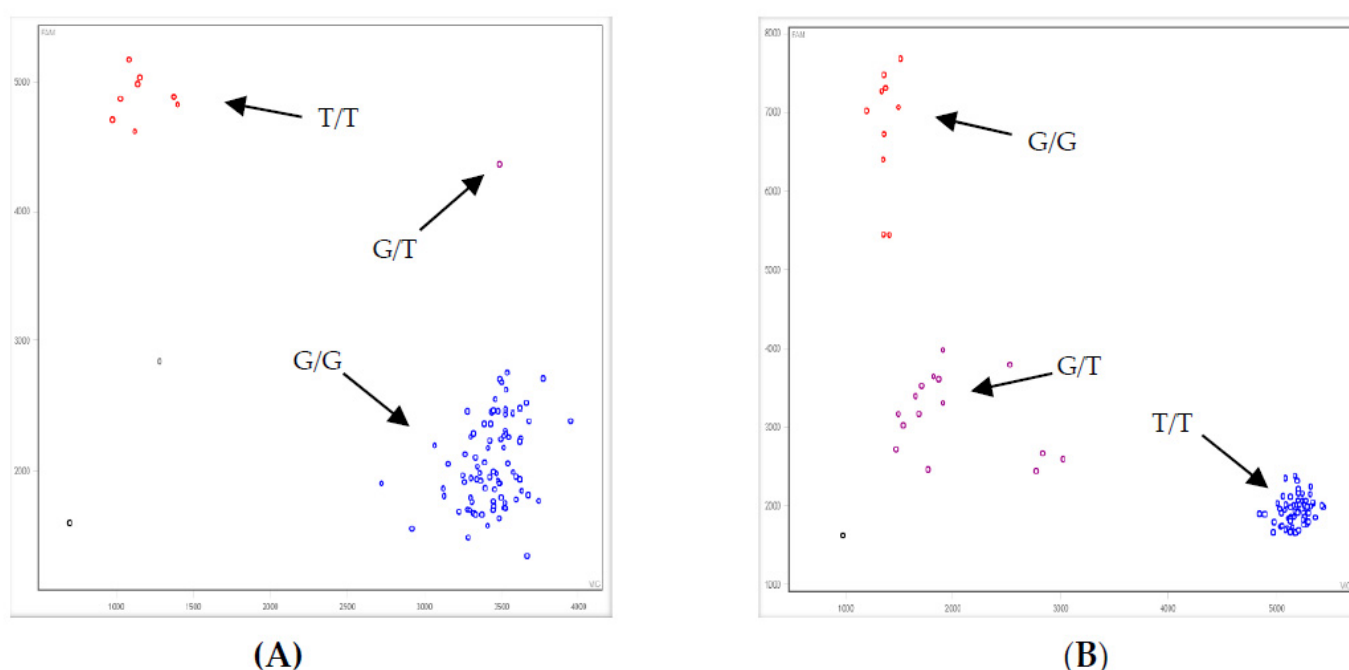


Figure 2. KASP assay of 93 selected F_4 plants for *S5* gene (A) and *Wx* gene (B). Note: (A) Homozygous G/G, T/T, and G/T represent the *S5-n*, *S5-i*, and heterozygous genotype, respectively. (B) Homozygous T/T, G/G, and G/T indicate the *Wx^b*, *Wx^a*, and heterozygous genotype, respectively.

Meanwhile, the above 62 candidate restorer lines were firstly crossed with Zhe08A during the spring of 2016. Subsequently, in the summer of the same year, extensive evaluations were conducted to assess the yield and rice quality of the resulting combinations. To be deemed successful, these combinations needed to satisfy special criteria: a yield increase of more than 10% compared to the control Jiayou5, and rice quality meeting the Grade 3 standard set by the relevant government department. Among the various crosses, the 10th restorer line exhibited exceptional performance when paired with Zhe08A. It not only surpassed the yield requirements but also met the specified rice quality standards. As a result of its exceptional performance, this particular combination was given the distinguished name of Zhehuizhi810.

