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A genotyping platform assembled with high-throughput DNA extraction, codominant functional markers, and automated CE system to accelerate marker-assisted improvement of rice

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Abstract The introgression of multiple genes into traditional cultivars using marker-assisted selection (MAS) in order to obtain favorable traits is an effective strategy to achieve improved rice lines. Genotyping of markers is a central component of the evaluation of germplasm and the selection of progeny lines. However, efficient DNA extraction and genotyping of large breeding populations still remain limiting factors in rice molecular breeding programs. This study has developed and validated a cost-effective, rapid (<1 h for 96 samples), and high-throughput (96-well format) total DNA-extraction method based on magnetic particle technology. To improve the grain-quality traits of two rice varieties, we have designed and employed an efficient codominant functional marker system (including *Wx*, *ALK*, *Chalk5*, and *fgr* genes), in combination with genotyping based on automated capillary electrophoresis. Rice lines with simultaneous improvement at multiple loci were obtained and found to have superior grain quality and to be fragrant. The genotyping pipeline established in this study represents an efficient, reliable, and precise platform for MAS.

Keywords Rice · DNA extraction · Marker-assisted selection · Grain quality · Genotyping

Introduction

Recent advances in biotechnology and genomics offer new opportunities for the application of molecular technologies in rice breeding programs that can accelerate the development of high-yielding varieties with improved grain quality and resistance to various biotic and abiotic stresses (Jain et al. 2010). The use of new technologies has allowed the identification and characterization of many valuable traits in rice, as well as the application of marker-assisted selection (MAS) for the transfer of desirable loci into breeding lines (Xu et al. 2012) including precise marker-assisted backcrossing. MAS can significantly enhance genetic gain, especially in cases where phenotypes are highly dependent on specific environmental conditions (Moose and Mumm 2008), where selection is time-consuming, or where traits such as grain quality and disease resistance are pivotal. In rice breeding programs, new improved rice varieties have been

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developed by the introgression of genes or quantitative trait loci (QTL) into traditional varieties using MAS (Jantaboon et al. 2011; Ni et al. 2011; Luo et al. 2012; Hari et al. 2013; Jiang et al. 2015; Septiningsih et al. 2015; Sheng et al. 2015).

The genotyping of target genes/QTLs with suitable molecular markers and array technologies has been developed progressively. The advent of DNA marker technology has offered the possibility of developing new approaches to breeding procedures (Tanksley 1983). And the markers based on different sizes of DNA products, such as SSR (simple sequence repeats), small insertions and deletions (InDels), and CAPS markers can be detectable using agarose electrophoresis, polyacrylamide gel electrophoresis (PAGE), and capillary separation (Kadirvel et al. 2015). Traditional genetic markers linked to genes of interest are extensively exploited for selection during rice breeding. However, such markers cannot serve as a diagnostic tool to determine whether a favored allele is present in different genetic backgrounds or in various germplasms. Furthermore, when traditional markers are used for MAS, they can give false positives due to genetic recombination, and the selection accuracy depends on their linkage distance to the targeted genes (Zhou et al. 2013). In contrast, functional markers are derived from polymorphic sites of genes that causally affect target trait variation, i.e., they are based on functional characterization of polymorphisms (Andersen and Lubberstedt 2003). Therefore, functional markers can act as a diagnostic tool in a targeted search of germplasm collections; they are also reliable for MAS as they are not affected by meiotic recombination and therefore do not show false positives. As long as the parents have the required polymorphism, functional markers offer the possibility of efficient fixation of alleles in populations.

Although genotyping technologies allow the screening of hundreds or thousands of rice samples, a common problem is the lack of a cost-effective, rapid, and high-throughput DNA-extraction method suitable for various genotyping technologies. The existing DNA-extraction methods and commercial kits are time-consuming, have low throughput, are low yielding, or are costly (Xin and Chen 2012; Li et al. 2013). Crude DNA-extraction methods for SSR analysis in rice are highly efficient; however, the final product contains humic materials and protein impurities that can inhibit PCR amplification, while their compatibility with other PCR-based marker systems has not been verified. A gDNA-extraction protocol for large-scale MAS breeding experiments should be evaluated not only in terms of purification, yield performance, and stability of the end product, but also in terms of time, effort, and cost.

Detection of PCR products is one of the crucial steps in genotyping samples. Conventional agarose-/acrylamide gel-based electrophoresis is time-consuming, costly, and labor intensive (Ramkumar et al. 2015). Parallel capillary electrophoresis (CE) is appropriate to avoid gel-based genotyping and improves analysis capability and efficiency as a result of the automation and high resolution associated with CE.

The objective of this study was to develop and implement a genotyping platform consisting of high-throughput DNA extraction, codominant functional markers, and an automated CE system. A rapid, high-throughput, and cost-effective genomic DNA (gDNA) extraction method for rice using the automated magnetic particle separation technology was developed. Simple and efficient functional markers for the rice grain-quality genes of *Wx*, *ALK*, *Chalk5*, and *fgr* were developed and validated. These functional markers were used to evaluate the different rice germplasms. Following this, we used a high-throughput, automated CE system to facilitate marker-assisted backcrossing to improve these grain-quality traits. Using this genotyping platform, we accelerated introgression of four genes for starch physicochemical properties, chalky properties, and fragrance traits and obtained improved lines with superior grain quality, demonstrating the reliability and value of this platform for large-scale MAS breeding.

