African Journal of Biotechnology Vol. 11(27), pp. 7016-7027, 3 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3810
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Application of high-resolution melting for variant scanning in chloroplast gene *atpB* and *atpB-rbcL* intergenic spacer region of Crucifer species

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Accepted 26 January, 2012

High-resolution melting (HRM) analysis is a rapid and sensitive method for single nucleotide polymorphism (SNP) analysis. In this study, a novel HRM assay was carried out to detect SNPs in the chloroplast gene *atpB* which encodes the beta subunit of the ATP synthase and *atpB* upstream intergenic region. The polymorphisms of the two fragments in intertribal samples from the Cruciferae family and within the species of *Brassica napus* were detected. Based on this results, we found that HRM were able to determine over 90% of the variants which included single or multiple variants and insertion-deletion polymorphisms (INDELs) and rendered possible genotyping of more closely spaced polymorphisms, although there were several false positives (FPs) and misclassification. Six haplotypes were identified in the intertribal materials. The analysis of 90 *B. napus* found five variation types and the variations were all located in the intergenic region. In conclusion, HRM analysis is a closed tube assay that is easy to perform and is a more effective approach to identify variant of chloroplast genes. This study will facilitate further functional investigations into the role of chloroplast genes in photosynthesis, phylogeny and molecular evolution.

Key words: *atpB* gene, chloroplast genome, crucifer, high-resolution melt curve analysis, SNP, INDEL.

INTRODUCTION

The chloroplast genome (cpDNA) in plants is highly conserved in size and structure, as well as gene content and linear order of genes among angiosperms (Biss et al., 2003). The lack of heteroplasmy and recombination has made it an attractive tool for phylogenetic, phylogeographic, and population genetic studies. Crucifer species exhibit a high level of genetic and phenotypic diversity (Martin et al., 2002). Therefore, a variety of molecular markers have been developed for the construction of cpDNA linkage maps in Cruciferae, including restriction fragment length polymorphism (RFLP) (Palmer et al., 1983), polymerase chain reaction-RFLP (PCR-RFLP) and chloroplast simple sequence repeat (cpSSR) (Allender et al., 2007). However, the density of the markers that have been detected is not sufficient for analysis of lower taxonomic levels. Furthermore, these methods are labour-intensive and require sophisticated technology. The most abundant form of genetic variation, single nucleotide polymorphism (SNP), may resolve this problem (Hess et al., 2000). The use of SNPs is expected to lead to a better understanding of the genetic basis for complex characteristics, such as plant productivity, development, and adaptation to stress. Therefore, this approach could be essential for genetic improvement programmes. Recently, many methods have been developed to genotype SNPs. High-resolution melting

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Abbreviations: HRM, High-resolution melting; **SNP,** single nucleotide polymorphism; **INDELs,** insertion-deletion polymorphisms; **FPs,** false positives.

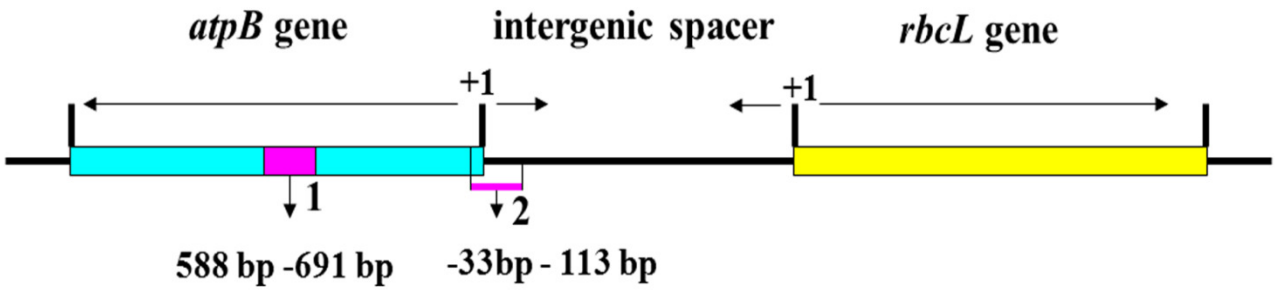

Figure 1. Regions of *atpB* gene investigated with HRM analysis. The first region (1) was in *atpB* gene at positions 588 bp to 691 bp and the second fragment (2) was mainly in the intergenic region between *atpB* and *rbcL* genes, upstream of *atpB* gene from -113 bp to 33 bp. The two regions are indicated by solid arrowheads, and the sequences of the primers used for PCR amplification are described in Table 1. Concerning the coding DNA reference sequence, nucleotide numbering uses the A of the ATG translation initiation start site as nucleotide +1; the GenBank accession numbers were provided when available.

