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Stacking of five favorable alleles for amylase content, fragrance and disease resistance into elite lines in rice (*Oryza sativa*) by using four HRM-based markers and a linked gel-based marker

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Abstract Marker-assisted selection (MAS) for qualitative traits such as grain quality and resistance to certain diseases has proven to be highly effective. Multiple genes responsible for various quality components and disease resistances can be simultaneously stacked to boost the performance and to lengthen the commercial lifespan of high-yield varieties. Grain quality genes (*fgr* and *Wx*) and three disease resistance genes (*Pita, Pik* and *Xa23*) have been well characterized and used in MAS breeding. However, stacking all of them together into a single variety has not been reported. We reported here the stacking of the five genes into elite lines in rice. We achieved this through the development of functional markers from causal mutations at *fgr, Wx* and *Pita*, a gene-targeted marker at *Pik* and the use of a linked marker for *Xa23*. We employed and optimized the high-resolution melting (HRM) analysis method for use as the genotyping platform of *fgr, Wx*, *Pik* and *Pita*. By combining high-throughput DNA isolation, multiplex and nested-PCR methods, we showed that HRM could serve as cost-effective, highly automated, moderate-throughput and reliable non-gel genotyping platform for a small-scale MAS program.

Keywords HRM · Genotyping · Gene stacking · MAS · Rice

Introduction

Marker-assisted selection (MAS) offers a very good tool for breeders to obtain quality traits and disease resistances provided they are inherited in a single-gene manner. Selection can be done on the genetic determinants of the targeted traits by using molecular markers. Once the traits are fixed, breeders can evaluate large numbers of progenies for yield performance in a conventional way. A combination of MAS and conventional evaluation could significantly improve the efficiency and precision of plant breeding and thus accelerate the breeding process and release of new varieties (Morris et al. 2009; Collard and Mackill 2008).

The linkage between markers and gene(s) controlling the targeted trait must be first established prior to the MAS process. Random DNA markers (RDMs)

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which are randomly distributed and linked to the targeted gene(s), as determined by genetic mapping, have been widely used since the 1980s (Tanksley 1983). The selection accuracy of RDMs depends on their distance or linkage level to the targeted genes. When a marker is developed from the polymorphism within the targeted gene, it is called a gene-targeted marker (GTM); when the marker is derived from the polymorphic site within the allele (functional sequence motif) causing phenotypic variation, it is a functional marker (FM; Andersen and Lübberstedt 2003). Since the GTMs and FMs are derived from the targeted functional genes, recombination should be extremely rare and this eliminates the worry about losing the target genes. Even more importantly, FMs are preferable and more breeder-friendly since they are derived from functional motifs representing corresponding phenotypic variations, and thus can be directly used under different genetic backgrounds without recalibration in the MAS process.

Another important issue associated with MAS is the analytical system employed for genotyping of the DNA polymorphisms of interest. The gel electrophoresis-based ("gel-based" for short) analytical system for distinguishing sequence length polymorphisms (SLPs), such as insertions/deletions (InDels) and simple sequence length polymorphisms (SSLPs or SSRs), is widely used as a basic analytical tool in crop genetics and molecular breeding. However, for single nucleotide polymorphisms (SNPs) and very short InDels (less than five bases), the gel-based approach has low capacity to cope since it is low-resolution. Even though some SNPs can be converted into length polymorphism markers with the assistance of restriction enzymes or allele-specific amplification, the conversion rate is low and time-consuming. To improve the limitation of gel electrophoresis in resolution and efficiency, several novel post-PCR methods such as single strand conformation polymorphism (SSCP), denaturing high-performance liquid chromatography (DHPLC) and high-resolution melting (HRM) analysis have been developed. In addition, several moderate- to high-throughput SNP genotyping platforms based on fluorescence-labeled oligos such as KASP^{TM} (LGC Genomics, www.lgcgenomics.com), TaqMan^{TM} (Applied Biosystems, www.appliedbiosystems.com) and Golden Gate^{TM} (Illumina, www.illumina.com), have been developed and employed in large-scale commercial breeding programs. However, these platforms would be less customizable and could not be cost-effective when applied to a small breeding program.

HRM was derived from a diagnostic module in quantitative PCR (Wittwer et al. 2003) and has been well evaluated. It provides a closed-tube, efficient and cost-effective alternative to gel electrophoresis in discriminating SNPs, InDels and SSRs (Wu et al. 2008; Wittwer 2009; Vossen et al. 2009). Several studies has been reported on exploiting HRM for mutation discovery (Jeong et al. 2012), genetic mapping (Lehmensiek et al. 2008), allele mining (Hofinger et al. 2009) and diversity analysis (Distefano et al. 2010) in plants, but none of them focused on employing HRM-based markers in plant MAS breeding practise.

Rice blast disease (caused by *Magnaporthe oryzae*, *M. oryzae*) and bacterial blight (BB; caused by *Xanthomonas oryzae pv. oryzae*, *Xoo*) are the most destructive diseases of rice (*Oryza sativa* L.) worldwide (Madamba et al. 2009). Development of resistant varieties is considered the most cost-effective and environment-friendly approach to protecting rice from the two diseases (Basavaraj et al. 2010; Singh et al. 2012), and varieties with adequate resistance to them are given higher priority in official certification in several provinces of China (Xiao et al. 2012). Moreover, *indica* rice with low to medium amylose content (AC) and fragrance is preferred by consumers in the south China region, even though such rice is much more expensive on the market. Marker-assisted pyramiding of resistance (R) genes or quantitative trait loci (QTLs) to provide broad-spectrum and durable resistance to BB and rice blast have been reported (Huang et al. 2012; Luo et al. 2012; Jiang et al. 2012; Miah et al. 2013), and pyramiding of desirable alleles such as the fragrance gene (*fgr*) and low AC gene (Wx^b) has also been carried out (Jin et al. 2010).

