A rare Waxy allele coordinately improves rice eating and cooking quality and grain transparency

Changquan Zhang1,2, Yong Yang1, Shengjie Chen2†, Xueju Liu2, Jihui Zhu1,2, Lihui Zhou2, Yan Lu2, Qianfeng Li1,2, Xiaolei Fan1,2, Shuzhu Tang1,2, Minghong Gu1,2 and Qiaoquan Liu1,2*

1. Key Laboratory of Crop Genomics and Molecular Breeding of Jiangsu Province, State Key Laboratory of Hybrid Rice, Yangzhou University, Yangzhou 225009, China
2. Key Laboratory of Plant Functional Genomics of the Ministry of Education/Jiangsu Key Laboratory of Crop Genetics and Physiology/Co-Innovation Center for Modern Production Technology of Grain Crops of Jiangsu Province, Yangzhou University, Yangzhou 225009, China
†Present address: State Key Laboratory of Agrobiotechnology, School of Life Sciences, The Chinese University of Hong Kong, Hong Kong, China
*Correspondence: Qiaoquan Liu (qqliu@yzu.edu.cn)

ABSTRACT

In rice (Oryza sativa), amylose content (AC) is the major factor that determines eating and cooking quality (ECQ). The diversity in AC is largely attributed to natural allelic variation at the Waxy (Wx) locus. Here we identified a rare Wx allele, Wxmw, which combines a favorable AC, improved ECQ and grain transparency. Based on a phylogenetic analysis of Wx genomic sequences from 370 rice accessions, we speculated that Wxmw may have derived from recombination between two important natural Wx alleles, Wxn and Wxb. We validated the effects of Wxmw on rice grain quality using both transgenic lines and near-isogenic lines (NILs). When introgressed into the japonica Nipponbare (NIP) background, Wxmw resulted in a moderate AC that was intermediate between that of NILs carrying the Wxb allele and NILs with the Wxmp allele. Notably, mature grains of NILs fixed for Wxmw had an improved transparent endosperm relative to soft rice. Further, we introduced Wxmw into a high-yielding japonica cultivar via molecular marker-assisted selection: the introgressed lines exhibited clear improvements in ECQ and endosperm transparency. Our results suggest that Wxmw is a promising allele to improve grain quality, especially ECQ and grain transparency of high-yielding japonica cultivars, in rice breeding programs.

Keywords: allelic variation, amylose content (AC), eating and cooking quality (ECQ), grain appearance, Oryza sativa L.

INTRODUCTION

Rice (Oryza sativa L.) is a major staple food and the main source of dietary carbohydrates for half of the world’s population. To meet the food requirements for the growing human population, continuous improvements in both rice yield and grain quality will be necessary (Tilman et al., 2011; Custodio et al., 2019). As rice is commonly consumed as cooked intact grains, both the eating and cooking quality (ECQ) and grain transparency (a measure of rice commercial and appearance quality) have been the key traits selected in released cultivars (Zhou et al., 2020).

The main macromolecules accumulating in a polished rice grain are starches (amounting up to 90% by dry weight). The starch composition of the endosperm, indicated by the grain amylose content (AC), is a key factor determining the...
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physicochemical properties and ECQ of rice grains (Fitzgerald et al., 2009; Li et al., 2016). Rice cultivars are routinely classified according to their AC: high (>25%), intermediate (20%–25%), low (10%–19%), very low or soft (3%–9%), and waxy or glutinous (<2%). Generally, cooked rice grains with a low or intermediate AC exhibit an elastic, sticky, and glossy texture, whereas high AC varieties are characterized by firm and separated grains after cooking (Li et al., 2016). Notably, consumer preferences for rice ECQ differ by region and culture. For instance, individuals from China, Japan, and Korea tend to prefer rice with low AC and sticky ECQ, whereas individuals from India and Bangladesh prefer high AC rice, with firm, separate grains when cooked (Misra et al., 2018; Custodio et al., 2019).

In the rice endosperm, amylose synthesis is largely regulated by the Wx locus located on chromosome 6, which encodes granule-bound starch synthase I (GBSSI) (Wang et al., 1990). The diversity of AC is largely attributable to allelic variation at the Wx locus (Tian et al., 2009; Biselli et al., 2014; Zhang et al., 2019a).

To date, at least eight Wx alleles, Wx<sup>α</sup>, Wx<sup>γ</sup>, Wx<sup>δ</sup>, Wx<sup>δ<sub>2</sub>mp</sup>, Wx<sup>θ</sup>, Wx<sup>α</sup> and Wx<sup>ws</sup>, and Wx<sup>n</sup> have been shown to be associated with the five possible amylose types observed in rice cultivars (Cai et al., 1998; Sato et al., 2002; Larkin and Park, 2003; Wanchana et al., 2003; Mikami et al., 2008; Liu et al., 2009; Yang et al., 2013; Zhang et al., 2019a). Most of these alleles have been successfully incorporated into modern rice cultivars, where Wx<sup>α</sup> (controlling low to very low AC) have been introgressed to breed good ECQ varieties known as soft rice. However, grain appearance for these soft rice varieties with AC values commonly lower than <13% is usually dull or opaque, and thus may not meet the commercial or appearance quality desired by consumers (Liu et al., 2009; Li et al., 2018). Therefore, there is a need to screen rice accessions and mine novel Wx alleles with appropriate AC (such as 13%–14%) from natural germplasm collections to help generate new cultivars with both good ECQ and grain transparency.

