High-throughput SNP Genotyping by Melting Curve Analysis for Resistance to Southern Root-knot Nematode and Frogeye Leaf Spot in Soybean

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Abstract

Melting curve analysis of fluorescently labeled DNA fragments is used extensively for genotyping single nucleotide polymorphism (SNP). Here, we evaluated a SNP genotyping method by melting curve analysis with the two probe chemistries in a 384-well plate format on a Roche LightCycler 480. The HybProbe chemistry is based on the fluorescence resonance energy transfer (FRET) and the SimpleProbe chemistry uses a terminal self-quenching fluorophore. We evaluated FRET HybProbes and SimpleProbes for two SNP sites closely linked to two quantitative trait loci (QTL) for southern root-knot nematode resistance. These probes were used to genotype the two parents and 94 F2 plants from the cross of PI 96354 × Bossier. The SNP genotypes of all samples determined by the LightCycler software agreed with previously determined SSR genotypes and the SNP genotypes determined on a Luminex 100 flow cytometry instrument. Multiplexed HybProbes for the two SNPs showed a 98.4% success rate and 100% concordance between repeats of two of the same 96 DNA samples. Also, we developed a HybProbe assay for the Rcs3 gene conditioning broad resistance to the frogeye leaf spot (FLS) disease. The LightCycler 480 provides rapid PCR on 384-well plate and allows simultaneous amplification and analysis in approximately 2 h without any additional steps after amplification. This allowed for a reduction of the potential contamination of PCR products, simplicity, and enablement of a streamlined workflow. The melting curve analysis on the LightCycler 480 provided high-throughput and rapid SNP genotyping and appears highly effective for marker-assisted selection in soybean.

Introduction

The availability of the 7.23x shotgun sequence of soybean [*Glycine max* (L.) Merr.] will facilitate the discovery of large numbers of single nucleotide polymorphisms (SNPs) such as the more than 37,000 SNPs that have already been identified in *Arabidopsis* (Schmid et al. 2003; Torjek et al. 2003), and the 1,703,176 SNPs and 479,406 Insertion/Deletions (InDels) markers that have been identified in rice (Shen et al. 2004). These SNP markers will offer high-resolution SNP marker coverage for most of the soybean genome and enhance mapping of important genes, positional cloning, and marker-assisted breeding. For

example, Choi et al. (2007) recently discovered 5,551 SNPs via resequencing of soybean sequence-tagged sites (STSs) and mapped an additional 1,141 SNP markers across 20 linkage groups along with the previously mapped 1,015 simple sequence repeat (SSR) markers and 709 restriction fragment length polymorphism (RFLP) markers (Song et al. 2004).

Marker-assisted breeding has been enhanced by the recent development of fast and highly automatable methods for SNP genotyping. One of the high-throughput approaches is the use of 'homogeneous' or 'closed tube' assays which do not require the processing or separation steps after PCR amplification (Gibson 2006; Mamotte 2006). These assays include the TaqMan assay (Livak 1999), melting curve analysis (Bennett et al. 2003), molecular beacons (Tyagi et al. 1998), scorpion probes (Thelwell et al. 2000), invader assay (Olivier 2005), and pyrose-

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quencing (Langaee and Ronaghi 2005). Of these various methods the TaqMan assay and melting curve analysis were designed for using real-time thermocyclers.

The TaqMan assay is based on the 5'-exonuclease activity of *Taq* polymerase to cleave two allele-specific probes complementary to the mutant and wild-type alleles (De La Vega et al. 2005; Livak 1999). The two probes are labeled with different fluorescent reporter dyes, but a common quencher dye. Cleavage separates a 5'-reporter dye from a 3'- quencher leading to a detectable fluorescent signal. Genotyping is determined by measurement of the fluorescence intensity of the two reporter dyes after PCR amplification. Examples of the use of TaqMan assay in plants include genotyping of soybean cyst nematode resistance gene *Rhg4* in soybean (Meksem et al. 2001) and for allelic discrimination in maize (Salvi et al. 2001).

Melting curve analysis is based on the thermal stability difference between matched and mismatched DNA duplexes (Bennett et al. 2003; Bernard et al. 1998). The melting temperature (Tm) reflects the thermal stability of a DNA duplex and depends on dsDNA length, GC content, and degree of complementarity between strands (Wetmur 1991). Thus, a probe/target duplex containing a destabilizing mismatch melts off at a lower temperature than when a probe/target duplex contains a perfect match.

SNP genotyping with melting curve analysis has been developed with two probe chemistries. One is the hybridization probe (HybProbe) format that produces fluorescence resonance energy transfer (FRET) when two fluorescently labeled oligonucleotide probes anneal to adjacent regions on the target (Bernard et al. 1998; De Angelis 1999). The "sensor" probe is designed to hybridize across the SNP site and is labeled with a LightCycler Red fluorophore at its 5'-end. The "anchor" probe is designed to hybridize to an adjacent site, is more thermally stable than the sensor probe, and is labeled with fluorescein at its 3'-end. When heated after PCR amplification, the sensor probe disassociates with the complementary template DNA (melts) and physically separates from the anchor probe, resulting in loss of fluorescent signal. Melting curve analysis with hybridization probe has already been applied for detecting disease resistance alleles in plants (Dufresne et al. 2004).

The other probe type is referred to as a SimpleProbe since it contains only a single-labeled sensor probe. The SimpleProbe is designed to specifically hybridize to a SNP site and is labeled with a specific, non-fluorescent quencher and fluorescein at its 5'-end or internally. When the probe is free in solution, emission of the reporter dye is reduced by quencher. However, once hybridized to its target, quenching is reduced and the probe emits a greater fluorescent signal. The SimpleProbe chemistry has been applied for SNP detection in human (Frances et al. 2005; Grannemann et al. 2005). However, the application of SimpleProbe chemistry in plants has not yet been reported.