Materials and methods

Plant materials

Rice seed, leaf, and root samples were obtained from an experimental field and stored at −80 °C until use. For high-throughput DNA-extraction and genotyping analysis, leaf samples of a wide range of genotypes were supplied by the National Engineering Research Center of Plant Space Breeding, South China Agricultural University. For controls, we used Basmati

370 (harboring *fgr* allele), Zhenshan 97 (harboring *Fgr*, *Wx-G*, and *Chalk5-T* alleles), Huahang 31 (harboring *ALK-TT*), and Minghui 63 (harboring *Wx-T*, *ALK-GC*, and *Chalk5-C*). In total, 16 indica rice cultivars (including the four control samples) were used for genotyping analysis and for optimizing PCR amplification of the four new markers.

The H467 line, stacked with favorable alleles, was developed in our laboratory and has excellent grain quality (Luo et al. 2014). We used this line here as the donor parent and two elite indica rice cultivars as recipient parents, Huazhan (released by CNRRI, China) and Yuefengxinzhan (released by GAAS, China). Two MABC populations were developed by crossing Huazhan or Yuefengxinzhan with H467; the F_1 progenies were checked using the new markers and selected plants were backcrossed to the recurrent parents (Huazhan or Yuefengxinzhan). We carried out MAS at the seedling stage in the $BC_1F_1 \sim BC_1F_3$ generations, and only individuals harboring the targeted genotypes were transferred to the field.

Optimization of sample-preparation process for the new DNA-extraction method

We selected multiple factors—including the size and weight of leaf pieces with or without manual agitation to evaluate the sample-preparation process (Table S1). Young leaf tissues were cut into pieces 5.0, 7.5, 12.5, or 15.0 mm in length, and the amount of samples that was used was either 40 or 80 mg. With minor modification, manual agitation treatment was performed as described by Ferreira Santos et al. (2014). We placed the samples and the stainless steel ball (4 mm in diameter) in each well of 96-deep-well plates (Axygen, Corning, NY, USA), sealed with a tight-fitting cap, and manually stirred for 30 s after transient freezing with liquid nitrogen. The effects of leaf size, weight, and agitation were evaluated according to the concentration and optical density OD260/OD280 of the extracted gDNA. Optimal preparation factors were used for the next step of extraction.

DNA-extraction protocol

The protocol is illustrated in Fig. 1. Leaf tissues (80 mg) were cut into small pieces (7.5 mm in length) and placed into a 96-deep-well plate (Axygen, Corning, NY, USA). Root samples were treated as the leaf samples, while seed samples were shelled and sliced in half. To each well, we added the following: 500 µl of extraction buffer (200 mM Tris–HCl [pH 7.8], 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid [EDTA], 0.5 % sodium dodecyl sulfate [SDS], and 2 % polyvinylpyrrolidone [PVP]-40). We then sealed the plate using a 96-well cap mat (Axygen, Corning, NY, USA) and incubated it at 75 °C for approximately 12 min in a thermostatic water bath. Lysates were then cooled in an ice bath and transferred to a 96-deep-well microtiter plate (Thermo Fisher Scientific, Waltham, MA, USA). To perform purification and elution, we used KingFisher™ Flex (Thermo Fisher Scientific, Waltham, MA, USA), which is based on magnetic particle technology (Fig. S1). A KingFisher Flex protocol (Table S2) was created using BindIt (Thermo Fisher Scientific, Waltham, MA, USA) and performed according to the manufacturer's instructions. Bio-magnetic particles (100 mg ml^{-1}; Huier Nano, Henan, China) were vortexed and mixed with isopropanol and lysates for gDNA to bind to the magnetic particles. Then gDNA was washed with washing buffer (60 % ethanol, 50 mM NaCl, 10 mM Tris–HCl [pH 8.0], 0.5 mM EDTA [pH 8.0], and 75 % ethanol). Finally, the gDNA was eluted in 100 µl of Tris–EDTA (TE) buffer (10 mM Tris–HCl, 1 mM EDTA [pH 8.3], and 100 µg ml^{-1} RNase A), and the DNA solutions were stored at −20 °C until further analysis.

This study compared the new protocol with three traditional DNA isolation protocols: the cetrimonium bromide (CTAB)-based method (Pervaiz et al. 2011; Xin and Chen 2012), the SDS-based method (Ahmed et al. 2009), and the MagAttract 96 DNA Plant Kit (Qiagen, Venlo, Netherlands). In all methods, 80 mg of leaf samples was used, and gDNA was dissolved in 100 µl of TE buffer or Buffer AE for MagAttract.

Evaluation of DNA yield and quality and its validation with SSR-PCR amplification

Total DNA was evaluated using a spectrophotometer ND-1000 (NanoDrop® Technologies, Wilmington, DE, USA). The ratio OD260/OD280 was used to estimate the purity of DNA. In addition, 5 µl of each gDNA sample was loaded and visualized on a 1 % agarose gel.

