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Improving cooking and eating quality of Xieyou57, an elite indica hybrid rice, by marker-assisted selection of the *Wx* locus

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Abstract Xieyou 57, an elite hybrid rice with high grain yield and broad eco-adaptability, is widely planted in China. Its cooking and eating quality, however, is unsatisfactory due to high-amylose content (AC). In this research, a molecular marker-assisted selection method was used to breed for low-amylose content through the modification of the *Wx* genes in both parents of Xieyou 57. The quality of the parent and hybrid lines were then compared before and after modification. Amylose content of GT-type hybrids derived from crosses of Xieqingzao A(GG) × 057(TT) or Xieqingzao A(TT) × 057(GG), was reduced to about 19% from about 26% in the original hybrid Xieqingzao A(GG) × 057(GG). Wide variation in amylose contents, however, was observed in these GT-type hybrids. With further improvements in both parental lines, the TT type hybrid of Xieqingzao A(TT) × 057(TT) contained even lower amylose (12.5%) with good uniformity and exhibited much better cooking and eating quality than the original hybrid of Xieqingzao A(GG) × 057(GG). Meanwhile, yield potential was also improved by increasing panicle size and grain number of the male sterile line. These results demonstrated the success in significantly improving the cooking and eating quality of hybrid rice while maintaining the good agronomical attributes of the parent lines by using molecular marker-assisted breeding in combination with conventional agronomical selection.

Keywords Hybrid rice · Quality · Amylose content · Marker-assisted selection · Granule-bound starch synthase · Rapid viscosity analysis

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Introduction

Since its initial released and promoted in China in 1976, hybrid rice has gained great popularity for its outstanding productivity, wide eco-adaptability and tremendous contributions to China's food security. While the benefit of high yield potential is highly recognized, the poor eating and cooking quality of most rice hybrids poses challenges in meeting consumer expectations. This issue is especially prominent for *indica* hybrid rice. Therefore, quality improvement has been highlighted as one of the key

objectives in hybrid rice breeding in order to satisfy the increasing market needs and consumption standards in recent years (Cheng and Li 2007). Breeders and producers start to pay more attention to the composite performance of rice hybrids and varieties in terms of high productivity, good quality, and high efficiency. Meanwhile, the breeding strategies place more emphases on specific improvements of parents using modern molecular methods in addition to the conventional methods that mainly depend on combination of parental genotypes.

Xieyou 57 is an elite *indica* hybrid developed and released in the beginning of the twenty-first century. It is grown in the mid-lower reaches of the Yangtze river featuring high yield and wide eco-adaptability. However, its poor cooking and eating quality and low seed setting rate in seed production has undermined its acceptance by consumers and the seed industry. The high-amylose content of its parents (27.3% in the restorer line 057 and 25.2% in the male sterile line Xieqingzao A) has been identified as the main cause of the poor quality of Xieyou 57, which makes the cooked rice fluffy, granular, and with unsatisfactory taste. The low yield performance and the associated high production costs in reproduction of the male sterile parent line and in production of hybrid seeds is resulted from the small panicle and low grain number per panicle of the female parent Xieqingzao A. All these obstacles became the major limitations for large scale plantation and adoption of this hybrid (Zhang et al. 2005).

Knowledge accumulated in the past three decades clearly shows that AC is the key factor affecting rice taste. AC is controlled by the waxy gene (*Wx*) which encodes the granule-bound starch synthase (GBSS). Wang et al. (1990) successfully cloned the *Wx* gene and intensively studied the regulation of its expression. They discovered that a G/T polymorphism at the 5′ leader intron splicing site caused profound changes in the production of mature mRNA for starch synthase and thus affected the AC. If the intron +1 base is a G, the intron would be recognized and then effectively spliced, which results in the accumulation of mature mRNA boosting the GBSS activity to a high level with consequent high AC. However, if the G mutates to T at the splicing site, the intron would not be recognized. Therefore, only a cryptic splicing site would be used and the splicing efficiency would be greatly reduced. This causes a low yield of mature mRNA, low GBSS activity, and considerably low AC (Wang et al. 1990; Cai et al. 1998, 2000). This phenomenon was also confirmed by Frances H et al. (1998), Hirano et al. (1998) and Isshiki et al. (1998). Cai et al. (1998) developed a CAPS marker (cleaved amplified polymorphic sequence, referred to as PCR–*AccI*) based on the G/T polymorphism and the flanking sequences that were recognized by endonuclease *AccI*. Using this method, the high-amylose (GG-type) and low-amylose (TT type) cultivars can be precisely distinguished from each other. In this research, we successfully modified the high-amylose trait of Xieyou 57 using this CAPS marker-assisted selection.