Fluorescent melting curve analysis is relatively simpler and a somewhat less expensive method than TaqMan assays for SNP genotyping. Melting curve analysis depends on only a single pair of hybridization probes or one SimpleProbe complementary to the wild-type sequence or the mutant sequence. Also, it is not necessary to design the probe to have a specific Tm because a notably different Tm on matched and mismatched target is only required (Gibson

2006). The cost per sample for the reagents and consumables for the melting curve analysis was approximately 60% of that for TaqMan assay in 96- and 384-well plates (Chantarangsu et al. 2007). However, unlike the TaqMan assay, HybProbe and SimpleProbe assays often require some degree of additional optimization (Chantarangsu et al. 2007; Houghton and Cockerill III 2006).

In this study, we developed melting curve assays to genotype several SNPs in soybean. Six of these SNPs were closely linked to the southern root-knot nematode [*Meloidogyne incognita* (Kofoid and White) Chitwood] (Mi) resistance quantitative trait loci (QTL) reported by Ha et al. (2007). Three SNPs in Satt358 source-sequences were located near a major Mi-resistant QTL on linkage group O (LG-O) and 3 SNPs in Satt199 source-sequences were located near a minor Mi resistant QTL on LG-G. These SNPs (SNP358 and SNP199) have been genotyped by allele-specific oligonucleotide hybridization assay with the Luminex 100 flow cytometer. Also, melting curve assays were developed for SNP markers associated with the *Rcs3* locus conditioning broad resistance to frogeye leaf spot (FLS) disease caused by *Cercospora sojina* Hara. Originally, these SNPs were identified in bacterial artificial chromosome (BAC) end sequences that were anchored with SSR markers Satt244 and Satt547 on LG-J (Missaoui et al. 2007). Allele-specific oligonucleotide probes for use on the Luminex 100 were reported for the InDel of AZ573TA150 and a SNP AZ573AG159 to provide a robust assay for marker-assisted selection (MAS) of the *Rcs3* gene using the Luminex 100.

Although SNP detection on the Luminex 100 flow cytometer eliminates the need for gel electrophoresis and manual gel tracking and allows high speed detection of SNP genotype, it requires post-PCR processing and handling steps. Here, we developed melting curve analysis with HybProbe and SimpleProbe chemistries, and compared the accuracy, efficiency, and cost of several PCR-based genotyping assays.

Materials and Methods

Plant materials and DNA preparation

Ninety-four F_2 plants from the cross of PI 96354 × 'Bossier' were used in the study of Mi. PI 96354 has a high level of resistance to Mi for both gall formation and nematode reproduction and Bossier is highly susceptible (Luzzi et al. 1987). This population was utilized in the RFLP mapping (Tamulonis et al. 1997), SSR mapping (Li et al. 2001), and discovery of SNPs (Ha et al. 2007) linked to the QTL conferring Mi resistance. DNA was extracted from individual parental genotypes and individual field-grown F_2 plants using the modified CTAB procedure of Keim et al. (1988) and diluted to 20 ng μL-1.

The study of FLS used 48 F_2 plants from the cross of 'Davis' × 'Blackhawk' previously used in the study of Missaoui et al. (2007). Davis possesses the *Rcs3* allele conditioning broad resistance to FLS and Blackhawk possesses the *rcs3* allele and is susceptible to FLS. Genomic DNA was extracted from lyophilized plant tissue using a 2% CTAB extraction buffers.

Table 1. Sequences of PCR primers, HybProbes, and SimpleProbes for melting curve analysis of SNP199 for LG-G and SNP358 for LG-O Mi resistance QTL and SNP AZ573AG159 for *Rcs3* detection of FLS resistance.

Name		5'-3' sequence[a]
SNP199	Forward primer	ACAGTAGCGTATGAATAAAGGT
	Reverse primer	TATAAATTCCTTTAGTCAATCGTGGG
	Sensor HybProbe	LC Red 670B-AAAAAGTAGTAGTAATTAAAACGTTTACAG**CC**TAA-Phosphate
	Anchor HybProbe	GGTCCATCATCAACTTACTCACAAAATTCATAAAG-Fluorescein
	SimpleProbe	Fluorescein-QC-AAAGTAGTAGTAATTAAAACGTTTACAG**C**CTAATA-Phosphate
SNP358	Forward primer	GATTCATTAACTTCCATTCTGGC
	Reverse primer	CAGAGTGCGGAGTAATTAAAC
	Sensor HybProbe	LC Red 610-AAT**A**CTTAGCT**G**TGCGCTTTATGTAACAATAC-Phosphate
	Anchor HybProbe	CCATTCTGGCACCAGAAATCATTATAAATGCTATCCT-Fluorescein
	SimpleProbe	ATCGTATTGTTACA XID AAAGCGCA**C**AGCTAAG-Phosphate
SNP	Forward primer	AGA CCA CTT CAA ACT CCA ACG
AZ573AG159	Reverse primer	ATT CTT AGG AGG ATG AGC AGG
	Sensor HybProbe	LC Red 640-GGT GTG GTT GTT TAA TGG CCA TTG T-Phosphate
	Anchor HybProbe	GCA AGT AAT TAA TTG GGG CTG AGT GAT ACA CG-Fluorescein

[a] The position of the SNP in probes is underlined and in bold
[b] LC Red 670: LightCycler Red 670, LC Red 610: LightCycler Red 610, LC Red 640: LightCycler Red 640
[c] Q: fluorescence quencher
[d] XI: the internal SimpleProbe label

HybProbe and SimpleProbe for *Meloidogyne incognita* (Mi) resistance

PCR primers and probes were designed with the LightCycler Probe Design software (Roche Applied Science, Indianapolis, IN, USA) (Table 1). For the SNP199 on LG-G, a 206-bp fragment was amplified with primers. The anchor HybProbe was labeled with fluorescein at the 3'-end. The sensor HybProbe, covering the target region, was labeled with the LightCycler Red 670 (LC Red 670) fluorescent dye at the 5'-end and blocked with phosphate at the 3'-end. The SimpleProbe for SNP199 was labeled with fluorescein at the 5'-end and blocked with phosphate at the 3'-end.