3.2. Breeding of Indica–Japonica Hybrid Rice Zheyoun810

During the spring of 2017, the initial stages of Zheyoun810's small-scale seed production commenced in Hainan. This process involved the restorer line Zhehuizhi810 and the male-sterile line Zhe08A. The seeds harvested from this endeavor were subsequently utilized

for both autonomous regional trials and official trials. In the summers of 2017 and 2018, an initial two-year autonomous evaluation trial of Zheyoud810 was conducted, yielding impressive results in terms of yield. This led to its recommendation for inclusion in official trials. In 2019, Zheyoud810 participated for the first time in the official organization's trials specifically focused on the single-season late japonica rice in the middle and lower reaches of the Yangtze River. Throughout these trials, Zheyoud810 exhibited exceptional comprehensive traits and successfully met the synchronization requirements between regional and production trials. Continuing into the following year, 2020, Zheyoud810 simultaneously engaged in the second year of regional and production trials, consistently delivering outstanding performance when compared to control Jiayou5 and meeting the rigorous approval standards. By 2021, the remarkable performance of Zheyoud810 had gained recognition and approval from the National Variety Approval Committee, earning the designation of National Approval for Rice 20210385 (Figure 3). This marked a significant milestone in the journey of Zheyoud810, affirming its excellence and potential contribution to the rice cultivation industry.

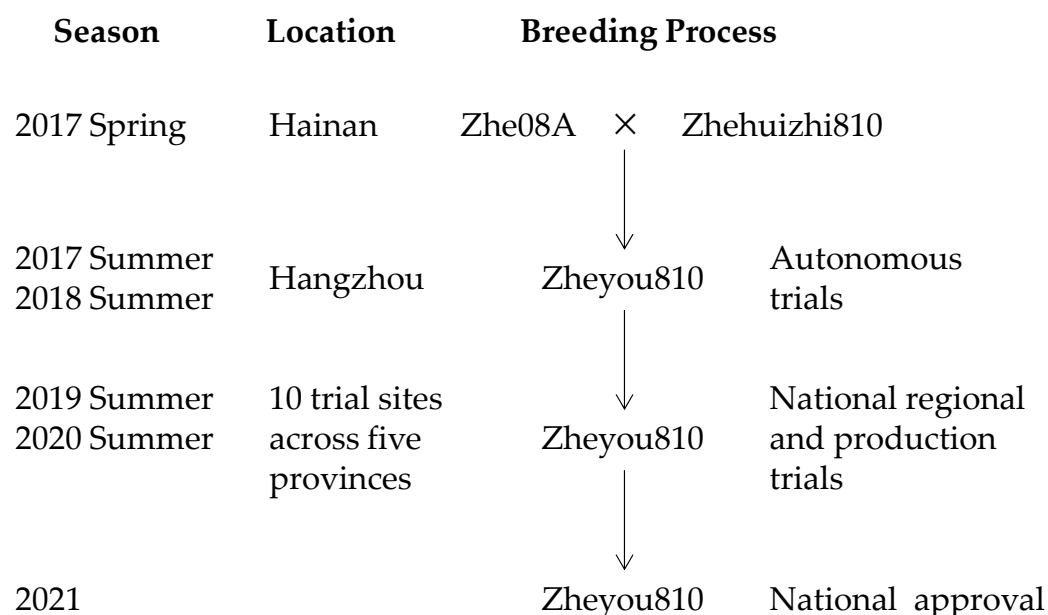


Figure 3. Breeding process of Zheyoud810.

3.3. Production Performance and Agronomic Traits of Zheyoud810

In the period from 2019 to 2020, Zheyoud810 underwent national single-season late japonica rice regional trials, participating in a total of 10 regional trials spread across five provinces (Table S1). Remarkably, this variety showed a remarkable increase in yield production throughout these trials. In 2019, Zheyoud810 exhibited a significant yield increase of 13.07% compared to Jiayou5, and in 2020, it continued to impress with a yield increase of 12.09%, both of which were considered highly significant (Figure 4). Over the course of the two-year evaluation, the average yield of Zheyoud810 reached 10,737.75 kg/ha, which was 12.58% higher than that of the control Jiayou5. It is worth mentioning that all trials showed increasing production (Table S2). During the production trials in 2020, Zheyoud810 continued to shine with an average yield of 11,777.25 kg/ha, which was 13.36% higher than that of Jiayou5, with all trials increasing production (Table S3). These findings further reinforced the exceptional yield potential of Zheyoud810.

