

Note

Development and validation of a PCR-based functional marker system for identifying the low amylose content-associated gene Wx^{hp} in rice

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Low amylose content (AC) is a desirable trait for rice (*Oryza sativa* L.) cooking quality and is selected in soft rice breeding. The Wx^{hp} allele was derived from a Yunnan rice landrace in China, Haopi, with low AC. To efficiently and rapidly utilize the low amylose content-associated gene Wx^{hp} in rice molecular breeding programs, we developed a tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method, according to the single-nucleotide variation of the Wx^{hp} . Four Wx^{hp} -specific primers were used to perform PCR assays using genomic DNA extracted from several rice varieties. Based on the band pattern of the amplified products after electrophoresis, this method can accurately distinguish three Wx^{hp} -related genotypes (i.e., Wx^{hp} homozygotes, Wx^{hp} heterozygotes, and wild-type), and the genotypes completely correspond to the appearance of mature endosperm. This method represents a novel approach that is both inexpensive and highly efficient and can be widely used for genotyping Wx^{hp} alleles in rice germplasm collections and may aid breeding programs with marker-assisted selection (MAS).

Key Words: rice (*Oryza sativa* L.), low amylose content, Wx^{hp} gene, tetra-primer ARMS-PCR.

Introduction

Rice (*Oryza sativa* L.), one of the most important food crops in the world, serves as staple food for over half the world's population. With increasing consumption levels and improvements in the quality of life, the demand for rice has changed from high yield to high quality; therefore, high-quality rice, particularly in terms of the eating quality, is now favored by consumers. Rice quality pertains to four major properties: appearance, processing characteristics, eating and cooking quality, and nutritional characteristics (Zhao *et al.* 2015). Eating and cooking quality is considered as the predominant quality index and is largely determined by the starch structure of the endosperm, which is the major edible part of rice grains. About 90% of the endosperm is starch, which has two molecular structures, namely amylose and amylopectin. Studies have shown that the amylose content is a key determinant of the eating and cooking quality of rice (Juliano 1992).

Soft rice with low amylose content is generally bright and translucent in appearance with a soft and elastic texture, and thus it is generally considered tasty and has high com-

mercial value (Zhu *et al.* 2004). The synthesis of amylose in rice is controlled by granule-bound starch synthase, which is encoded by the *Waxy* (Wx) gene (Nelson and Rines 1962). To date, a number of Wx alleles have been identified in rice germplasm resources, which include Wx^a (Sano 1984), Wx^b (Wang *et al.* 1995), Wx^{in} (Mikami *et al.* 2008), Wx^{mw} (Mao *et al.* 2017), Wx^{op} (Mikami *et al.* 1999), Wx^{hp} (Liu *et al.* 2009), Wx^{mq} (Sato *et al.* 2002), $Wx1-1$ (Ando *et al.* 2010), and wx (Inukai *et al.* 2000). Wx^{hp} , an allele originally identified in Yunnan Province, China, confers relatively low amylose content (9.7%) to the Haopi rice variety. In addition, an examination of the coding region revealed that the Wx^{hp} allele carries an A to G change at nucleotide position 77 in the fourth exon, resulting in an Asp (165)/Gly (165) substitution. Consequently, gene transcription is reduced, and granule-bound starch synthase enzyme activity is also inhibited, resulting in significantly lower amylose content (Liu *et al.* 2009). The rice varieties carrying this low amylose content-associated gene exhibit an opaque endosperm phenotype (cloud-like, milky white, and slightly less transparent), and it is therefore also called an opaque endosperm mutation.

The sequence differences in the Wx alleles have been extensively investigated. Cai *et al.* (2002) designed restriction site *AccI*-specific PCR to distinguish the Wx^a and Wx^b genotypes; by combining *AccI*-PCR with a pair of sequence-tagged site (STS) markers, Mao *et al.* (2017) developed a method

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to identify Wx^b , Wx^{in} , and Wx^{mw} . Sato *et al.* (2002) and Chen *et al.* (2009) established dominant PCR and cleaved amplified polymorphic sequences (CAPS) markers based on the mutation sites in exon 4 of the Wx^{mq} for genotyping. The disadvantage of these dominant markers is that they are not capable of effectively distinguishing homozygous and heterozygous mutants, whereas the use of restriction endonucleases with CAPS markers is often cumbersome and costly.