For the SNP358 on LG-O, a 250-bp fragment was amplified with primers. The anchor HybProbe was labeled with fluorescein at the 3'-end. The sensor HybProbe was labeled with the LC Red 610 fluorescent dye at the 5'-end and blocked with phosphate at the 3'-end. The SimpleProbe for SNP358 was internally labeled as substitute for one naturally occurring base (TIB MOLBIOL, Adelphia, NJ, USA). All probes were designed to match the Bossier allele.

SNP genotyping assay for *Meloidogyne incognita* (Mi) resistance

All HybProbe and SimpleProbe PCR reactions were performed in 384-well plates with a total volume of 3 μL per well on the LightCycler 480 instrument (Roche Applied Science, Indianapolis, IN, USA) and conducted as asymmetry. Asymmetric PCR produces more copies of the strand complementary to the probe and reduces competitive binding (Gameau et al. 2005). The PCR reaction mixture for the HybProbe assay consisted of 20-30 ng of genomic DNA, 1 μM of SNP199 for-

ward primer, 0.5 μM of SNP199 reverse primer (0.5 μM of SNP358 forward primer and 1 μM of SNP358 reverse primer), 0.2 μM of each HybProbe, and 0.75x of LightCycler 480 Genotyping Master mix containing a modified Taq DNA polymerase, reaction buffer, MgCl₂, and dNTPs (Roche Diagnostics, Indianapolis, IN, USA).

For multiplex assays, the PCR reaction contained two primer sets and two fluorescently labeled probe sets in a single reaction. Each 3 μL reaction contained 20-30 ng of genomic DNA, 1 μM of SNP199 forward primer, 0.5 μM of SNP199 reverse primer, 0.5 μM of SNP358 forward primer, 1 μM of SNP358 reverse primer, 0.2 μM of each anchor probe sets, 0.1 μM of each sensor probe sets, and 1x of LightCycler 480 Genotyping Master mix.

The PCR reaction mixture for the SimpleProbe assay consisted of 20-30 ng of genomic DNA, 1 μM of SNP199 forward primer, 0.5 μM of SNP199 reverse primer (1 μM of SNP358 forward primer, 0.5 μM of SNP358 reverse primer), 0.2 μM of SimpleProbe, 1.0 mM MgCl₂, and 0.5x of LightCycler 480 Genotyping Master mix.

After an initial denaturation of 5 min at 95 ℃, 45 PCR cycles were performed with 10 s of denaturation at 95 ℃, 15 s of annealing at 55 ℃, and 20 s extension at 72 ℃. A final melting cycle was performed by raising the temperature to 95 ℃ for 2 min, lowering the temperature to 40 ℃ for 2 min, and increasing the temperature to 85 ℃ with continuous fluorescent acquisition followed by a cool down to 40 ℃. The fluorescence signal (F) was plotted in real time against temperature (T) to produce melting curves for each sample. Melting curves were then converted into negative derivative curves of fluorescence with respect to temperature (-dF/dT) by the LightCycler Data Analysis software (Roche Diagnostics, Indianapolis, IN, USA). The software then groups similar melting curves and automatically calls genotypes based on melting standards for known genotypes in the experiment or software-defined melting standards.

HybProbe assay for the *Rcs3* gene for resistance to frogeye leaf spot (FLS)

Assays were optimized for a SNP AZ573AG159 closely linked to *Rcs3* resistance gene. PCR primers and probes were designed with the LightCycler Probe Design software (Roche Applied Science, Indianapolis, IN; Table 1). The anchor HybProbe was labeled with fluorescein at the 3'-end. The sensor HybProbe, covering the target region, was labeled with the LC Red 640 (Roche Applied Science, Indianapolis, IN, USA) at the 5'-end and blocked with phosphate at the 3'-end.

PCR reactions were performed in a 384-well plate with a total volume of 3 μL per well in the LightCycler 480 instrument. PCR reaction mixture consisted of 20-30 ng of genomic DNA, 1 μM of forward primer, 0.5 μM of reverse primer, 0.2 μM of each HybProbe, and 0.75x of LightCycler 480 Genotyping Master mix. After an initial denaturation of 5 min at 95 ℃, 45 PCR cycles were performed with 10 s of denaturation at 95 ℃, 15 s of annealing at 55 ℃, and 20 s extension at 72 ℃. A final melting cycle was performed by raising the temperature to 95 ℃ for 2 min, lowering the temperature to 40 ℃ for 2 min and increas-

SNP199A209G
↓
5'./TATTAGGCTGTAAACGTTTTAATTACTACTACTTTTTTACTTTATGAATTTTGTGAGTAAGTTGATGATGGACC/.3'

