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Evaluation of High-Resolution Melting for Gene Mapping in Rice

Jinshan Li · Xuming Wang · Ruixian Dong · Yong Yang · Jie Zhou · Chulang Yu · Ye Cheng · Chengqi Yan · Jianping Chen

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Abstract In this study, high-resolution melting (HRM) analysis was evaluated for gene mapping in rice with sequence-tagged site (STS) and simple sequence repeat (SSR) markers. A total of 103 out of 353 normal STS and SSR markers revealed polymorphic melting curves among the parental genotypes, and 12 of these were successfully used to genotype the F_2 mapping population for HRM analysis. Additional electrophoresis findings demonstrated that HRM genotyping matched with traditional electrophoresis results. To optimize the HRM-marker screening efficiency, different HRM reaction conditions were evaluated. A 5-μl touchdown-polymerase chain reaction (PCR) system provided no significant improvement in screening efficiency but in a 10-μl touchdown-PCR system, the marker screening efficiency increased by 75%. Twenty-one markers were obtained for mapping purposes under the optimized reaction conditions. This study indicates that HRM analysis can speed up the gene mapping progress in rice, while saving a lot of manpower.

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J. Li · R. Dong
College of Chemistry and Life Sciences,
Zhejiang Normal University,
Jinhua 321004, People's Republic of China

J. Li · X. Wang · R. Dong · Y. Yang · J. Zhou · C. Yu · Y. Cheng · C. Yan · J. Chen (✉)
State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control, MOA Key Laboratory for Plant Protection and Biotechnology, Zhejiang Provincial Key Laboratory of Plant Virology, Institute of Virology and Biotechnology, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, People's Republic of China
e-mail: jpchen2001@yahoo.com.cn

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Introduction

Rice (*Oryza sativa* L.) is a staple cereal crop grown worldwide, providing food for almost half of the world's population (Zhu et al. 2009). However, diseases, such as bacterial blight, have caused severe reductions in rice yield (Zhao et al. 2010). To improve resistance and raise the yield, great efforts have been made to clone resistance genes in rice and other plants (Feng et al. 2009; Zhao et al. 2009; Leng et al. 2010). Many resistance genes have been cloned and now contribute to improve disease resistance; one example is gene *Xa21*, which confers resistance to almost all Indian and Philippine races of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Ronald et al. 1992). Map-based cloning is the most commonly used method for cloning new genes (Araújo et al. 2009). Until recently, map-based cloning in rice has relied heavily on the use of gel-based methods, which are both time consuming and hazardous to human health. New systems are therefore urgently needed to increase simplicity and save time. Currently, there are several methods for detecting nucleotide polymorphism. The first method is based on fluorescence resonance energy transfer (FRET), which is fast and high throughput, using TaqMan probe and molecular beacon (Yesilkaya et al. 2006). However, it can only detect specific polymorphic sites with clear background, and the probes are expensive. The second method uses molecular hybridization, such as oligonucleotide ligation assay (OLA), dynamic allele-specific hybridization (DASH) and invasive cleavage assay, which also need sequence-specific probes and have similar shortcomings to the first method (Prince et al. 2001; Black et al. 2006). The

third category is based on a combination of PCR and other methods, including denaturing high-performance liquid chromatography (DHPLC), single-strand conformation polymorphism (SSCP), and denaturing gradient gel electrophoresis (DGGE). This type of method is based on analysis of PCR products through columns and gels without any sequence-specific probes (Aguirre-Lamban et al. 2010; Galeano et al. 2009; Lu et al. 2007). However, running costs are high, the DHPLC apparatus is expensive, and both SSCP and DGGE have relatively low throughputs and are difficult to operate. A number of new technologies have been developed to detect single nucleotide polymorphisms (SNPs). Genetic Bit Analysis (GBA), pyrosequencing, SNPlex, and Masscode system have been relatively popular (Gupta et al. 2001; Li et al. 2009; Tobler et al. 2005). Technically, these methods are advanced and effective, but their costs are relatively high. Hence, they are not suitable for carrying out large-scale projects at the present time.