In the study conducted between 2019 and 2020, a total of 240 plants underwent cultivation across 10 distinct trial zones, each with three repetitions. The main focus was to analyze the agronomic characteristics of Zheyoud810. When combining data from both years, it was observed that Zheyoud810 displayed comparable effective panicle counts and fruiting rates to the control Jiayou5, measuring at 243.0×10^4 /ha and 82.2%, respectively.

However, Zheyoun810 exhibited a longer average growth period of 153.2 days over the two-year span, which surpassed Jiayou5 by 4.4 days. Furthermore, Zheyoun810 showcased elevated plant height, longer panicles, and a higher grain count per panicle in comparison to Jiayou5. The measurements were recorded at 125.5 cm for plant height, 22.0 cm for panicle length, and 229.9 grains per panicle, respectively. These distinctive traits contribute to the high yield potential of Zheyoun810, despite its slightly lower thousand-grain weight of 24.7 g compared to the control variety Jiayou5 (Table 3).

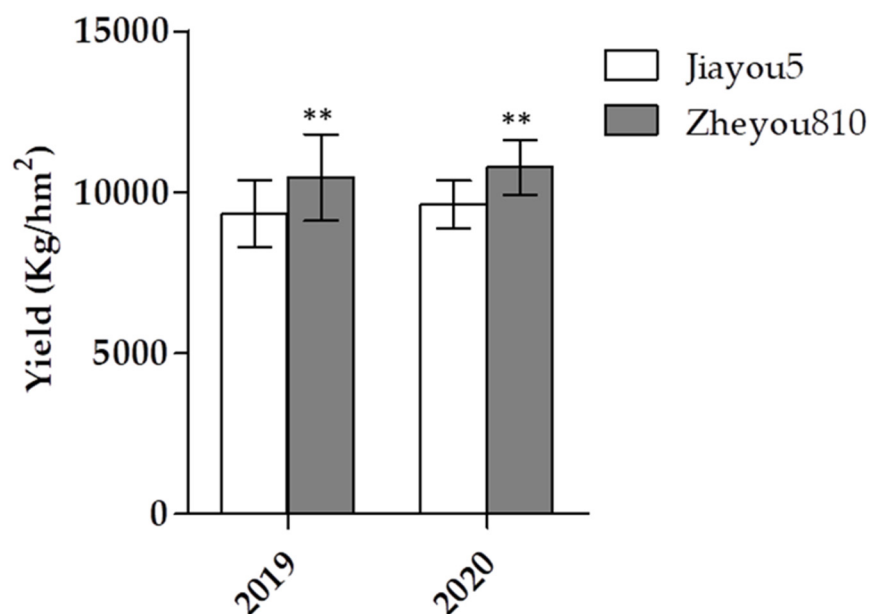


Figure 4. Yield of Zheyoun810 undertaken by national regional trials in 2019 and 2020, respectively. Note: Data are the means and SD (n = 10), ** $p < 0.01$, Student t -test.

Table 3. The agronomic traits performance of Zheyoun810 in 2019 and 2020, respectively.

Var	Year	GP	EP	PH	PL	NFGP	SS	TGW
ZY	2019	151.5 ± 9.3	244.5 ± 49.5	126.6 ± 5.1	22.1 ± 0.8	244.8 ± 51.4	85.1 ± 3.6	25.3 ± 1.6
JY	2019	147.5 ± 8.6	248.8 ± 33.6	109.7 ± 4.6	19.1 ± 1.1	168.0 ± 41.2	85.8 ± 2.1	29.1 ± 1.1
ZY	2020	154.9 ± 7.2	241.5 ± 37.5	124.4 ± 4.3	21.9 ± 1.8	215.0 ± 40.9	79.3 ± 7.7	24.0 ± 0.9
JY	2020	150.1 ± 6.4	246.2 ± 41.2	109.3 ± 3.5	19.7 ± 0.9	176.6 ± 39.8	86.4 ± 5.4	28.7 ± 1.2
ZY	Mean	153.2 ± 8.3	243.0 ± 87.0	125.5 ± 4.7	22.0 ± 2.6	229.9 ± 46.2	82.2 ± 5.7	24.7 ± 1.3
JY	Mean	148.8 ± 6.6	247.5 ± 54.2	109.5 ± 5.3	19.4 ± 1.9	172.3 ± 63.4	86.1 ± 6.8	28.9 ± 1.0

Note: Var, Variety; ZY, Zheyoun810; JY, Jiayou5; GP, Growing period (d); EP, Effective panicles (10^4 /ha); PH, Plant height (cm); PL, Panicle length (cm); NFGP, Number of filled grains per panicle; SS, Seed setting rate (%); TGW, Thousand grain weight (g); CK, Control variety Jiayou5.