A.
```
        3'                                              5' 3'                                        5'
        AATCCGACATTTGCAAAATTAATGATGATGAAAAA  GAAATACTTAAAACACTCATTCAACTACTACCTGG
     P/                  Sensor probe                                    Anchor probe
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B.
```
        3'                                    5'
        ATAATCCGACATTTGCAAAATTAATGATGATGAAA
     P/
```

Fig. 1. Melting curve analysis design strategy for SNP199 genotyping.
A. HybProbe. The sensor probe is labeled with LightCycler Red 670 from the 5' end, which includes the SNP site. The probe is completely complementary to the Bossier sense strand. Anchor probe is labeled with fluorescein at the 3'-end. The physical proximity of the LightCycler Red 670 and fluorescein labels results in fluorescence resonance energy transfer (FRET) when the probe is hybridized. The probe melting temperature is monitored as temperature is increased.
B. SimpleProbe. A single probe containing the SNP site is labeled with quencher and fluorescein at 5'-end. When the probe is free in solution, emission of the fluorescein dye is reduced by quencher. Once hybridized to target, quenching is reduced and probe emits a large fluorescent signal. P: phosphate, Q: fluorescence quencher, F: fluorescein, R: LightCycler Red 670.

ing the temperature to 85 ℃ with continuous fluorescent acquisition followed by a cool down to 40 ℃.

Results

Design probes for *Meloidogyne incognita* (Mi) resistance

The major Mi-resistance QTL is closely linked to SSR marker Satt358 on LG-O and the minor Mi-resistance QTL is located between Satt199 and Satt012 on LG-G (Ha et al. 2004; Li et al. 2001). In the sequence of the Satt358 flanking region, three SNPs were previously identified at sequence position 83, 91, and 190 (Ha et al. 2007). Also, two SNPs were identified at sequence position 202, and 209 in the sequence of the Satt199 flanking region.

Fig. 2. SNP genotyping of SNP358 and SNP199 by melting curve analysis with HybProbes.
A. Melting peaks for SNP358 on LG-O. The melting peak profiles of plants homozygous for the PI 96354 allele show a melting peak at 59.36 °C. The melting peak profiles of plants homozygous for the Bossier allele show a melting peak at 64.90 °C. The melting peak profiles of the heterozygous plants show melting peaks at 59.42 and 65.18 °C.
B. Melting peaks for SNP199 on LG-G. The melting peak profiles of plants homozygous for the PI 96354 allele show a melting peak at 58.97 °C. The melting peak profiles of plants homozygous for the Bossier allele show a melting peak at 62.91 °C. The melting peak profiles of the heterozygous plants show melting peaks at 59.04 and 62.98 °C.
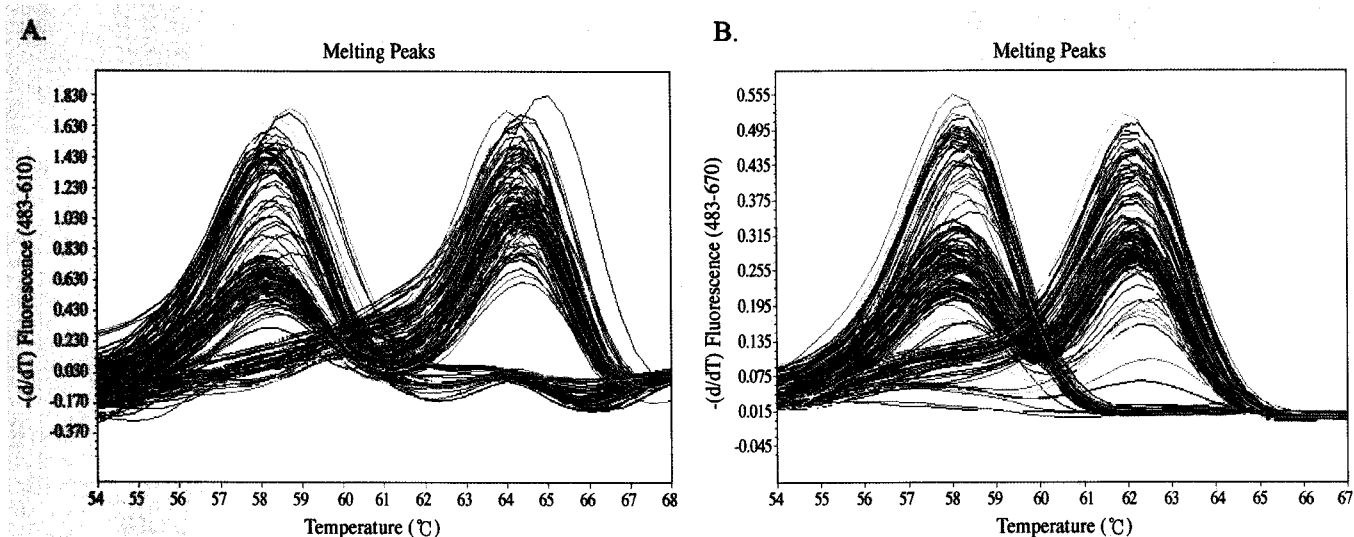
Fig. 3. Multiplex SNP genotyping by melting curve analysis for two SNP sites that are linked to the LG-O (SNP358) and LG-G (SNP199) Mi resistance QTL. A multiplex assay contains both SNP358 and SNP199 primer and HybProbe sets in a single reaction using two different fluorescence channels (610 nm and 670 nm, respectively) of the instrument. The resulting melting peaks for SNP358 (A) and SNP199 (B) are shown for 192 samples, which are two repeats of 96 samples including the two parents and 94 F2 plants from the cross of PI 96354 × Bossier.

On LG-G, one SNP (A/G) at the nucleotide position 209 was used to design probes. The placements of HybProbe and SimpleProbe on the template DNA are illustrated in Fig. 1. For the Hybprobe the 35-mer 5'-LC Red 670 labeled sensor probe formed a match with the Bossier (susceptible to Mi) DNA (SNP209G). The 3'-fluorescein-labeled anchor probe was designed with higher Tm than the sensor probe and remains annealed to the template while the sensor probe is heated through the characteristic Tm for that allele. Instead of the two probes required for HybProbe assay, a SimpleProbe assay requires only a single probe that contains the SNP site (Fig. 1). The singular probe uses a terminal self-quenching fluorescein at the 5'-end. Like the HybProbe assay, the SimpleProbe was designed to form a perfect match with the Bossier DNA.