Over the last 15 years, a number of R genes and grain quality genes have been isolated in rice (Bryan et al. 2000; Yuan et al. 2011; Zhai et al. 2011; Wang et al. 1990; Bradbury et al. 2005). The availability of gene sequences has allowed the development of functional markers for these traits. Table 1 lists the gel-based FMs developed from the cloned rice key genes or QTLs of agronomic importance. These provided the foundation for the present research.

The major objectives of this study are (1) to develop and optimize HRM-based FMs for *Wx*, *fgr* and *Pita*,

Table 1 Partial list of key isolated rice genes and their gel-based FMs

Gene	Chromosome	Function description	FNP	FM type	Reference
Wx	6	Largely controls AC	G/T SNP at +1 position of intron 1	CAPS	Cai et al. 1998
fgr	8	Gene for rice fragrance	8-bp deletion and three SNPs in exon 7	InDel	Sakthivel et al. 2009
S_5^n	6	Wide compatibility gene	136-bp deletion flanking the start codon	InDel	Yang et al. 2009
Pik	11	Dominant R gene to blast disease	T/G SNP at base 2,944 of *Pik-1*	CAPS	Zhai et al. 2011
Pita	12	Dominant R gene to blast disease	G/T SNP at codon 918	AS-PCR	Wang et al. 2007
Pit	1	Dominant R gene to blast disease	A/G SNP at codon 780	AS-PCR	Hayashi et al. 2010
xa5	5	Recessive R gene to BB	TC/AG SNP at codon 39	CAPS	Iyer and McCouch 2007
GS3	3	Major QTL for grain size	C/A SNP in exon 2	CAPS	Fan et al. 2009

FM functional marker, *FNP* functional nucleotide polymorphism, *CAPS* cleaved amplified polymorphic sequence, *AS-PCR* allele-specific polymerase chain reaction

and a GTM for discriminating the blast resistance alleles from the susceptible ones at the *Pik* locus, (2) to identify favorable alleles (Wx^b, *fgr*, *Pita* and *Pik*) from rice germplasm by using these HRM-based markers, and (3) to stack Wx^b, *fgr*, *Pita*, Pik^p (a resistance allele of the *Pik* locus) with a broad-spectrum BB resistance gene *Xa23*. The results demonstrate that HRM-based markers are cost-effective, breeder-friendly and well suited for MAS breeding, and that the breeding lines harboring five key genes that contributed to good grain quality and resistance to blast and BB would be excellent parents for further marker-assisted improvement programs.

Materials and methods

Plant materials

Nipponbare (genotype Wx^b-*Fgr-pita-pik* according to GRAMENE database), Basmati 370 (carrying *fgr*; Sakthivel et al. 2009), Taifenzhan (carrying Pik^p; Zhai et al. 2011), Shengbasimiao (released by our group, carrying *Pita* according to a previous assay) and R 173 (a restorer line developed by our group, harboring Wx^a) were used as references for the development of HRM-based markers.

In total, 88 non-waxy rice lines (Table S1), including the five lines mentioned above, were chosen for genotyping and for measuring AC and fragrance; three of them, Bataixiangzhan, Taifenzhan and Xinhuangzhan, were selected as crossing parents for marker-assisted stacking. For each of the 88 rice lines, equal amounts of leaf tissue from three plants were mixed for DNA isolation, and equal numbers of seeds of the same three plants were mixed for determining grain quality traits.

Development of HRM-based markers

The nested-PCR approach was used for HRM-based markers to ensure high specific amplification of the targeted regions, and the internal amplicons for SNP genotyping were kept shorter than 100 bp. Internal primer pairs anchoring functional nucleotide polymorphisms (FNPs) for *Wx, Pita* and *fgr* described in Table 1 were first picked with Primer3 software(Untergrasser et al. 2012).

The *Pik* locus is composed of two adjacent NBS-LRR (nucleotide binding site–leucine-rich repeat) class genes, and at least four alleles including *Pik*, Pik^p, Pik^m and *Pi1* conferring broad-spectrum resistance to blast disease have been isolated (Zhai et al. 2011; Ashikawa et al. 2008; Yuan et al. 2011; Hua et al. 2012). Based on the alignment of DNA sequences from resistant alleles (Genebank accessions: HM048900.1, HM035360.1, AB462325.1 and HQ606329.1) to the susceptible allele in Nipponbare (LOC_Os11g46210), we found that several polymorphisms within the second NBS-LRR element (*Pik-2*)

can be used to distinguish all the resistant alleles from the susceptible ones. From these polymorphisms, a C/T SNP ("C" for susceptible and "T" for resistant) was picked for developing internal primer pairs, and here "*Pik*" is simply used to refer to these resistance alleles at this locus.

In order to reduce the costs, we implemented multiplex PCR at the first-round amplification, and external primer pairs covering internal amplicons for all four genes were picked by using the MPprimer tool (Shen et al. 2010). All the nested-PCR primer pairs for HRM analysis were synthesized by Sangon (Shanghai, China) and purified with HPLC (high-performance liquid chromatography).

High-throughput DNA isolation, PCR and HRM analysis with the LightScanner® platform

We performed a high-throughput preparation procedure of rice genomic DNA from rice leaf samples for PCR reaction as described by Wang et al. (2013), with some modifications. About 5 mg of leaf tissue from each rice sample was transferred to a 96-well PCR plate, and 100 μL of rapid DNA isolation solution (10 mM Tris–HCl, pH 9.5; 1 mM EDTA; 100 mM KCl; 5 % Tween-20) was added to each well. The 96-well DNA plate was sealed and then incubated for 15 min at 95 °C in the PCR amplifier. After incubation, 0.5–1 μL of DNA extraction solution from the DNA plates was transferred to PCR plates with a 96-pin applicator.

The first round of nested PCR is multiplex PCR, and amplification was performed in 10-μL volumes containing 5 μL 2× PCR MasterMix (Bioteke, Beijing, China), 0.3 μM of each primer of all four external pairs and 0.5–1 μL of DNA extraction solution; the PCR profile was 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min. After amplification, the PCR products were diluted to 20× with TE buffer.

