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Full Length Research Paper

A screening method for detecting simple sequence repeat (SSR) polymorphism of *Zea mays* using high-resolution melting-curve analysis

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As a very important molecular marker technique, simple sequence repeat (SSR) is widely used in marker assistant selection and maize DNA fingerprint. High-resolution melting-curve (HRM) analysis is a new technique to detect the sequence differences in polymerase chain reaction (PCR) amplicons, which is quicker, more accurate and effective than electrophoresis analysis. In this study, we developed a HRM system to detect SSR polymorphism of *Zea mays*. We successfully used this method to identify genotypes of F2 generation and detecting the SSR polymorphism in different maize inbred lines, therefore, this HRM-SSR system can be used to substitute the electrophoresis after SSR PCR amplification.

Key words: High-resolution melting-curve, SSR, *Zea mays*.

INTRODUCTION

Currently, the simple sequence repeat (SSR) technique is the most widely used molecular marker method used in crop QTL exploration, marker assistant breeding as well as deoxyribonucleic acid (DNA) fingerprint (Sharopova et al., 2002; Maccaferri et al., 2007; Wu and Huang, 2007; Jia et al., 2009; Qiu et al., 2010; Xu et al., 2011; Yu et al., 2011). The SSR method is improving based on relevant researches (Robinson et al., 2004; Chapman et al., 2009; Qiu et al., 2010; Wen et al., 2010); however, those researches are still limited in efficiency since electrophoresis has to be eventually employed to detect polymerase chain reaction (PCR) products. Either agarose gel electrophoresis or PAGE is required after SSR-PCR, leading to a low working efficiency. In addition, the detection resolution is restricted by these electrophoretic methods. High-resolution electrophoresis equipment based on capillary electrophoresis (CE) is available now, but the investment for this apparatus is huge and not very practical in a general laboratory.

High-resolution melting-curve (HRM) analysis is an advanced technique invented in 2003. The technique is used to detect DNA diversity (Wittwer et al., 2003) and has been widely used in clinical chemistry, epidemical analysis, plant genomics and molecular biology (White et al., 2007; Croxford et al., 2008; Wu et al., 2008, 2009; Dagar et al., 2009; Hofinger et al., 2009; Li et al., 2010). HRM detection is based on the principle that melting curves change during the process of DNA denaturation based on the primary sequence. The operation can be done immediately after PCR without electrophoresis and in the same reaction tube. In comparison to electrophoresis, the detecting time is less and the resolution is higher.

Since it is effective to distinguish single base difference, the technique is widely used in the detection of single nucleotide polymorphism (SNP) and indels (insertion/deletion) mutations of known gene fragments. But the application of the technique to SSR site

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Abbreviations: SSR, Simple sequence repeat; **HRM,** high-resolution melting-curve; **PCR,** polymerase chain reaction; **CE,** capillary electrophoresis; **SNP,** single nucleotide polymorphism.

Table 1. SSR primers.