On LG-O, two SNPs at positions 83 (T/A) and 91 (A/G) were used to design the sensor HybProbe. The 32-mer 5'-LC Red 610 labeled sensor probe formed a match with the Bossier DNA (SNP83A and SNP91G). Our initial SimpleProbe also was designed to cover the two SNP sites (SNP83A and SNP91G), but the probe did not succeed in creating a melting curve. The SimpleProbe carrying an internal label for SNP358 was redesigned to include only one SNP site (SNP91G) for the Bossier DNA (Table 1).

HybProbe assay for *Meloidogyne incognita* (Mi) resistance

The FRET HybProbes were first applied to determine the SNP genotypes of the two parents and 94 F_2 plants from the cross of PI 96354 × Bossier. The genotypes of these lines and their parents were previously characterized by non-homogeneous methods including SSR markers and a direct hybridization assay using the Luminex 100 flow cytometer (Ha et al. 2007; Li et al. 2001). Fig. 2 shows a typical result of the melting

curve analysis for SNP358 and SNP199 at the completion of 45 cycles of PCR. For SNP358 marker on LG-O the plants containing PI 96354 allele yielded melting peaks at 59.36 ℃ (S.D. = 0.17) and the plants containing Bossier allele yielded melting peaks at 64.90 ℃ (S.D. = 0.29). The sensor probe formed a mismatch with the PI 96354 sequence, which resulted in lower Tm of the probe by 5.5 ℃ than a perfectly match with the Bossier sequence. The heterozygous samples generated both peaks at 59.42 (S.D. = 0.14) and 65.18 ℃ (S.D. = 0.18). For SNP199, the plants containing the PI 96354 allele created melting peaks at 58.97 ℃ (S.D. = 0.18) and the plants containing the Bossier allele created melting peaks at 62.91 ℃ (S.D. = 0.21). Also, the sensor probe of SNP199 formed to match with the Bossier sequence, resulted in higher Tm of the probe by 3.9 ℃ than a mismatch with the PI 96354 sequence. In addition, both peaks at 59.04 (S.D. = 0.13) and 62.98 ℃ (S.D. = 0.15) were observed in heterozygous individuals. The SNP genotypes of all samples determined by the LightCycler software agreed with previously determined SSR genotypes and SNP genotypes determined on a Luminex 100 flow cytometry instrument. There were no negative results or unknown declarations, and only a single analysis was necessary. In this study, two SNP sites of sensor probe in SNP358 marker created higher ΔTm than one SNP site of sensor probe in SNP199 marker. However, the value of ΔTm did not affect the correct genotype calling.

In addition, we evaluated a multiplex assay containing both SNP358 and SNP199 primers and HybProbe sets in a single reaction using a different fluorescence channel (610 nm and 670 nm, respectively) of the instrument (Fig. 3). We ran two repeats of 96 samples containing two parents and 94 F_2 plants. A total of 384 data points (96 samples × 2 primers × 2 repeats) were obtained in a single plate. Six SNPs out of a potential of 384
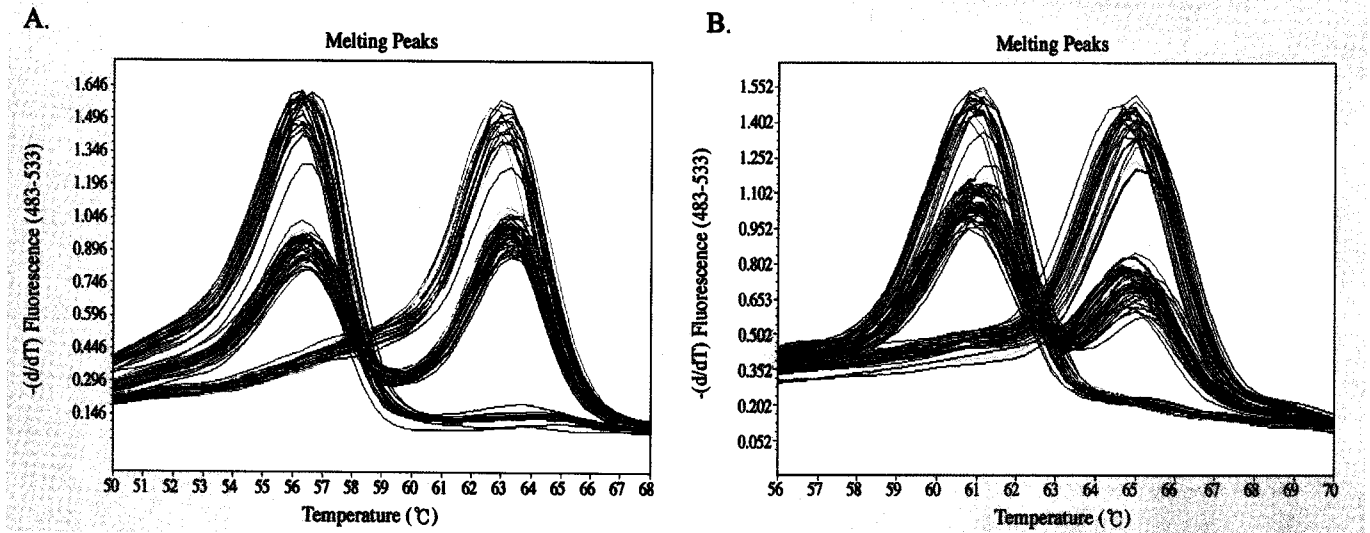
A.

B.

Fig. 4. SNP genotyping for the LG-O (SNP358) and LG-G (SNP199) Mi resistance QTL by melting curve analysis with SimpleProbes.
A. Melting peaks for SNP358 on LG-O. The melting peak profiles of plants homozygous for the PI 96354 allele show a melting peak at 56.08 °C. The melting peak profiles of plants homozygous for the Bossier allele show a melting peak at 63.12 °C. The melting peak profiles of the heterozygous plants show melting peaks at 56.28 and 63.33 °C.
B. Melting peaks for SNP199 on LG-G. The melting peak profiles of plants homozygous for the PI 96354 allele show a melting peak at 61.03 °C. The melting peak profiles of plants homozygous for the Bossier allele show a melting peak at 65.03 °C. The melting peak profiles of the heterozygous plants show melting peaks at 60.94 and 65.24 °C.