HRM is a post-PCR technique involving amplification and melting of double-stranded DNA (dsDNA). The changes in dsDNA are monitored by fluorescence changes and displayed as melting curves because the intercalating fluorescent dye can only bind to dsDNA (Mao et al. 2007). The software clusters the test population into different genotype groups based on the different shapes of the melting curves (Tindall et al. 2009). Compared with the other methods, HRM analysis has considerable advantages. It only requires standard PCR reagents, needs no post-PCR handling, and the only additional reagent is a suitable dsDNA binding dye. In HRM analysis, PCR products are analyzed without gels and hazardous chemicals, such as ethidium bromide (EB). It also has the advantage that the melting process is faster, and data analysis can be performed automatically in a few minutes (Vossen et al. 2009). Furthermore, the sensitivity of HRM is very high; even a single base-pair difference can be detected (Reed and Wittwer 2004). Because of these benefits, HRM has already been used in many investigations including the detection of SNP mutations (Margraf et al. 2006; Wu et al. 2008), RNA editing (Chateigner-Boutin and Small 2007), identification of transgenic plants (Akiyama et al. 2009), varietal identification (Mackay et al. 2008), and food traceability (Ganopoulos et al. 2010; Jaakola et al. 2010). HRM analysis has also been successfully used for genetic mapping in white lupin, barley, apple, and almond (Chagné et al. 2008; Croxford et al. 2008; Lehmensiek et al. 2008; Wu et al. 2009). It has also been used to develop a genetic map of SNP markers in *Cryptomeria japonica* (Ujino-Ihara et al. 2010).

HRM has been proved suitable for detecting not only SNP markers but also STS and SSR markers (Croxford et al. 2008; Mackay et al. 2008). In this study, HRM analysis is employed to develop markers with currently widely used SSRs and STSs, to help make full use of those existing resources to improve the efficiency of rice gene mapping and associated tasks.

Materials and Methods

Plant Materials and DNA Extraction

The *Japonica* rice cultivar Y73 that is highly resistant to bacterial blight and the highly susceptible *indica* rice cultivar IR24 were used to create the test mapping population for HRM analysis. The F_1 progeny was acquired from the cross of the two rice cultivars. The F_2 progeny was generated from selfing of F_1 progeny. DNA was extracted from samples for HRM analysis using a modified CTAB method described by Wu et al. (2008). The DNA solution was diluted to 100 ng/μl and stored at 4°C for use.

STS and SSR Markers

The SSR markers used for HRM analysis were synthesized according to information at http://www.grammene.org/microsat/, and the STS markers used for HRM analysis were adopted from http://www.ncbi.nlm.nib.gov (Supplemental Table 1).

Amplification and HRM Analysis

The HRM curve was acquired and analyzed on 384-well plates using the LightCycler 480 real-time PCR system (Roche, Basle, Switzerland). To save costs, a 5-μl reaction system was employed as follows (final concentrations given): 0.2 U/μl *Taq* DNA polymerase (Takara, Dalian, Liaoning, China), 1× PCR buffer, 0.25 mM dNTP each, 0.8 μM each primer, 5 ng/μl DNA template, 2 mM Mg^{2+}, 1× EvaGreen (Biotium, San Francisco, CA), and made up to 5 μl with deionised water.

The HRM reaction procedure and melting analysis were performed as follows: a 5-min initial denaturation followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s and extension at 72°C for 25 s. The amplification cycles were immediately followed by the high-resolution melting steps: 95°C for 1 min, cooling to 40°C for 1 min, and then the temperature was raised to 60°C. Subsequently, the temperature was raised to 95°C with 25 fluorescent acquisitions per degree Celsius at this step.

PCR Verification and Electrophoresis

PCR was performed on the Eppendorf PCR System. The 20-μl total reaction consisted of 1× Master Mix, 0.8 μM

each primer, 5 ng/μl DNA template, and made up to 20 μl with deionised water (final concentrations given). The reaction procedure was performed as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 40 s, annealing at 58°C for 40 s, and extension at 72°C for 30 s, and a final extension at 72°C for 7 min.

