The Application of High Resolution Melting in the Analysis of Simple Sequence Repeat and Single Nucleotide Polymorphism Markers in a Pea (*Pisum sativum* L.) Population

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Abstract

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The aim of this study was to verify the high resolution melting (HRM) method in the analysis of single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) markers in pea (*Pisum sativum* L.). A recombinant inbred line population, Carneval × MP1401, was tested for three SNP and 103 SSR markers. HRM analysis was conducted on a LightScanner 96 instrument with LC Green dye. The melting curve shape permitted two polymorphic genotypes to be distinguished. The results were confirmed by gel electrophoresis. Three SSR markers were sequenced and analysed by the melting prediction software. The results confirmed the presence of one polymerase chain reaction (PCR) product with two melting domains. Sequence tagged site (STS) markers produced specific products: Psat_EST_00189_01_1 (300 bp), Pis_GEN_18_2_1 (400 bp), Pis_GEN_7_1-2_1 (600 bp). Amplicons contained one, four and seven single nucleotide polymorphisms, respectively. Melting curve differences enabled the population genotyping except for Psat_EST_00189_01_1 where resolution was too low. Primers for Psat_EST_00189_01_1 were redesigned to obtain a shorter (100 bp) PCR product which increased the resolution. The number of SNPs and amplicon length are crucial for HRM resolution. The HRM method is fast and has a high throughput. The melting analysis of 96 samples takes less than 10 min. Agarose gel analysis confirmed the reliability of HRM, which eliminates laborious post-PCR analysis.

Keywords: genotyping; high resolution melting; molecular markers; *Pisum sativum* L.

DNA analysis via high resolution melting (HRM) is a relatively new technique. It is useful for genotyping, mutation scanning and sequence matching. HRM is based on the properties of fluorescent dyes which bind only to double-stranded DNA. These dyes emit strong fluorescence when they bind to DNA. When the sample is heated, DNA slowly denatures and releases the fluorescent dye, which causes the fluorescence to decrease. When the temperature reaches the melting point of the fragment being analysed, a rapid decrease in fluorescence is observed. HRM instruments measure the fluorescence during the entire heating time to generate a melting curve. The melting temperature of a DNA duplex depends on its length and sequence; thus, different sequences generate different melting curves (Reed *et al.* 2007). The new generation of saturation dyes (LCGreen®, EvaGreen®) allows detection of SNP polymorphism and small deletions or insertions. These dyes enable high saturation of double-stranded DNA, which is crucial for obtaining high resolution. The utility of the HRM technique has been confirmed by genotyping various plant species, such as *Medicago sativa* L. (Han *et al.* 2012), *Zea mays* L. (Yu *et al.* 2011), *Oryza sativa* L. (Li *et al.* 2011), *Lupinus albus* L. (Croxford *et al.* 2008), *Hordeum vulgare* L. (Hofinger *et al.* 2009) and *Brassica rapa* L. (Lochlainn *et al.* 2011). HRM is an alternative to the time consuming and laborious gel techniques; however, in the field of plant genetics it still requires further testing and improvement. There are few reports of simple sequence repeat (SSR) markers analysed by HRM.

The aim of this study was to assess if HRM is suitable for single nucleotide polymorphisms (SNP) and SSR marker analysis in pea (*Pisum sativum L.*). The recombinant inbred line (RIL) population Carneval × MP1401 was tested for several SNP and SSR markers.

MATERIAL AND METHODS

Samples of the seeds of the Carneval × MP1401 population were provided by Dr B. Tar'an and Dr T. Warkentin from the University of Saskatchewan, Canada. Both parents of the population are semi-leafless and have short internodes. The plants of 88 F_7 inbred lines were analysed (Tar'an *et al*. 2003).

Primer pairs for SNP markers were developed in the framework of the 6th EU FP Grain Legumes Integrated Project (GLIP). The primer information is available on the following website: http://bioweb.abc.hu/cgi-mt/pisprim/pisprim.pl. One pair of primers was redesigned using the LightScanner Primer Design software (Idaho Technology, Salt Lake City, USA). The Pea Microsatellite Consortium, set up by Agrogene, France, developed primer pairs for the SSR markers. Most of the markers tested in this study have been used for mapping (Loridon *et al*. 2005).