data points (1.6%) produced no data (no result) and seven SNPs out of 378 remaining SNPs (1.9%) could not be assigned genotypes with the automated allele calling software and were initially designated as "unknown". These seven SNPs were assigned genotypes manually based on the melting peaks of the parental DNAs included in the plate. Also, we evaluated the reproducibility of SNP genotype calls by comparison the repeated DNA samples. When a genotype call was made for both DNAs, the concordance was 100% for all samples.

SimpleProbe assay for *Meloidogyne incognita* (Mi) resistance

The SimpleProbes were also applied to genotype the same 94 F₂ plants from the cross of PI 96354 × Bossier. For the SNP358 marker on LG-O, the plants homozygous for PI 96354 allele showed melting peaks at 56.08 ℃ (S.D. = 0.16) and the plants homozygous for Bossier allele showed melting peaks at 63.12 ℃ (S.D. = 0.16) (Fig. 4). The SimpleProbe formed a perfectly match with the Bossier sequence, which resulted in higher *T*m of the probe by 7.0 ℃ than a mismatch with the PI 96354 sequence. The heterozygous plants yielded both peaks at 56.28 (S.D. = 0.13) and 63.33 ℃ (S.D. = 0.13). For the SNP199 marker on LG-G, the plants homozygous for PI 96354 allele created melting peaks at 61.03 ℃ (S.D. = 0.17) and the plants homozygous for Bossier allele created melting peaks at 65.03 ℃ (S.D. = 0.18). Also, the SimpleProbe of SNP199 was developed to match with the Bossier sequence, having higher *T*m of the probe by 4.0 ℃ than a mismatch with the PI 96354 sequence. In addition, both peaks at 60.94 (S.D. = 0.16) and 65.24 ℃ (S.D. = 0.18) were observed in heterozygous individuals. Although both SNP358 and SNP199 contained one SNP site in the

SimpleProbes, the SNP358 SimpleProbe created a higher Δ*T*m than the SNP199 SimpleProbe. This result suggested that the number of SNP sites in probe was not responsible for the differences in the Δ*T*m. This Δ*T*m depends on the length of the probe, the type of mismatch the neighboring nucleotides, and the position of the mismatch (Bernard et al. 1998).

The melting peak shapes and fluorescence values of SNP358 with the internally labeled SimpleProbe were not different from those of SNP199 with the 5'-end labeled SimpleProbe. For SNP358, however, the internally labeled SimpleProbe worked very well when compared with the 5'-end labeled SimpleProbe (data not shown). All the genotyping results from SimpleProbes for SNP358 and SNP199 were identical with the results of the HybProbes. However with the SimpleProbe assays only one SNP can be genotyped per reaction, which prevents multiplex assays in its current format.

HybProbe assay for resistance to frogeye leaf spot (FLS)

Table 2. Comparison of reagent costs per data point between two melting curve analyses.

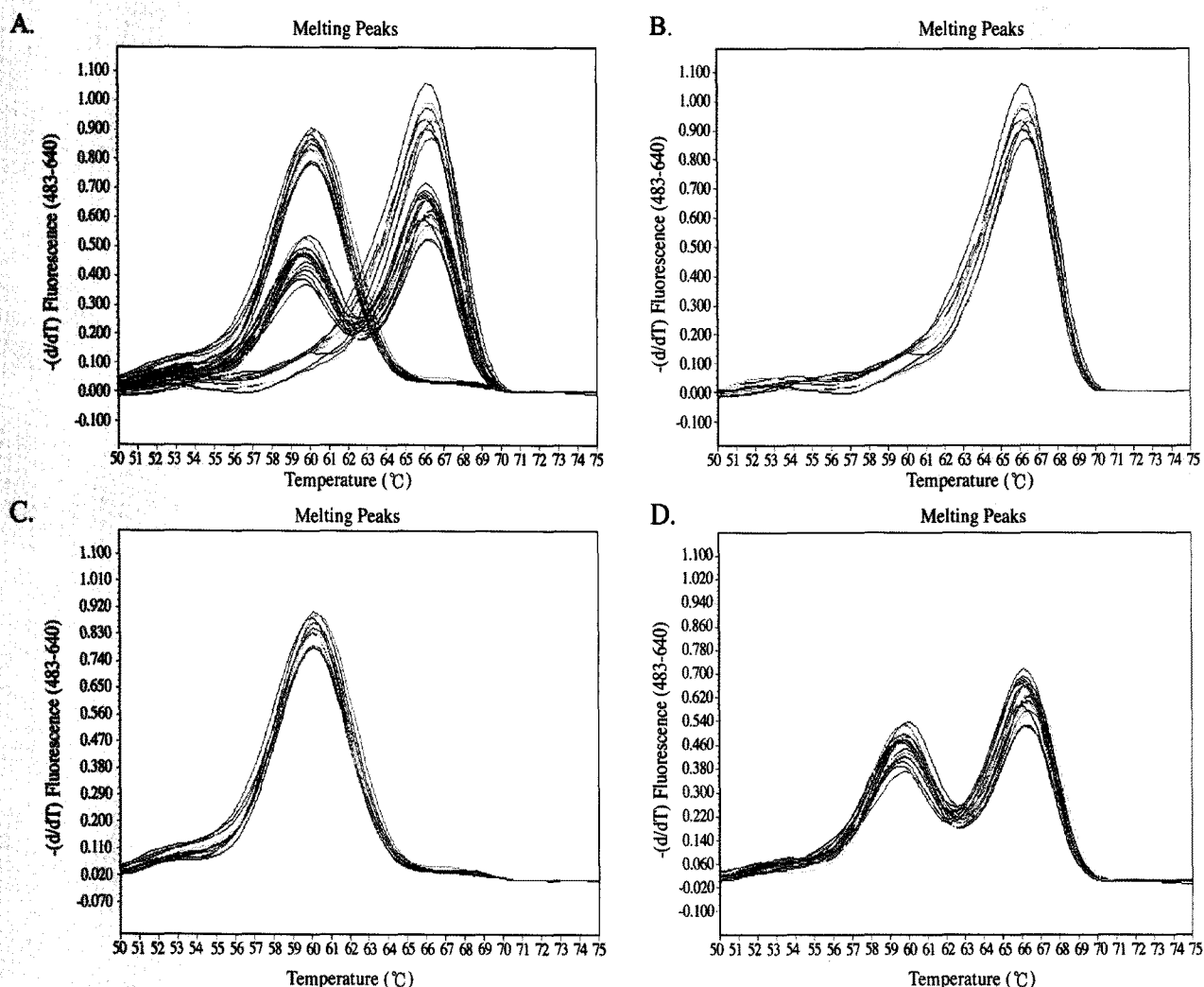| Items	Cost (USD $) / data point		
	SimpleProbe (1 SNP / reaction)	HybProbe (1 SNP / reaction)	HybProbe (2 SNPs / reaction)
PCR primers	0.002	0.002	0.002
SimpleProbe	0.017	-	-
HybProbe	-	0.025	0.025
Genotyping master mix	0.055	0.082	0.055
384 well plate	0.021	0.021	0.011
Total	0.095	0.13	0.093

Fig. 5. Melting peak profiles of 48 F₂ plants derived from Davis and Blackhawk where data from the HybProbe assay is from A) the entire population, B) those individuals that share the same "G" allele as the FLS-resistant parent Davis, C) the individuals sharing the "A" allele with the FLS-susceptible parent Blackhawk, and D) the heterozygous plants.

To develop HybProbe assay we used a SNP AZ573AG159 closely linked to *Rcs3* resistance gene. The sensor probe was formed to match with the Davis resistant allele (AZ573G159) and was labeled with the LC Red 640 fluorescent dye at the 5'-end and blocked with phosphate at the 3'-end. The HybProbes were applied to determine the SNP genotypes of 48 F₂ plants from the cross of Davis × Blackhawk. Fig. 5 shows the melting peak profiles of the HybProbe assay. The plants homozygous for Davis allele created melting peaks at 66.05 ℃ (S.D. = 0.15) and the plants homozygous for Blackhawk allele created melting peaks at 60.05 ℃ (S.D. = 0.11). Also, both peaks were observed in heterozygous individuals. The SNP genotypes obtained from the HybProbe assay showed 100% congruent with previously determined SSR genotypes and SNP genotypes determined on a Luminex 100 instrument (Missaoui et al. 2007).

