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RESEARCH ARTICLE

Function-assisted selection for cold-resistant rice varieties with aroma in high-latitude regions

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ABSTRACT

Rice (*Oryza sativa* L.) varieties in high-latitude regions exhibit a pronounced characteristic of narrow genetic basis. Hybridization between geographically distant *japonica* and locally cultivated varieties can lead to slower stability in offspring due to significant genetic differences in parent lines. The increasing precision in functional gene mapping and cloning, combined with the use of molecular markers, allows the targeted introduction of desirable genes and reduces the reliance on field selection methods. However, this method does not analyze functional differences between parental genes, and the selected lines are disconnected from field production and even variety approval, hindering their potential for market adoption. Therefore, this study explored gene sequence polymorphism and developed specific detection markers with the aim to address the needs in rice production in cold regions. A stable new rice line named 'Songkegeng 119' with aroma and cold tolerance was created by aggregating the aroma gene *BADH2* (*betaine aldehyde dehydrogenase 2*) from the high-quality 'Wuxianggeng 9' in Jiangsu Province and the cold-tolerance gene *CTB4a* (*cold tolerance at booting stage 4a*) from the main cv. Songgeng 12 in Heilongjiang Province. 'Songkegeng 119' underwent various tests and was approved in 2022. The phenotype and genomic analysis of 'Songkegeng 119' indicated that function-assisted breeding could accurately and efficiently screen for desired functional genes. This can be combined with the selection of agronomic traits to obtain high-quality, high-yield, stress-tolerant, and aromatic rice varieties. This finding provides theoretical support for the polymerization of more functional genes in the future, and also serves as a practical test for the theory of function-assisted selection breeding.

Key words: Aroma, breeding by design, cold tolerance, function-assisted selection, *Oryza sativa*, rice.

INTRODUCTION

Heilongjiang Province is located between latitude 43°N and 53°N, and is the world's highest latitude rice production area, particularly known for its high-quality rice production (Fujino et al., 2008; Liu et al., 2018). However, in recent years, the newly bred varieties have exhibited drawbacks in yield, quality and resistance, hindering the breeding of breakthrough varieties. The narrow genetic basis is a significant problem in rice breeding in Heilongjiang Province (Liu, 2012; Tang, 2020). However, introducing rice germplasm from distant genetic origins may make it challenging to stabilize the offspring (Shen et al., 2017). Different geographical environments present unique challenges, indicating that the traits prioritized in the process of variety breeding will vary depending on the location and environment. Therefore, a reasonable molecular design breeding process needs to be formulated based on the breeding objectives. Also, the required target functional genes should be tracked and imported. The frost-free period in Heilongjiang province is less than 150 d, breeding rice

varieties here prioritizes the characteristic of cold tolerance. Low temperatures can reduce the budding rate in the seedling stage, while also reducing the seed setting rate or even significantly reducing yield in the booting stage. According to statistics, rice production in China is reduced by approximately 3-5 million tons annually due to low temperatures (Zhu et al., 2015). Therefore, cold tolerance in rice is a major concern requiring attention in all stages of rice growth and development in cold regions (Li et al., 2022).