The second round of PCR amplification was performed in 10-μL volumes containing 5 μL 2× PCR MasterMix, 0.3 μM of each primer of an internal pair, 0.5 μL 20× EvaGreen (Biotium Inc., USA) and 0.5–1 μL of 20× diluted products of the first-round amplification. The PCR profile was 95 °C for 3 min, followed by 20 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 10 s, then one cycle of 95 °C for 2 min, and then ramped down to 40 °C at 0.1 °C/s and holding for 1 min to randomly form double-stranded DNA. After the final holding, 20 μL of mineral oil was added to each well. Subsequently, the plates were centrifuged at 2,000 rpm for 2 min and high-resolution melting performed using the LightScanner® instrument (Idaho Technology Inc., USA). The data analysis was done as described by Hofinger et al. (2009).

Evaluation of disease resistance and grain quality traits

We evaluated Xinhuangzhan, Taifenzhan, Bataixiangzhan and their stacking lines against 32 isolates of *M. oryzae* by individual inoculation as described by Xiao et al. (2012). Evaluation of BB resistance was carried out according to the leaf-clipping method in heading stage, and lesion length representing disease score was measured as described by Zhou et al. (2009). The assessment of grain quality traits was done by measuring amylose content and fragrance as described by Jin et al. (2010).

Marker-assisted stacking of Wx^b, *fgr*, *Pita*, Pik^p and *Xa23*

Selection of Wx^b, *fgr, Pita* and Pik^p was done using the HRM-based markers developed in this study. The selection of *Xa23* was carried out by using a random linked SSR marker, RM206, which is 1.9–1.3 cM distant from the *Xa23* locus (Pan et al. 2003; Zhou et al. 2009). We tried to design a HRM-based marker for discriminating RM206's (CT) repeat polymorphism, but its melting curves did not behave as expected and were indistinguishable. We thus had to use gel-based RM206 for selecting *Xa23*. Notably, both *Xa23* and *Pik* (LOC_Os11g46210) are on the long arm of chromosome 11, and RM206 locates between them; the physical distance from *Pik* to RM206 is 5.5 Mb, and hence the approximate genetic distance from *Xa23* to *Pik* is 23.5 cM [(1.9 + 5.5)/0.255], representing about 23.5 % recombination rate.

We first crossed Xinhuangzhan with Taifenzhan and their F1 ($C_{XT}F1$) plants were checked by markers. The selected F1 plants were then crossed with Bataixiangzhan to produce the multi-crossed F1 ($C_{XTB}F1$). MAS was run at the seedling stage of $C_{XTB}F1$ and $C_{XTB}F2$, and only the individuals harboring the targeted genotypes were selected and

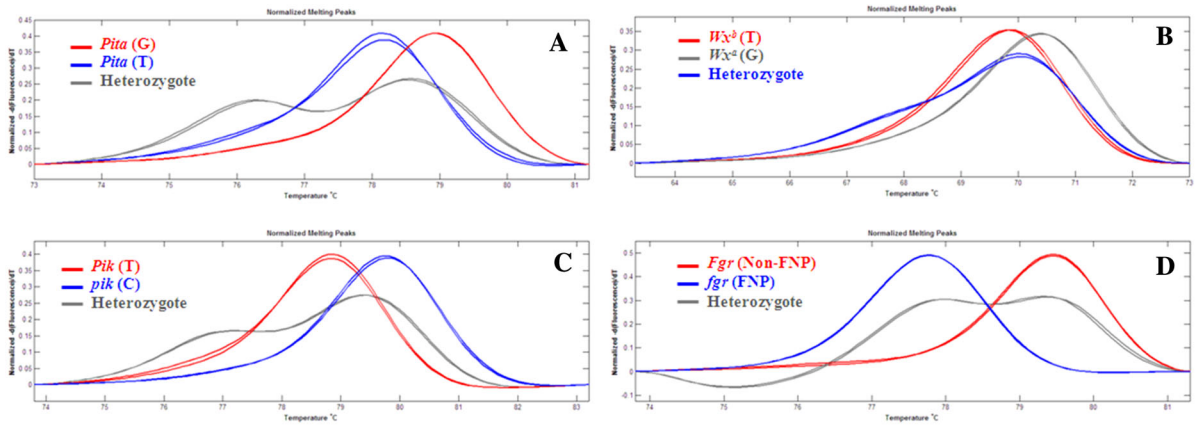

Fig. 1 Normalized differential plots of internal amplicons of the four markers. The melting peaks of two references and their homozygote are showed with different *colors* in each plot, and the genotype of each homozygote is given in *parentheses* following the corresponding allele. **a** Plot of 65-bp internal amplicon of *Pita*-G/T. **b** Plot of 72-bp internal amplicon of *Wx*-a/b. **c** Plot of 57-bp internal amplicon of *Pik2*-C/T. **d** Plot of internal amplicon of *fgr*-E7FNP, FNP indicating 8-bp deletion and three SNPs

transferred to the field. To eliminate the selection errors of *Xa23* resulting from recombination events, all transferred plants were infected with *Xoo* at the heading stage.

Results

Development of HRM-based markers

To achieve a distinguishable melting curve for each of the three genotypes at a given marker, the internal amplicon flanking the targeted SNPs must be shorter than 100 bp, as recommended by the manufacturer. All nested-PCR primer pairs of the four genes for HRM analysis are listed on Table S1.

Due to the highly sensitive nature of HRM analysis, optimization to achieve high uniformity and specificity is critical for successful HRM experiments. Since we carried out nested PCR, the amplification specificity and uniformity of the second-round PCR could be easily obtained and the gradient PCR optimization procedure was not necessary. In our experience, the important thing was to ensure enough amplification products in the first-round PCR; otherwise a small peak before the specific products' melting peaks was likely to be observed and led to false genotyping (data not shown). In our multiplex- and nested-PCR protocol, the first round was carried out with 40 cycles of annealing at 55 °C (primer length = 22 ± 2 bp), and the second round was amplified with 20 cycles of annealing at 58 °C. By using this amplification protocol, PCR was automatically performed, and the high-resolution melting curves were very stable and distinguishable for the markers we currently developed (more than 20 markers; data not shown).