3.4. Rice Quality of Zheyoun810

Wx^a regulates the development of elevated amylose content, resulting in rice typically containing over 25% amylose, with some varieties reaching beyond 30%. On the other hand, Wx^b predominantly appears in glutinous rice variants, which exhibit significantly lower amylose content compared to rice strains containing Wx^a that typically ranges between 15% to 18%. According to the cooking rice variety quality standards set forth in the People's Republic of China's agricultural industry (NY/T 593-2021), rice amylose content falling within the 13–20% range qualifies as premium quality rice, and a range of 13–18% designates it as Grade 1 premium quality rice. In the years 2019 and 2020, Zheyoun810 underwent comprehensive analysis and evaluation conducted by the Center for Food Quality Supervision and Testing (Wuhan) Ministry of Agriculture and Rural Affairs PRC. The determined amylose content stood at 16.6% and 15.7%, respectively, both

aligning with the benchmark for Grade 1 premium quality rice. Furthermore, Zheyoud810 exhibited a commendable head rice rate and transparency that fulfilled the criteria for Grade 1 premium quality rice. The two-year assessment yielded results for other essential rice quality indicators: gel consistency at 61 mm and 77 mm, alkali dissipation value at 6.2 and 6.1, and chalkiness degree at 3.6% and 4.6%, which fulfilled the criteria for Grade 3 premium quality rice. These findings finally led to the classification of Zheyoud810 as a third-tier edible rice variety (Table 4).

Table 4. Quality performances of Zheyoud810.

Index	2019	2020	Grade
Amylose content (%)	16.6	15.7	1
Gel consistency (mm)	61	77	3
Alkali dissipation value	6.2	6.1	3
Head rice rate (%)	72.1	72.2	1
Chalkiness degree (%)	3.6	4.6	3
Transparency	1	1	1

4. Discussion

The KASP marker technology has revolutionized breeding programs due to its efficient and precise detection of genetic variations, significantly impacting various crops such as cotton, maize, soybean, and wheat, where it has played a crucial role in the development of new varieties possessing improved traits [15]. For instance, researchers Rasheed et al. identified a major QTL linked to grain yield in wheat using KASP markers [16], and Jagtap et al. used KASP markers to expedite the breeding of heat-resilient maize [17]. A study successfully developed and validated a KASP assay linked to low Kunitz trypsin inhibitor (KTI) levels in soybean seeds, which holds promise for marker-assisted breeding to facilitate the incorporation of low KTI content in soybean seeds [18]. In cotton, ten validated KASP markers have been used to enhance Verticillium wilt resistance [19]. Additionally, this technology has also made notable strides in rice breeding programs, where it has been used to identify markers associated with disease resistance, grain quality, and yield-related traits. For example, several studies have reported the successful use of KASP markers for identifying blast resistance genes, including *Pi2* and *Pi9* [20,21]. KASP markers have also been utilized to identify markers associated with other important diseases, such as bacterial blight [22] and bakanae disease [23]. Additionally, KASP markers have been used to identify markers associated with grain quality, such as amylose content [24–26], cooking quality [27], and aroma [28,29]. In this study, KASP markers were employed to screen restorer lines of the *Wx^b* type with low amylose content, which has subsequently led to significant enhancements in rice quality. Moreover, KASP markers have been utilized to identify genetic markers that are closely linked to yield-related traits in rice. For example, KASP markers have been used to identify markers linked to panicle length [30,31], grain size [32], and grain weight [33]. This study mainly uses KASP markers to improve yield by enhancing seed setting rate, a significant yield-related trait.