At present, several cold-tolerance genes have been cloned in different developmental stages of rice. The genes responsible for cold resistance are different from those responsible for cold tolerance. For example, *COLD1* (*chilling-tolerance divergence 1*), a gene mainly expressed in the vegetative stage, is located on chromosome 4 and encodes a G-protein signaling regulator. It senses low temperatures by interacting with the G protein α subunit RGA1 (resistance gene analog 1), activates Ca channels, and enhances the cold tolerance of rice by enhancing the activity of G protein GTPase (Ma et al., 2015). The gene *Ctb1* (*cold tolerance at booting stage 1*), located on chromosome 4 in rice, plays a crucial role in regulating cold tolerance in rice during the booting stage. This gene encodes an F-box protein, which interacts with E3 ubiquitin ligase subunit Skp1, helping respond to cold tolerance (Saito et al., 2010). The *CTB4a*, another rice seedling cold-tolerance gene located on chromosome 4, plays a vital role in encoding a leucine-rich repeat receptor-like kinase. This gene interacts with the ATP synthase β subunit AtpB, and positive regulation of *CTB4a* gene expression can increase ATP synthase activity and ATP content, leading to improvements in rice cold tolerance and seed emergence rate (Zhang et al., 2017). In addition, many genes have been related to cold tolerance in rice, such as *HAN1*, *bZIP73*, and *qLTG3-1*, which are located on different chromosomes (Fujino et al., 2008; Liu et al., 2018; Mao et al., 2019). Currently, the research progress on the cold tolerance of rice is relatively slow due to challenges in experimental conditions and environmental impact. Therefore, the number of cloned genes related to cold tolerance is relatively limited (Li et al., 2025). However, compared with *indica* rice, *japonica* rice is cultivated in higher latitudes and exhibits greater cold tolerance. Similarly, the cold tolerance of northern *japonica* is also stronger than that of southern *japonica* (Huang et al., 2012; Guo et al., 2020).

Introducing the genetic background of southern *japonica* rice varieties, combined with targeted gene introduction and molecular design, can help accelerate the breeding process and develop new rice varieties (lines) with improved genetic diversity and enhanced cold resistance. This study aimed to improve rice varieties for cold regions using a function-assisted selection breeding system guided by gene function requirements. It leveraged a high-quality *japonica* rice 'Wuxianggeng 9' (WXG9) from Jiangsu Province as the maternal variety and a high-quality disease-resistant rice cv. Songgeng 12 (SG12) from Heilongjiang Province as the gene donor. The cold tolerance and aroma functional genes were tracked and screened using molecular markers, and finally a new cold-tolerant and aroma rice line 'Songkegeng 119' (SKG119) was obtained. The variety approval of Heilongjiang Province was completed in 2022. The feasibility of breeding breakthrough new varieties by molecular design was verified in practice.

MATERIALS AND METHODS

Selection of parent varieties and variety characteristics

Selection of maternal variety. The maternal 'Wuxianggeng 9' (WXG9) was bred by the Jiangsu (Wujin) Institute of Rice Research using the pedigree method, with 'Xiushui 04'/'Wuyugeng 3' as a female parent and 'Wuxianggeng 1' as a male parent. It was approved by Jiangsu Province in 1999. The WXG9 is an early-maturing *japonica* rice variety when planted in the adapted area. But if planted in long day environment, such as Heilongjiang province, it cannot heading normally. It is a typical erect panicle-type southern *japonica* variety with strong stem and lodging resistance. The rice is good in quality and aroma, with comprehensive properties (Figure 1).

Selection of paternal variety. The paternal 'Songgeng 12' (SG12) was bred by the Biotechnology Research Institute of Heilongjiang Academy of Agricultural Sciences through the pedigree method. This variety was obtained after crossing the long-grain *japonica* 'Song 93-8' as a female parent and the stress- and disease-resistant 'Tong 306' as a male parent in 1994. It was approved by Heilongjiang Province in 2008. The plant height of the variety is 98 cm, the panicle length is 18 cm, the number of grains per panicle is about 115, the

1000-grain weight is approximately 25 g, and the main stem has 14 leaves. This variety takes 137 d from seedling to maturity in suitable areas (Figure 1).

