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High resolution agarose-based system for single-tube genotyping of *fgr* and *Waxy* genes in rice: MAGE to displace PAGE?

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Abstract

Aroma and amylose content are the key determinants of grain quality and commercial value in rice (*Oryza sativa* L.). Here, we report a novel agarose-based multiplex polymerase chain reaction (PCR)-assay for the unambiguous identification of genes controlling aroma (*fgr*) and amylose content (*Wx*) traits in rice. The multiplex assay was developed following the validation of two previously reported gene-specific primers, namely, the *fgr*-SNP and *Wx*-SSR. Twenty aromatic and non-aromatic rice genotypes with a wide range of variation in amylose content were used in this study. Optimization of primer concentration and critical parameters for thermal cycling, inclusive of cycle number and annealing temperature, were performed to obtain optimal results for the multiplex amplification. The resulting amplification products displayed well-differentiated allelic variants of both *fgr* and *Wx* genes on a 3% MetaPhor agarose gel, at the cost of less than $0.15 per sample. MetaPhor agarose gel electrophoresis (MAGE) was effectively employed as a genotyping method which offers an alternative to polyacrylamide gel electrophoresis (PAGE); a system, commonly used in rice research, that can be technically challenging and time-consuming. Without the need of expensive probes or specialized equipment, this newly developed multiplex assay is suitable for researchers, whose studies are dependent on mass genotyping, and in molecular laboratories with limited resources.

Keywords: Amylose content; aroma; *fgr* gene; MetaPhor agarose gel electrophoresis; multiplex PCR; rice; *Waxy* gene.
Abbreviations: EAP_External antisense primer; ESP_External sense primer; IFAP_Internal fragrant antisense primer; INSP_ Internal non-fragrant sense primer; MAGE_MetaPhor agarose gel electrophoresis; PAGE_Polyacrylamide gel electrophoresis; QTL_ Quantitative trait locus; SNP_Single nucleotide polymorphism; SSR_Simple sequence repeat; Wx_Waxy.

Introduction

The invention of PCR in the mid-1980s radically transformed all branches of life science. It has become an indispensable tool for a wide array of molecular biology applications, such as cloning, genotyping and sequencing (Amheim et al., 1990; Garibyan et al., 2013; Mullis 1990). Multiplex PCR is a variant of PCR in which two or more regions of DNA are simultaneously amplified in a single reaction. Since its first description in 1988, multiplexing has been consistently gaining popularity as it offers several notable advantages over the singleplex assays, which includes significant reduction in the cost and time of an analysis; and decrease in the amount of template DNA and PCR components required to obtain a same set of results (Butler et al., 2001; Enifro et al., 2000; Palais et al., 2005). Recent years have witnessed considerable progress in the development of a rapid and sensitive multiplex PCR assay in rice improvement programmes (Khush et al., 2001; Masouleh et al., 2009; Pessoa-Filho et al., 2007; Salgotra et al., 2011; Wang et al., 2014). One prime example is illustrated in the study conducted by Masouleh et al. (2009) which demonstrated the reliability of MALTI-TOF mass spectrometry system in distinguishing five important commercial traits in rice, namely plant height, aroma, amylose content, gelatinization temperature and blast resistance. Another substantial example is potrayed in the study reported by Salgotra et al. (2011) on an efficient capillary electrophoresis (CE)-based multiplex assay for the detection of aroma and bacterial blight resistance genes. Nevertheless, these reported assay systems have yet to be well standardized and are often used with appurtenances, such as fluorescent-labelled primers, which are expensive and not readily adopted for routine analysis, particularly in smaller and intermediate-sized laboratories common in less-developed countries.

The phenomenal economic growth in Asian countries during the mid-2000s, particularly in China and India, has boosted a demand for high-quality rice. Since then, rather than solely focusing on the improvement of rice yield, breeders have paid considerable attention to its quality

improvement. Consequently, researchers have devoted a large part of their studies to the improvement of major quality components in rice. In one of our previously published article on the determination of amylose content in rice, we have provided an essential evaluation of the potential of MAGE as an alternative to PAGE (Cheng et al., 2012). Separately, in our recently published QTL study, the MAGE system was used as a platform for the parental polymorphism screening which involved hundreds of SSR markers, as well as for the genotyping of the F_2 mapping population (Cheng et al., 2014). Our previous findings demonstrated that MAGE could be an effective tool for mass genotyping. In the present study, we sought to develop a novel single-tube multiplex assay to identify the functional polymorphisms of a single nucleotide polymorphism (SNP) in the *fgr* gene and a microsatellite (SSR) in the *Wx* gene using MAGE.