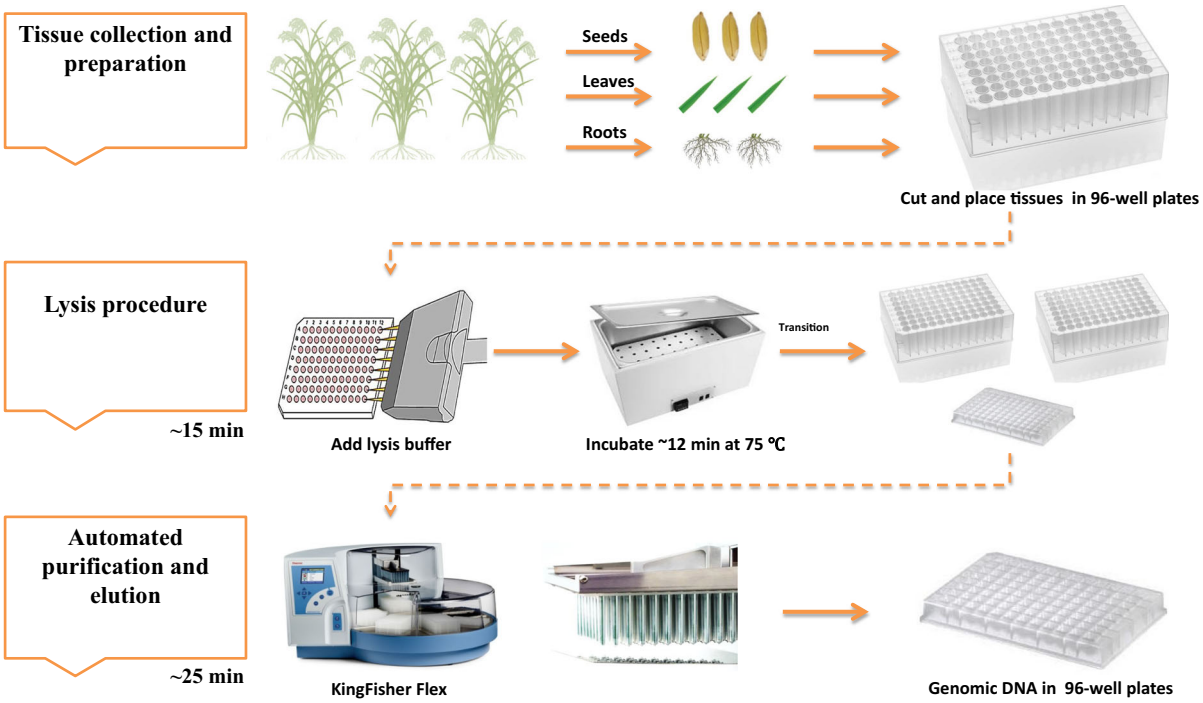

Fig. 1 Flow diagram of main steps of the new protocol for DNA extraction from various rice tissues

To determine the reliability and applicability of the new DNA-extraction method, we used 10 pairs of SSR markers (RM224, RM190, RM22, RM307, RM71, RM19, RM208, RM481, RM85, and RM432), randomly selected from the Gramene database (http://www.gramene.org/), for PCR amplification. PCR was performed in a total volume of 10 µl, containing 5 µl 2 × AceTaq® Master Mix (Vazyme Biotech, Nanjing, China), 0.4 µM of each primer, and 50 ng of gDNA template. All reactions were performed using the Gene Amp PCR System 9700 Thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). PCR conditions were as follows: 5 min at 94 °C, 33 cycles at 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. Amplified products were separated by electrophoresis through 8 % polyacrylamide gel (PAGE), and bands were revealed using the silver-staining procedure. Electrophoretic banding patterns were recorded, and the photographs were taken with a digital camera.

Primers design for the functional molecular markers

The genomic sequences of the *fgr*, *Wx*, *Chalk5,* and *ALK* genes were obtained from GenBank. Sequence alignment was analyzed using CLC Sequence Viewer 7.6 (http://www.clcbio.com/), and polymorphic sites were identified according to the published information. We used the tetra-primer ARMS-PCR system described by Ye et al. (2001) for marker development. The inner primers were targeted to mutation or polymorphic sites, such as SNPs or short InDels, and one of the outer primers was used for the initial amplification. The outer primers also created a control band in the PCR. The amplification using outer and inner primers was carried out in one reaction tube, and products of different lengths were separated by gel electrophoresis. Target sequences were submitted to the Primer 1 online tool (http://primer1.soton.ac.uk/primer1.htm) for primer design (Collins and Ke 2012).

PCR with four new functional markers and detection of amplified fragment

PCR amplification was performed in a 15 µl reaction mixture containing 7.5 µl 2 × AceTaq Master Mix, 5 pM of each primer, and 1 µl gDNA (∼50 ng). The PCR conditions were: initial denaturation at 95 °C for 5 min, 33 cycles of denaturation at 95 °C for 30 s, annealing at 55 or 57 °C (see Table S3) for 30 s, and extension at 72 °C for 45 s. As a final step, the reaction

mixture was incubated at 72 °C for 5 min. The PCR products were separated on a 2 % agarose gel. High-throughput genotyping for MAS was carried out using the Fragment Analyzer CE system (Advanced Analytical Technologies, Inc.), according to the manufacturer's instructions. Alleles were visualized and discriminated using PROSize 2.0 software (Advanced Analytical Technologies, Inc.).