In the present study, we identified and characterized a novel Wx allele, Wx<sup>mw</sup>, from a low AC rice landrace. We present here a detailed analysis of Wx<sup>mw</sup> with respect to amylose synthesis as well as its evolutionary relationship with other Wx alleles. Our results suggest that Wx<sup>mw</sup> will be instrumental in breeding new rice varieties, especially in japonica rice, with improved ECQ and a transparent grain appearance.

RESULTS

A landrace rice with relatively low AC and good transparency

After screening many rice accessions for desirable combinations of grain traits, we discovered that the rice landrace Mowanngu (MWG) from southern China had a relatively low AC (14.1%). Under natural air-drying conditions, MWG grains exhibited a transparency and separated grains after cooking (Li et al., 2016). Notably, consumer preferences for rice ECQ differ by region and culture. For instance, individuals from China, Japan, and Korea tend to prefer rice with low AC and sticky ECQ, whereas individuals from India and Bangladesh prefer high AC rice, with firm, separate grains when cooked (Misra et al., 2018; Custodio et al., 2019).

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**Mowanngu harbors a novel Wx allele, Wx<sup>mw</sup>**

To identify the putative gene responsible for low AC in MWG grains, we crossed the MWG variety to the japonica cultivar Nipponbare (NIP), which carries the Wx<sup>α</sup> allele and has an AC of 16.02%. We phenotyped 500 individuals from the resulting segregating F<sub>2</sub> population and selected 92 individuals with MWG-like low AC for genetic mapping. We determined that the causal locus responsible for the low AC mapped to the short arm of chromosome 6. We then selected 418 individuals with low AC from 2 000 individuals derived from the F<sub>2</sub> progeny of informative F<sub>2</sub> lines for fine mapping, which narrowed down the location of the causal locus between markers Chr 608 and Chr 613 (Figure 1E). The Wx locus was included in the mapping interval, highlighting Wx as an obvious candidate (Figure 1E–F). We therefore sequenced the entire Wx gene (8 073 bp) in the MWG background, and identified a single A-to-C substitution on exon 6 (EX6–62) relative to the Wx<sup>α</sup> allele from NIP (Figure 1F). This A-to-C substitution was also present in the Wx<sup>α</sup> allele from IR64, associated with intermediate AC. However, Wx<sup>α</sup> also included another single nucleotide polymorphism (SNP) in the first intron (Int 1–1) that distinguished Wx<sup>α</sup> (with a G) from Wx from MWG (with a T). Furthermore, the Wx allele from MWG did not share any functional SNPs with the other sequenced Wx<sup>α</sup>, Wx<sup>δ</sup>, Wx<sup>δ<sub>2</sub>mp</sup>, or Wx<sup>θ</sup> alleles (Figure 1F).

These results indicated that the newly cloned Wx allele from the MWG accession combines the T polymorphism at the Int 1-1 position, as in Wx<sup>α</sup>, with the C polymorphism in Ex 6-62, as in Wx<sup>α</sup>. Therefore, the relatively low AC exhibited by MWG may be attributable to the combination of these two SNPs. We will refer to the Wx allele from MWG as the novel haplotype Wx<sup>mw</sup> (Wx from Mowanngu). Previous association analyses of diverse rice collections had demonstrated a significant association between low AC and both the Int 1–1 and Ex 6–62 SNPs at the Wx locus (Larkin and Park, 2003; Hoai et al., 2014). However, how the Wx<sup>mw</sup> haplotype affects rice grain quality has not been investigated. In the following experiments, we thus compared the functional consequences associated with the Wx<sup>mw</sup> allele with those of the Wx<sup>α</sup> and Wx<sup>α</sup> alleles, also resulting in low AC.

**Wx<sup>mw</sup> contributes to low AC and improved rice ECQ**

To validate the role of the novel Wx<sup>mw</sup> allele in establishing a low AC content, we introduced three constructs (Figure S2), carrying the entire Wx<sup>mw</sup>, Wx<sup>α</sup>, and Wx<sup>α</sup> genomic regions from MWG, NIP and NG46, respectively, into the glutinous near-isogenic line (NIL) NIP(wx), carrying the null wx allele in the NIP background. Under natural air-drying conditions,
transgenic grains carrying the Wx<sup>mmw</sup> transgene showed a better transparency than transgenic grains with the Wx<sup>mp</sup> transgene (Figure 1G). Transgenic NIP(Wx<sup>mp</sup>)-Wx<sup>mmw</sup> grains had a significantly lower AC (12%–13%) relative to NIP(Wx<sup>mp</sup>)-W xb transformants, although their AC remained higher than that of NIP(Wx<sup>mp</sup>)-Wx<sup>mp</sup> transgenic grains (Figure 1H). The gradient texture of rice grains followed a negative correlation with AC values across the transformants, as evidenced by the GC data (Figure 1H, I), but we observed no significant differences in gelatinization temperature (GT) between NIP(Wx<sup>mp</sup>)-Wx<sup>mmw</sup> and NIP(Wx<sup>mp</sup>)-Wx<sup>mp</sup> transgenic grains (Table S3).