The normalized differential plot of these four internal amplicons (Fig. 1) shows clearly distinguished genotypes of the reference samples described above. From the melting curves, we can see that the larger melting peaks shift (T_m shift), and the genotypes can be more easily and accurately distinguished, particularly for homozygotes. In comparison with SNPs, the FNP at *fgr* locus consists of an 8-bp InDel, and three SNPs significantly increase the T_m shift (1.8 °C; Fig. 1d), giving rise to easier differentiation. For the three SNPs, the T_m shifts could be associated with their internal amplicons' length. Both SNPs at *Pita* and *Wx* were G/T, the *Pita*-G/T's internal amplicons were 65 bp and the SNP led to a ca. 0.8 °C shift (Fig. 1a); but in the *Wx*-a/b's amplicons (72 bp), the SNP resulted merely in a ca. 0.5 °C shift (Fig. 1b). The internal amplicon of the marker *Pik2*-C/T was 57 bp and the shift effect of its C/T SNP was 1 °C (Fig. 1c), larger than that of the other two SNPs.

In our experience of amplifying very small amplicons (40–70 bp) with nested PCR, we found that nearly all the G or C to A or T SNPs could be easily

converted to distinguishable HRM curves with T_m shifts larger than 0.5 °C.

Identification of favorable alleles from rice germplasm with HRM-based markers

It is much easier to introduce favorable alleles from elite lines or released cultivars than from unadapted germplasm because of linkage drag problems. To identify *Wx*^{b}, *fgr*, *Pita* and *Pik*, we checked the genotype status of 88 rice lines used in this study. Most of them were released as cultivars in the past 10 years by Guangdong province, China; meanwhile, we re-measured the amylose content and fragrance of these lines in order to validate the FMs for *Wx* and *fgr*. Assay results of genotype, AC and fragrance are summarized in Table 2, and the details are given in the Supplementary Material (Table S1).

Among the 88 lines, *Wx*^{b} and *Pita* are relatively abundant and several lines carry both alleles together, while the distribution frequency of *fgr* is very low and only three lines carry the homozygous *fgr*. The high allele frequency of *Wx*^{b} and *Pita* could be explained by the emphasis on grain quality improvement and wide use of *Pita* in rice breeding programs in south China. Thirteen lines were determined to harbor resistant allele(s) at the *Pik* locus, including Taifenzhan which was previously reported carrying *Pik*^{p} (Zhai et al. 2011), and allele details of the other 12 lines need to be further characterized.

For the *Wx* and *fgr* loci, genotype status correlated well with the corresponding phenotype variations; the AC of lines carrying homozygous *Wx*^{b} (60 lines, mean AC 15.15 %) was far below those carrying homozygous *Wx*^{a} (24 lines, mean AC 25.34 %), and only grains of these lines with homozygous or heterozygous *fgr* were tested for fragrance.

Stacking *Wx*^{b}, *fgr*, *Pita*, *Pik*^{p} and *Xa23*

The *Pita* gene has been widely used in rice breeding programs worldwide and it is feasible to improve blast resistance by pyramiding *Pita* with other major resistance genes (Wang et al. 2007; Miah et al. 2013). The allele *Pik*^{p} was reported to confer province-specific blast resistance and was tested to have good resistance to isolates from Guangdong province (Wang et al. 2009). Our goal here was to stack *Wx*^{b},

Table 2 Assay summaries of 88 rice germplasms for amylose content, fragrance and genotype status at four loci

Genotype	Number	AC (%)[a]	Genotype	Number	Fragrance[b]	Genotype	Number	Genotype	Number
Wx^a	24	25.34 ± 2.41	*fgr*	3	+++	*Pita* (R[c])	43	*Pik* (R[c])	13
Wx^b	60	15.15 ± 1.56	*Fgr*	83	–	*pita* (S[c])	44	*pik* (S[c])	75
Heterozygous	4	18.13 ± 3.94	Heterozygous	2	+–	Heterozygous	1	Heterozygous	0

[a] Mean ± standard error, tested by using grains generated in the late season of 2010; [b] +++, +– and –indicates that all grains are fragrant, parts of grains are fragrant, all grains are non-fragrant, respectively; [c] *R* resistant and *S* susceptible

Table 3 Amylose content, fragrance and genotype status of the three crossing parents

Accession	Genotype					AC (%)	Fragrance
	Wx-a/b	*fgr*-E7FNP	*Pita*-G/T	*Pik2*-C/T	RM206		
Taifenzhan	Wx^a	*Fgr*	*pita*	Pik^p	*xa23*	22.97[b]	–
Bataixiangzhan	Wx^b	*fgr*	*Pita*	*pik*	*xa23*	15.75	+++
Xinhuangzhan	Wx^b	*−Fgr*	*pita*	*−pik*	*Xa23*	16.16	–