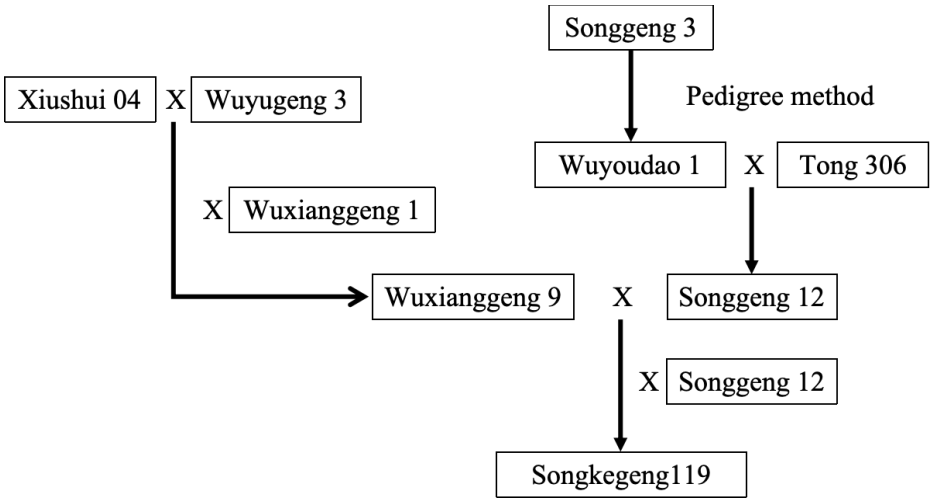


Figure 1. Pedigree map of 'Songkegeng 119' (SKG119) breeding.

Functional gene screening and molecular marker development

Based on the target functional gene type, the functional gene database of the China National Rice Data Center (https://www.ricedata.cn/gene/) was used to retrieve the full-length gene sequence. International Rice Genome Sequencing Project (IRGSP) 1.0 was set as the reference genome. Primers were designed in the flanking region of functional genes to amplify the gene sequences of parents (Table 1), and the amplified products were sequenced with both primers. The forward and reverse sequence assembly and multiple sequence alignment were performed using the DNAMAN software (version 9, Lynnon Corporation, San Ramon, California, USA). First, the genetic differences between parents were obtained by sequencing and aligning complete gene sequences. Then, the difference in gene function between parents was compared and analyzed according to the sequence of the coding region in the reference genome. Finally, the differential functional genes that could be used in breeding were determined.

For the sequence differences between functional genes, corresponding molecular markers were developed for offspring detection. When the difference sequence was a large fragment InDel, molecular markers were directly developed for detection according to the product size. However, when the difference locus was a single-nucleotide polymorphism (SNP) or a small InDel, competitive allele-specific polymerase chain reaction (KASP) markers were developed for offspring detection. Marker detection was used to track the separation of functional genes and screen the offspring containing the target genes.

Table 1. Candidate genes and amplification primers.

Gene	ID	Forward-Primer	Reverse-Primer	Start position	Product length (bp)
COLD1	Os04g0600800	ACGCCCCATCTTTGAATCGT	GTGACGGCCCGAATAAAACG	30311574	5343
Ctb1	Os04g0619300	TGCCGCCAATAGGAAGAGTG	CCACCGACATTTGCACAGTG	31458951	3520
CTB4a	Os04g0132500	ATTGTCAGTGCCACACCCAA	GGTTCCGAACTGACTGCTGA	2034480	4623
HAN1	Os11g0483000	TGACCCTCCCAAGATGAGCA	AGTTGGCATAGGGGTAGAGC	16985426	2421
bZIP73	Os09g0474000	GGGCAAACCATCAAGGCATC	GCGAAAAGCTCACCGTCATG	18122627	1172
qLTG3-1	Os03g0103300	TGGCCTCCAACTCAATCGTC	ACGATTGGGACCGATGGATC	219979	1313
BADH2	Os08g0424500	GCTCCGTGGGAAGAGGAAAA	TCCTTCACTAACAACGGGCC	20379823	5178