Results and Discussion

Selection and validation of gene-specific primers

Primer sequences and concentrations are usually the most crucial elements to multiplex PCR as they would determine the overall yield of each amplicon. In order for a multiplex reaction to work efficiently, the selected primer pairs for all amplified regions have to be compatible (Schoske et al., 2003). In this study, the multiplex assay was developed by firstly selecting two sets of published gene-specific primers; *fgr*-SNP and *Wx*-SSR (Table 1). The chromosomal positions of these primers have been confirmed in our recently published rice framework map (Cheng et al., 2014). The presence of a distinctive yet pleasant aroma is arguably one of the most sought-after quality attributes of rice. A volatile compound, 2-acetyl-1-pyrroline, is the distinct component responsible for fragrant in rice (Bergman et al., 2001). Bradbury et al. (2005) reported that aroma is controlled by a recessive gene (*fgr*) on chromosome 8 that contains of an 8-bp deletion and three SNPs. Consequently, they had successfully developed a perfect aromatic marker system based on allele specific amplification (ASA), which consists of the four primers used in this study.

Amylose content is considered the key determinant of the processing and eating quality of rice. Amylose synthesis is catalysed by the granule-bound starch synthase encoded by the *Waxy* (*Wx*) gene which is located on chromosome 6 (Bligh et al., 1995). Ayres et al. (1997) reported two nucleotide polymorphisms that are associated with the *Wx* gene, explained by a (CT)*n* SSR and a G–T SNP located at the 5'-leader intron splice site. The *Wx*-SSR marker was selected to be used in this study following its effectiveness in discriminating high amylose varieties from intermediate and low amylose varieties (Cheng et al., 2012). It could be utilised in rice breeding programmes, which aims, to improve the most preferred varieties that possess unfavourably high amylose content, such as Malaysian local high-yielding fragrant rice, the MRQ74, which could be further improved by lowering its high amylose content. The three key components that determine cooking and eating quality of rice are aroma, amylose content, and cooked grain elongation (Amarawathi et al., 2008; Cheng et al., 2014; Ge et al., 2005). The latter trait, which was examined in our previous work, is not included in the present study due to the inconsistency and incompatibility of its currently available molecular markers. One of these is a SNP marker system developed by Ramkumar et al. (2010). Named DRR-GL, this marker system was reported to target the functional polymorphism for *GS3*; a gene located on chromosome 3 which is responsible for 80-90% of the variation in grain length. The use of this marker was unsuccessful in our genotypic and phenotypic screening of a set of 53 rice varieties. Additionally, no QTL for cooked grain elongation was detected on rice chromosome 3 in our recent QTL study (Cheng et al., 2014).

Thermal cycling parameters and optimization

Besides primer optimization, critical parameters for thermal cycling were also examined in order to obtain optimal results for the multiplex amplification. To optimise the cycle number, the template DNA was amplified with four different sets of cycle number: 30, 35, and 38 and 40 cycles. Multiplex loci delivered good signal intensities from 35 and more cycles; accordingly the minimal adequate number of cycles 35 was chosen for the final protocol. Amplifications were performed using two gradient thermal cyclers of different manufacturers, one from Eppendorf and the other from BioRad. Both machines performed well and yielded similar and reproducible results. According to Butler et al. (2001), multiplex PCR optimization is more challenging compared to singleplex reactions because more than one primer-annealing event must occur simultaneously without interfering with each other. In order for a multiplex reaction to work efficiently and with high sensitivity, the primer annealing temperature must be similar, and excessive regions of complimentarily between the primers should be avoided to prevent the formation of primer dimers. In the present study, both selected gene-specific primers have the same annealing temperatures (55°C), as a result, the task of achieving uniform amplification results across the targeted loci was simplified. The level of primer concentration tested for both the *fgr*-SNP and *Wx*-SSR primers ranged between 0.4 µM to 0.6 µM. Our observation indicated that the usage of an equal amount of primer concentration (of 0.5 µM) resulted in a low intensity of bands within some of the amplified products of *Wx*-SSR, in comparison to the products of *fgr*-SNP. This might be due to the unequal number of primers within each primer sets viz. two primers in *Wx*-SSR and four primers in fgr-SNP. As a countermeasure, we reduced the level of primer concentration for the *fgr*-SNP primer from 0.5 µM to 0.4 µM, which thence resulted in an increase of band intensity for the *Wx*-SSR products. The remaining parameters are the same as in singleplex PCR reactions, as shown in Table 2.