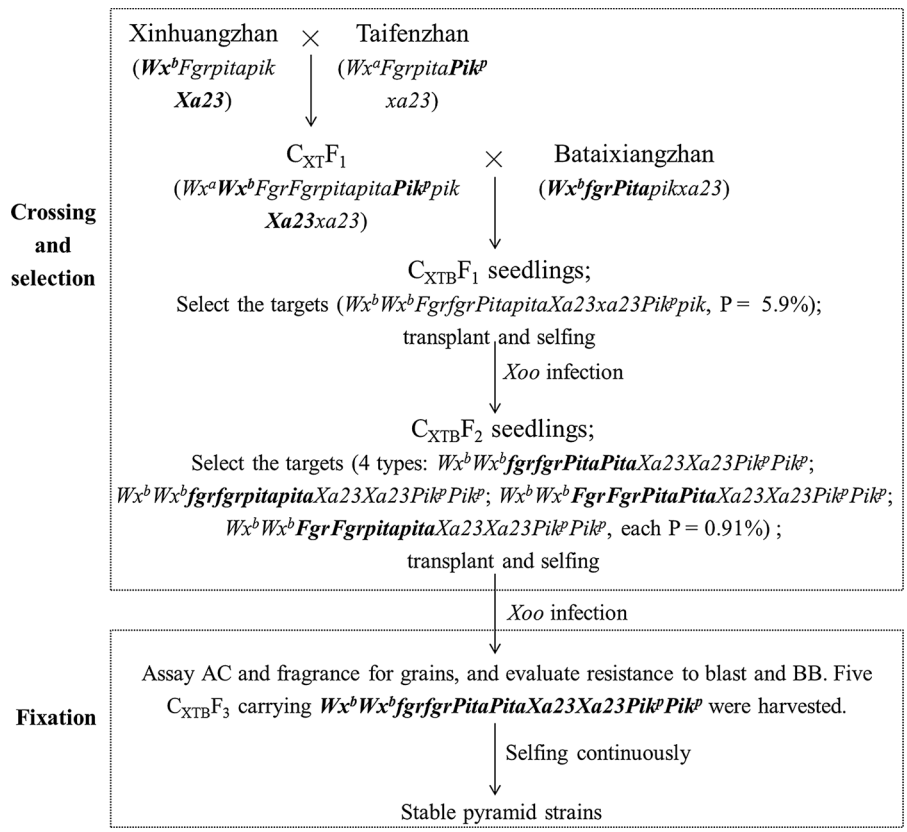

Fig. 2 Flowchart of marker-assisted pyramiding process

fgr, Pita, Pik^p and *Xa23* into new lines with good grain quality and adequate resistance to blast and BB.

We selected Taifenzhan as the donor of *Pik*^{p} and the line Bataixiangzhan as the donor of *Pita* and *fgr*; and an elite line, Xinhuangzhang, which was released in 2011, was used as the donor for *Xa23*, the most promising and completely dominant R gene to BB (Zhou et al. 2009). Grain quality measurement and genotypes of the three elite *indica* lines at the five targeted loci are shown in Table 3.

The marker-assisted stacking process was as described in Fig. 2. Since *Xa23* is linked to *Pik*^{p}, the theoretical probability of a recombinant gamete (*Xa23Pik*^{p} or *xa23pik*) produced by the $C_{XT}F1$ would be $P = 0.5 \times 23.5\ \%$, and thus the probability of the allele combination $Wx^bWx^bFgrfgrPitapitaXa23xa23Pik^ppik$ arising in $C_{XTB}F1$ would be $P = (0.5 \times 0.5 \times 23.5\ \%) = 5.9\ \%$. We checked 108 $C_{XTB}F1$ individuals and identified five with the targeted genotype; *Xoo* infection showed that all the five individuals were well resistant to BB, with lesion length <1 cm.

These $C_{XTB}F1$ individuals were transplanted and harvested and generated five $C_{XTB}F2$ populations;

from each population, 384 individuals were analyzed at four loci, *Pita*, *fgr*, *Pik* and *Xa23*. In total, 7,680 marker data points were analyzed. Four types of allele combination, Wx^bWx^b–(*fgrfgrPitaPita*, *fgrfgrpitapita*, *FgrFgrPitaPita* and *FgrFgrpitapita*) –*Xa23Xa23Pik*^{p}-*Pik*^{p}, were selected and in total 61 selected plants (14 + 15 + 15 + 17; Table S2) were transplanted. The probability of each of the four combinations is equivalent to $P = (0.5 \times 0.5 \times 0.5 \times 76.5\ \%)^2 = 0.91\ \%$ and the 61 selected targets were close to the expected number ($4 \times 1{,}920 \times 0.91\ \% = 69$).

At heading stage, *Xoo* infection revealed that one false positive expressed high susceptibility to BB in the type III plants (Fig. S1), implying that a recombination likely occurred between RM206 and *Xa23*. The erroneously selected plant was discarded when harvesting $C_{XTB}F2$ plants individually, resulting in 60 plants comprising four homozygous genotypes on the target loci.

Assessment of the stacking lines for amylose content, fragrance and disease resistance

Seeds derived from the 60 selected $C_{XTB}F2$ plants, along with their crossing parents, were used to determine amylose content, fragrance and resistance to blast and BB (Table 4). At the *Wx* locus, only the crossing parent Taifenzhan carrying Wx^a displayed high AC (24.25 %), and the other two crossing parents as well as their 60 offspring carrying Wx^b were found to have low AC (around 15 %). Similar to *Wx*, all four plant types harboring the *Xa23* gene were assessed to be very resistant to BB (lesion length < 1 cm). Type I (genotype $Wx^bWx^bfgrfgrPitaPitaXa23Xa23Pik^pPik^p$) and type II (genotype $Wx^bWx^bfgrfgrpitapitaXa23Xa23Pik^pPik^p$) harboring the *fgr* gene from Bataixiangzhan were fragrant, while the other two types were non-fragrant. Both type I and III, carrying two target blast R genes, *Pita* and *Pik*^{p}, were evaluated to have a broader resistance spectrum (96.88 and 100 %) compared to the other two types and crossing parents (84.38 to 87.5 %). Since the effects of other potential R genes or QTLs were not excluded from the evaluation of blast resistance in this study, the exact pyramiding effect of *Pita* and *Pik*^{p} could not be precisely determined. Further assessment may be needed by using near-isolated lines.

In the 14 type I $C_{XTB}F3$ families, all the five favorable target alleles were fixed. Their field performance was surveyed, and five of them showing preferred plant type were harvested separately for continuous self-crossing to generate stable pyramid strains.