The *S5-n* gene has been successfully applied since its cloning in 2008. This gene fragment deletion is a common genetic variation that is widely present in wide-compatibility rice and exhibits certain genetic diversity. Therefore, using the *S5-n* gene fragment deletion as a molecular marker, it is possible to genotype different individuals in an F_2 population, study the relationship between genotype and phenotype, and also evaluate and screen rice germplasm resources. The marker has also been used to distinguish F_2 strains from a high-generation stable *indica* restorer line and to detect hybrid rice materials [34]. The wide-compatibility characteristics and resilience of three *indica-japonica* intermediary wide-compatibility restorer lines, CH58, C84, and C927, have also been studied [35]. The lines were found to carry the *S5-n* gene and exhibit higher seed-setting rates than the control *japonica* restorer line, indicating the potential for improved fertility in *indica-japonica* hybrids [32]. Furthermore, the broad-compatibility gene *S5-n* has been introduced into

the maintainer line Zhen5B and the restorer line SIR2622 using molecular marker-assisted breeding, resulting in significant improvements in the fertility of *indica-japonica* hybrids [36]. Based on the research conducted, it is evident that the introduction of the *S5-n* molecular marker into restorer lines improves the hybrid vigor of the *indica-japonica* hybrid F₁. To enhance the selection efficiency of *S5-n* and minimize costs, this study developed a high-throughput KASP marker for *S5-n* detection. Subsequently, the selected restorer lines with the *S5-n* molecular marker were crossed with the sterile *japonica* line Zhe08A. As a result, a new variety called Zheyoun810 was successfully bred, which displayed normal fertility and excellent comprehensive traits, laying the foundation for subsequent high-energy detection of *S5-n*.

The “Waxy gene” (*Wx*) plays a pivotal role in controlling amylose content, and its multiple alleles have led to improvements in rice quality [11,37–39]. In order to address the issue of high amylose content, the *Wx^a* gene was replaced with the *Wx^b* gene using molecular markers, leading to a decrease in chain starch content and a notable enhancement in cooking and eating quality [11,40]. For instance, Liu et al. utilized PCR-AccI molecular markers to introduce *Wx^b* into the *indica* hybrid rice parents Longtefu and Zhenshan97 [11]. Similarly, Chen used molecular marker-assisted selection to obtain a high-quality early *indica* rice line Ganzayou57, which exhibited both high yields and low amylose content [36]. Also, the *Wx^b* gene was inserted into Teqing and Xieyou 57, leading to significant improvements in cooking and eating qualities [11,40]. This approach is also applicable to the *indica-japonica* hybrid rice in our research, as shown in the successful testing of *Wx^b* genotype lines from *indica* restorer lines against the *japonica* sterile Zhe08A genotype *Wx^b*. In rice quality evaluation, greater gel viscosity corresponds to superior rice quality. Previous studies have shown a negative relationship between amylose content and gel viscosity. In this research, the introduction of the *Wx^b* gene ensured a uniform *Wx* locus genotype in the hybrid. This simultaneously reduced the amylose content and increased gel viscosity, thereby elevating the hybrid to a premium rice quality level. The Center for Food Quality Supervision and Testing (Wuhan), Ministry of Agriculture and Rural Affairs PRC, assessed the rice quality as Grade 3 Premium Rice, significantly enhancing the effectiveness of rice quality selection.

In this research, we initially conducted field-based comprehensive agronomic trait selection and identified 93 exceptional individuals from a pool of approximately 900 individual plants. Subsequent analysis using *S5* KASP detection revealed a genotype distribution of 82:1:8 among these 93 plants. Notably, a significant proportion exhibited the *S5-n* genotype, which might be attributed to its potential to enhance fruiting rates, thus simplifying the selection process from the initial 900 plants. Additionally, by employing *Wx* KASP detection, we observed a genotype ratio of 69:15:9 among the samples. While the proportion of heterozygotes almost matched the theoretical 12.5%, the ratios of pure homozygous *Wx^b* and *Wx^a* largely diverged. This discrepancy might be attributed to the non-random selection of the 93 individuals based on agronomic traits. Further investigation is required to ascertain the specific agronomic traits associated with *Wx^b* within this particular parental cross combination.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture13091807/s1>, Table S1: Information of 10 testing sites, including longitude, latitude, and altitude. Table S2: Yield and Main Agronomic Economic Traits of Zheyoun810 in Various Trial Sites in Regional trials of 2019 and 2020. Table S3: Yield, Growth Period, and Main Agronomic Economic Traits of Zheyoun810 in Various Trial Sites in production trial of 2020.

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