Tetra-primer ARMS-PCR is a novel method for detecting single nucleotide polymorphisms (SNPs) and was widely used in rice molecular breeding (Chen *et al.* 2013, Ramkumar *et al.* 2015, Zhou *et al.* 2013). Because Taq DNA polymerase lacks of 3'→5' exonuclease activity, allele-specific primers containing a mismatch at their 3'-terminus can only be extended at low speed, and the reaction stops when a large number of mismatched bases are present and thus the formation of 3'-terminus nucleotide phosphodiester bonds is less likely to occur. Therefore, these primers can be used to specifically amplify a single allele (Ye *et al.* 2001). The present study utilized this principle and designed primers based on the A-to-G change that occurs in the fourth exon (at nucleotide position 77) of Wx^{hp} . Using the genomic DNA extracted from rice plants of multiple varieties, the specificity and efficiency of these primers were tested, and the corresponding phenotypes were also determined. Our results indicate that this method is convenient, accurate, and inexpensive; thus, it may be applicable to MAS and genotyping Wx^{hp} in rice germplasm collections.

Materials and Methods

Plant material

Three test varieties (Haopi, Haomuxi and Huangbansuo) containing the low amylose content-associated gene Wx^{hp} were used in this study. Six varieties for multiple alleles of Wx , namely, Hanhui 49 (Wx^a), Huhan 1509 (Wx^b), IR64 (Wx^{in}), Mowangu (Wx^{mw}), Nangeng 46 (Wx^{mq}), and Guixiangsinuo (wx). A F_2 segregating population contains 192 individuals, derived from a cross between Huhan 1509 and Huangbansuo (a soft rice variety), was used to validate the newly developed ARMS-PCR markers. A mini-core collection of rice germplasm, including 170 Chinese landraces and 100 water-saving and drought-resistant rice varieties (Wu *et al.* 2015), was genotyped to detect polymorphisms of the functional marker.

All these materials were provided by the Shanghai Agrobiological Gene Center, China. Seeds were sown in the spring of 2018 at The Zhuanghang Experimental Station of The Shanghai Academy of Agricultural Sciences, and the plants were maintained using conventional cultivation practice.

Primer design and synthesis

Based on the A-to-G change in the fourth exon (at nucleotide position 77) of Wx^{hp} (accession number: AF141954) (Liu *et al.* 2009), Primer Premier 5.0 software (<http://www.premierbiosoft.com/>) was employed to design the primers according to the principle of tetra-primer ARMS-PCR (Supplemental Table 1). The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd., China.

DNA extraction, PCR amplification, and electrophoretic separation

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Genomic DNA extraction was performed as previously described (Murray and Thompson 1980). Each PCR amplification system (20 μ L) contained the following: 1 μ L rice genomic DNA as template, 10 μ L Taq PCR Mastermix (TIANGEN Biotech (Beijing) Co., Ltd., China), 0.5 μ L of each primer (10 μ M) and 7 μ L H_2O . The PCR reaction conditions were as follows: pre-denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min; and then a final extension at 72°C for 5 min. The PCR products (8 μ L) were visualized by a gel imager after electrophoretic separation on a 3% agarose gel.

Sequence analysis of the single-nucleotide variation of Wx^{hp} gene

Genomic DNAs from nine rice varieties were amplified by using primers Wx-hp-OUT F and Wx-hp-OUT R (Supplemental Table 1). The PCR products were purified and the sequences confirmed by BioSune (Shanghai) Co., Ltd., China. DNA sequences were aligned using Clustal X 1.8 (Thompson *et al.* 1997).

External characterization of the endosperm

The detection functionality of the four ARMS-PCR primers designed in this study was verified by assessing the external features of the endosperm (Fig. 1). The genotypes of starch endosperms were visually identified after drying,