Discussion

HRM has been used for closed-tube and fast discrimination of nucleotide polymorphisms such as SNPs, InDels and SSRs in a range of plants (Wu et al. 2008; Studer et al. 2009; Distefano et al. 2010). Here we showed again that HRM could serve as an alternative to the gel-based method for genotyping in a moderate-throughput manner in MAS. In many cases of marker-assisted practises, only a few polymorphic markers need to be genotyped on thousands of plant samples. The HRM-based method is a reasonable platform for such applications due to its low cost and ease of use. With instrumentation like LightScanner® 96/384

Table 4 Assessment of four $C_{123}F3$ populations for amylose content, fragrance and resistance to BB and blast

Genotype	Blast resistance spectrum (%)[a]	BB resistance score[b]	AC (%)[b]	Fragrance
Type I ($Wx^bWx^bfgrfgrPitaPitaXa23Xa23Pik^pPik^p$)	96.88	0.85 ± 0.21	14.82 ± 1.13	+++
Type II ($Wx^bWx^bfgrfgrpitapitaXa23Xa23Pik^pPik^p$)	84.38	0.83 ± 0.21	15.30 ± 2.29	+++
Type III ($Wx^bWx^bFgrFgrPitaPitaXa23Xa23Pik^pPik^p$)	100.00	0.80 ± 0.23	14.94 ± 1.62	–
Type IV ($Wx^bWx^bFgrFgrpitapitaXa23Xa23Pik^pPik^p$)	87.50	0.87 ± 0.19	13.46 ± 1.80	–
Xinhuangzhan ($Wx^bWx^bFgrFgrpitapitaXa23Xa23pikpik$)	87.50	0.83 ± 0.18	15.37	–
Taifenzhan ($Wx^aWx^aFgrFgrPitaPitaxa23xa23Pik^pPik^p$)	84.38	16.6 ± 4.43	24.25	–
Bataixiangzhan ($Wx^bWx^bfgrfgrPitaPitaxa23xa23pikpik$)	87.50	14.9 ± 5.26	16.04	+++

[a] Blast resistance spectrum (%) = (number of incompatible isolates/number of total tested isolates) × 100 %; in total 32 isolates were tested; [b] AC (%) was tested by using grains generated in the late season of 2012

(Idaho Technology), LightCycler® 480 (Roche Molecular Systems) and iCycler® (Bio-Rad Laboratories), HRM analysis can be performed in 96- or 384-well plates, giving it much higher throughput than the gel-based method; more importantly, HRM analysis is a closed-tube method and therefore can significantly reduce the chance of the experimenters being exposed to hazardous chemicals.

HRM relies upon the extremely precise monitoring of the alteration of fluorescence as a double-strand DNA melts. Saturated intercalating fluorescent dyes and monitoring instruments are necessarily required in HRM analysis, and the high costs of the two items could limit the application of HRM for checking a large number of samples in plant breeding programs. In our case, with recycled 96-well amplification plates, the cost of the first-round multiplex amplification for HRM analysis was basically equivalent to the amplification of regular PCR plates, and that of the second-round amplification using EvaGreen was about 35 RMB (5.5 USD) per plate. Altogether, the total cost of nested-PCR amplification was around 55 RMB (9 USD) per plate or 0.6 RMB (0.1 USD) per data point. The costs would be affordable for a general breeding laboratory analyzing thousands of samples in a short time window during the growing season.

Unlike its constant length, the melting curve (or T_m) of a DNA duplex is susceptible and easily affected by the PCR reaction system, hence maintaining high uniformity is of great importance for HRM analysis. To obtain successful HRM analysis, the standard amplification protocol commonly recommended to continually optimize the PCR reaction, to use similar amounts of high quality genomic DNA, to use calibrated pipettes, to keep reaction volumes at 20 μL or above and so on. Here, we avoided the tedious optimization procedure through nested-PCR amplification, and significantly reduced time-consuming template preparation by using a highly-automated rapid DNA isolation protocol. Consistent with previously reported studies (Lochlainn et al. 2011; Carillo et al. 2011), nested amplification provides reliable and stable melting plots in our study. Of course, due to the nature of the HRM analysis, genotyping errors were difficult to avoid completely, especially where the T_m shift is small ($\leq$0.5 °C), as with the G/T FNP at the *Wx* locus in the present study. These errors can be reduced or even eliminated by using internal temperature calibrators as described by Gundry et al. (2008).

According to our marker development experience, the HRM-based marker conversion rate for G or C to T or A SNPs and InDels could be nearly 100 %, but for SSRs was very low. Because of the replication slippage problem, the amplification specificity of most repeated sequences was not very good, even when nested-PCR amplification was performed, and this problem also resulted in multiple allelic SSRs being widely distributed. Markers derived from SNPs and/or InDels of functional genes are mainly biallelic, and HRM is much more reliable with biallelic markers than multiple allelic SSR markers. Based on our experience with SSR markers, we do not recommend HRM be used with SSR markers.

It has been proven that FMs are very useful in plant breeding, enhancing the precision, accuracy and reliability of identification and selection of target gene(s) with minimum effort, time and cost (Iyer and McCouch 2007; Perumalsamy et al. 2010; Salgotra et al. 2012). In our study, selection and stacking of favorable alleles was carried out by using gel-based RDM (RM206), HRM-based GTM (*Pik2*-C/T) and FMs (*Wx*-a/b, *Pita*-G/T and *fgr*-E7FNP). Obviously, selection using FMs or GTMs is more preferable since it is recombination-free and does not need extra phenotypic validation. With advances in functional genomics, more and more functional gene-derived FMs or GTMs will be developed and used in plant breeding programs.

Introgression of multiple genes into elite lines or high-yielding varieties can be achieved in a more straightforward way if these alleles of interest are fixed into one donor. Our goal in the marker-assisted pyramiding process is to develop new lines carrying favorable alleles for good grain quality and adequate resistance to blast and BB. From this study, we were able to generate a total of 14 lines in which all five targeted genes were fixed. Since these pyramids were derived from elite lines, their plant types were more desirable and less yield penalty was observed. With these lines as donors, efforts are being made to integrate the five genes into our current high-yield lines and hybrid parents.