Variety phenotype and yield evaluation

Rice is soaked and germinated around mid-April, and then sown in dry conditions in greenhouses. Transplant to paddy fields for planting after the three leaves stage. Water and fertilizer management were carried out in accordance with the local paddy field management methods. The randomized block method was used in the variety comparison test, and three replicates were set. The plot comprised eight rows with a 30 cm spacing between rows. The plants within each row were spaced 12 cm apart. The area of the plot was approximately 20 m^2, and it was surrounded by at least six rows of a protection zone. Ten individual plants were randomly selected in the plot to determine several key characteristics: Plant height, panicle length, grain number per panicle, and tiller number. The average value for each trait was obtained. All agronomic and grain quality traits were measured according to the standards set forth in the Standard Evaluation System for Rice (IRRI, 2002). The final yield was calculated according to the water content of 14.5% after threshing. The weight of 1000 seeds was calculated at random. Cold tolerance was evaluated during the booting stage. When the pulvinus distance between the flag leaf and the inverted second leaf is about 2 cm ~ 4 cm, the plants were placed in an artificial climate chamber for 7 d of low-temperature treatment. The conditions are constant temperature of 16 °C with light treatment for 14 h, light intensity of 3000 lx, dark treatment for 10 h. When finished, move the plants to out-door environment and the number of empty grains were investigated after ripening as the evaluation standard of cold resistance. The identification of blast resistance adopts the natural infection method of inoculating pathogenic bacteria in the field. The flavor of the strain was identified using the KOH immersion method (Yang et al., 2019). To vaporize the component of aroma, 10 mL 1.7% solution of KOH were added to 2 g decorticated grains.

Whole-genome sequencing, assembly, and sequence alignment of varieties

Mixed sampling of individual leaves was carried out at maturity. The samples were re-sequenced according to the standard protocol provided by the Illumina platform. The original data were filtered according to the standard for next-generation sequencing analysis, and the clean reads of each sample were obtained for subsequent analysis (https://support.illumina.com.cn/). The sequencing results were compared with the reference genome using BWA-mem2 (V2.2) software (Li and Durbin, 2009). Sequence filtering was performed using Samtools (Li, 2011). The SNP and InDel mutation detection was performed using the HaplotypeCaller algorithm in Genome Analysis Toolkit (GATK v3.8), and mutation sites were filtered through the default value officially specified by GATK (McKenna et al., 2010). The final mutation results were used for chromosome fragment analysis and mapping. The reference genome for sequence alignment was IRGSP 1.0 of Nipponbare (Kawahara et al., 2013). Two R software packages, RIdeogram and tidyverse, were used to draw the chromosome introgression fragment map (Wickham et al., 2019; Hao et al., 2020).