Confirmation of genotype by Sanger sequencing

To evaluate the efficiency and accuracy of the genotyping arrays, selected DNA samples of the 16 rice accessions, including a positive and negative control, were amplified with the outer-primer pair, and the amplicons were sequenced using the Sanger protocol (Life Technologies, https://www.thermofisher.com/). We compared the results obtained by T-ARMS-PCR with those determined by Sanger sequencing to confirm the genotype of the four target alleles.

Evaluation of grain-quality traits

Dried rice grains were stored at room temperature for one month prior to the evaluation of grain-quality traits. Amylose content was determined as described by Perez and Juliano (1978). The assessment of fragrance and non-fragrance traits was determined using a sensory test as described by Jin et al. (2010). The gelatinization temperature was indirectly measured by the alkali spreading extent method, as described by He et al. (2006). The percentage of grain with chalkiness (PGWC) and the degree of endosperm chalkiness (DEC) were evaluated according to Liu et al. (2011).

Results

Development and evaluation of the new gDNA-extraction method

In this study, the effects of leaf size and weight, as well as of manual agitation, were investigated in order to optimize sample preparation. In total, sixteen treatment combinations were used for evaluating (Table S1). The results showed that leaf size has a significant impact on DNA yield and purity and that the optimal length of leaf pieces was 7.5 mm. Under the same conditions, a higher DNA quantity was obtained using 80 mg of tissue compared with 40 mg. Therefore, the optimal conditions of sample preparation were the use of 80 mg of leaf tissue cut into pieces of 7.5 mm in length, while agitation treatment was removed from the procedure to simplify the protocol.

As shown in Table S4, the average concentration of extracted gDNA eluted in TE buffer was 432.90 ng μl^{-1} (i.e., 43.29 μg in total) from leaf samples, 321.36 ng μl^{-1} (i.e., 32.14 μg in total) from seed samples, and 323.56 ng μl^{-1} (i.e., 32.36 μg in total) from root samples. The yields of the new protocol did not differ significantly from those of the SDS-based method (597.77 ng μl^{-1}), were higher than those of MagAttract (165.33 ng μl^{-1}), but lower than those of the CTAB-based method (1,136.08 ng μl^{-1}). The coefficient of variation of gDNA concentration obtained from the new protocol (CV = 0.333) was much lower than that obtained from the CTAB-based method (CV = 0.572) and MagAttract (CV = 0.597), indicating higher yield stability. The purity of gDNA extracted by the CTAB-based or SDS-based methods was very high, since the use of phenol–chloroform enabled successful separation of gDNA from proteins. The gDNA extracted from various tissues using the new protocol generated an OD260/OD280 ratio of 1.81–1.84 and an OD260/OD230 ratio higher than 1.50, indicating high purity. However, the average OD260/OD280 and OD260/OD230 ratios for gDNA extracted by MagAttract were 1.71 and 1.11, respectively, showing the presence of protein impurities and residual salt contamination.

In addition to yield, purity, and performance, we also investigated the relative simplicity, duration, and cost of the different total DNA-extraction methods (Table 1). The new protocol did not require any grinding or the use of liquid nitrogen or any other hazardous reagent, providing a simple and safe method for gDNA extraction, compared with the CTAB-based and SDS-based methods, as well as MagAttract. Additionally, the magnetic-bead technology allowed for the development of a fully automated, high-throughput (96-sample format), cost-effective (lower than $100 per 96 samples), and time-saving gDNA-extraction protocol (<1.0 h/96 samples).

The quality of gDNA was further evaluated by 1.0 % agarose gel electrophoresis (Fig. S2), which showed that the gDNA extracted using the new

Table 1 Summarization of seven different methods for genomic DNA extraction from frozen rice leaves

Method	Technology	Grinding	Liquid nitrogen	Hazardous reagents	Cost for 96 samples[a]	Total time for 96 samples (h)	Automation or manual	References
CTAB-based	Traditional (liquid extraction)	Yes	Yes	Phenol–chloroform	Low	3.0–5.0	Manual	Pervaiz et al. (2011)
SDS-based	Traditional (liquid extraction)	Yes	Yes	Phenol–chloroform	Low	3.0–5.0	Manual	Ahmed et al. (2009)
MagAttract 96 DNA Plant Core Kit	Magnetic beads	Yes	Yes	Guanidine hydrochloride/guanidine thiocyanate	Medium	1.5–2.0	Manual or automated	Manufacturer's protocol
NucleoMag 96 Plant	Magnetic beads	Yes	Yes	Sodium perchlorate	Medium	1.5–2.0	Manual or automated	Manufacturer's protocol
E.Z.N.A Plant DNA kit	Silica based	Yes	Yes	Guanidine hydrochloride	High	2.5–3.0	Manual	Manufacturer's protocol
DNeasy 96 Plant Kit	Silica based	Yes	Yes	Guanidine hydrochloride	High	1.5–2.0	Manual	Manufacturer's protocol
New protocol	Magnetic beads	Not required	Not required	None	Low	<1.0	Automated	This study