The SNP reaction was performed as follows: 0.5× LCGreen dye (Idaho Technology), 1× buffer GoTaq flexi (Promega, Madison, USA), 1.5mM $MgCl_2$ (Promega), 1mM dNTP (ThermoFisher Scientific, Waltham, USA), primer 1 (1μM), primer 2 (1μM), 0.6 U GoTaq polymerase (Promega), 100 ng/μl BSA (Sigma-Aldrich, St. Louis, USA), 25 ng DNA. The touchdown PCR profile was: 16 × (95°C (3 min), 60°C (60 s), 72°C (60 s)) – the annealing temperature was reduced by 2°C every two cycles until 46°C was reached; then 19× (94°C (30 s), 45°C (60 s), 72°C (60 s)); 72°C (4 min); hold at 10°C.

The SSR reaction was performed as follows: 0.5× LCGreen dye (Idaho technology), 1× buffer GoTaq flexi (Promega), 1.5mM $MgCl_2$ (Promega); 0.6mM dNTP (ThermoFisher Scientific); primer 1 (0.2μM); primer 2 (0.2μM); 0.6 U GoTaq polymerase (Promega), 25 ng DNA. PCR profile: 95°C (3 min); 35× (95°C (30s), required T_M (60 s), 72°C (60 s)); and 72°C (5 min). HRM analysis was conducted on a LightScanner 96 instrument (Idaho Technology). Melting profiles were tested by uMELT, the online software for melting curve predictions (Dwight *et al*. 2011).

Restriction enzymes able to distinguish between two genotypes were identified using the dCAPS 2.0 software (Neff *et al*. 2002). The digestion with restriction enzymes was performed as follows: 0.5 U restriction enzyme – *Vsp*I, *Hin*1II or *Cfr13*I (ThermoFisher Scientific), 1× buffer – Orange, Green or Tango, respectively (ThermoFisher Scientific), 5 μl PCR reaction mixture. Temperature profile: 37°C (1 h), 65°C (20 min).

RESULTS AND DISCUSSION

The HRM analysis requires an appropriately optimized PCR reaction. Specificity is more important than efficiency in such analyses. One hundred and three SSR and three STS markers were tested. Product quality was tested by agarose gel electrophoresis. Only clean amplicons were subsequently tested by HRM. Yu *et al*. (2011) used polyacrylamide gel electrophoresis to identify clean SSR markers in maize for further HRM analysis. In the present study, clean products for 65 SSR primers were obtained. Fourteen of them were polymorphic in the parental lines and were tested in the mapping population (Figure 1). The PSMPSAA72 marker was additionally tested via gel electrophoresis to confirm the HRM output. The HRM results were consistent with the agarose gel results (Figure 2). Distefano *et al*. (2012) used HRM to analyse SSR markers in *Citrus*. They confirmed the HRM outputs by capillary electrophoresis. HRM identified more genotypes than capillary electrophoresis because of SNP polymorphisms within monomorphic SSR markers. We identified samples that did not match to any of the parental genotypes and were marked as unknown (Figure 1d–g). We hypothesize that these amplicons contain additional mutations. The HRM analysis of markers PSMPSAA72, PSMPSAA153, PSMPSAA170 and PSMPSAD135 resulted in melting curves with two regions of rapid fluorescence decrease. These regions can be presented as melting peaks in a different kind of graph. Such a shape suggests the presence of two PCR products with different melting temperatures in the sample. This observation was inconsistent with the gel electrophoresis, which resulted in one clean band for each marker. We hypothesised that the sequences of these markers comprised two melting domains: an AT rich domain with a lower T_M and a GC rich domain with a higher T_M. Such a possibility has been described in literature for fragments of eukaryotic translation initiation factor 4E (Hofinger *et al*. 2009). Markers PSMPSAA72, PSMPSAA153 and PSMPSAD135 were sequenced to test this hypothesis. The obtained sequences were tested by uMELT for melting curve predictions (Dwight *et al*. 2011). The results of the HRM analysis matched those predicted by uMELT,