Materials and methods

Plant materials

Rice line 057, the restorer used in production of hybrid Xieyou 57, was first converted from the GG-type to the TT type at the *Wx* locus. In this conversion 057 was used as the receiver in the initial cross and as the recurrent parent in the following backcrosses. Another restorer line Yanhui 559 of low AC and good quality was used as the donor to 057. Meanwhile Xieqingzao B, the maintainer line of the male sterile line Xieqingzao A, was also converted from GG-type to the TT type at the *Wx* locus. In this conversion, Xieqingzao B was used as the receiver and recurrent parent. The corresponding donor was a maintainer line ND42 with low AC, good quality and large panicle size.

Modification of parental lines 057 and Xieqingzao A

Rice line 057 and the donor Yanhui 559 was crossed, and then backcrossed with 057 as the recurrent parent for four generations. During the backcrosses, agronomic characteristics of selected plants were maintained as similar to 057 as possible except that the low AC trait was maintained by PCR–*AccI* marker-assisted selection. After self-fertilization of the BC_4F_1 generation, stable lines with low AC and good agronomic traits were obtained. The original restorer line 057, and the improved line were referred to as 057(GG) and 057(TT), respectively.

Another cross was made between Xieqingzao B and the donor parent ND42. Xieqingzao B was used as the recurrent parent in backcrosses to obtain BC_2F_1 generation. Due to the small panicle and low seed setting phenotype of Xieqingzao B, only two generations of backcrossing were performed in order to retain the big panicle and high grain setting features of the donor parent ND42. During backcrosses, individuals with low AC (selected by PCR-*AccI*), big panicle and more grains were selected, and other characteristics were maintained as close as possible to that of Xieqingzao B. Finally, elite TT genotype lines with low AC and better agronomic traits were obtained through self-fertilization. The recurrent parent Xieqingzao B and the modified line were referred to as XB(GG) and XB(TT), respectively. The introgression of the TT waxy region into Xieqingzao A was carried out by crossing Xieqingzao A with selected XB(TT) followed by consecutive backcrossing. The starting Xieqingzao A and the improved version were designated as XA(GG) and XA(TT), respectively.

The XA(GG) and XA(TT) were crossed with 057(GG) and 057(TT) to generate four different combinations, XA(GG)/057(GG), XA(GG)/057(TT), XA(TT)/057(GG), and XA(TT)/057(TT). These lines were used in the following research.

PCR–*AccI* marker analysis and GBSS activity determination

Total DNA was extracted according to the method of Lu and Zheng (1992). The PCR–*AccI* marker analysis was performed as described by Cai et al. (2002). The forward and reverse primers for PCR were 5′-GCT TCACTTCTCTGCTTGTG-3′ and 5′-ATGATTTAA CGAGAGTTGAA-3′, respectively. The PCR product is a 460 bp fragment containing the *Wx* gene leader intron splicing site. Upon digestion with *AccI* restriction enzyme, the amplified PCR product of the GG-type gave rise to two fragments of 403 and 57 bp. However, the PCR product of the TT type could not be digested with *AccI* and only a 460 bp band was revealed after electrophoresis. The heterozygous GT-type showed the 460 and 403 bp bands simultaneously after digestion.

The GBSS activity was determined according to Nakamura et al. (1989). Briefly, fresh developing seeds of plants 10 days after fertilization were collected for crude enzyme extraction and activity determination. Heat-inactivated crude enzyme was used as the control in this analysis. The enzyme activity was expressed as nmol substrate per min pre gram fresh weight.