S/N	Name	Sequence		S/N	Name	Sequence	
1	umc1269	F	TATATTAGAGGCACCTCCCTCCGT	13	umc1165	F	TATCTTCAGACCCAAACATCGTCC
		R	AGCTGCTTCAGCGACTTTGG			R	GTCGATTGATTTCCCGATGTTAAA
2	bnlg439	F	TTGACATCGCCATCTTGGTGACCA	14	bnlg339	F	CCAACCGTATCAGCATCAGC
		R	TCTTAATGCGATCGTACGAAGTTGTGAA			R	GCAGAGCTCTCATCGTCTTCTT
3	phi96100	F	AGGAGGACCCCAACTCCTG	15	umc1173	F	ATCCGCCAAAAAGGGGAAAA
		R	TTGCACGAGCCATCGTAT			R	TAGAAGTAGCACACGCGCCG
4	bnlg1520	F	TCCTCTTGCTCTCCATGTCC	16	phi080	F	CACCCGATGCAACTTGCGTAGA
		R	ACAGCTGCGTAGCTTCTTCC			R	TCGTCACGTTCCACGACATCAC
5	bnlg161	F	GCTTTCGTCATACACACACATTCA	17	phi112	F	TGCCCTGCAGGTTCACATTGAGT
		R	ATGGAGCATGAGCTTGCATATTT			R	AGGAGTACGCTTGGATGCTCTTC
6	phi048	F	GCAAACCTTGCATGAACCCGATTGT	18	umc2188	F	CGCCAACATGATTAACTTGCTATC
		R	CAAGCGTCCAGCTCGATGATTTC			R	ATTTTCAGTCTGGGTACTTGAGCG
7	phi119	F	GGGCTCCAGTTTTCAGTCATTGG	19	umc1380	F	CTGCTGATGTCTGGAAGAACCCT
		R	ATCTTTCGTGCGGAGGAATGGTCA			R	AGCATCATGCCAGCAGGTTTT
8	phi109642	F	CTCTCTTTCCTTCCGACTTTCC	20	phi056	F	ACTTGCTTGCCTGCCGTTAC
		R	GAGCGAGCGAGAGAGATCG			R	CGCACACCACTTCCCAGAA
9	umc1160	F	CGTTTGATATGATGTGGAGATTCG	21	phi072	F	ACCGTGCATGATTAATTTCTCCAGCCTT
		R	AAGCTTGTGAATGTTCTGGATGTC			R	GACAGCGCGCAAATGGATTGAACT
10	phi116	F	GCATACGGCCATGGATGGGA	22	bnlg1702	F	TTATCATCAAATGGAGGACACG
		R	TCCCTGCCGGGACTCCTG			R	AAAGACACACGCTAATGGGC
11	umc1590	F	CAGAGTCTGATAGTCCGAACCCAG	23	phi022	F	TGCGCACCAGCGACTGACC
		R	GTAAAGCTCACAGCTTCCGACAG			R	GCGGGCGACGCTTCCAAAC
12	phi115	F	GCTCCGTGTTTCGCCTGAA				
		R	ACCATCACCTGAATCCATCACA				

polymorphism has not yet been reported. Considering the advantages of HRM, this study focused on using HRM to detect SSR site polymorphism, screening maize SSR primers used in HRM detection and establishing a highly efficient system to detect maize SSR polymorphism.

MATERIALS AND METHODS

Plant materials

Test materials included several maize inbred lines: Mo17, B73, 78599, Ye-478, Dan-340 and HangzaoIV, which are model species of 5 maize main families in China, and a maize hybrid, Jidan27, and its parent inbred lines.

SSR primer screening

23 pairs of SSR primers, with diverse polymorphism and only one specific, clear and stable amplification band were screened (Table 1). Information on SSR primers' sequences are on Maize GDB (http://www.maizegdb.org.ssr.php). SSR primers used in the experiment were synthesized by Beijing Dingguo Biotech Co. ltd.