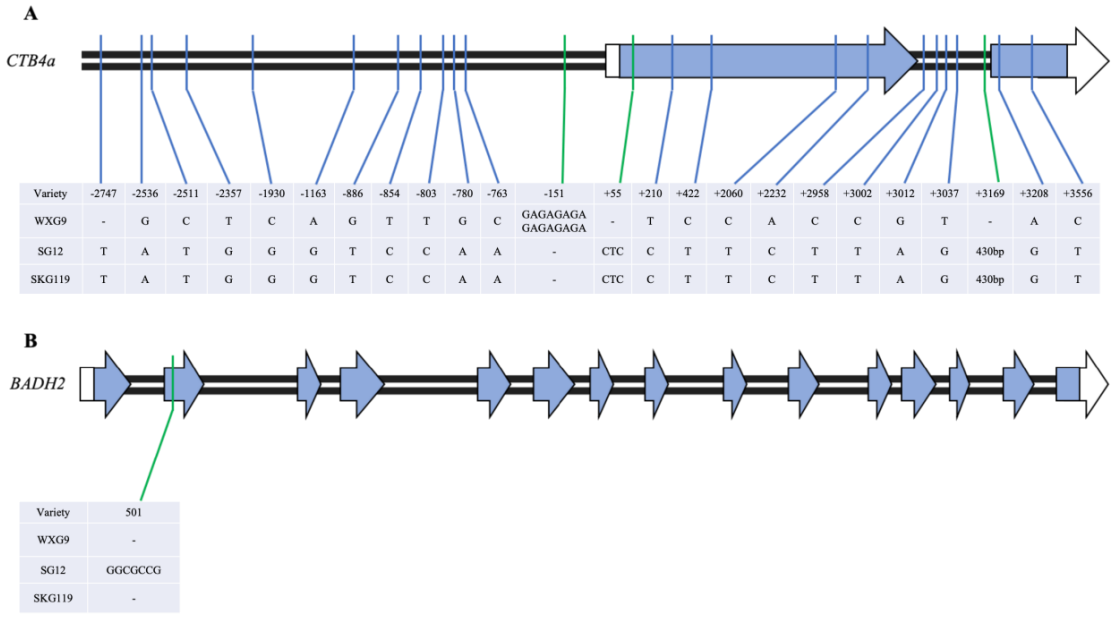
RESULTS

Screening of cold-tolerance and aroma functional genes

We first set the cold-tolerant and aroma rice variety as the breeding goal to aggregate the excellent genes in southern *japonica* and cold regions. A series of genes related to cold tolerance, such as *COLD1*, *Ctb1*, *CTB4a*, *HAN1*, *bZIP73*, and *qLTG3-1*, were screened by searching for related functional genes (Table 1). The study results obtained by designing primers, sequencing, and analyzing these functional genes between parents showed that the cold tolerance gene *CTB4a* located on chromosome 4 had multilocus differences between parents (Figure 2A). Combined with relevant reports, the functional region of the gene was found to be mainly SNP mutation located at –2536 bp, –2511 bp, –1930 bp, and –780 bp in the promoter and 2060 bp region in the gene (Zhang et al., 2017). The sequence alignment revealed that SG12 contained the strong cold-tolerance allele, namely the strong cold-tolerance haplotype (*CTB4a*$^+$). The WXG9 did not include any cold-tolerance-related SNP (*CTB4a*$^-$). However, the sequences of other candidate genes related to cold tolerance in parents were completely consistent, indicating that no functional differences existed between parents.

Further, we sequenced and analyzed the gene to clarify the sequence differences of the *BADH2* gene in parents (Chen et al., 2008). The results revealed a 7-bp deletion sequence in the second exon of the *BADH2* gene in WXG9, which led to abnormal gene coding (Figure 2B). Also, betaine aldehyde dehydrogenase could not be produced. Further, 4-aminobutyraldehyde oxidation was terminated, and 2-acetyl-1-pyrroline was

accumulated to produce flavor. The screening of these functional genes can identify the target genes of function-assisted selection breeding and provide basic gene and variation information for the development of subsequent molecular markers and generation tracking.



Variety	-2747	-2536	-2511	-2357	-1930	-1163	-886	-854	-803	-780	-763	-151	+55	+210	+422	+2060	+2232	+2958	+3002	+3012	+3037	+3169	+3208	+3556
WXG9	-	G	C	T	C	A	G	T	T	G	C	GAGAGAGA GAGAGAGA	-	T	C	C	A	C	C	G	T	-	A	C
SG12	T	A	T	G	G	G	T	C	C	A	A	-	CTC	C	T	T	C	T	T	A	G	430bp	G	T
SKG119	T	A	T	G	G	G	T	C	C	A	A	-	CTC	C	T	T	C	T	T	A	G	430bp	G	T

Variety	501
WXG9	-
SG12	GGCGCCG
SKG119	-

Figure 2. Sequence differences in the target functional genes: *CTB4a* (A) and *BADH2* (B). WXG9: 'Wuxianggeng 9'; SG12: 'Songgeng 12'; SKG119: 'Songkegeng 119'.

Development of molecular markers and generation tracking

According to the variation of the *BADH2* gene between parents, marker primers were designed in the second exon of *BADH2*, and KASP-specific fluorescent marker primers were connected upstream. Although many SNPs of the *CTB4a* gene existed, they were relatively close, and the possibility of chromosome exchange between these markers was low. Therefore, only KASP marker primers were designed for one SNP (Table 2).

Table 2. Primers for screening and tracking *BADH2* and *CTB4a* genes.