Evaluation of cooking and eating quality

The AC, gelatinization temperature (valued as alkali spreading, ASV), and gel consistency were determined according to the methods included in the Agricultural Industry Standard NY/T593-2002 issued by the Ministry of Agriculture, P. R. of China. Rapid viscosity analysis (RVA) was carried out using a 3-D rapid viscosity analyzer (Newport Scientific, Australia) according to the recommended procedures of American Association of Cereal Chemists (Reddy et al. 1994).

Assessment of agronomic performance

For agronomic performance assessment of hybrids with and without improvements a randomized block design was used. Two parental lines and four hybrids with three replications each were included in the test. Each plot contained three rows with 11 plants per row. The plant spacing was 14 cm in the row and 23 cm between the rows. Observation and recording of rice growth and data processing were performed according to Huang et al. (2006).

Results

Wx genotype, GBSS activity and AC of the recipient and donator parents

PCR–*AccI* analysis showed that all the *Wx* loci of the recipients 057(GG), XB(GG), and their hybrid Xieyou 57 [Xieqingzao A(GG) × 057(GG)] were GG-type (Fig. 1, Lane1, 2, and 9), while those of the donors Yanhui 559 and ND42 were TT type (Fig. 1, Lane 4 and 5). Details of GBSS activity in the developing seeds 10 days after fertilization and AC in the mature seeds are shown in Table 1. The GG-type samples consistently exhibited higher AC and stronger GBSS activity than the TT type samples. Thus, the molecular marker faithfully reflected the differences in genotype which determines the difference in enzymatic activity

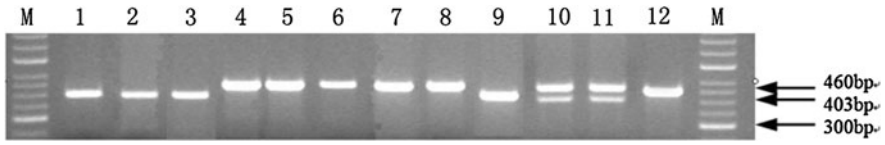

Fig. 1 PCR–*Acc I* detection of the *Wx* genotype *Lanes 1–10*: 057(GG), XB(GG), XA(GG), Yanhui 559, ND42, 057(TT), XB(TT), XA(TT), XA(GG) × 057(GG), XA(GG) × 057(TT), XA(TT) × 057(GG), XA(TT) × 057(TT). *Lane M* 50 bp ladder marker

Table 1 *Wx* genotype, GBSS activity, and amylose content of parental lines

Variety	XB(GG)	057(GG)	Yanhui 559	ND42	Xieyou 57
Wx genotype	GG	GG	TT	TT	GG
GBSS activity (nmol/g min)	9.4 ± 0.25	10.2 ± 0.65	5.1 ± 0.14	4.9 ± 0.52	11.0 ± 0.52
Amylose content(%)	25.0 ± 0.31	27.3 ± 0.32	14.3 ± 0.29	11.4 ± 0.23	26.1 ± 1.53

Data presented as mean ± stand deviation

and in the target trait AC. However, the GBSS activity and AC varied to some degree even in the varieties with same GG or TT genotype (Table 1).

Parent's molecular marker-assisted modification

The recipient and donor lines were crossed and backcrossed following the modification process described in the material and method section. The F_1 generation which is in a GT heterozygous genotype, was backcrossed separately with their recurrent parents, 057(GG) and XB(GG), and generated two *Wx* genotypes GG and GT. By PCR–*AccI* marker recognition and selection, the GG-type plants were discarded and the GT-type individuals that had nearly identical phenotype to the recurrent parent were backcrossed consecutively with the recurrent parent. The modification of 057(GG) was achieved by one generation of cross, four generations of recurrent backcross, and multiple rounds of selfing. Six stable lines that had the TT genotype and agronomic characteristics similar to the recurrent parent 057(GG) were selected. Of the six lines, ZH171 was most identical to 057(GG) and was designated as 057(TT) (Lane 6 in Fig. 1). The modification of XB(GG) was achieved by one generation of cross, two generations of recurrent backcross, and multiple generations of selfing. Five stable plants were selected based on the TT *Wx* genotype, larger panicle, and the highest similarity of agronomic characteristics to the recurrent parent XB(GG). Of the five, line nh136 was most identical to XB(GG) except for panicle size and was designated as XB(TT) (Lane 7 in Fig. 1). Meanwhile, the TT *Wx* genotype was transferred from XB(TT) into the isogenic male sterile line XA(TT) by crossing and recurrent backcrossing with XA(GG) (Lane 8 in Fig. 1). The AC of the two improved lines, 057 (TT) and XB(TT), were reduced to 14.3 and 11.9% from 27.3 to 25.0%, respectively. The GBSS activities dropped to 5.8 and 5.0 nmol/g min from 10.2 to 9.4 nmol/g min, respectively. The AC of XA(TT) was also reduced from 25.2 to 10.7% (Table 2).