CTAB cetyltrimethylammonium bromide, *SDS* sodium dodecyl sulfate

[a] High cost, >$150; medium cost, $100–150; low cost, <$ 100

protocol had uniform brightness and no obvious tails (Fig. S2-IV), similar to that extracted using the SDS-based method (Fig. S2-II). DNA bands from MagAttract were relatively blurred with some residual contaminations at the fore end of the lanes (Fig. S2-III). For further validation, we amplified the DNA with RM224, RM190, and RM22 (Fig. S3). The comparison of amplification patterns clearly revealed that their intensity and sharpness was qualitatively and quantitatively similar. Furthermore, when we used additional SSR markers to amplify the gDNA from the 96 rice accessions extracted using the new protocol, the results showed that the bands were clear, sharp, and uniform (Fig. S4), confirming the high quality and adequate yield of DNA.

PCR-based functional markers using the T-ARMS-PCR system

We selected *fgr*, *Wx*, *Chalk5,* and *ALK* for development of functional markers. The *fgr-E7-FM* allele is the result of an 8-bp deletion; *fgr* contains 3 SNPs and encodes a putative betaine aldehyde dehydrogenase 2 (Saihua et al. 2008; Shao et al. 2013). In the Wx-G/T marker, the G/T polymorphism in intron 1 of *Wx* is responsible for most of the variation in amylose content (Ayres et al. 1997; Cai et al. 1998; Chen et al. 2008). The consensus T/A SNP at −485 bp of the 5′UTR of *Chalk5* has a significant association with white belly phenotype (Li et al. 2014). The marker ALK-GC/TT was developed to distinguish two contiguous SNPs (GC/TT) in the *ALK* gene; these SNPs have a very strong association with gelatinization temperature (Bao et al. 2006; Umemoto and Aoki 2005; Waters et al. 2006). We utilized this available sequence information to design primer pairs for the four functional markers (Table S3). The different sizes of the allele-specific amplicons from the targeted functional polymorphic regions were designed to be greater than 25 bp so that they could be easily separated on agarose gels. To enhance allelic specificity in Wx-G/T, ALK-GC/TT, and Chalk5-T/C, a deliberately mismatched base (indicated by italicized lowercase letters in Table S3) was incorporated at the

3′ terminus of the specific primers using the Primer 1 tool. Annealing temperatures, a key factor in the amplification system, were optimized (see Table S3).

Evaluation and allele identification of the functional markers

PCR amplicons of the expected sizes were generated at all loci, and the amplification profiles showed clearly distinguishable genotypes in the reference samples (Fig. 2). All the PCR products were clearly resolved and sized by 2 % agarose gel electrophoresis, allowing easy identification of different genotypes. Heterozygotes and homozygotes were unambiguously assigned from the gel profile, suggesting both high sensitivity and specificity. The sizes of the DNA fragments amplified with the primers were as follows: fgr-E7-FM, 360/368-bp control fragment, 215-bp deletion allele, and 188-bp insertion allele; Wx-G/T, 287-bp control fragment, 202-bp T allele, and 137-bp G allele; ALK-GC/TT, 293-bp control fragment, 192-bp GC allele, and 144-bp TT allele; and Chalk5-T/C, 298-bp control fragment, 150-bp T allele, and 196-bp C allele (Fig. 2). A collection of sixteen rice varieties was genotyped using the four markers (Table S5), and the results were further confirmed by Sanger sequencing. There was 100 % concordance between the results from the two methods. Among the 16 accessions, the *Wx-T* allele was relatively abundant, whereas the frequency of *fgr* or *Chalk5-C* allele was low. Several varieties carried two or more favorable alleles, such as Xiangyaxiangzhan (*Wx-T* + *fgr* + *ALK-TT*). In conclusion, our functional markers based on the T-ARMS-PCR system offer a simple, cost-effective, highly sensitive, and specific alternative for the detection of the four alleles of major grain-quality genes.

Establishment of MAS program with high-throughput genotyping

As the Huazhan and Yuefengxinzhan varieties are widely cultivated in south China and may possess an elite genetic background, we selected them as the recurrent parents and crossed them with the H467, a favorable-alleles-stacking line that carries the alleles of *fgr, Wx*^{b}, and *chalk5*. In these experiments, grain quality was verified to confirm phenotypes (Table 2). In summary, Huazhan has a high gelatinization temperature (ASV = 2) and poor chalk properties (PGWC = 17.57, DEC = 8.9). Yuefengxinzhan has a high amylose content (AC = 27.66 %). Both varieties