Gene	Forward-Primer1	Reverse-Primer	Product length (bp)
BADH2	GAAGGTGACCAAGTTCATGCTGCGCCGGGCGCCGTC GAAGGTCGGAGTCAACGGATTGCGCGCGCGCCG	TACGGAACACACGCACGCGG	114
CTB4a	GAAGGTGACCAAGTTCATGCTCCATTGGTTTGCTTGCGAAGC GAAGGTCGGAGTCAACGGATTCCATTGGTTTGCTTGCGAAGG	AAATCCCATGAAATGCGCGG	592

First, we used WXG9 as a female parent and SG12 as a male parent for hybridization to obtain F_1 seeds. The F_1 hybrid was used as a female parent and SG12 as a male parent for backcrossing in the winter of 2009 in Sanya, Hainan Province, to ensure that the offspring varieties could easily adapt to photoperiod and temperature conditions in high-latitude areas, and also to introduce more excellent characteristics of local varieties. After that, continuous self-pollination was started with the BC_1F_1 generation, and marker tracking was used simultaneously to screen for aroma and cold-tolerance genes, thereby reducing the size of the offspring population. The aroma of individuals was verified after maturity. Considering the factors such as field yield performance, the self-bred offspring of two genes labeled as heterozygous or homozygous *BADH2* and *CTB4a*$^+$

were retained. After years of continuous screening, an offspring line with excellent comprehensive field traits and homozygous for two target genes was obtained in BC_1F_7 in 2013 and named as 'SKG119'. From 2017 to 2018, a 2 yr field variety comparison test demonstrated that the comprehensive performance, including yield and disease resistance, was excellent. The variety subsequently received official approval from Heilongjiang Province in 2022 (Figure 3).

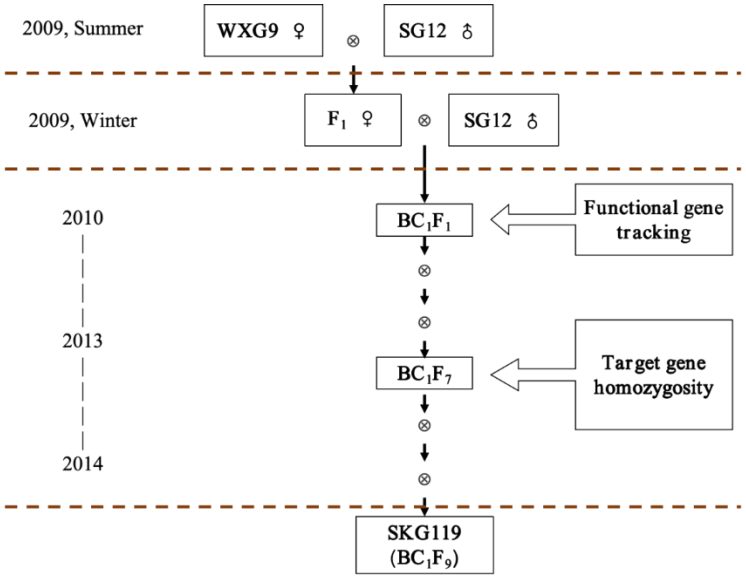


Figure 3. 'Songkegeng 119' (SKG119) breeding roadmap. WXG9: 'Wuxianggeng 9'; SG12: 'Songgeng 12'.

Detection of disease resistance and cold tolerance, and field evaluation

SKG119 took about 146 d from seedling to maturity in the adaptive area. It had 14 leaves on the main stem, 102.1 cm plant height, 19.4 cm spike length, 111.7 grains per panicle, about 2.6 grain length: Width ratio and 26.3 g 1000-grain weight. Besides participating in the variety approval test, the variety also participated in the inoculation identification of rice blast resistance for two consecutive years. The results showed that both the average leaf blast grade and the spike neck blast grade were 3-5. The identification of cold tolerance over three consecutive years revealed that the average empty grain rate of varieties after cold treatment was 20.5%. The analysis of grain quality for two consecutive years showed that the average brown rice percentage of the variety was 81.8%, head rice rate was 67.4%, chalkiness rate was 2.5%, chalkiness degree was about 0.3%, amylose content was approximately 18.34%, gel consistency was 77 mm, and crude protein content was approximately 6.77%. The average yield identification result for three consecutive years was 8486.7 kg hm^{-2}.