PCR and HRM system

The PCR amplification system and HRM detection system used in

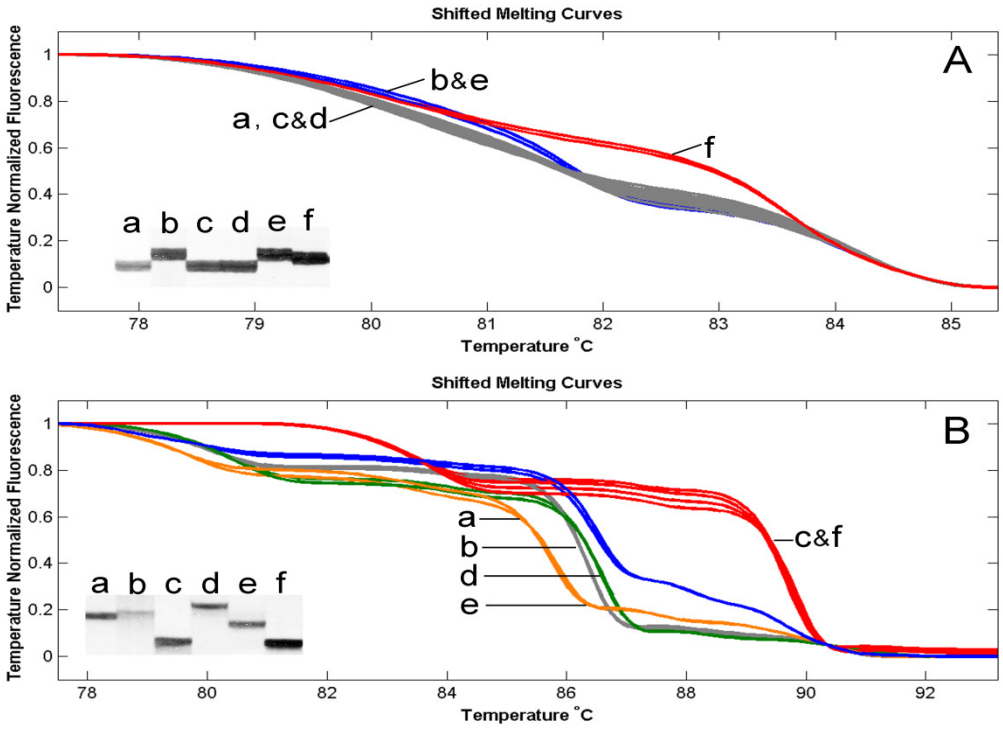

Figure 1. SSR site polymorphism detected by HRM. Curves a, b, c, d, e and f represent Mo17, Dan-340, B73, Ye-478, HangzaoIV and 78599, respectively. Left lower parts of Figure 1A and 1B display the PAGE results of SSR primer (umc1160 and umc1590) amplification products matching the melting curves.

this study were according to Li et al. (2010) HRM-PCR detection system with a little modification. Briefly, PCR amplification system (20 µL) consisted of 5 µL rTaq Premix (Dalian Takara Biotech Co. ltd.), 1 µL 20×EvaGreen (Biotium Co. ltd., USA), 1 µL 5 µM primers, 50 ng maize DNA templates and 15 µL mineral oil.

The PCR program was the following: predenaturation at 95℃ for 5 min; denaturation at 95℃ for 30 s, annealing at 60℃ for 45 s, extension at 72℃ for 1 min, 35 cycles; extension at 72℃ for 10 min; denaturation at 95℃ for 30 s; reducing the temperature by 0.1℃/s to 40℃. PCR products were detected using a LightScanner (Idaho Technique Inc.) according to the method by Hofinger et al. (2009).

RESUTLS

SSR primer screening

PAGE was first performed to verify the fidelity of 23 pairs of primers. All primers produced amplification products with clear bands. Each primer amplified a single specific band from a variety of maize and presented with polymorphisms in different maize varieties (Figure 1).

HRM detection on SSR sites of maize inbred lines

After the amplification of the screened SSR primers, HRM detection was performed to the inbred lines Mo17, B73, 78599, Ye-478, Dan-340 and HangzaoIV. As shown in Figure 1, each pair of primers could clearly detect the SSR sites of polymorphism. And the results were consistent with those by electrophoresis, which suggests that HRM-SSR detection system is feasible in maize.

Maize genotype detected by HRM-SSR system

Detection on hybrid variety Jidan27 and its parents showed the HRM-SSR detection system could distinguish male parent, female parent and hybrid variety by melting curves (Figure 2). Thus HRM-SSR detection system can be applied to identify individual genotypes in maize F2 generation.

DISCUSSION

In this study, HRM was used for the detection of SNPs and indels was used for the detection of SSR site polymorphisms. The HRM system studied was a modified one based on previous methods (Wittwer et al., 2003; Hofinger et al., 2009; Li et al., 2010). As was confirmed by Li et al. (2010), HRM detection using Evagreen dye led to a lower cost and a higher accuracy. This lays a