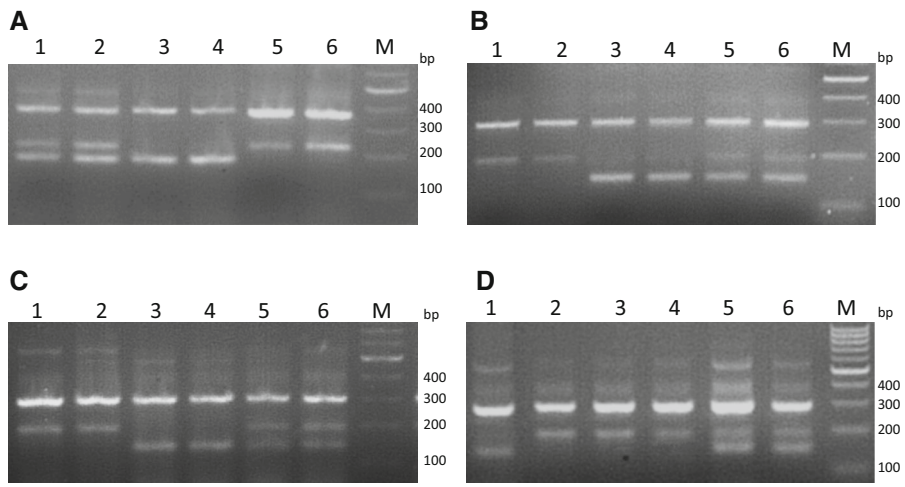

Fig. 2 Amplification products of the selected gene markers. **a** fgr-E7-FM marker for the *fgr* gene. *Lanes 1* and *2* heterozygotes with the genotype *fgr/FGR*. *Lanes 3* and *4* homozygotes with the genotype *FGR/FGR*. *Lanes 5* and *6* homozygotes with the genotype *fgr/fgr*. **b** Wx-GT marker for the *Wx* gene. *Lanes 1* and *2* homozygotes with the genotype T/T. *Lanes 3* and *4* homozygotes with the genotype G/G. *Lanes 5* and *6* heterozygotes with the genotype T/G. **c** ALK-GC/TT marker for the *ALK* gene. *Lanes 1* and *2* homozygotes with the genotype GC/GC. *Lanes 3* and *4* homozygotes with the genotype TT/TT. *Lanes 5* and *6* heterozygotes with the genotype GC/TT. **d** Chalk5-T/C marker for the *Chalk5* gene. *Lane 1* homozygote with the genotype T/T. *Lanes 2, 3*, and *4* Homozygotes with the genotype C/C. *Lanes 5* and *6* heterozygotes with the genotype T/C. M = 100 bp DNA ladder

Table 2 Performance of principal grain-quality traits in the three parental and improved breeding lines

Cultivar/improved breeding lines	Genotype	AC (%)	ASV	PGWC (%)	DEC (%)	Fragrance
Huazhan	Wx^b	14.17	2	17.57	8.9	–
H467	$fgr + Wx^b + chalk5$	14.92	3	11.11	4.4	+++
Yuefengxinzhan	$chalk5 + alk$	27.66	5	11.07	2.8	–
Huazhan-198-6-2	$fgr + Wx^b + chalk5$	14.36	2	9.12	2.1	+++
Huazhan-199-1-3		18.01	2	8.99	2.6	+++
Huazhan-202-4-4		15.67	2	9.13	4.1	+++
Huazhan-204-3-6		14.08	2	10.06	5.5	+++
Yuefengxinzhan-220-3-2	$fgr + Wx^b + chalk5 + alk$	16.05	5	10.93	3.3	+++
Yuefengxinzhan-223-5-1		16.14	4	10.54	4.1	+++
Yuefengxinzhan-225-2-6		17.26	6	3.20	0.5	+++

AC amylose content, *ASV* alkali spreading value (1–7), *PGWC* percentage of grain with chalkiness, *DEC* degree of endosperm chalkiness

+++, fragrant; –, non-fragrant

are non-fragrant. H467 is fragrant and has a good amylose content and chalk properties, although ASV is low.

Two MABC populations were developed as shown in the flowchart in Fig. S5. The F_1 plants were determined using the markers, and selected F_1 plants were backcrossed with the recurrent parent Huazhan or Yuefengxinzhan. To reduce the cost and time of gel electrophoresis, we used the Fragment Analyzer CE system, which is an automated system capable of running 96 capillaries, to analyze the PCR amplification products (Fig. S6). This analysis obtained clear separation of the specific allele amplicons with the expected size peaks. Thus, MAS was carried out at the seedling stage of BC_1F_1–BC_1F_3, and only individuals harboring the targeted genotypes were selected and transferred to the field. After the screening of heterozygosity with the four markers, 11 F_1 plants were selected from the cross of Huazhan and H467, and 6 from the cross of Yuefengxinzhan and H467. They were backcrossed with the recurrent parents, and 94 plants of the Huazhan/H467 BC_1F_1 generation and 104 plants from the Yuefengxinzhan/H467 BC_1F_1 were generated. Seventeen Huazhan/H467 BC_1F_1 plants were found to have heterozygous alleles at *fgr* and *Chalk5* and to be homozygous for the Wx^b locus. Nine Yuefengxinzhan/H467 BC_1F_1 plants were heterozygous at *fgr*, Wx^b, and *alk* and homozygous at *chalk5*. Plants with all the target genes in the homozygous condition were obtained in BC_1F_2 and selfed to produce BC_1F_3 lines. In our MABC procedure, we also took into account phenotypic similarity to the recurrent parent. With this MAS scheme, four lines derived from the MABC population of Huazhan/H467 and three lines from Yuefengxinzhan/H467 were selected from the BC_1F_3 generation; all of the selected lines were homozygous for target genes based on genotyping with the four new functional markers.