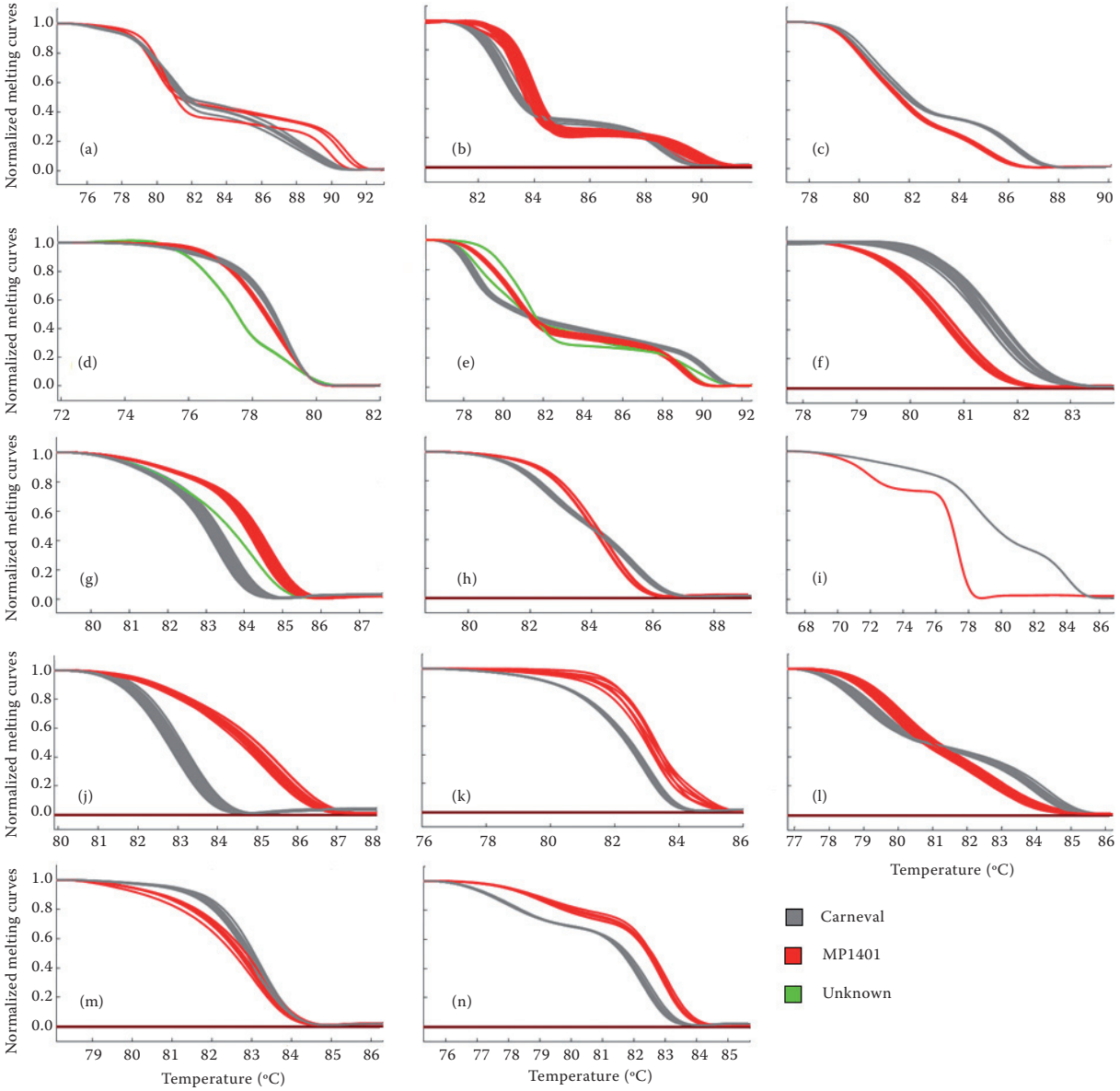

Figure 1. Melting curves of simple sequence repeat (SSR) markers (parental and population samples): (a) – PSMPSAA72, (b) – PSMPSAA153, (c) – SMPSAA170, (d) – PSMPSAD61, (e) – PSMPSAD135, (f) – PSMPSAB30, (g) – PSMPSAB47, (h) – PSMPSAA278, (i) – PSMPSAA238, (j) – PSMPSAA355, (k) – PSMPSAA179, (l) – PSMPSAC21, (m) – PSMPSAC27, (n) – PSMPSAD135; grey lines represent the Carneval genotype, red lines represent the MP1401 genotype and green lines represent unknown samples (samples that did not match the parental genotypes)