In a complementary approach, we generated two NILs, NIP(Wx<sup>mmw</sup>) and NIP(Wx<sup>mp</sup>), in the NIP background, by introgressing the Wx<sup>mmw</sup> and Wx<sup>mp</sup> alleles from MWG and NG46, respectively. We confirmed that both NILs carried only a small segment around the Wx region from the donor genomes with published molecular markers (Zhang et al., 2011), and exhibited an overall plant morphology that was comparable with that of their recurrent parent NIP(Wx<sup>b</sup>) (Figure S3). As expected, brown rice from the NIP(Wx<sup>mmw</sup>) line exhibited a slightly darker endosperm when compared with NIP(Wx<sup>mp</sup>) grains, but had improved appearance relative to NIP(Wx<sup>mp</sup>) grains (Figure 2A). In addition, NIP(Wx<sup>mmw</sup>) grains had an AC that was intermediate between NIP(Wx<sup>b</sup>) and NIP(Wx<sup>mp</sup>) (Figure 2B), and exhibited very similar soft GC values and rapid viscosity analysis (RVA) curves to NIP(Wx<sup>mp</sup>) grains (Figure 2D; Tables S4, S5). Differential scanning calorimetry (DSC) analysis indicated that both NIP(Wx<sup>mmw</sup>) and NIP(Wx<sup>mp</sup>) had lower GT than NIP(Wx<sup>b</sup>) (Table S4). Importantly,
the sensory property test showed that cooked NIP(Wx<sup>mw</sup>) rice grains had a slightly lower taste value relative to cooked NIP(Wx<sup>mp</sup>) grains, but ranked higher than cooked NIP(Wx<sup>fb</sup>) grains (Figure 2C). Together, these results confirmed that Wx<sup>mw</sup> is a novel Wx allele with a behavior distinct from the Wx<sup>fb</sup> and Wx<sup>mp</sup> alleles, and suggest that Wx<sup>mw</sup> may constitute a suitable genetic material for breeding new rice cultivars with improved grain appearance and ECQ.

**Wx<sup>mw</sup> endosperm has lower GBSSI activity than Wx<sup>fb</sup> endosperm**

We next compared the expression levels of the Wx<sup>mw</sup>, Wx<sup>fb</sup>, and Wx<sup>mp</sup> alleles in developing seeds from NIP and the respective NILs. All genotypes accumulated similar transcript levels of mature Wx messenger RNA (mRNA) during the grain-filling process (Figure 2E), ruling out Wx transcriptional output as the source for the distinct grain properties.
observed in each genotype. We then turned to the enzymatic activity of GBSSI, the Wx-encoded enzyme. GBSSI activity broadly followed the same pattern over the course of seed development in all three genotypes, but with notable differences as well. Indeed, GBSSI activity derived from the Wx<sup>mp</sup> allele was the lowest measured in our experiments; the Wx<sup>mm</sup> allele in NIP(Wx<sup>mw</sup>) resulted in higher GBSSI activity relative to NIP(Wx<sup>mp</sup>), but remained lower than that of GBSSI from Wxb in the NIP background (Figure 2F).

To determine if GBSSI activity corresponded with protein levels, we performed sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of GBSSI (Figure 2G) and total seed proteins (Figure 2H) from immature seeds. GBSSI protein levels clearly varied between NIP and the two NILs, with the most protein accumulating in NIP, followed by NIP(Wx<sup>mm</sup>) and finally NIP(Wx<sup>mp</sup>) (Figure 2G, H). Collectively, these results indicate that the A–C SNP detected in exon 6 (Ex 6–62) in Wx<sup>mm</sup>, which results in a Ser to Tyr substitution (Figure 1E), may decrease GBSSI levels and/or GBSSI activity, leading to a reduction in AC of Wx<sup>mm</sup> endosperm.

**Wx<sup>mm</sup>** rice grains are transparent

The degree of transparency exhibited by milled rice is generally a reflection of AC and moisture content (Zhang et al., 2017; Li et al., 2018). In addition, safe storage of mature rice grains typically requires a moisture content lower than 14%.

Therefore, we compared grain appearance along a moisture gradient, generated via gradual air-drying. Our starting materials consisted of rice grains from three NILs in the NIP background, and carrying the alleles wx, Wx<sup>mp</sup> or Wx<sup>mm</sup>, respectively. As shown in Figure 3A and Table S7, grains with a high moisture content (over 18%), even glutinous grains from NIP(wx), exhibited a transparent appearance. However, glutinous NIP(wx) endosperms rapidly became dull or waxy with decreasing seed moisture, while NIP(Wx<sup>mp</sup>) grains turned opaque when their moisture content fell below 14%. Notably, NIP(Wx<sup>mm</sup>) grains only became opaque when their moisture content dropped below 12%.