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References

Andersen JR, Lübberstedt T (2003) Functional markers in plants. Trends Plant Sci 8(11):554–560

Ashikawa I, Hayashi N, Yamane H, Kanamori H, Wu J, Matsumoto T, Ono K, Yano M (2008) Two adjacent nucleotide-binding site-leucine rich repeat class genes are required to confer *Pikm*-specific rice blast resistance. Genetics 180:2267–2276

Basavaraj SH, Singh VK, Singh A, Singh A, Singh A, Yadav S, Ellur RK, Singh D, Gopalakrishnan S, Nagarajan M, Mohapatra T, Prabhu KV, Singh AK (2010) Marker-assisted improvement of bacterial blight resistance in parental lines of Pusa RH10, a superfine grain aromatic rice hybrid. Mol Breed 26:293–305

Bradbury LMT, Fitzgerald TL, Henry RJ, Jin QS, Waters DLE (2005) The gene for fragrance in rice. Plant Biotechnol J 3:363–370

Bryan G, Wu KS, Farrall L, Jia YL, Hershey HP, McAdams SA, Faulk KN, Donaldson GK, Tarchini R, Valent B (2000) A single amino acid difference distinguishes resistant and susceptible alleles of the rice blast resistance gene *Pi-ta*. Plant Cell 12:2033–2046

Cai XL, Wang ZY, Xing YY, Zhang JL, Hong MM (1998) Aberrant splicing of intron 1 leads to the heterogeneous 5′UTR and decreased expression of waxy gene in rice cultivars of intermediate amylose content. Plant J 14(4):459–465

Carillo S, Henry L, Lippert E, Girodon F, Guiraud I, Richard C, Dubois Galopin F, Cleyrat C, Jourdan E, Kralovics R, Hermouet S, Lavabre-Bertrand T (2011) Nested high-resolution melting curve analysis a highly sensitive, reliable, and simple method for detection of *JAK2* exon 12 mutations–clinical relevance in the monitoring of polycythemia. J Mol Diagn 13(3):263–270

Collard BCY, Mackill DJ (2008) Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Phil Trans R Soc B 363:557–572

Distefano G, Carus M, La Malfa S, Gentile A, Wu SB (2010) High resolution melting analysis is a more sensitive and effective alternative to gel-based platforms in analysis of SSR–an example in citrus. PLoS ONE 7(8):e44202

Fan CC, Yu SB, Wang CR, Xing YZ (2009) A causal C-A mutation in the second exon of *GS3* highly associated with rice grain length and validated as a functional marker. Theor Appl Genet 118:465–472

Gundry CN, Dobrowolski SF, Martin YR, Robbins TC, Nay LM, Boyd N, Coyne T, Wall MD, Wittwer CT, Teng DH (2008) Base-pair neutral homozygotes can be discriminated by calibrated high-resolution melting of small amplicons. Nucleic Acids Res 36:3401–3408

Hayashi K, Yasuda N, Fujita Y, Koizumi S, Yoshida H (2010) Identification of the blast resistance gene *Pit* in rice cultivars using functional markers. Theor Appl Genet 121:1357–1367

Hofinger BJ, Jing HC, Hammond-Kosack KE, Kanyuka K (2009) High-resolution melting analysis of cDNA-derived PCR amplicons for rapid and cost-effective identification of novel alleles in barley. Theor Appl Genet 119:851–865

Hua L, Wu J, Chen C, Wu W, He X, Lin F, Wang L, Ashikawa I, Matsumoto T, Wang L, Pan Q (2012) The isolation of *Pi1*, an allele at the *Pik* locus which confers broad spectrum resistance to rice blast. Theor Appl Genet 125:1047–1055

Huang B, Xu JY, Hou MS, Ali J, Mou TM (2012) Introgression of bacterial blight resistance genes *Xa7*, *Xa21*, *Xa22* and *Xa23* into hybrid rice restorer lines by molecular marker-assisted selection. Euphytica 187:449–459

Iyer AS, McCouch SR (2007) Functional markers for *xa5*-mediated resistance in rice (*Oryza sativa* L.). Mol Breed 19:291–296

Jeong HJ, Kwon JK, Pandeya D, Hwang J, Hoang NH, Bae JH, Kang BC (2012) A survey of natural and ethyl methane sulfonate-induced variations of *eIF4E* using high-resolution melting analysis in *Capsicum*. Mol Breed 29:349–360

Jiang HC, Feng YT, Bao L, Lin X, Gao GJ, Zhang QL, Xiao JH, Xu CG, He YQ (2012) Improving blast resistance of Jin 23B and its hybrid rice by marker-assisted gene pyramiding. Mol Breed 30(4):1679–1688

Jin L, Lu Y, Shao Y, Zhang G, Xiao P, Shen S, Corke H, Bao J (2010) Molecular marker assisted selection for improvement of the eating, cooking and sensory quality of rice (*Oryza sativa* L.). J Cereal Sci 51:159–164

Lehmensiek A, Sutherland MW, McNamara RB (2008) The use of high resolution melting (HRM) to map single nucleotide polymorphism markers linked to a covered smut resistance gene in barley. Theor Appl Genet 117(5):721–728

Lochlainn SO, Amoah S, Graham NS, Alamer K, Rios JJ, Kurup S, Stoute A, Hammond JP, Ostergaard L, King GJ, White PJ, Broadley MR (2011) High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. Plant Methods 7:43

Luo Y, Sangha JS, Wang S, Li Z, Yang J, Yin Z (2012) Marker-assisted breeding of *Xa4*, *Xa21* and *Xa27* in the restore lines of hybrid rice for broad-spectrum and enhanced disease resistance to bacterial blight. Mol Breed 30:1601–1610