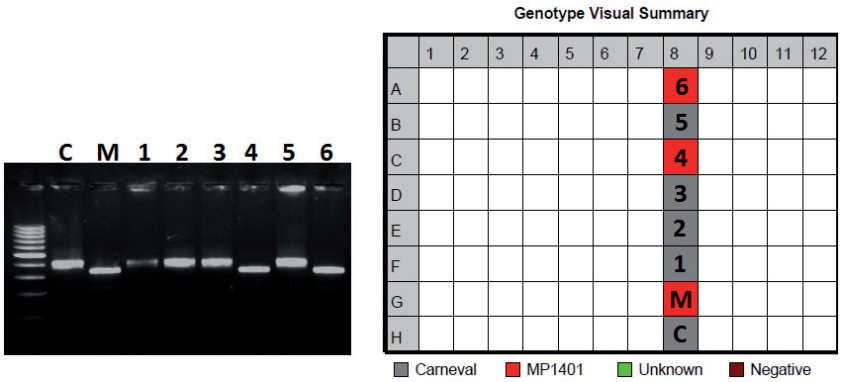

Figure 2. High resolution melting (HRM) analysis of marker PSMPSAA72 confirmed by gel electrophoresis; C – Carneval; M – MP1401; 1 to 6 – population samples; Unknown is a sample that did not match the parental genotypes; Negative is a sample that did not amplify

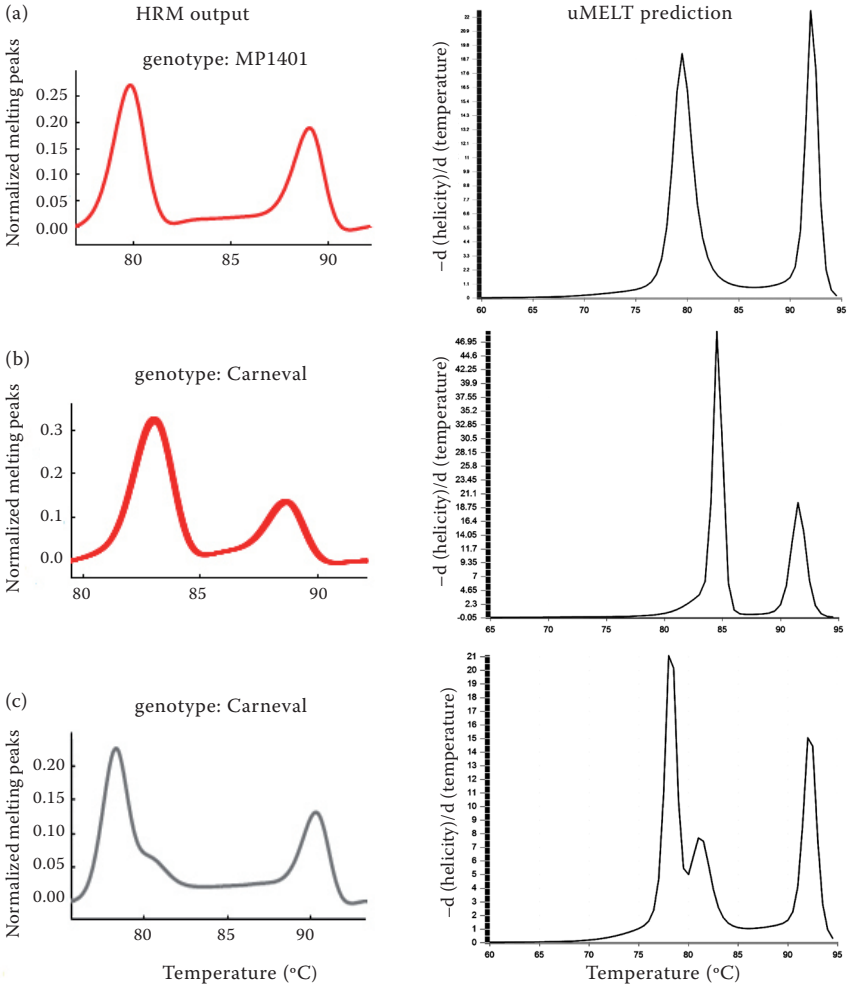

Figure 3. High resolution melting (HRM) analysis of simple sequence repeat (SSR) markers (melting peaks) and melting prediction results by uMELT: (a) – PSMPSAA72, (b) – PSMPSAA153, (c) – PSMPSAD135

which confirmed the presence of two melting domains within the analysed amplicons (Figure 3).