We then turned to scanning electron microscope (SEM) observations of transverse grain sections. In either high or extremely low moisture content (2 h and 24 h after drying treatment), we noticed many small holes in the core of starch granules within NIP(wx) glutinous endosperm (Figure 3B, F), although we failed to see similar structures in starch granules from transparent NIP(Wxb) grains (Figure 3C, G). We detected no holes under high moisture conditions in NIP(Wx<sup>mm</sup>) or NIP(Wx<sup>mp</sup>) grains (Figure 3D, E), although holes did appear later under low moisture. We quantified the area covered by holes in both genotypes: the gaps in NIP(Wx<sup>mm</sup>) grain starch granules comprised a smaller area (0.11 ± 0.05 μm<sup>2</sup>) when compared to the gaps in NIP(Wx<sup>mp</sup>) rice grains (0.25 ± 0.04 μm<sup>2</sup>) (Figure 3H, I). We observed a similar phenomenon in transgenic rice lines in the...
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NIP(wx) background expressing the various Wx alleles under investigation here, lending support to the contribution of Wx alleles to grain appearance (Figure S4). In agreement with our earlier studies (Zhang et al., 2017, 2019b), these results indicate that the improvement of endosperm transparency in Wx<sup>mw</sup> grains may be due to smaller air pockets in the center of starch granules caused by their slightly higher AC, when compared to soft Wx<sup>mp</sup> rice.

**Wx<sup>mw</sup> may have derived from natural recombination between Wxb<sup>s</sup> and Wxin**

To examine the relationship between Wx<sup>mw</sup> and other cloned Wx alleles, we compared the full-length Wx gene in a panel of 370 worldwide rice accessions (Zhang et al., 2019a; Table S8). Only four rice accessions (including MWG) belonging to the tropical japonica subpopulation carried the Wx<sup>mw</sup> allele. We next constructed a phylogenetic tree of all sequenced Wx alleles, which divided the 370 rice accessions into four groups (Figure 4A). Notably, the Wx<sup>mw</sup> allele clustered with the Wx<sup>n</sup> and Wx<sup>b</sup> alleles, indicating a close genetic relationship between these three alleles. We then calculated the average genetic distances between distinct Wx alleles. As expected, Wx<sup>mw</sup> appeared to be much closer to both Wx<sup>n</sup> (0.000 52) and Wx<sup>b</sup> (0.000 44) relative to all other alleles (Table S6). In a recent study, we had proposed that the Wx<sup>b</sup> and Wx<sup>n</sup> alleles may have evolved from the same haplotype (Wx<sup>n</sup>–I) from the ancestral Wx<sup>y</sup> allele (Zhang et al., 2019a). Therefore, we hypothesize here that the Wx<sup>mw</sup> allele may have arisen from a spontaneous recombination between the Wxin and Wxb alleles during the later stages of rice domestication (Figure 1B). In fact, several studies have determined that the Wx locus is a recombination hotspot in rice cultivars (Inukai et al., 2000; Muto et al., 2016).

![Figure 4](https://www.jipb.net/resource/4.1420/727240/4a.png)

**Figure 4. Phylogenetic analysis and proposed evolutionary relationship among various Wx alleles in rice**

(A) Neighbor-joining phylogenetic tree based on full-length Wx genomic sequences from 370 rice accessions. The rice accessions and their Wx genotypes are given in Table S8. (B) Wx<sup>mw</sup> is derived from the recombination between Wx<sup>n</sup> and Wx<sup>b</sup> alleles. Both alleles are evolved from the type I haplotype (Wx<sup>y</sup>–I) of the ancestral Wx<sup>y</sup> allele.
**DISCUSSION**

**Natural variation and modification of the Wx locus**

Starch synthesis-related genes play essential roles in controlling rice ECQ, and Wx is the most important gene affecting this trait (Tian et al., 2009). The ever-increasing demand for rice varieties with high ECQ is driving research toward the identification of natural Wx alleles and/or artificially modifying Wx to meet the requirements of breeding programs. To date, most of the observed diversity in AC can be linked back to natural allelic variation at the Wx locus. Over the past four decades, at least eight Wx alleles have been identified (Hoai et al., 2014; Zhang et al., 2019a). In this study, we described a rare Wx allele, designated Wx\textsuperscript{mw}, that fine-tuned AC by exhibiting moderate GBSSI activity. Wx\textsuperscript{mw} is a promising target for improving either ECQ or grain transparency in rice breeding programs, especially for temperate japonica rice.

Among SNPs from various Wx alleles, the G/T SNP at the splicing donor site of the first intron (Int 1–1) is considered to be the key factor underlying the observed variation in AC (Wang et al., 1995). This Int 1–1 SNP can, alone or in combination with other SNPs or InDels (Figure 1E), explain the function associated with available Wx alleles (Wanchana et al., 2003; Zhang et al., 2019a). For example, the Wx\textsuperscript{a} allele is a combination of T at Int 1–1 and A at Ex 6–62 (Wx exon 6 SNP) and is strongly associated with low AC (15%–18%) accessions (Wang et al., 1995; Isshiki et al., 1998). Similarly, the Wx\textsuperscript{ab} allele consists of G at Int 1–1 and C at Ex 6–62 and is highly represented among intermediate AC (18%–22%) accessions (Mikami et al., 2008). The newly Wx\textsuperscript{mw} allele cloned in this study appears to combine T at Int 1–1, as in Wx\textsuperscript{a}, and C at Ex 6–62, as in Wx\textsuperscript{ab}. Separately, these two SNPs result in a decrease in Wx expression or GBSSI activity (Wang et al., 1995; Larkin and Park, 2003); it is therefore satisfying to see that their combination in the recombined Wx\textsuperscript{mw} allele also reduces GBSSI activity and thus leads to decreased AC relative to the Wx\textsuperscript{a} allele.