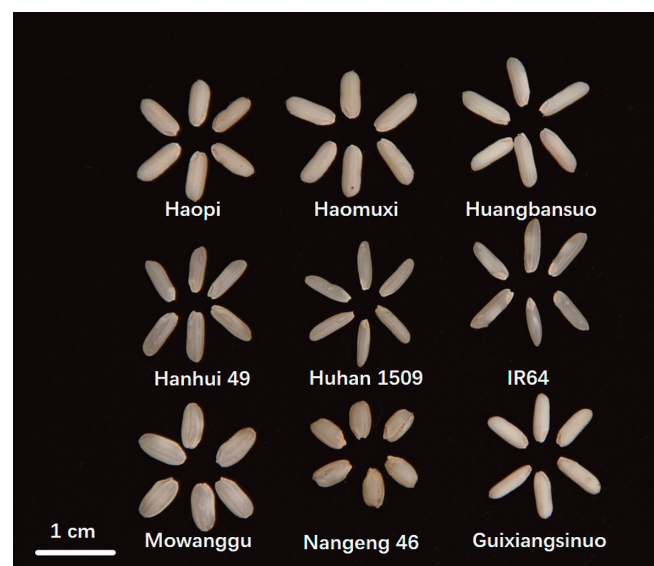


Fig. 1. Rice grains appearance of nine cultivars for various Wx gene alleles. The white scale bar is 1 centimeter.

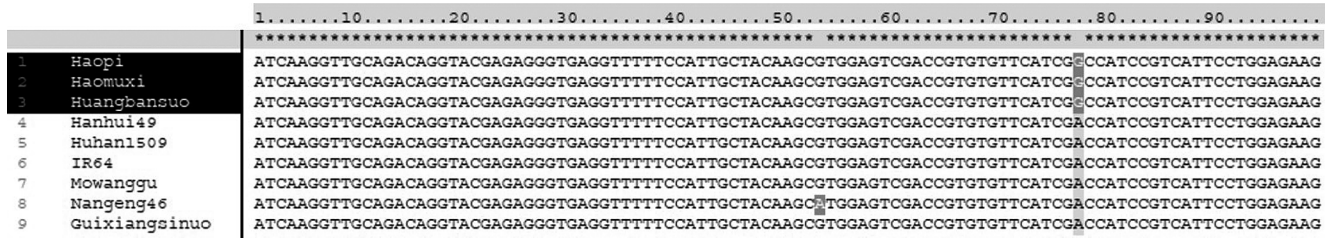


Fig. 2. Sequence analysis of the single-nucleotide variation of the Wx^{hp} gene. Sequence alignment showing an A-to-G substitution at nucleotide position 77 of the 4th exon region between Wx^{hp} allele varieties (Haopi, Haomuxi and Huangbansuo) and non- Wx^{hp} allele varieties (Hanhui 49, Huhan 1509, IR64, Mowanggu, Nangeng 46 and Guixiangsinuo).

threshing, and hulling the rice materials. Individuals whose grain endosperm was all transparent (cloud-like and milky white) were confirmed to be wild-type (with no Wx^{hp} allele); individuals with poorly transparent (opaque endosperm phenotype) grains were identified as Wx^{hp} homozygous plants; and individuals displaying a mixture of these two endosperm phenotypes are usually Wx^{hp} heterozygous plants.

Determination of amylose content

The AC of rice endosperm starch was estimated using the concanavalin A-based method as described previously (Gibson *et al.* 1996, Yun and Matheson 1990). Standard samples with different amylose levels were purchased from Megazyme International Ireland Limited (Wicklow, Ireland).

Results

The development of markers for the specific detection of the Wx^{hp} SNP site

To further verify the SNP site of the Wx^{hp} gene, PCR fragments corresponding to the 4th exon region of Wx gene were amplified from nine cultivars and were sequenced (Fig. 2). Through multiple sequence alignment, an A-to-G substitution at nucleotide position 77 of the 4th exon was detected in Wx^{hp} allele rice varieties (Haopi, Haomuxi and Huangbansuo), which was consistent with result of Liu *et al.* (2009). Another SNP site (G-to-A change at nucleotide position 53 of the 4th exon) in Nangeng 46 was identical with that of the previously reported Wx^{mq} gene (Sato *et al.* 2002). Thus, the SNP site specific markers allow the detection of the Wx^{hp} allele.