Genome re-sequencing and genetic map drawing

We re-sequenced the variety and its parents to intuitively display the source of chromosome fragments in SKG119. A total of 229 804 574 clean reads were obtained, with an average Q30 of 94.20%. The comparison analysis with the reference genome showed that the average proportion of the comparison to the reference genome was 98.92% and the average coverage depth of the sample was about 21X (Table 3). The variation comparison revealed 1 642 958 SNPs and 297 873 InDels among the parents. After screening and gene annotation, variations located in intergenic and intron regions were excluded. The chromosome fragment introgression map was drawn according to the source of variations in the offspring. The results showed that the 3, 4, 6, 7, 9, and 10 chromosomes were mainly the recurrent parent SG12 chromosome fragments and the other chromosomes retained larger maternal chromosome fragments (Figure 4). The introduction of recurrent parent fragments ensured that the line could adapt to the specific environment in Heilongjiang Province and had the potential for incorporating desirable traits such as disease resistance, high quality, and high yield in the male parent; however, extensive gene screening and verification were needed.

Table 3. Statistics of alignment between varieties and reference genome.

ID	Total reads	Q30	Mapped	Properly mapped	Average depth	Coverage ratio 1X	Coverage ratio 5X	Coverage ratio 10X
		%	%	%				
SG12	69 701 408	92.83	99.01	96.74	20	99.97	98.38	94.12
WXG9	87 568 502	94.49	99.12	92.52	24	99.98	97.60	88.87
SKG119	72 534 664	95.27	98.62	91.37	20	99.98	95.46	84.69

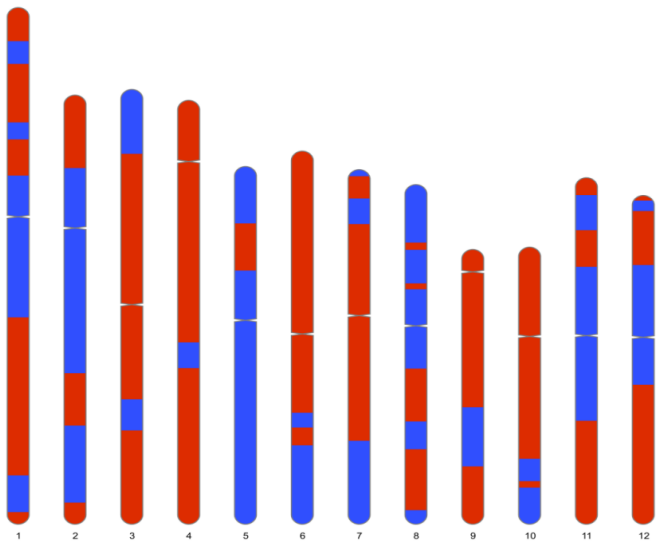


Figure 4. 'Songkegeng 119' (SKG119) chromosome introgression fragment map. From left to right are chromosomes 1-12. The red region is the chromosome fragment from 'Songgeng 12' (SG12), and the blue region is the chromosome fragment from 'Wuxianggeng 9' (WXG9).