PCR products and purified HRM amplification products were separated through gels. SSR products were electrophoresed through 8% polyacrylamide for about 6 h. The gels were then stained with silver for 3 min and scanned. STS products were electrophoresed on 3% agarose gels for 20 min. The gels were stained with EB for 40 min and visualized under ultraviolet.

Optimization of HRM Analysis

Two new HRM protocols were compared to the original method. In one method, a 5-μl total reaction (similar to that in the original method) and a touchdown-PCR were used. The touchdown-PCR was conducted as follows: a 5-min initial denaturation followed by 45 cycles of denaturation at 95°C for 15 s, annealing from 62°C to 56°C for 15 s (the annealing temperature decreased by 1°C every cycle for the first 7 cycles and the remaining cycles at 56°C), extension at 72°C for 25 s, and then the high-resolution melting step was done as before.

In another method, a 10-μl total reaction and a touchdown-PCR protocol were used. The 10-μl reaction contained 0.2 U/μl *Taq* DNA polymerase, 1× PCR buffer, 0.25 mM dNTP each, 0.8 μM each primer, 5 ng/μl DNA template, 2 mM Mg^{2+}, 1× EvaGreen and then made up to 10 μl with deionised water (final concentrations given). The touchdown-PCR procedure and the high-resolution melting steps were done as in the first protocol.

Results and Discussion

Screening Polymorphic HRM-markers with Y73, IR24 and their F_1 Progeny

The markers used for the HRM analysis were all regular SSR and STS markers obtained from the related web sites. In total, 353 SSR and STS markers were synthesized for HRM-marker development. The parents Y73, IR24 and their F_1 progeny were used to screen the markers showing polymorphic melting curves by HRM, and 103 polymorphic markers were acquired for further studies (Fig. 1). The other 250 markers did not show polymorphic melting curves.

HRM analysis can detect SNP differences, and therefore, it can also detect STS and SSR polymorphism between parents and their F_1 progeny (Croxford et al. 2008; Mackay et al. 2008). The majority of the 250 markers did not show polymorphic melting curves because they were not polymorphic between IR24 and Y73. Additionally, negative amplification appeared for a few markers since the reaction annealing temperature of 58°C was not suitable for all of them.

Testing Polymorphic HRM-Markers with a F_2 Test Population

To verify whether the 103 polymorphic markers could be used for gene mapping, a F_2 test mapping population of a suitable size was created. Twenty-two progenies were selected from the F_2 together with the two parents. All 103 markers showing polymorphic melting curves between the parents and their F_1 progeny were tested using the 5-μl reaction volume and normal PCR system. The results showed that the melting curves of 12 markers were clustered into the three parental genotype groups by the

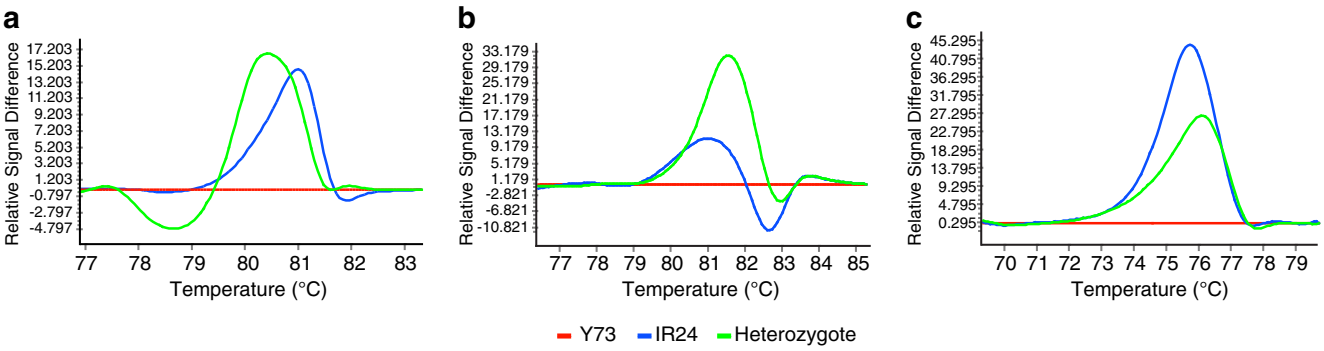

Fig. 1 HRM data presented as difference plots for three examples of the 103 polymorphic markers. The Y73 parental genotype was chosen as the baseline and is coded *red*. The second parent, IR24 is coded *blue* and their F_1 genotype is coded *green*. Markers are **a**, R04M35; **b**, R04M95; **c**, RM7193