Wx has been modified by conventional mutagenesis and biotechnological methods such as RNA interference (RNAi) or gene editing by clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nuclease Cas9 (Liu et al., 2003; Ma et al., 2015; Lin et al., 2017). For instance, the Wx\textsuperscript{mq} and Wx\textsuperscript{mp} alleles were generated by N-methyl-N-nitrosourea-induced mutagenesis (Sato et al., 2002; Yang et al., 2013). Another 13 Wx mutants were induced by treatment with sodium azide, yielding mutant grains with typically opaque endosperm and very low AC ranging from 1.5% to 7.1% (Lin et al., 2017). In terms of the modulation of GBSSI, our previous studies showed that GBSSI activity is very sensitive to amino acid changes (Liu et al., 2014; Zhang et al., 2017), and thus, it is very difficult to generate mutants with moderate AC by editing the Wx coding sequence directly. Recent studies have generated a series of glutinous rice mutants by editing the Wx coding sequence using CRISPR/Cas9 (Ma et al., 2015; Zhang et al., 2018).
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Likewise, we have created a series of rice lines with fine-tuned AC levels by editing the Wx promoter core regions via CRISPR/Cas9 editing (Huang et al., 2020).

Relationship between AC and endosperm transparency

Several other regulators can also regulate Wx expression at the transcriptional level and thus generate rice grains with low AC but opaque or chalky endosperm. These regulators include dull endosperm 1 (Du1, Zeng et al., 2007), Du3 (Ishihiki et al., 2008), quantitative AC (qAC2, Takemoto-Kuno et al., 2015), floury endosperm 2 (Fio2, Wu et al., 2015), and nuclear factor YB1 (OsNF-YB1, Bello et al., 2019). Rice grain transparency is an important contributing index to the measure of rice quality that affects rice appearance and commercial quality (Zhou et al., 2020). Previous studies have determined that irregular, small and loosely packed starch granules are the main causes behind rice floury or chalky endosperm (Wu et al., 2015; Zhang et al., 2017; Chen et al., 2020). Regarding opaque endosperm, such as in the low AC rice Haopi (carrying the Wx<sup>op</sup>/hp allele), SEM results indicated that grains presented small empty spaces between starch granules, as well as small holes at the center of starch granules that might be associated with reduced grain transparency (Liu et al., 2009). However, the relationship between AC and grain transparency is unclear. We recently generated several low AC rice lines with dull endosperm by modifying GBSSI activity and established that holes inside starch granules were responsible for the dull appearance of rice grains (Zhang et al., 2017, 2019b). In this study, we observed a similar dullness, especially in the Wx<sup>mp</sup> endosperm, and further demonstrated that this dull grain appearance was negatively correlated with AC from the analysis of NILs and transgenic rice lines carrying various Wx alleles (Figures 4, S4). Finally, we established that the hole size within the core of starch granules increased as moisture content decreased and adversely affected the opaque appearance (Figure 4).

Starch granules consist of two different glucose polymers: amylose and amylepectin. Amylose molecules appear to disperse among amylepectin molecules and locate primarily in the amorphous zones in a random coiled form. Previous work on starch granules in cereals has shown that amylose is mainly in the centers (cores) of the granules (Glarign et al., 2006; Cai et al., 2014). Our recent studies indicated that grain transparency was positively correlated with AC, while negatively correlated with the area of cavity within starch granules (Zhang et al., 2017, 2019b). Considering that even a glutinous rice grain can have a transparent phenotype when the moisture content is sufficiently high (>18%), we predicted that AC plays a key role in endosperm appearance and may form a stable and well-distributed structure with both water and amylopectin (Zhang et al., 2019b). Once the AC drops below a given threshold (<14%), the starch granule structure will become unstable and its semi-crystalline structure may retract in the center of the starch granule, resulting in the appearance of holes. Indeed, we recently discovered that amylepectin may function together with amylose to form a stable semi-crystalline structure and a transparent endosperm in transgenic RNAi rice lines against soluble starch synthase II-2 (SSSIII-2), even with extremely low AC (Li et al., 2018).

Use of Wx<sup>mw</sup> to improve rice grain quality by MAS

Rice cultivars with varied AC values are an agronomic necessity to accommodate the preferences of different countries and regions (Custodio et al., 2019; Zhou et al., 2020). Currently, soft rice with low AC is preferred in some regions in China, where soft indica cultivars usually carry the rare Wx<sup>mp</sup>/hp allele (Larkin and Park, 2003; Liu et al., 2009) and soft japonica cultivars carry the Wx<sup>mp</sup> allele, which was generated through artificial induction (Yang et al., 2013). However, all soft rice cultivars bearing these alleles usually also display a dark or dull endosperm appearance and are sometimes too sticky after cooking. Our data demonstrated that japonica rice with the Wx<sup>mw</sup> allele had a higher AC than either Wx<sup>mp</sup> or Wx<sup>op</sup>. In addition, the presence of the Wx<sup>mw</sup> allele improved endosperm transparency. Moreover, the taste of the new Wx<sup>mw</sup> rice lines generated here improved greatly when compared to other cultivars such as NIP and 2611. Therefore, the newly identified Wx<sup>mw</sup> allele may constitute a useful genetic resource to improve ECQ and endosperm appearance, especially in temperate japonica rice cultivars, to meet the demands of consumers in specific regions.