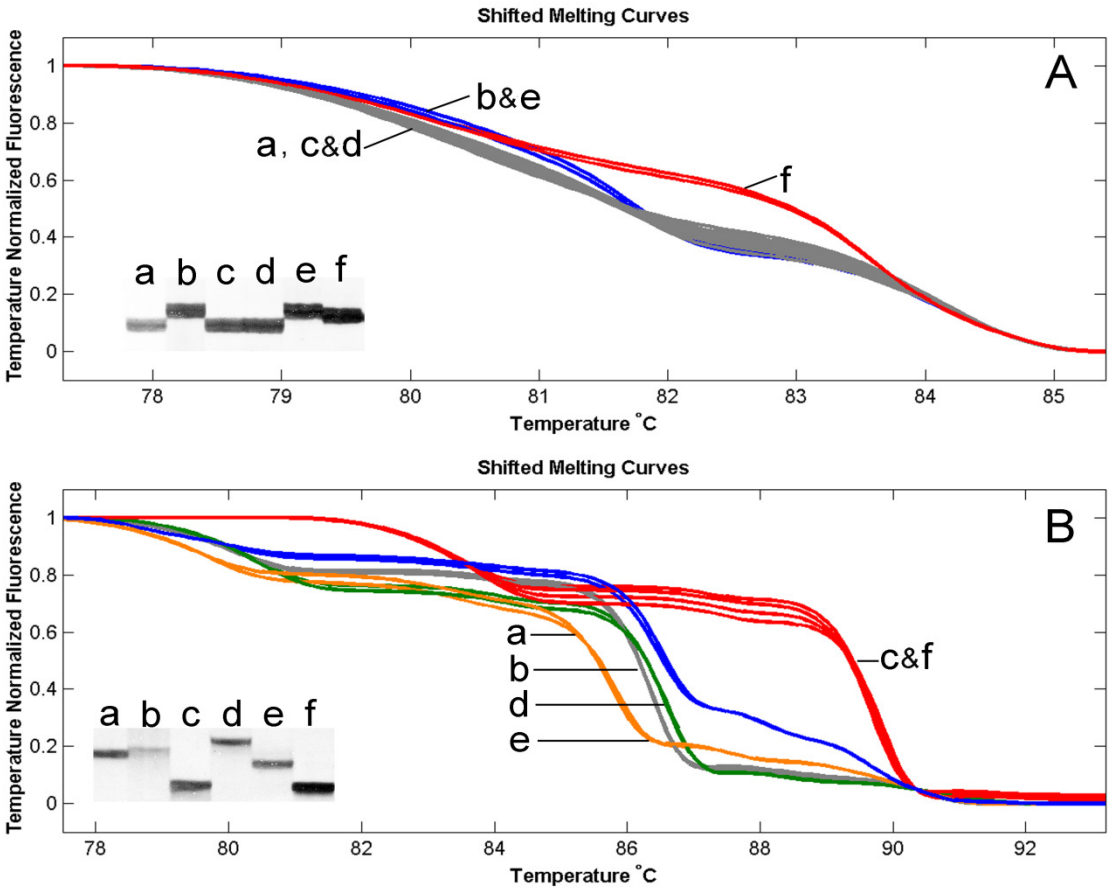

Figure 2. Genotyping of Maize (Jidan27) F2 plants by HRM with a SSR primer (bnlg1520). Top parts display normalized melting curves of the amplicons of 10 F2 plants, of which the genotypes P1 (A144), P2 (Si287), and heterozygote are identified. Bottom parts show derivative melting peaks of the same amplicons

foundation for further research.

In terms of the detection on SSR site polymorphism, the melting curves varied simply due to the variation of DNA lengths. This was because SSR primer amplification fragments for each inbred line were different in length. According to Hofinger et al. (2009), more SNPs per amplification results in better HRM detection. This study demonstrated that HRM is absolutely feasible to detect SSR site differences.

In the process of screening SSR primers, we simply selected those with only a single amplification band for further HRM detection, but those with more than one band were ignored simply because they might cause multiple complex melting curves, which make it difficult to recognize different inbred lines clearly. The above is not a problem considering that it is not difficult to screen those SSR primers with only a single amplification band among massive primers. The application of the HRM-SSR system on maize is effective, but its usefulness for other organisms may be limited, especially those with few SSR primers.

In addition, we establish a maize genotype detection system based on HRM-SSR. The system was able to identify hybrid variety Jidan 27 and its parent inbred lines (Figure 2) because its two parents' SSR amplification fragments were different in length and the hybrid Jidan 27 displayed two amplification fragments. In the HRM-PCR process, the two amplification products formed hybrid double strands, causing the melting curves to shift markedly, differing from two parents' melting curves. Thus, all three genotypes could be distinguished by this method.

According to Li et al. (2010), a new HRM methodology was established to detect rice genotype. The principle is that HRM acts effectively in SNP detection by amplifying and detecting the genome fragments with multiple SNP sites. In this study, we employed some known SSR primers in maize and ignored SNP site inquiry as well as the design and verification of the corresponding PCR amplification primers, enabling the SSR method to be widely promoted and applied. It is assumed that if a SNP were present in the amplicons from SSR primers, HRM-SSR accuracy would be negatively affected. We did not run into the trouble in this study. But the potential problem

should not be ignored.

In conclusion, this study focused on the HRM technique used in the detection of maize SSR site. The limitations of electrophoresis based methods were avoided by using this HRM based strategy. At the same time, the high-resolution property of HRM contributes to improving the efficiency of SSR detection, making the technique promising for promotion and application.

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