According to the principle of the tetra-primer ARMS-PCR technology, a forward inner primer (Wx-hp-IN F) and reverse inner primer (Wx-hp-IN R) were designed with a G at their 3'-terminus, which correspond to the A-to-G SNP at nucleotide position 77 of the fourth exon. In addition, to enhance the specificity of the primers, a C-to-A mismatch and a G-to-T mismatch were each introduced into the 3rd positions from 3'-terminus of the primers. Furthermore, we took several factors into consideration to select the two primer pairs from a list generated by Primer Premier 5.0 software such as the melting temperature (T_m), the complementary

situation, and whether the length difference is big enough to be visualized by gel electrophoresis (Fig. 3, Supplemental Table 1). PCR using these primers always produces a 665-bp band as DNA quality control, and for Wx^{hp} homozygous and Wx^{hp} heterozygous plants, one additional band (325 bp) and two additional bands (382 bp and 325 bp) were amplified.

Genotyping the Wx^{hp} and amylose content determination

Genomic DNA extracted from rice plants of nine varieties was tested by the tetra-primer ARMS-PCR amplification, and 3% agarose gel electrophoresis revealed two bands (Supplemental Fig. 1). With two outer primers, Wx-hp-OUT F and Wx-hp-OUT R, a 665-bp band was detected in all tested samples, suggesting that the DNA was suitable for PCR amplification. One additional 382-bp band, produced by primers Wx-hp-IN F and Wx-hp-OUT R, was detected in three Wx^{hp} homozygous varieties (Haopi, Haomuxi and Huangbansuo). For the other varieties, the smaller band was amplified by the forward outer primer Wx-hp-OUT F and reverse outer primer Wx-hp-IN R. To verify the accuracy of

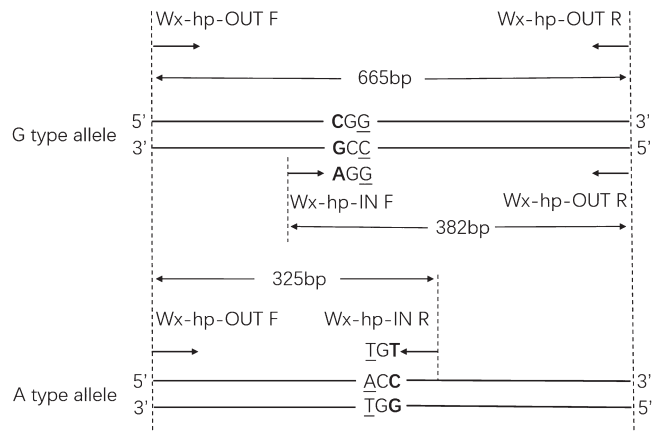


Fig. 3. The primer design strategy of the tetra-primer ARMS-PCR system. The SNP of interest is a A/G mutation; mismatches introduced at the 3'-terminus (underlined) and the 3rd nucleotide position (3'-terminus, bolded) of Wx-hp-IN F and Wx-hp-IN R ensured the amplification specificity. The genotype-specific PCR product sizes for Wx^{hp} allele (G allele) and non-mutated allele (A allele) are 382 bp and 325 bp, respectively, and both band patterns contain a 665 bp band.

Table 1. Wx genotypes and amylose content in tested varieties

Varieties	Genotype of Wx	Amylose content/%
Haopi	$Wx^{hp} Wx^{hp}$	9.6
Haomuxi	$Wx^{hp} Wx^{hp}$	10.1
Huangbansuo	$Wx^{hp} Wx^{hp}$	10.3
Hanhui 49	$Wx^a Wx^a$	23.2
Huhan 1509	$Wx^b Wx^b$	15.4
IR64	$Wx^{in} Wx^{in}$	19.2
Mowanggu	$Wx^{mw} Wx^{mw}$	14.3
Nangeng 46	$Wx^{mq} Wx^{mq}$	10.8
Guixiangsinuo	$wx wx$	2.1

our tetra-primer ARMS-PCR strategy, we also monitored the endosperm phenotype (appearance and the starch content composition) of mature grains (Table 1). Our results indicate that PCR amplification band patterns completely correspond to the observed phenotypic features. Therefore, the ARMS-PCR markers can accurately identify whether the varieties of a rice germplasm collection contain the low amylose content-associated gene Wx^{hp} .