Assessment of major grain-quality traits in the improved lines

The main agronomic traits of the MABC lines and their recurrent parental varieties were compared in the field, and nonsignificant differences were identified. Grain-quality traits including amylose content, gelatinization temperature/ASV, chalkiness-related parameters, and fragrance were assessed (Table 2). The seven improved lines harbor allele *fgr* and were assessed to be fragrant, whereas the recurrent parental varieties (Huazhan and Yuefengxinzhan) were non-fragrant. Only Yuefengxinzhan carrying Wx^a displayed a high amylose content (27.66 %). The other two crossing parents, as well as their improved lines carrying Wx^b, were found to have low amylose content (14–15 %). Other grain-quality properties were significantly improved in the MABC lines of Huazhan with PGWC ranging from 8.99 to 10.06 and DEC ranging from 2.1 to 5.5. For MABC improved lines of Yuefengxinzhan, high ASV and excellent chalk

properties were retained (Fig. 3). In particular, Yuefengxinzhan-255-2-6 was demonstrated to have superior grain quality with an amylose content of 17.26 %, an ASV of 6, a PGWC of 3.2, and a DEC of 0.5, as well as being fragrant (Table 2; Fig. 3j). The plant potentially has the characteristics to make a significant breakthrough as a high-quality rice line.

Discussion

Plant genotyping is a rapidly advancing field, and different marker systems are available for plant studies (Batley 2015). In rice and most other crops, PCR-based markers such as simple sequence repeats (SSR or microsatellites) are preferred for use in MAS because of their high levels of polymorphism, reliability and technical simplicity, their requirement for relatively small amounts of DNA, and their low cost (Sun et al. 2010). However, crop improvement is expected to move to a more precise approach in the future. Thus, combining useful functional markers in a controlled cross for varietal development is expected to become an integral part of breeding. There are several high-throughput SNP (single-nucleotide polymorphism) genotyping platforms commercially available today that use SNPs as markers, such as Fluidigm Dynamic Arrays, Douglas Scientific Array Tape, and the LGC automated system for KASP markers (Thomson 2014). These platforms, however, are expensive, lack flexibility, and are not of practical use for small to medium-sized laboratories (see Table S6). Routine deployment of trait-specific SNP markers requires flexible, low-cost systems for genotyping smaller numbers of SNPs across large breeding populations; the T-ARMS-PCR-based allele genotyping technology appears to be suitable as it achieves genotyping of multiple samples accurately and cost-effectively (Table S6). The functional markers developed in this study are user-friendly and also can be directly utilized in other laboratories and performed using traditional gel electrophoresis, even though without the CE system.

However, the purity and quality requirements of DNA vary with PCR principles. We tested other rapid gDNA-extraction protocols and identified unstable results, as well as relatively low PCR efficiency for the T-ARMS-PCR. Furthermore, DNA extraction is a major problem in molecular breeding programs, since it is labor intensive and increases the cost of MAS. Numerous DNA-extraction protocols have been developed based on the standard phenol–chloroform extraction protocol, which is a time-consuming method (Karakousis and Langridge 2003). In this