Marker-assisted selection has been instrumental in addressing the limitations of AC measurements and producing improved super-rice with high yield and superior quality (Zeng et al., 2017). In this study, we developed an allele-specific DNA marker based on the A/C SNP in exon 6 and generated two NILs in two distinct backgrounds (NIP and 2661) with the help of MAS. The cultivar 2661 is high-yielding, and the derived NIL showed the same improved ECQ and grain appearance as when Wx<sup>mw</sup> was introgressed into the NIP background, which underscores the advantages of the Wx<sup>mw</sup> allele for improving rice grain quality. The use of SNP markers based on Kompetitive Allele-Specific PCR (KASP) has recently been shown to be suitable for high-throughput automated genotyping for rice breeding, and functional SNPs between different Wx alleles can be used for the development of KASP markers (Yang et al., 2019). The molecular marker RM190 can be used to distinguish between Wx<sup>mp</sup> and Wx<sup>op</sup> alleles in some rice cultivars (Biselli et al., 2014). Moreover, RM190 can be used to distinguish between Wx<sup>mw</sup> and Wx<sup>op</sup> in some cultivars. Thus, both RM190 and allele-specific DNA markers can be used in rice breeding, depending on the Wx allele polymorphism between different rice cultivars.

CONCLUSION

The AC of endosperm starch is the key factor determining rice grain quality, especially ECQ and grain transparency. Of the many genes involved in starch biosynthesis in the rice
endosperm, Wx is largely responsible for AC, and thus ECQ (Tian et al., 2009; Zhang et al., 2019a). The major goal of rice grain quality breeding programs is not only good ECQ but also transparent endosperm appearance. Here, we cloned Wx\textsuperscript{mm}, a rare Wx allele responsible for a favorable AC, improved ECQ and grain transparency. Notably, we successfully harnessed the Wx\textsuperscript{mm} allele to breed new rice cultivars with moderate AC and good grain appearance via MAS. Our results suggest that Wx\textsuperscript{mm} is a promising allele for grain quality improvement in rice breeding programs, especially for improving ECQ and grain transparency in high-yielding japonica cultivars.

### MATERIALS AND METHODS

#### Plant materials and growth conditions

We used a number of rice (Oryza sativa) accessions in this study. These accessions included a set of six varieties with different Wx alleles and AC for comparison of grain qualities (Figure S1; Table S8): the tropical japonica landrace MWG, carrying the novel Wx\textsuperscript{mm} allele; the three temperate japonica cultivars NIP, carrying the Wx\textsuperscript{b} allele, Guanglingxiangnuo (GLXN, carrying the wx allele), and Nangeng 46 (NG46, carrying the Wx\textsuperscript{mp} allele); and the two indica cultivars IR64 (carrying the Wx\textsuperscript{a} allele) and Teqing (TQ, carrying the Wx\textsuperscript{a} allele). We also worked with the other temperate japonica cultivar 2661 (carrying the Wx\textsuperscript{b} allele) and two NILs, NIP(wx) and NIP(Wx\textsuperscript{mm}), for functional analyses. The NILs NIP(wx) and NIP(Wx\textsuperscript{mm}) were generated by introgression of the wx and Wx\textsuperscript{mm} alleles, respectively, in the NIP background as described previously (Zhang et al., 2019a). We also used a panel of 370 worldwide rice accessions (including the above rice varieties) (Table S8) from our previous study (Zhang et al., 2019a). We cultivated all rice plants under natural field conditions at experimental stations located in Yangzhou (Jiangsu Province, 32°23′ N). For analysis of rice phenotypes and grain quality, we planted the NILs in triplicate during the summer season in Yangzhou, and harvested seeds at maturity from 20 plants in the middle of each plot before allowing the seeds to air-dry. For gene expression and GBSSI protein assays, we collected rice seeds at different development stages from three biological replicates.

#### Construction of the mapping population and near-isogenic lines (NILs)

For genetic analysis and fine mapping of the candidate gene for low AC in the MWG background, we generated an F\textsubscript{2} population by crossing MWG and NIP. For gene functional analyses, we used the NIL NIP(wx) as the recipient for transformation. For gene expression and grain quality analyses, we generated the NIL NIP(Wx\textsuperscript{mm}) in the NIP background by introducing the Wx\textsuperscript{mm} allele from the donor MWG, followed by seven rounds of backcrossing, assisted with the molecular markers listed below (Figure S3A). To evaluate the effects of Wx\textsuperscript{mm} on breeding programs, we introgressed the Wx\textsuperscript{mm} allele into the japonica cultivar 2661 (with a high yield potential, originating from Jiangsu Province) followed by six rounds of backcrossing to 2661, yielding the new NIL 2661(Wx\textsuperscript{mm}) (Figure 5). We planted all rice materials mentioned above during the summer season in Yangzhou (Jiangsu, China, 32°23′N) or during the winter season in Lingshi (Hainan, China, 18°30′N).