During the recurrent backcrossing, AC was measured in different lines at the same generation (BC_4F_2) of 057(GG). Table 3 shows that the AC value is dependent mainly on the *Wx* genotype as the differences in AC were significant at the 0.01 level between different genotypes despite variations observed among plants with the same *Wx* genotype. These results indicate that in addition to major *Wx* gene, the AC might be affected by other minor modifier genes. Greater variations were observed in GT heterozygous plants.

Cooking and eating quality of the modified and the original hybrids

The comparison of cooking and eating quality before and after modification among parents and their hybrids showed that the hybrid of XA(GG)/057(GG) [marked as XY57(GG)] with GG *Wx* genotype (Lane

Table 2 Quality measurements of original and improved parental varieties and their hybrids

Line or hybrid	Amylose content (%)	Gelatinization temperature (ASV)	Gel consistency (mm)
057(GG)	27.3 ± 0.32	6.8 ± 0.09	45.8 ± 1.12
057(TT)	14.3 ± 0.63**	5.7 ± 0.25**	69.60 ± 5.79**
XB(GG)	25.0 ± 0.31	4.2 ± 0.14	33.3 ± 2.32
XB(TT)	11.9 ± 0.39**	4.1 ± 0.12	87.1 ± 4.40**
XA(GG)	25.2 ± 0.52	5.0 ± 0.18	40.2 ± 5.20
XA(TT)	10.7 ± 0.34**	4.0 ± 0.11**	74.0 ± 6.93**
XY57(GG)	26.1 ± 1.53A	5.6 ± 0.44Aa	36.5 ± 2.25C
XY57(GT)	19.3 ± 1.72B	5.3 ± 0.37Aa	47.5 ± 5.53B
XY57(TG)	19.2 ± 2.52B	5.1 ± 0.24ABa	51.5 ± 7.36B
XY57(TT)	12.5 ± 0.45C	4.5 ± 0.52Bb	83.8 ± 5.71A

Data presented as mean ± standard deviation

** Significantly different at 0.01 probability level for comparisons of 057(TT) with 057(GG), XB(TT) with XB(GG) and XA(TT) with XA(GG), respectively. Data within a column marked by the same upper and lowercase letters indicate no significant difference at 0.01 and 0.05 probability levels for multiple comparisons in all hybrids

Table 3 The AC values of three *Wx* genotypes in modified progenies BC_4F_2

Genotype	Number of plants	AC (mean ± SD)	AC variation range (%)
GG	65	24.7 ± 1.97A	20.4–29.4
GT	96	22.4 ± 2.41B	17.7–28.5
TT	106	13.8 ± 1.43C	10.8–17.5

Data within a column followed by different letters indicate significant difference at 0.01 level