Cost and time analysis

Low consumable reagent cost per data point, short assay time, and streamlined workflow are important factors for MAS since breeders usually need to assay only a few markers across thousands of individuals. Table 2 presents the comparison of reagent costs between two melting curve analysis assays with

Fig. 6. Comparison of running time among the three genotyping methods.

SimpleProbe and HybProbe chemistries. The costs were based on the 3 μL volume of PCR reagents and 30 nmol scale of probe synthesis in this study. The reagent cost for SimpleProbe assay was approximately USD $0.095/ data point and 73% of that for simplexed HybProbe assays. However, 2-plex assays of HybProbes reduced the costs of genotyping master mix and plates which resulted in a cost per data point similar to the SimpleProbe assays.

We also estimated the time requirements for completing the SNP genotyping assay (Fig. 6). There were no different time requirements between SimpleProbe and HybProbe assays. Both assays took the amount of time (~ 2 hours) for DNA amplification, melting curve analysis, and genotype calling using the LightCycler 480. However, direct hybridization assay using Luminex 100 flow cytometer platform required approxinately 4.5 h for SNP genotyping and SSR assay using ABI 377 took about 5 h to obtain complete data (Ha et al. 2007).

Discussion

In this study, we have developed and evaluated two methods that allow SNP genotyping in a single step. The methods rely on differences in melting temperatures between matched and mismatched DNA duplexes (Bennett et al. 2003; Bernard et al. 1998). Many genotyping applications using melting curve analysis have been reported in human and plant (Dufresne et al. 2004; Frances et al. 2005; Liew et al. 2006). Here, we compared HybProbe and SimpleProbe assays to identify Mi and FLS resistant and susceptible genotypes during rapid-cycle DNA amplification in soybean.

First, we designed and evaluated HybProbes and SimpleProbes to detect two SNPs sites closely linked to Mi-resistant QTL on LG-O and LG-G. The designs of all HybProbes were successful. However, the initial design of SimpleProbe covering the two SNP sites (SNP83A and SNP91G) on LG-O did not succeed to create melting curves that differentiated the genotypes. These results were consistent with the study of Murugesan et al. (2005) that tested both SimpleProbes and HybProbes to genotype approximately 100 SNPs distributed in 30 different genes in human. They reported that HybProbes have performed more consistently, reliably, and predictably than SimpleProbes. In some cases, the high strength of cross-complementarity bonds between oligos ($\Delta G > -7000$) may affect proper function of SimpleProbe. In addition, HybProbes are preferred over SimpleProbes when the SNP is located in GC-rich areas because a guanine on the reverse complementary strand in positions -1, 0, and 1 relative to the SimpleProbe label have the potential to reduce the fluorescent signal of the SimpleProbe probe (Gameau et al. 2005). However, SimpleProbe assays are easy to design because the molecular neighborhood can be neglected (Grannemann et al. 2005). Also, there are some important considerations for designing effective probes. The sequence variation can be anywhere under the sensor probe of HybProbe or SimpleProbe, but not closer than four bases from either end. A mismatch at the termi-

nal positions has a smaller effect on the ΔTm. The G:T mismatch is quite stable and results in low ΔTm (Bernard et al. 1998). Instead of designing a probe with a G or T mismatch, the C or A mismatch on the opposite strand should be selected. Another consideration is the amplicon size. An amplicon between 100 and 200 base pairs is recommended for SimpleProbe chemistry and multiplexed HybProbes (Gameau et al. 2005).