Three STS markers (Psat_EST_00189_01_1, Pis_GEN_18_2_1, Pis_GEN_7_1-2_1) were tested in the parental lines. They produced specific 300, 400 and 600 bp products, respectively. Sequencing of the amplicons revealed single nucleotide polymorphisms (Table 1). The STS markers were tested in the mapping popula-

Table 1. Detected single nucleotide polymorphisms within sequence tagged site markers and restriction enzymes used for digestion

Marker name	Amplicon length (bp)	SNP position	Genotype		Cutting enzyme
			Carneval	MP1401	
Psat_EST_00189_01_1	300	103	G	A	*Hin*1II
Pis_GEN_18_2_1	400	142	A	G	
		207	T	C	*Vsp*I
		214	G	A	
Pis_GEN_7_1-2_1	600	127	A	G	
		136	A	T	
		146	C	T	
		365	T	C	*Cfr*13I
		466	G	T	
		500	C	G	
		508	G	C	

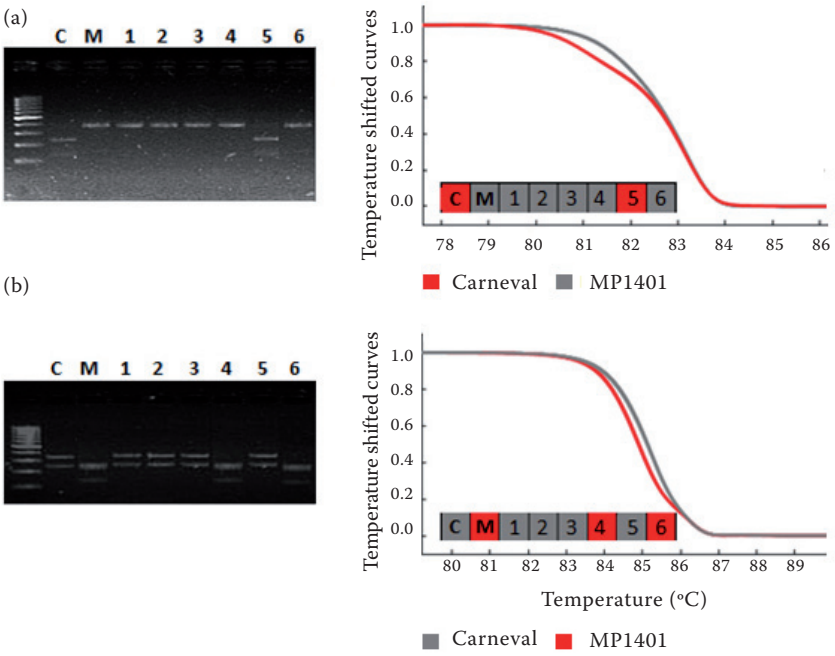

Figure 4. Sequence tagged site (STS) markers genotyping – comparison between cleaved amplified polymorphic sequence (CAPS) analysis and high resolution melting (HRM): (a) – Pis_GEN_18_2_1; (b) – Pis_GEN_7_1-2_1; (c) – Carneval; M – MP1401; 1 to 6 – population samples