9 in Fig. 1) conferred high GBSS activity (11.0 nmol/g min), high AC (26.1%, Table 2), low gel consistency (36.5 mm, Table 2), difficult to cook and cooked rice is fluffy. The two hybrids with a single improved parent, XA(GG)/057(TT) and XA(TT)/057(GG), were GT heterozygous genotype and designated as XY57(GT) and XY57(TG) (Lanes 10 and 11, Fig. 1). Their GBSS activities were reduced to 7.03 and 6.33 nmol/g min, and their AC values dropped to 19.3 (Table 2) and 19.2% (Table 2), respectively. Their gel consistencies were softer than original hybrid XY57(GG). These two hybrids had similar AC values indicating that the AC was determined mainly by hybrid genotype and that the maternal effect (or cytoplasm effect) was minimal. Therefore, contribution of the parental germplasm with the same *Wx* genotype to the amylose content of hybrids was not significantly different regardless of its role in the crosses, either as male or female parent. However, the amylose content varied remarkably among the hybrid combinations with GT genotype. For example, the AC was 17.2–21.8% for XY57(GT) and 16.0–22.8% for XY57(TG) with standard deviations of 1.72 and 2.52%, respectively. This further indicated that the GT or TG heterozygous hybrids had less uniformity in amylose contents. For the hybrids of two modified parents XA(TT)/057(TT), designated as XY57(TT), the GBSS activities and AC were reduced to 4.9 nmol/g min and 12.5% (Table 2). Meanwhile, the gel consistency (83.8 mm, Table 2) was significantly softer than the original hybrid (36.5 mm, Table 2). The hybrid XY57 (TT) was tender, cohesive, glossy and easy to cook. More interestingly, this TT homozygous genotype had a smaller standard deviation (0.45%) and narrower amplitude of variations (11.9–13.0%) in AC than that of GT and TG heterozygous hybrids, suggesting that less AC uniformity observed in heterozygous hybrids was caused by gene separation. Gelatinization temperature didn't vary correspondingly with the trend of changes in AC (Table 2). Comparison of the gelatinization temperature among parents and hybrids showed that *Wx* gene had no significant impact on ASV. The AC-determining genes may be independent to genes determining

the gelatinization temperature. On the other hand, gel consistencies were characterized by coordinated changes with *Wx* genotype and AC values. The trend of gel consistency was TT, GT and GG from high to low. Therefore, introduction of TT genotype into hybrid rice could increase gel consistency and soften cooked rice.

Rapid viscosity analyses of cooked rice provided further evidence for the improvement of cooking and eating quality of the new hybrid XY57(TT). As compared to the original hybrid XY57(GG) and the single parent improved combination XY57(GT) and XY57(TG), the new hybrid XY57(TT) from double improved parents exhibited higher peak viscosity, higher breakdown value, lower recovery value and lower decline value (Table 4), all of which are changes indicative of better cooking and eating quality. Whereas, these quality indicators of the original hybrid were at the opposite end of the spectrum, the heterozygous hybrids XY57(TG) and XY57(GT) fell between as expected.

Comparison of the agronomic performance

In the comparative studies of agronomic traits of the parental varieties and their hybrids, the restorer lines had no significant difference in most traits except the lower tiller number for 057(TT) than 057(GG) (Table 5). The improved XB(TT) showed obvious increases in panicle length and grain number per panicle. At the same time, other traits (e.g. combining ability) were inherited from XB(GG). For the four hybrids, no significant differences were observed in growth cycle period, plant height, tiller per plant, and seed setting rate. The panicle length and grains per panicle in hybrids XY57(TG) and XY57(TT) were enhanced significantly (difference at 0.05 probability levels). For XY57(TT) from improved male and female parents, although the 1,000-grain weight was slightly reduced, panicle's characteristics and yield per plant were considerably improved. In general, the progenies from double improved parents exhibited significant enhancement in yield and improvement in cooking and eating quality.

Discussion

Quality trait such as AC is a characteristic endosperm trait having complicated genetic and environmental controlling mechanisms. Although the endosperm is the offspring of a diploid plant, its development and formation is controlled by triploid interactive alleles. The homogeneity and consistency of quality traits in germplasm are profoundly affected by genetic segregation and gene dosage of the major controlling genes. Furthermore, some minor or modifier genes as well as the interactions between genetic and environmental factors exert more influences in the magnitude and quantity of the quality traits. This complexity makes quality trait breeding more difficult and conventional methods less effective. Applying the PCR–*AccI* molecular marker which is directly linked to the *Wx* gene to the improvement of the amylose content trait, the genotypes can be determined during the whole growth stage and the AC of

Table 4 Starch viscosity characteristics of original and improved parental varieties and their hybrids [unit: rapid viscosity unit (RVU)]