software (Fig. 2a–c). For the other markers, more than three kinds of genotypes appeared and the melting curves of some samples were not clustered into any of the three parental genotype groups.

To confirm whether the HRM genotyping results were consistent with traditional methods, an additional regular PCR and electrophoresis were performed for some markers. The results demonstrated that the samples coded red as parent Y73 genotype by HRM showed Y73 banding patterns in electrophoresis. The samples coded blue as IR24 genotype by HRM showed IR24 banding patterns, while the samples coded green as F_1 progeny genotype by HRM showed heterozygous banding patterns (Fig. 2d–i). These results indicated that the HRM genotyping results were consistent with the traditional electrophoresis procedure.

By analyzing the amplification curves and melting curves, we found that these 12 markers have some similar characteristics. The C_T values of these markers are no more than 30 cycles, there were few nonspecific products, and no exceptional melting peaks were found when performing *T*m calling analysis. In addition, these markers gave clear major bands in subsequent electrophoresis of the extracted PCR products.

For the other 91 markers, part of their melting curves were not clustered into any of the three parental genotypes. Nonspecific products were generated during amplification, leading to unreadable melting curves and smear banding in electrophoresis. It is reasonable to believe that the nonspecific products affected the analysis of melting curves, because the fluorescent dye can bind to any dsDNA and lead to confused HRM results (Mao et al. 2007).

Optimization of HRM Reaction Conditions

Several reports about HRM analysis have discussed the optimization of HRM reaction conditions, and most of them focused on the choice of proper annealing temperatures (Erali and Wittwer 2010; Mackay et al. 2008). Except for proper annealing temperature, DNA quality is also an important factor for HRM analysis, and we therefore confirmed the DNA quality of all samples before further tests. In order to raise the screening efficiency of HRM-markers, we introduced touchdown-PCR to allow correct annealing temperatures for many markers to be used in the same run and to increase the specificity of the amplification (Korbie and Mattick 2008). Additionally, different reaction total volumes for HRM analysis were compared in this optimization process, which few studies have examined.

To estimate the HRM-marker screening efficiency, the 103 polymorphic markers were evaluated by the