#### DNA extraction, sequencing, and population analyses

To determine the genetic background of each NIL, we used 120 molecular markers distributed over the 12 rice chromosomes, as described previously (Zhang et al., 2011). We amplified the Wx locus by PCR using three primer pairs (Table S1) from total genomic DNA extracted from NIP or MWG. We sequenced the PCR products and aligned their sequences using the software ClustaX (Larkin et al., 2007). For bioinformatics analyses, we made use of Wx genomic sequences (5,318 bp) from 370 rice accessions (Table S8). We identified the various Wx allelic types found in these accessions according to our previous study (Zhang et al., 2019). We carried out phylogenetic and molecular evolutionary analyses using MEGA version X (Kumar et al., 2018).

#### Plasmids and rice transformation

For functional complementation analysis, we PCR-amplified a 8 kbp genomic fragment covering the entire Wx locus with primers WxG1 and WxG2 (Table S1) from NIP, MWG, and NG46, respectively. Polymerase chain reactions were carried out in a 50 μL reaction volume using PrimeSTAR® HS DNA Polymerase (Takara, Kyoto, Japan). Amplification conditions consisted of 35 cycles at 98°C for 10 s, 4 min at 68°C, followed by 2 min at 72°C. We cloned the resulting PCR products into the pCAMBIA1300 vector at the BamHI and KpnI sites. We introduced the constructs p1300-Wx\textsuperscript{b}, p1300-Wx\textsuperscript{mm}, and p1300-Wx\textsuperscript{mp} (Figure S2A) into Agrobacterium (Agrobacterium tumefaciens) strain EHA105 and transformed the gluttonous NIL NIP(wx) via Agrobacterium-mediated
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transformation (Liu et al., 1998). We determined transgene copy number in transgenic rice with the SYBR Green Real-Time PCR Kit (TaKaRa, TB Green™ Premix Ex Taq™ GC) and a real-time PCR detection system (Bio-Rad CFX96), as described by (Ding et al., 2004). For real-time quantitative PCR, we employed 10 ng of genomic DNA as a template, and amplified the DNA samples with two independent primer sets specific for Wx and an endogenous single-copy molecular marker (R10M10; Table S1).

RNA extraction and real-time quantitative PCR analysis

We extracted total RNA from developing seeds at 5, 10, 15, 20, and 25 d after flowering (DAF) with the RNAprep pure Plant Kit (Tiangen, Beijing), according to the manufacturer’s protocol. We synthesized first-strand complementary DNAs (cDNAs) using the PrimeScript™ RT reagent Kit (TaKaRa), and performed real-time quantitative PCR analysis with the SYBR Green real-time quantitative PCR method. We performed three technical replications per template and with three biological replicates for each sample to obtain an average value for relative expression levels. OsACTIN1 was used as the internal control. All primer sets are listed in Table S1. We analyzed results using the 2−ΔΔC(t) method (Livak and Schmittgen, 2001).

Assay of GBSSI protein and activity

We collected developing seeds at 5, 10, 15, 20, and 25 DAF, and then ground them to powder in liquid nitrogen after removing husks and embryos. We used the resulting powders for GBSSI activity assay as described previously (Liu et al., 2014). For total seed protein analysis, we took a 100 mg aliquot of the powder from 20 DAF seeds for protein extraction, as described by (Yamagata et al., 1982). Moreover, we extracted starch granule-bound GBSSI as described by (Liu et al., 2014), followed by separation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, according to standard procedures.

Grain quality analyses

For grain transparency analysis, we harvested mature seeds and dried them in a drying oven (40 °C) for 2, 4, 6, 8, 12, and 24 h before milling to white rice. We measured the moisture content of milled grains using a halogen moisture analyzer (Metttler Toledo MJ33, Switzerland). For grain physicochemical quality analyses, we air-dried and milled mature seeds to white rice. We ground a fraction of the polished rice grains into 20 DAF seeds to white rice. We subjected all data to one way analysis of variance and Student’s t-test analysis, using the SPSS 16.0 statistical software program. Results with a probability value of P < 0.05 were considered statistically significant.

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AUTHOR CONTRIBUTIONS

Q.Q.L., C.Z., and M.G. conceived and designed the experiments. C.Z., Y.Y., Y.L., and S.T. performed the experiments. C.Z., S.C., J.Z., and X.L. analyzed the data. Q.F.L. and X.F. prepared the seed samples. Z.C. and Q.Q.L. wrote the manuscript. All authors have read and approved of the manuscript.