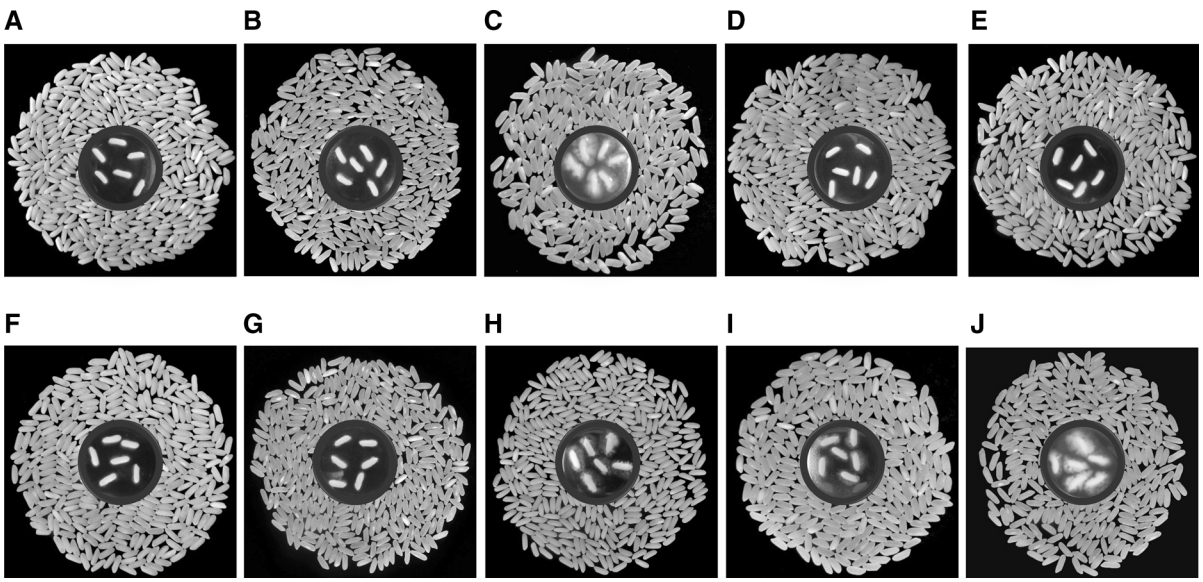

Fig. 3 The appearance and ASV parameter of milled rice of the three parent varieties and in the improved lines. **a** Huazhan, **b** H467, **c** Yuefengxinzhan, **d** Huazhan-198-6-2, **e** Huazhan-199-1-3, **f** Huazhan-202-4-4, **g** Huazhan-204-3-6, **h** Yuefengxinzhan-220-3-2, **i** Yuefengxinzhan-223-5-1, and **j** Yuefengxinzhan-225-2-6

study, we successfully developed a rapid, simple, and high-throughput protocol for rice gDNA extraction. The automated nucleic-acid procedure workstation based on magnetic particle technology allowed us to decrease effort, while the 96-sample format improved productivity. Using our experience of the 'crude extract' approach (Yang et al. 2007) and the extraction buffer introduced by Edwards et al. (1991), we eliminated freeze-drying with liquid nitrogen, grinding, and phenol–chloroform treatment in order to simplify the procedure and increase safety. The results showed that DNA could be extracted from more than 8×96 samples per day per operator with minimum hands-on time, high consistency, and reduced probability for cross-contamination or any other manual errors. Additionally, the optimizations of sample-preparation process and extraction protocol are supposed to be introduced to other automatic DNA isolation platforms, or performed combined with just a simple manual magnetic-bead separating block in different laboratories.

In the new gDNA-extraction protocol, SDS was used to lyse rice tissues quickly and magnetic particles to adsorb DNA from crude lysate. Impurities, such as proteins, detergents, and other PCR inhibitors, were removed by ethanol washing, while gDNA was eluted in TE to ensure its efficiency in various PCR-based genotyping analyses. Furthermore, our method was successfully developed into a high-throughput 96-well format that is compatible with the SNP array in the Fragment Analyzer™ CE system or LightScanner® 96 HRM system, which can further increase the efficiency of MAS on a larger scale.

Market survey data suggest that efforts to develop rice varieties with improved cooking and eating qualities have high economic returns (Son et al. 2014). At least 18 different quality types of rice are favored around the world; however, a combination of slender grain, low amylose content, low gelatinization temperature, and fragrance is most popular (Calingacion et al. 2014). Based on genetic understanding and availability of markers for such traits, MAS can facilitate the development of new varieties with good eating qualities. However, most previous studies using MAS only employed a single gene to improve the eating quality of rice (Jin et al. 2010). The present study is the first report to attempt simultaneous improvement for multiple characteristics, eating, cooking, and sensory qualities of two rice varieties through selection for favorable alleles of *Wx*, *ALK*, *Chalk5*, and *fgr* genes. Our study demonstrates the development of a robust approach for MAS to introduce and fine-tune grain-quality traits into rice lines that have diverse genetic backgrounds.

New functional markers for *fgr* were successfully used to transfer alleles from fragrant rice to the elite rice varieties, Huazhan and Yuefengxinzhan. This is an important step, given the general preference of consumers for fragrant rice. Two major genes involved in the starch metabolic pathway—*Wx* that encodes granule bound starch synthase and *ALK* that encodes soluble starch synthase IIa—have been demonstrated to effectively control amylose content and gelatinization temperature, respectively (Yan et al. 2014). In the present study, we showed again that functional markers based on polymorphisms in these genes could be used to improve the quality of conventional rice using MAS. *Chalk5* encodes a vacuolar H^+-translocating pyrophosphatase that influences grain chalkiness in rice and two consensus SNPs located in the promoter might partly account for the differences in *Chalk5* mRNA levels that contribute to natural variation in grain chalkiness (Li et al. 2014). Although grain chalkiness is a complex trait controlled by multiple genetic factors and influenced by the environment (Sun et al. 2015), our study showed that pyramiding *Chalk5* into a rice cultivar using SNP-based functional markers resulted in a significant improvement in chalk properties.

Introgression of multiple genes into elite lines or high-yielding varieties can be achieved in a more straightforward way if the alleles of interest are fixed in one donor (Luo et al. 2014). We exploited this approach in the present study through use of H467, which carries multiple favorable alleles ($fgr + Wx^b + chalk5$), as the donor parent. This approach proved to be an effective strategy for the targeted selection of four grain-quality traits with multiple markers. The introgression lines were also selected rigorously and were expected to carry as many favorable alleles as possible in order to retain similar agronomic traits as the recipient parent. In this study, we found that it was possible to accelerate the backcross-breeding program and to make it more cost-effective by screening the BC_1F_5 improved breeding lines using a combination of phenotypic selection and marker-assisted foreground selection. Our results demonstrate the successful development of a

genotyping platform consisting of high-throughput DNA extraction, codominant functional markers, and an automated CE system to accelerate marker-assisted improvement of rice. The genotyping platform is reliable and may be applied in large-scale MAS breeding.

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