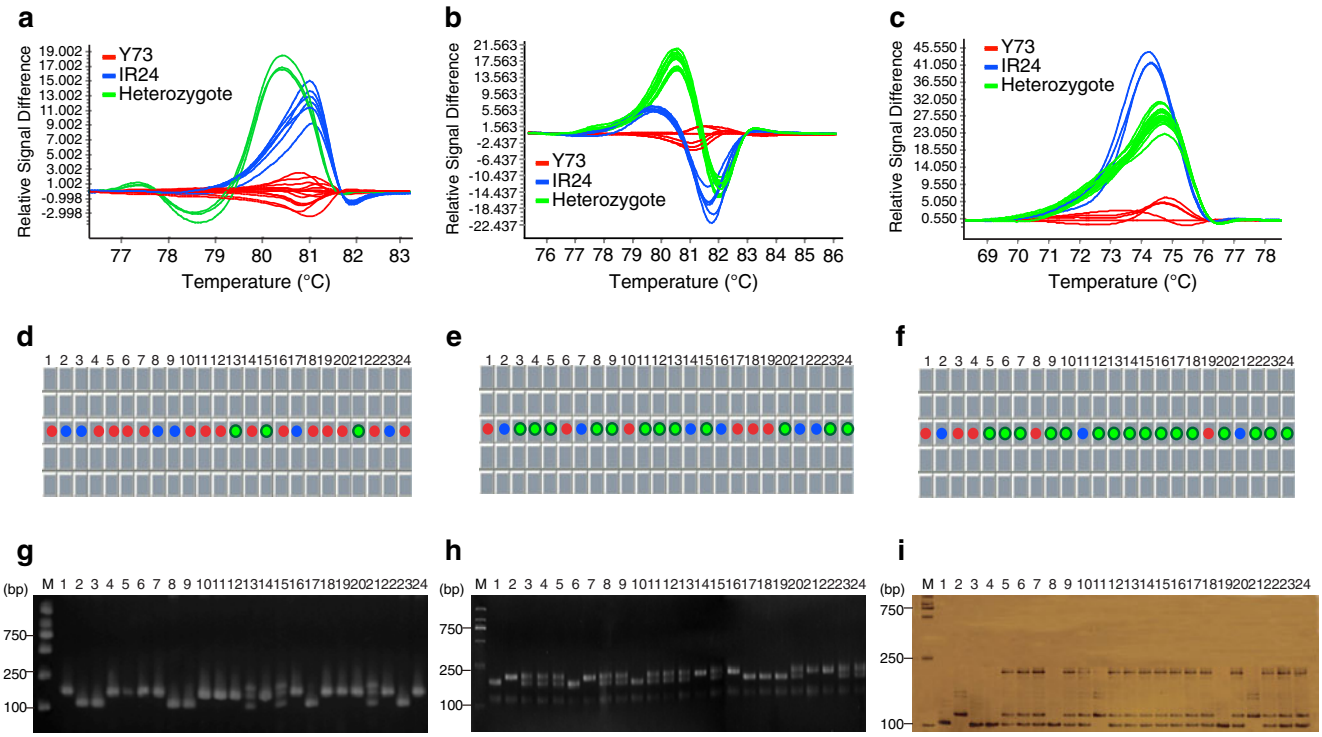

Fig. 2 HRM genotyping results and corresponding electrophoresis genotyping results of the F_2 population. Normalized and shifted melting curves of three representative markers are shown in the *upper panel*; the corresponding sample tables are shown in the *middle panel*, color-coded to show the genotyping results by HRM; the corresponding electrophoretic genotyping results are shown on the *lower panel*. Samples are numbered: 1-parent Y73; 2-parent IR24; 3–24: the 22 F_2 individuals. DNA size marker is shown. **a**, **d** and **g**, marker R04M35; **b**, **e** and **h**, marker R04M95; **c**, **f** and **i**, marker RM7193