Agarose-based genotyping

The multiplex products of twenty selected genotypes were separated by agarose-based electrophoresis on a 3% MetaPhor agarose gel for 3 h at 80 V (Fig 1). A brief description of the twenty selected genotypes together with the details of the singleplexes and multiplex PCR results are listed in Table 3.

According to Bradbury et al., (2005), a 355 bp band in the *fgr*-SNP analysis indicated a PCR product amplified from the non-fragrant allele by the External Antisense Primer (EAP) and Internal Nonfragrant Sence Primer (INSP), while a 257 bp band indicated a PCR product amplified from the *fgr* allele by the External Sence Primer (ESP) and Internal Fragrant Antisence Primer (IFAP). In the present study, six selected genotypes (MRQ74, MR50, Basmati 370, Domsia, KDML 105 and Y1304) showed bands of ~250 bp indicating fragrant genotypes. The remaining 14 genotypes produced ~350 bp bands indicating non-fragrant genotypes. The observed ~580 bp sized bands for all the genotypes representing

Table 1. Information of primers employed for polymorphism assays.

Trait	Locus	Chro	Marker type	Primer sequence (5'-3')	Tm (°C)	Ref
Aroma	*fgr*	8	SNP	EAP – AGTGCTTTACAAAGTCCCGC ESP - TTGTTTGGAGCTTGC TGATG IFAP - CATAGGAGCAGCTGAAATATATACC INSP - CTGGTAAAAAGATTATGGCTTCA	55	Bradbury et al., (2005)
Amylose content	*Waxy*	6	SSR	F' - CTTTGTCTATCTCAAGACAC R' - TTGCAGATGTTCTTCCTGATG	55	Ayres et al., (1997)

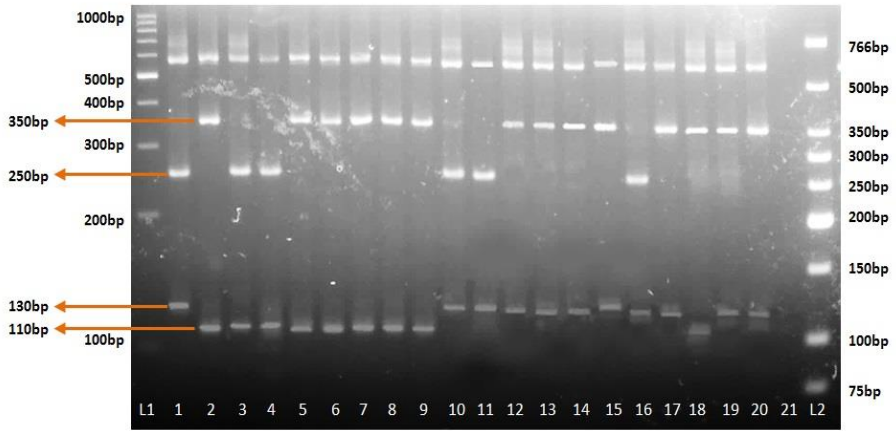

Fig 1. Amplified *fgr*-SNP and *Wx*-SSR products separated using 3% MetaPhor gel electrophoresis at 80 V for 3 h. L1, 100 bp ladder; L2, 25 bp ladder; 1, Basmati 370; 2, Ria; 3, MRQ50; 4, MRQ74; 5, Mahsuri; 6, Mahsuri Mutant; 7, MR167; 8, MR84; 9, MR106; 10, Domsia; 11, Khaw Dawk Mali 105; 12, MR219; 13, MR220; 14, MR232; 15, MR263; 16, Y1304; 17, IR64; 18, Setanjung; 19, Pulut Siding; 20, Pulut Hitam; 21, ddH_2O