Genotyping Wx^{hp} in a Huhan 1509 × Huangbansuo F_2 population

To verify the functionality of our ARMS-PCR markers, genomic DNA was extracted from 192 individual tillering-stage plants of a Huhan 1509 × Huangbansuo F_2 population. Genotyping the Wx^{hp} gene indicated a total of three band patterns, i.e., 665 bp and 325 bp for Wx^{hp} homozygous plants (GG); 665 bp, 382 bp, and 325 bp for Wx^{hp} heterozygous plants (GA); and 665 bp and 325 bp for non- Wx^{hp} allele wild-type plants (AA) (Supplemental Fig. 2). Statistical analysis (chi-square test, $\chi^2 = 0.46 < \chi^2_{0.05} = 5.99$) showed that the separation ratio of the three genotypes, 46GG:94GA:52AA, was in line with the 1:2:1 Mendelian single-gene segregation ratio. This ratio was also confirmed by phenotypic observation of the mature endosperm. Therefore, these four ARMS-PCR primers are codominant markers, which means they can effectively distinguish three different genotypes of the low amylose content associated gene Wx^{hp} in rice and thus may be utilized in marker-assisted breeding.

Genotyping Wx^{hp} using the mini-core collection of rice germplasm

To further validate the specificity of our tetra-primer ARMS-PCR system for genotyping Wx^{hp} , thereby identifying new Wx^{hp} carried accessions, we applied our newly developed markers to 270 varieties from a mini-core set of Chinese rice germplasm collection. Surprisingly, all accessions amplified the non- Wx^{hp} allele band pattern (665 bp and 325 bp), revealed that the Wx^{hp} gene has not been widely used in rice breeding program in China.

Discussion

Soft rice is a new type of high-quality rice with moderate

amylose content and unique cooking quality; it is therefore favored in the international market. Yunnan soft rice is *indica* rice, and its low-amylose content phenotype, naturally occurring in wild rice, is conferred by the Wx^{hp} allele (Liu *et al.* 2009). With such an excellent genetic tool for breeding high-quality soft *indica* rice varieties, the efficient identification and selection for the Wx^{hp} genotype has become an urgent challenge.

The development of functional markers can dramatically speed up the breeding program. Tetra-primer ARMS-PCR is a PCR derivative method based on standard PCR and ARMS-PCR that has been established to detect single-nucleotide mutations. Compared to traditional ARMS-PCR, the simultaneous amplification using two outer primers and two inner primers can detect mutant and normal alleles in a single PCR assay (Ye *et al.* 2001). The position of primers at the 3'-terminus must be aligned with the SNP of interest (same or complementary base), and that a mismatch must be artificially introduced at the 3rd nucleotide position from the 3'-terminus to enhance the specificity of the primers, therefore preventing the non-perfect-match extension (Little 1995). In this study, we embraced the tetra-primer ARMS-PCR principle and the mismatch-adding rule, introducing artificial mismatched bases at the 3rd nucleotide position of the 3'-terminus of the primers, namely, C-to-A for Wx -hp-IN F and G-to-T for Wx -hp-IN R. Testing a set of varieties, we demonstrated the functionality of these four primers, and our system appeared to be relatively more convenient, accurate and economical than the CAPS markers developed by Liu *et al.* (2009), thus representing a more suitable system for the large-scale screening of Wx^{hp} in MAS breeding programs. Furthermore, the absence of positive band in the mini-core collection of rice germplasm noted that the Wx^{hp} allele was only present in very few Yunnan landraces in China, such as Haopi, Haoanmen, and Haomuxi (Liu *et al.* 2009). Due to the poor transportation conditions for local ethnic groups and linkage-drag of important agronomic traits (such as tall and weak stem, long duration, low yield, and susceptibility to many diseases), the Wx^{hp} allele has not been used in breeding process in China. The new markers in present study could benefit breeding programs by introducing the Wx^{hp} allele into other elite cultivars to obtain high-quality rice and/or new materials for food industrial applications.

The advantage of the new markers is that it can detect the Wx^{hp} gene during the seedling stage, thus improving the operability and predictability of our breeding programs. Our results also suggest that the novel molecular markers with identify-SNP abilities are likely to play an important role in the selection of rice traits (e.g., high-yield, high-quality and disease-resistance) and in pyramiding multiple beneficial genes in the breeding programs.

Author Contribution Statement

Y L, LJ L, GL L and XQ Y conceived and designed the

experiments. AN Z, FM W, JH W, JG B, DY K and FY Z performed the experiments. Y L and GL L analyzed the data. Y L wrote the paper. All authors read and approved the final manuscript.

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