The SimpleProbes and HybProbes for SNP358 and SNP199 we developed were validated by genotyping the two parents and 94 F_2 plants from the cross of PI 96354 × Bossier. Both assays generated distinct melting peaks for homozygous resistant and homozygous susceptible plants, whereas both peak were observed in heterozygous individuals. The SNP genotypes of all samples determined by the melting curves analysis agreed with previously determined SSR genotypes and SNP genotypes determined on a Luminex 100 instrument (Ha et al. 2007; Li et al. 2001). Furthermore, the 2-plex HybProbes assay for SNP358 and SNP199 in a single reaction was analyzed against 96 samples and DNA was plated twice into separate wells in a 384-well plate (total of 192 wells), resulting in a total of 384 data points (192 wells x 2 SNPs/well). Overall 98.4% of the 384 data points succeeded in generating melting curves. The precision for automated SNP genotype calls was 98.1% and the remaining 1.9% of genotype calls were assigned a genotype manually (total of automated + manual calls = 100%). The genotype concordance between the duplicate DNA samples was 100%. A study of β-globin genotypes with the multiplex FRET hybridization assay on the LightCycler had a 99% of PCR success rate a genotype call drop-out rate around 1% for all loci (Vrettou et al. 2004). Also, multiplexed melting temperature analysis showed 100% sensitivity and specificity for genotyping of β-globin mutations (Lin et al. 2004). Another homogeneous TaqMan assay showed 98% successful data points and 94% accuracy in soybean (Meksem et al. 2001). Giancola et al. (2006) reported an average of 98.9% successful data points and an average of 99.8% concordance between two replicates for three SNPs in *Arabidopsis thaliana*.

The cost of SNP genotyping using SimpleProbe was approximately USD $0.095 per data point, while the cost for simplex HybProbe assay was USD $0.13 in our study. Genotyping of SNPs by multiplexed assays reduces the time, cost, and amount of DNA template. This multiplex genotyping technique on the LightCycler 480 is currently possible for up to three SNPs in a single well. In our study, two pairs of PCR primers were used to amplify the target DNA template and two pairs of probes were simultaneously used to hybridize to their PCR products (2-plex HybProbe assay). Melting curve analysis was carried out and genotypes were determined. Therefore, the reagent cost for 2-plex HybProbes was USD $0.093 per data point which resulted in the lowest reagent cost of the three methods (Table 2).

The cost for HybProbe and SimpleProbe assays was less than the Luminex 100 assay (USD $0.15) or SSR assay (USD $0.20) (Ha et al. 2007). Furthermore, the 384-well plate of the LightCycler 480 allows simultaneous amplification and analysis in ~2 hours without any work after amplification. In compari-

son, the direct hybridization assay using Luminex 100 required hybridization and instrument running steps and the SSR assay also required an electrophoresis step after amplification. The closed tube assay with the LightCycler 480 reduced post-amplification steps and provides an automated platform.

Although real-time thermocyclers are more expensive than conventional thermocyclers and development of effective fluorescent probes requires some optimization in the creation of new assays, melting curve analysis has some distinct advantages for MAS application. These include: i) elimination of time-consuming post-PCR steps; ii) reduction of the potential contamination of regents with PCR products; iii) simplicity; and iv) enablement of streamlined workflow (Mamotte 2006).

In this report we describe SNP genotyping assays based on melting curve analysis for the identification of two QTL for resistance to southern root-knot nematode and the *Rcs3* gene for resistance to FLS. These closed tube assays provide rapid SNP genotyping that are highly repeatable and precise for use in MAS in soybean.

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