DISCUSSION

The analysis of a large number of functional genes has revealed a change in the molecular design of rice varieties from the original breeding concept to an efficient, convenient, and operable mature technology. However, quantitative trait genes are influenced by the genetic background such as epistatic and additive effects (Wang et al., 2018; Wei et al., 2024). Even after marker-assisted selection introduces a desired gene into the target genome, some uncertainty remains (Wang et al., 2019). Moreover, different alleles of a single gene can exhibit distinct expression levels and varying functional outcomes (Li et al., 2020). The analysis of gene function mainly focuses on acquisition/deletion and resistance/sensitivity. Only a few studies have reported on the differences between alleles, gene additivity, and epistasis, which has also become a hidden barrier between gene function research and molecular breeding, reducing the accuracy and predictability of variety design. Therefore, strengthening the study of different alleles of functional genes and their regulatory pathways can ensure the operability of molecular design breeding. In breeding practice, tracking and screening of superior gene resources and accurately understanding the function of target genes are essential.

At present, the molecular design strategies for rice varieties vary, with different approaches tailored to specific target genes and genetic backgrounds. These include multi-gene polymerization approaches: One based on introducing multiple gene donors into a single recipient variety, and another involving multi-gene without considering the influence of background (Chen et al., 2023). Designing a scientific and reasonable technical route is necessary due to different breeding objectives, parents, and functional genes. For example, the simultaneous introduction of multiple target functional genes from a single donor can help continuously track multiple genes. When creating a segregating population, the scale of the population is crucial to facilitate

the screening of target introduction fragments. If the simultaneous introduction of target genes is not feasible, separate introgression lines for each gene can also be constructed and then combined through hybridization, eventually allowing for gene polymerization. The two target genes in this study were from two parents. However, considering the adaptability of the genetic background of southern *japonica* in high latitudes, we added a backcross with northern *japonica* SG12 to improve the background recovery rate, thereby increasing the selection efficiency of the designed varieties. It is believed that several factors, including the difficulty of introducing the target gene, controlling population size, and the duration of functional gene screening, must be fully considered in the early stage of variety design to ensure the selection of excellent comprehensive trait strains that contain the target gene.

In this study, we propose a function-assisted breeding system to achieve precise molecular design breeding. Starting from the molecular design of varieties, we selected and mined the target functional genes and then determined the tracking strategy for molecular markers and the gene polymerization method according to the source of the functional genes. Finally, a new rice line with both aroma and strong cold-tolerance genes and other excellent comprehensive agronomic traits was screened, which successfully passed the variety approval. As a practical application of function-assisted breeding technology, this study selected two genes from parents for screening and introduction, which is a primary application of this technology. A better understanding of functional alleles, combined with advanced molecular markers, can enable the creation of more complex gene introduction populations and efficient selection of desired traits through high-throughput molecular marker tracking, leading to more efficient and accurate molecular design breeding.

CONCLUSIONS

In this study, a breeding strategy was formulated for parent selection by analyzing the functional differences in genes between varieties. The molecular design of cold-tolerant and aromatic rice varieties in high-latitude was completed, and the breeding of a new rice variety was accomplished using this strategy. We first selected the northern cold-tolerant *japonica* and the southern high-quality aromatic *japonica* as the parents in molecular design breeding to introduce more abundant genetic variations and excellent genes. We then obtained the differences in the main cold-tolerance and aroma genes between the parents through sequencing analysis. Molecular markers, based on functional gene differences and the parental source, were developed for tracking and screening desired functional genes. After years of selfing, a stable new rice line 'Songkegeng 119' containing cold-tolerance and aroma genes was obtained, which passed the variety approval in 2022. The identification of disease resistance, cold tolerance, and yield showed that the new line performed well, indicating that the function-assisted breeding strategy proposed in this study had a certain application value. An upgraded version of this strategy can be developed in the future, which can be used to screen more functional genes during rice molecular design breeding.

Author contributions

Conceptualization: M.F-C. Methodology: Z.G-M. Software and writing-original draft: W.R-S. Validation: D.L-W. Formal analysis: L.K., Z.W. Investigation: L.H. Resources: T.Y-Q. Data curation: W.Y-L. Writing-review & editing: D.G-H. Visualization: Y.G. Supervision: M.J-T. Project administration and funding acquisition: M.F-C. All co-authors reviewed the final version and approved the manuscript before submission.

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