Low amylose content (AC) is a desirable trait for rice (Oryza sativa L.) cooking quality and is selected in soft rice breeding. The Wxhp 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 Wxhp 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 Wxhp. Four Wxhp-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 Wxhp-related genotypes (i.e., Wxhp homozygotes, Wxhp 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 Wxhp alleles in rice germplasm collections and may aid breeding programs with marker-assisted selection (MAS).

**Key Words:** rice (Oryza sativa L.), low amylose content, Wxhp gene, tetra-primer ARMS-PCR.
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 $Wx^{mw}$ 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 ($Wx^{mw}$), Mowanggu ($Wx^{in}$), Nangeng 46 ($Wx^{mw}$), and Guixiangsino (wx). A F2 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 Agricultural 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**

Genomic DNA extraction was performed as previously described (Murray and Thompson 1980). Each PCR amplification system (20 μL) contained the following: 1 μL rice genomic DNA as template, 10 μL Taq PCR Mastermix (TIANGEN Biotech (Beijing) Co., Ltd., China), 0.5 μL of each primer (10 μM) and 7 μL H2O. 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 μL) 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. 2. Sequence analysis of the single-nucleotide variation of the \( W_{xhp} \) gene. Sequence alignment showing an A-to-G substitution at nucleotide position 77 of the 4th exon region between \( W_{xhp} \) allele varieties (Haopi, Haomuxi and Huangbansuo) and non-\( W_{xhp} \) allele varieties (Hanhui 49, Huhan 1509, IR64, Mowanggu, Nangeng 46 and Guixiangsinuo).

**Thresholding 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 \( W_{xhp} \) allele); individuals with poorly transparent (opaque endosperm phenotype) grains were identified as \( W_{xhp} \) homozygous plants; and individuals displaying a mixture of these two endosperm phenotypes are usually \( W_{xhp} \) 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 \( W_{xhp} \) SNP site**

To further verify the SNP site of the \( W_{xhp} \) gene, PCR fragments corresponding to the 4th exon region of \( W_{x} \) 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 \( W_{xhp} \) 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 \( W_{xmq} \) gene (Sato et al. 2002). Thus, the SNP site specific markers allow the detection of the \( W_{xhp} \) allele.

According to the principle of the tetra-primer ARMS-PCR technology, a forward inner primer (\( W_{xhp}-\text{IN F} \)) and reverse inner primer (\( W_{xhp}-\text{IN R} \)) 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 (Tm), 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 \( W_{xhp} \) homozygous and \( W_{xhp} \) heterozygous plants, one additional band (325 bp) and two additional bands (382 bp and 325 bp) were amplified.

**Genotyping the \( W_{xhp} \) 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, \( W_{x-hp-OUT F} \) and \( W_{x-hp-OUT R} \), 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 \( W_{x-hp-IN F} \) and \( W_{x-hp-OUT R} \), was detected in three \( W_{xhp} \) homozygous varieties (Haopi, Haomuxi and Huangbansuo). For the other varieties, the smaller band was amplified by the forward outer primer \( W_{x-hp-OUT F} \) and reverse outer primer \( W_{x-hp-IN R} \). 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 \( W_{xhp} \) 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.](image-url)
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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