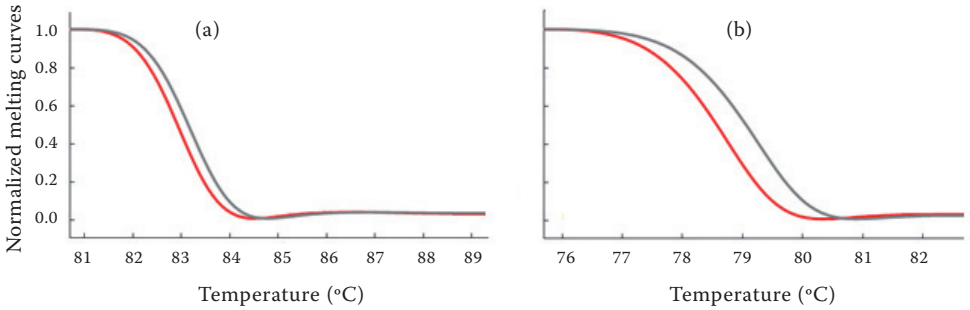

Figure 5. High resolution melting (HRM) analysis of sequence tagged site (STS) marker Psat_EST_00189_01_1 in the parental lines; (a) – 300 bp amplicon; (b) – 100 bp amplicon

tion via digestion with restriction enzymes. Genotypes were easily distinguishable after gel electrophoresis, except for Psat_EST_00189_01_1. The markers were then tested by the HRM method in the parental lines and in six population samples. The HRM outcome matched the electrophoresis results (Figure 4). It was impossible to genotype the Psat_EST_00189_01_1 marker based on the melting curve because of the very small difference between the two variants. Probably, the amplicon for this marker was too long (300 bp with only one SNP in comparison with 400 bp with three SNPs for Pis_GEN_18_2_1 and 600 bp with seven SNPs for Pis_GEN_7_1-2_1). According to Hofinger *et al.* (2009), a higher number of SNPs within an amplicon increases the resolution. Primers were redesigned to obtain a shorter PCR product (approximately 100 bp) (LightScanner Primer Design Software, Idaho Technology, Salt Lake City, USA). The difference in the melting curves of the parental genotypes was much more pronounced and allowed the correct genotyping of the mapping population. The analysis of SNP within the shorter amplicon resulted in better resolution (Figure 5).

CONCLUSIONS

The HRM method has many advantages. It is very fast and has a high throughput: it can be performed in less than 10 min. There is no need for time-consuming gel electrophoresis following the PCR reaction. It is also accurate and repeatable (Han *et al.* 2012). HRM is also reliable: we confirmed our results using agarose gel electrophoresis. Distefano *et al.* (2012) and Li *et al.* (2011) also confirmed the reliability of HRM. This method is safer for researchers than gel techniques, because there is no need to use toxic acrylamide or mutagenic ethidium bromide, which is the most common dye used for agarose gel electrophoresis. HRM eliminates the need for amplicon sequencing and the purchase of the restriction enzyme sets to visualize SNP polymorphisms. It is also possible to analyse SNPs that do not form restriction sites. SNP analysis techniques are developing very rapidly. Genotyping by next-generation sequencing (NGS) is being increasingly adopted for discovery applications (Semagn *et al.* 2014). In this case SNP identification

requires a reference genome which is not available for all species (including pea). Van *et al*. (2013) overcame this constraint by sequencing genomes of two cultivars. New-generation sequencing is a very effective approach to identify a large number of new SNPs, but it generates huge amounts of data which need further processing. This technique is not suitable for the analysis of many samples. An alternative to NGS is Kompetitive Allele Specific PCR (KASP). This technique has a broad range of applications (quality control, QTL mapping, allele mining). KASP is a uniplex technology based on allele specific oligo extension and fluorescence resonance energy transfer for signal generation (Semagn *et al*. 2014). This approach requires the knowledge of the DNA sequence to design primers for known SNP. HRM does not provide the information about sequence but it enables discovery of new polymorphisms. We successfully analysed a 600 bp amplicon containing seven SNPs; however, it was impossible to genotype a 300 bp amplicon containing one SNP. More SNPs within an amplicon increased the HRM resolution, which is consistent with the results of Hofinger *et al*. (2009). Conversion of 300 bp amplicon into 100 bp product containing one SNP increased the resolution of HRM. There are few references regarding the analysis of SSR markers by HRM in plant species (Li *et al*. 2011; Yu *et al*. 2011; Distefano *et al*. 2012). In this study we confirmed the utility of HRM for SSR markers in genotyping of a pea RIL population. We also proved the presence of the two melting domains within one amplicon for three analysed SSR markers (PSMPSAA72, PSMPSAA153 and PSMPSAD135), which was reported by Hofinger *et al*. (2009) for STS markers.

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