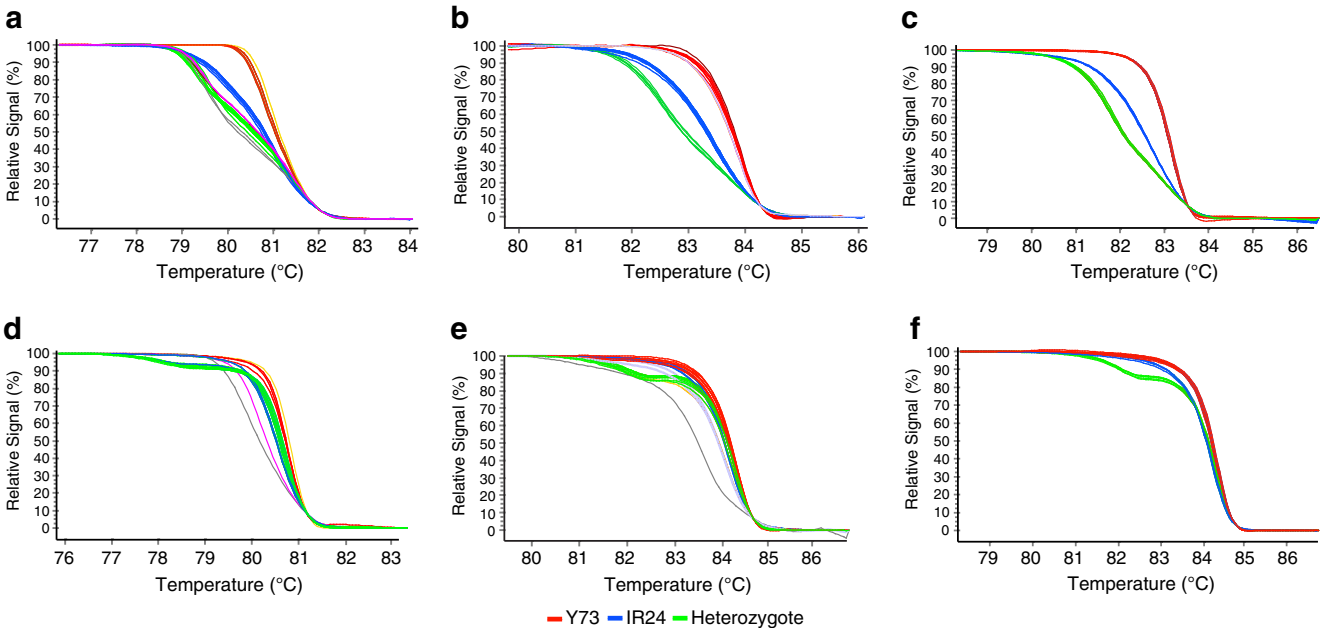

Fig. 3 HRM data from the three different experimental protocols presented as normalized and shifted melting curves. The *upper graphs* display the melting curves of R04M123 amplicons, and the *lower graphs* are for R08D77. Reaction conditions: **a** and **d**, a 5-μl reaction system with normal PCR procedure; **b** and **e**, a 5-μl reaction system with touchdown-PCR procedure; **c** and **f**, a 10-μl reaction system with touchdown-PCR procedure

F_2 test mapping population under two optimized conditions (see Materials and Methods). Those 12 markers previously identified were also successful for genotyping the F_2 test mapping population using both the two optimized HRM analysis methods. For the remaining 91 markers, none were useful for genotyping the F_2 test population using the first HRM analysis protocol used; however, nine markers successfully genotyped this test mapping population using the second HRM analysis protocol described in the Materials and Methods section (Fig. 3c and f).

In the first protocol for optimization, touchdown-PCR was the only change from the original experiment (Fig. 3a and d), and a slight improvement of the melting curve grouping was observed (Fig. 3b and e). When 10-μl total reaction volume and touchdown-PCR were both used (the second protocol for optimization), HRM-marker screening efficiency increased by 75%, and 9 new markers were identified (compare Fig. 3c and f in the second protocol for optimization with Fig. 3a and d in the original protocol and Fig. 3b and e with the 5-μl total reaction volume in the first protocol for optimization). These results suggest that touchdown-PCR can help to improve the stability of the reaction system, and this improvement is more effective when using a larger reaction system (10 μl).

Although the 5-μl reaction volume for HRM analysis was less stable than the 10-μl reaction volume, it contributes to lower costs. Since a large number of rice SSRs and STSs are available, it is acceptable to sacrifice some marker screening efficiency to gain lower costs. Similarly, touchdown-PCR can also help to increase the specificity of the amplification. In the touchdown-PCR system, the annealing temperature decreases gradually from a high temperature to a lower one. The high initial annealing temperature can improve the specificity of the amplification, and the lower final annealing temperature can assure a positive amplification. In the two protocols for optimization, even the concentration of the *Taq* DNA polymerase was half and the amplification was still adequate since the final annealing temperature was set lower than the normal annealing temperature (data not shown). This indicates that the amount of *Taq* enzyme can be further reduced, thereby further reducing costs. The lower final annealing temperature may help *Taq* DNA polymerase and primers to bind to DNA templates.

Conclusion

This is the first report of HRM analysis for gene mapping with SSRs and STSs in rice. Twenty-one markers successfully genotyped the test mapping group in a stable and economical reaction system. By comparing different PCR systems, it was found that a higher volume reaction combined with touchdown-PCR could improve the performance but will increase the cost per reaction. In optimized protocols for cost and reproducibility, HRM can be a simple and convenient method for gene mapping in rice.

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