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SUPPORTING INFORMATION

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Figure S1. Grain physicochemical characteristics of six rice varieties carrying different Wx alleles

Grain appearance and physicochemical characteristics of six rice varieties carrying different Wx alleles. (A) Gelatinization temperature (GT) of rice flours. (B) Rapid viscosity analysis (RVA) profiles of purified rice starches from mature grains. Rice varieties: tropical japonica landrace Mowanggu (MWG); three temperate japonica cultivars Nipponbare (NIP); Guangdongxiangnuo (GLXN) and Nangeng 46 (NG46); two indica cultivars IR64 and Teqing (TQ). Values labelled with different lowercase letters are significantly different by one-way ANOVA with multiple comparisons (p < 0.05). The error bars indicate standard deviation (SD).

Figure S2. T-DNA structure of the constructs used for rice transformation and PCR verification of transgenic rice

(A) T-DNA structure of the constructs used for rice transformation. The intact Wxmp, Wxmw or Wxmp genomic fragments inserted between HindIII and KpnI in the T-DNA region are derived from NIP, MWG or NG46, respectively. The indicated “T-G(A)-A(C)” in the Wx coding region corresponds to polymorphic nucleotides from the single nucleotide polymorphism (SNP) int1-1, Ex4-53 and Ex6-62, respectively, as shown in Figure 1F. ATG and TGA represent the start and stop codons of Wx, respectively; RB and LB indicate right and left borders, respectively; 3SS and 3SS polA mean the promoter and polA sequences of the cauliflower Mosaic Virus (CaMV) 3SS gene, respectively. (B) PCR verification on transgenic rice plants. We amplified the hygromycin resistance gene to validate transgenic plants. WT: receptor parent NIP(wx), NIP(wx)-Wxb, NIP(wx)-Wxmw and NIP(wx)-Wxmp transformants carrying the Wxb, Wxmw or Wxmp transgene in the NIP(wx) background, respectively.

Figure S3. Construction of near isogenic lines (NILs) carrying different Wx alleles and phenotypic and allele identification of NILs in the japonica NIP background

(A) Construction of near isogenic lines (NILs) carrying different Wx alleles. MAS, molecular marker-assisted selection. (B) Phenotypic and (C, D) allele identification of NILs in the japonica NIP background. (B) Plant morphology at maturity. (C, D) Allele-specific primer PCR (AS-PCR) for the detection of Wxmw (C) and Wxmp (D), respectively. NIP or NIP(Wx) represents the japonica cultivar Nipponbare as the recurrent parent carrying Wxb allele, while MWG and NG46 are the donors with the Wxmw or Wxmp allele, respectively.

Figure S4. Endosperm appearance of milled rice and morphology of grain transverse sections of the glutinous line NIP(wx) and transgenic derivatives by scanning electron microscopy

(A) Milled rice under dry conditions. (B-D) Transverse sections of mature grains from the glutinous line NIP(wx). (E-G) 31 Transverse sections of mature grains from NIP(wx)-Wxmw transformants. (H-J) Transverse sections of mature grains from NIP(wx)-Wxmp transformants. (K-M) Transverse sections of mature grains from NIP(wx)-Wxmw transformants. Red arrows indicate holes within starch granules.

Table S1. Primers used in this study

Table S2. Pasting properties of flours from different rice varieties

Data represent means ± standard deviations, n = 2. For each column in the same flour or starch samples, values displaying different lowercase letters are significantly different by one-way ANOVA with multiple comparisons (p < 0.05). Rice varieties: tropical japonica landrace Mowanggu (MWG); three temperate japonica cultivars Nipponbare (NIP); Guangdongxiangnuo (GLXN) and Nangeng 46 (NG46); two indica cultivars IR64 and Teqing (TQ).

Table S3. Thermal properties of mature rice grains from transgenic rice and its wild type

The data represent means ± standard deviation, n = 3. To, Tp, Tc, and ΔH indicate onset temperature, peak temperature, conclusion temperature, and enthalpy of gelatinization, respectively. Means with different lowercase letters in each column for the same cultivar are significantly different by one-way ANOVA with multiple comparisons (p < 0.05).

Table S4. Taste value and physicochemical properties of the mature grains from NILs carrying different Wx alleles

Data represent means ± standard deviation, n = 3. To, Tp, Tc, and ΔH indicate onset temperature, peak temperature, conclusion temperature, and enthalpy of gelatinization, respectively. Means with different lowercase letters in each column for the same cultivar are significantly different by one-way ANOVA with multiple comparisons and student’s t-test analysis (p < 0.05).

Table S5. Pasting properties of rice flours from near-isogenic lines (NILs) in the Nipponbare (NIP) and 2661 backgrounds

Data represent means ± standard deviation, n = 2. For each column in the same cultivar background, values with different lowercase letters are significantly different by one-way ANOVA with multiple comparisons and student’s t-test analysis (p < 0.05).

Table S6. Average genetic distances between groups carrying various Wx alleles

Population average distances were calculated from SNPs in 370 accessions (Table S8) using the bootstrap method.
Table S7. Moisture content of rice grains from different NILs after gradient drying as shown in Figure 3A
The mature seeds were dried directly in a drying oven (40°C) for 2, 4, 6, 8, 12, and 24 h and then milled to white rice for measurement of moisture content. Data represent means ± standard deviations, n = 2. For each column, values with different lowercase letters are significantly different by one-way ANOVA with multiple comparisons (p < 0.05).

Table S8. List of rice accessions used in this study