Madamba MRS, Sugiyama N, Bordeos A, Mauleon R, Satoh K, Baraoidan M, Kikuchi S, Shimamoto K, Leung H (2009) A recessive mutation in rice conferring non-race-specific resistance to bacterial blight and blast. Rice 2:104–114

Miah G, Rafii MY, Ismail MR, Puteh AB, Rahim HA, Asfaliza R, Latif MA (2013) Blast resistance in rice: a review of conventional breeding to molecular approaches. Mol Biol Rep 40:2369–2388

Morris M, Dreher K, Ribaut JM, Khairallah M (2009) Money matters (II): costs of maize inbred line conversion schemes at CIMMYT using conventional and marker-assisted selection. Mol Breed 11:235–247

Pan HJ, Wang CL, Zhao KJ, Zhang Q, Fan YL, Zhou SC, Zhu LH (2003) Molecular mapping by PCR-based markers and marker-assisted selection of Xa23 a bacterial blight resistance gene in rice. Acta Agronomica Sin 29(4):501–507

Perumalsamy S, Bharani M, Sudha M, Nagarajan P, Arul L, Saraswathi R, Balasubramanian P, Ramalingam J (2010)

Functional marker-assisted selection for bacterial leaf blight resistance genes in rice (*Oryza sativa* L.). Plant Breed 129:400–406

Sakthivel K, Rani NS, Pandey MK, Sivaranjani AKP, Neeraja CN, Balachandran SM, Madhav MS, Viraktamath BC, Prasad G, Sundaram RM (2009) Development of a simple functional marker for fragrance in rice and its validation in Indian Basmati and non-Basmati fragrant rice varieties. Mol Breed 24(2):185–190

Salgotra RK, Gupta BB, Millwood RJ, Balasubramaniam M, Stewart CN Jr (2012) Introgression of bacterial leaf blight resistance and aroma genes using functional marker-assisted selection in rice (*Oryza sativa* L.). Euphytica 187:313–323

Shen ZY, Qu WB, Wang W, Lu YM, Wu YH, Li ZF, Hang XY, Wang XL, Zhao DS, Zhang CG (2010) MPprimer: a program for reliable multiplex PCR primer design. BMC Bioinform 11:143

Singh VK, Singh A, Singh SP, Ellur RK, Choudhary V, Sarkhel S, Singh D, Gopala Krishnan S, Nagarajan M, Vinod KK, Singh UD, Rathore R, Prasanthi SK, Agrawal PK, Bhatt JC, Mohapatra T, Prabhu KV, Singh AK (2012) Incorporation of blast resistance into 'PRR78', an elite Basmati rice restorer line, through marker assisted backcross breeding. Field Crop Res 128:8–16

Studer B, Jensen LB, Fiil A, Asp T (2009) "Blind" mapping of genic DNA sequence polymorphisms in *Lolium perenne* L. by high-resolution melting curve analysis. Mol Breed 24:191–199

Tanksley S (1983) Molecular markers in plant breeding. Plant Mol Biol Rep 1:3–8

Untergrasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012) Primer3—new capabilities and interfaces. Nucleic Acids Res 40(15):e115

Vossen RH, Aten E, Roos A, den Dunnen JT (2009) High-resolution melting analysis (HRMA): more than just sequence variant screening. Hum Mutat 30(6):860–866

Wang Z, Wu Z, Xing Y, Zheng F, Guo X, Zhang W, Hong M (1990) Nucleotide sequence of rice *waxy* gene. Nucl Acids Res 18:5898

Wang Z, Jia Y, Rutger JN, Xia Y (2007) Rapid survey for presence of a blast resistance gene *Pi-ta* in rice cultivars using the dominant DNA markers derived from portions of the *Pi-ta* gene. Plant Breed 126:36–42

Wang L, Xu X, Lin FC, Pan Q (2009) Characterization of rice blast resistance genes in the *Pik* cluster and fine mapping of the *Pik-p* locus. Phytopathology 99(8):900–905

Wang HN, Chu ZZ, Ma XL, Li RQ, Liu YG (2013) A high through-put protocol of plant genomic DNA preparation for PCR. Acta Agron Sin 39(7):1200–1205

Wittwer CT (2009) High-resolution DNA melting analysis: advancements and limitations. Hum Mutat 30(6):857–859

Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ (2003) High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 49:853–860

Wu SB, Wirthensohn M, Hunt P, Gibson J, Sedgley M (2008) High resolution melting analysis of almond SNPs derived from ESTs. Theor Appl Genet 118:1–14

Xiao WM, Yang QY, Wang H, Duan J, Guo T, Liu YZ, Zhu XY, Chen ZQ (2012) Identification and fine mapping of a major *R* gene to *Magnaporthe oryzae* in a broad-spectrum resistant germplasm in rice. Mol Breed 30:1715–1726

Yang Y, Wu J, Chen Z, Wang L, Li J, Liu X, Lu Y (2009) Mining rice new germplasm containing S_5^n gene by functional molecular marker and sequencing. Chin Sci Bull 54(18):3258–3264

Yuan B, Zhai C, Wang W, Zeng X, Xu X, Hu H, Lin F, Wang L, Pan Q (2011) The *Pik-p* resistance to *Magnaporthe oryzae* in rice is mediated by a pair of closely linked CC-NBS-LRR genes. Theor Appl Genet 122:1017–1028

Zhai C, Lin F, Dong Z, He X, Yuan B, Zeng X, Wang L, Pan Q (2011) The isolation and characterization of *Pik*, a rice blast resistance gene which emerged after rice domestication. New Phytol 189(1):321–334

Zhou YL, Xu JL, Zhou SC, Yu J, Xie XW, Xu MR, Sun Y, Zhu LH, Fu BY, Gao YM, Li ZK (2009) Pyramiding *Xa23* and *Rxo1* for resistance to two bacterial diseases into an elite *indica* rice variety using molecular approaches. Mol Breed 23:279–287