the positive control from the *fgr*-SNP primers, amplified by the two external primers EAP and ESP. The amplified products for *Wx*-SSR ranged from 110 to 130 bp in length. All the nine tested varieties with high amylose content showed bands of ~110 bp, while the other eleven varieties with other amylose classes showed bands of ~130 bp. All of the genotypes analysed were homozygous for the *Wx*-SSR allele. Based on the present study, the combination of *fgr*-SNP and *Wx*-SSR identification could be used to differentiate between aromatic and non-aromatic varieties and also high amylose varieties from varieties of the other amylose classes. This combination of primer sets could be utilized as an effective tool in marker-assisted selection breeding programmes to develop fragrant rice with intermediate amylose content. The cost of using the MAGE system is relatively low. The two main ingredients, Metaphor agarose and standard agarose, cost approximately $7.00 per gel, can be used to obtain at least 30 data points. In this study, 60 data points were observed within a single gel, summing up the cost of each gel per data point to approximately $0.12. According to the study conducted by Asif et al. (2008), MetaPhor agarose gel could be reuse up to five to six times in microsatellites screening. MAGE is, practically, a more economical alternative, as it is operable without the need of experts, and can be performed using the standard electrophoresis system. In view of the protocols, MAGE is far more superior to PAGE MAGE is far more superior to PAGE in that it is more user-friendly with its ability to produce thicker and more stable gels. Additionally, it has a safer framework, wherein, there is zero usage of acrylamide, a carcinogenic chemical. Overall, MAGE is a promising system, which could be utilized as an effective alternative to PAGE, and other expensive genotyping systems, such as cappilary electrophoresis and MALTI-TOF mass spectrometry (Cheng et al., 2014). The latter systems are deemed too costly, due to their pricey equipment and consumables, for mass routine genotyping analyses in laboratories with limited resources.

Materials and Methods

Germplasms

A set of twenty genotypes including two local check varieties (MRQ74 and MR219) was used in this study. Three of them were globally popular fragrant rice namely, Basmati 370, Domsia and Khaw Dawk Mali 105 (KDML105). Seeds were obtained from the Malaysian Agricultural Research and Development Institute (MARDI).

Phenotyping for quality traits

The presence and absence of aroma was determined by sensory test using the procedure of Sood and Siddiq (1975) with minor modifications. Approximately 100g of young rice leaves were placed into a ventilation vial containing 10 ml of 1.7% KOH and incubated at room temperature for 10 min. The samples were then smelled and rated for aroma by a panel of three experts in scale of 1 to 3, where 1 was non-aromatic and 3 was highly aromatic. The test was repeated thrice for each of the samples. The selected genotypes were classified as waxy (0–2%); low amylose (below 18%); intermediate amylose (18–24%) and high amylose (above 24%) based on the data provided by MARDI (Table 3). The apparent amylose content (%) was estimated according to Juliano (1971) with minor modifications. A set of 30 polished grains were ground to fine powder and sieved through a 0.40 mm screen. Rice flour weighing 40 mg was extracted overnight in an amylose-iodine solution. The pH of the solution was stabilized with corresponding amount of acetic

Table 2. Thermal cycling parameters and optimization for singleplex and multiplex reactions.

Optimization	Singleplex (*fgr*)	Singleplex (*Wx*)	Multiplex (*fgr* and *Wx*)
Thermal cycling profile	Modification of Bradbury et al., (2005)	Modification of Ayres et al., (1997)	Final optimized Protocol
First Denaturing	95°C; 5 min	95°C; 4 min	95°C; 5
Cycle number	30	35	35
Denature	95°C; 1 min	94°C; 0.75 min	95°C; 1 min
Anneal	55°C; 1.5 min	55°C; 0.5 min	55; 1 min
Extend	72°C; 2 min	72°C; 1 min	72; 2 min
Final extension	72°C; 5 min	72°C; 5 min	72°C; 5 min
Concentration of each primers	0.5 µM	0.5 µM	0.4 µM (*fgr*); 0.5 µM (*Wx*)
Concentration of $MgCl_2$	1.5 mM	1.5 mM	1.5 mM
Concentration of dNTP	0.2 mM	0.2 mM	0.2 mM
Concentration of DNA template	0.25 µg	0.25 µg	0.25 µg
Final reaction volume	10 µl	10 µl	10 µl

Table 3. Description of genotypes and results of singleplexes and multiplex PCR.