Rice line	Peak viscosity	Hot broth viscosity	Breakdown value	Cold broth viscosity	Decline value	Time of peak	Recovery
057(GG)	2204b	1787a	417d	3272a	1068a	6.27a	1485a
057(TT)	2804a	1602b	1202a	2607b	−197e	6.2a	1005b
XB(GG)	1383e	758d	625c	1769d	386b	5.4c	1011b
XB(TT)	1483d	1063c	420d	1870c	387b	5.93b	807d
XY57(GG)	2549b	1845a	704d	3457a	908a	5.93a	1612a
XY57(GT)	2297c	1354b	943c	2548b	251b	5.6b	1194b
XY57(TG)	2061d	908c	1153b	1589d	−472c	5.53b	681d
XY57(TT)	3384a	1357b	2027a	2149c	−1235d	5.53b	792c

Data within a column followed by the same lowercase letters indicate no significant difference at 0.05 probability level

Table 5 Comparison of the agronomic traits of improved parental varieties and their hybrids

Line or hybrid	Days to harvesting(d)	Plant height (cm)	Tillers/ plant	Panicle length (cm)	Grains/ panicle	Seed setting (%)	Grain weight (g/1000)	Yield (g/plant)
057(GG)	139	113.6	4.7	22.4	144.3	86.1	25.6	14.3
057(TT)	140	116.0	4.0	21.7	166.2	92.2	24.6*	15.1
XB(GG)	118	70.0	5.3	18.5	82.9	87.0	27.1	10.0
XB(TT)	120	70.9	4.7	22.5*	107.9*	83.9	27.2	12.1*
XY57(GG)	143a	122.2a	5.3a	24.0a	145.5a	92.7a	26.9a	19.2a
XY57(GT)	142a	122.1a	5.8a	24.0a	133.0a	93.7a	27.4a	21.1ab
XY57(TG)	143a	122.6a	4.7a	26.4b	198.1b	92.7a	26.0b	22.4ab
XY57(TT)	144a	120.4a	4.9a	27.0b	232.2b	92.6a	25.8b	26.7b

* Significant difference at 5% probability level between original and improved parent. Data within a column followed by the same lower case letters indicate no significant difference at 5% probability levels

the progeny can be analyzed accurately. Thus, marker-assisted selection can markedly enhance the efficiency of rice quality trait breeding (Cai et al. 2002; Zhang et al. 2005; Chen et al. 2008).

Using PCR–*AccI* molecular marker-assisted selection, the restorer line 057(GG) and the male sterile line XA(GG) were improved in amylose content. At the same time, the grain number per panicle of

XA(TT) increased significantly. AC of the hybrids produced from single improved parent was reduced to intermediate levels. However, a wide divergence in the AC traits was observed in the GT or TG heterozygous hybrids which resulted in wide variation in rice quality. Due to the homozygous nature of *Wx* locus (T/T), XY57(TT) from double improved parents exhibited remarkable improvement not only in the eating and cooking quality but also in the homogeneity among the grains. In addition, the improved XA(TT) with larger panicle and higher grain number per panicle was very useful in enhancing the multiplication of the male sterile line, the production of hybrid seed, and the potential productivity of the hybrid. In this research we not only focused on the key issue of AC but also took the whole quality improvement into consideration as such an integrated technical approach was deployed to combine molecular marker-assisted selection and conventional field selection. Such integrated breeding strategies should have broad applications and prospects in the improvement of crops.

Zhou et al. (2003) had reported that the *Wx* gene determined the AC, gelatinization temperature, and gel consistency of rice. However, Sun et al. (2005) and Liu et al. (2006) found no sensible link between gelatinization temperature and *Wx* genotype. Our results provided further supports to the latter statement. In addition, our results demonstrated that down-regulation of the amylase content caused reduction of 1,000-grain weight, which confirmed the observations by Zhou et al. (2003) and Liu et al. (2006). There might be a QTL (quantitative trait locus) near the *Wx* locus that could be responsible for the phenotype of grain weight variation in the range of 5.2–8.6% (Yu et al. 1997). The reduction of 1,000-grain weight may be affected by this QTL. Meanwhile, from a biochemical perspective, down-regulation of AC synthesis probably resulted in negative effect on the weight of seed because AC was the major component of endosperm. Obviously, more should be learned before a conclusion can be made.

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