Variety	Aroma	Amylose class	Singleplex (*fgr*) bp	Singleplex (*Wx*) bp	Multiplex (*fgr* & *Wx*) bp
MRQ74	Yes	High	~250	~110	~250; ~110
MRQ50	Yes	High	~250	~110	~250; ~110
Mahsuri	No	High	~350	~110	~350; ~110
Mahsuri Mutant	No	High	~350	~110	~350; ~110
MR167	No	High	~350	~110	~350; ~110
MR84	No	High	~350	~110	~350; ~110
MR106	No	High	~350	~110	~350; ~110
Ria	No	High	~350	~110	~350; ~110
Setanjung	No	High	~350	~110	~350; ~110
Basmati 370	Yes	Intermediate	~250	~130	~250; ~130
Domsia	Yes	Intermediate	~250	~130	~250; ~130
IR64	No	Intermediate	~350	~130	~350; ~130
MR219	No	Intermediate	~350	~130	~350; ~130
MR220	No	Intermediate	~350	~130	~350; ~130
MR232	No	Intermediate	~350	~130	~350; ~130
Y1304	Yes	Low	~250	~130	~250; ~130
KDML105	Yes	Low	~250	~130	~250; ~130
MR263	No	Low	~350	~130	~350; ~130
Pulut Siding	No	Waxy	~350	~130	~350; ~130
Pulut Hitam	No	Waxy	~350	~130	~350; ~130

acid. The absorbance was recorded at 620 nm in a spectrophotometer calibrated with a control solution containing no rice flour. The AC was estimated using a standard curve developed from known quantities of purified potato amylose.

DNA isolation and primer selection

The seeds were sown and grown in the greenhouse and leaves of 4-week-old seedlings were harvested for DNA isolation. Genomic DNA was extracted using genomic DNA extraction kit according to the manufacturer's protocol (Qiagen, USA). The quality of DNA samples was examined using a 1% agarose gel prepared in 1xTAE (Tris-acetate-ethylenediaminetetraacetic acid). Two sets of gene-specific markers, *fgr*-SNP and *Wx*-SSR, were used for the unambiguous identification of genes controlling aroma and amylose content traits in selected rice germplasm. These primers were selected on the basis of an extensive literature search. Table 1 displays the information of primers used in this study.

PCR amplification

Using the *fgr*-SNP and *Wx*-SSR primers, two individual singleplex PCR assays for were modified and subsequently combined into the multiplex PCR format for the simultaneous detection of *fgr* and *Wx* genes in a single tube. Amplifications were performed using two gradient thermal cyclers of different manufacturers (Eppendorf, Germany and BioRad, USA). Table 2 shows the thermal cycling profiles for singleplexes and multiplex PCR along with the optimized parameters.

Analysis of polymorphisms

The multiplex products were separated by agarose-based electrophoresis on a 3% MetaPhor agarose gel in 1xTAE buffer at 80 V for 3h, stained with SYBR green (Invitrogen, USA) and visualised under UV transillumination (Alpha-Imager). The size of the amplification products was estimated by comparisons to 25-bp (Promega, USA) and 100-bp DNA ladders (New England Biolabs, USA).

The high resolution 3% MetaPhor gel was prepared by a slow sprinkle of 1.5 g of MetaPhor agarose (Lonza, USA) and 1.5 g standard agarose (First Base, Singapore) into 100 ml of 1xTAE buffer with rapid stirring. The agarose was then soaked in the buffer for 15 min before heating to prevent the solution from foaming during heating. The solution was heated in a microwave at medium power for 2 min before reheating at high power for 1 min. Once the molten gel was cast and solidified, it was placed at 4 °C for 20 min prior to use.

Conclusion

Through the successful development of a reliable (MAGE)-based single-tube assay in our present study, functional polymorphisms of the *fgr* and *Wx* genes can now be identified simultaneously; this would play an important role in fuelling the development of high quality rice. MAGE is an efficient and cost-adept alternative to PAGE and also to high throughput genotyping systems. Greater efforts should be channelled on the publicity of the advantages of this genotyping system, especially to researchers whose studies are dependent upon mass genotyping and in laboratories with limited resources.

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