curve (HRM) analysis is a new method that has been identified as a powerful, rapid, and simple way for genotyping and detection of polymorphisms and mutations (De Leeneer et al., 2008). HRM analysis measures the dissociation of double-stranded DNA from a PCR product amplified in the presence of a saturating fluorescence dye which enables differentiation of PCR products based on their dissociation behavior as they are subjected to increasing temperatures (Studer et al., 2009). It has been widely used to genotype plant and human nuclear genes (De Koeyer et al., 2010). However, there are few reports describing the scanning of chloroplast (cp) genes. Our lab has used HRM analysis to search for SNPs and insertion-deletion polymorphisms (INDELs) of the cp gene *accD*. We found that the detection efficiency depends on variation degree of the gene in the population detected (has been submitted to another journal). Therefore, the potential for use of HRM analysis to scan cp gene variants of intergenic region is considerable.

Studies indicated that (cp) gene *atpB* located in the large single-copy region of the plastid genome (Hu et al., 2011) and it codes for the beta subunit of the ATP synthase (Zurawski et al., 1982). Moreover, the *atpB* gene and intergenic spacer region between *rbcL* genes have been used successfully in phylogenetic studies at higher taxonomic levels (Hoot et al., 1999).

However, little attention has been given to develop plastid (chloroplast) SNP markers for *Brassica* and its close relatives. In our study, we used HRM analysis to detect the *atpB* gene variations among different taxa of Cruciferae and intraspecies of *B. napus*. Our results demonstrated six haplotypes within the detected fragment at intertribal level and detected five variation types within *B. napus* species. This study also demonstrated that HRM analysis is an effective approach to identify variants of cp gene.

METHODS AND MATERIALS

Plant materials

Two sets of plant specimens were sampled to detect the *atpB* gene variation in the cp genome at different taxonomic levels. The first set was composed of 48 representative accessions from 13 species and 7 genera of the tribe Brassiceae and Arabideae (Electronic Supplementary Material S 1). This set was used to detect the *atpB* variance at the intertribal, intergeneric and interspecific levels. The second set sampled 90 accessions of cultivars of *B. napus* (Electronic Supplementary Material S 2) to evaluate the ability of HRM analysis for detecting intraspecific variation.

DNA extraction

The fresh young leaves of each accession were collected for extracting total DNA using a previously reported method (Guillemaut and Maréchal-Drouard, 1992). DNA samples with absorbance ratios A260/A280 of about 2.0 were used in this experiment. Pure DNA samples were diluted to 50 ng μL^{-1} and stored at -20°C until needed.

PCR analysis

The primers were designed according to the HRM analysis protocol. The sequence of GenBank GQ861354 was the reference sequence. Concerning the coding DNA reference sequence, nucleotide numbering uses the A of the ATG translation initiation start site as nucleotide +1; the GenBank accession numbers were provided when available.

Two regions were chosen for the HRM assay. The first region was within the *atpB* gene (588 bp to 691 bp) and the second fragment was the intergenic spacer between *atpB* and *rbcL* genes. There was at least one SNP existing in the two regions at the intraspecies level for *B. napus*, and more SNPs were found at a higher taxonomic level. When designing primers, we chose all the primer pairs with the annealing temperature in common. As a result, we can amplify different fragment in one plate under the same PCR conditions. The primer sequences and their description are reported in Figure 1 and Table 1.

PCR was performed in a total volume of 20 µL using Biomed 2×

Table 1. Primers used for HRM analysis of the *atpB* gene.

Amplicon	Primer sequence (5'–3')	Region (bp)	Location	Expectedsize (bp)	GC%
1	F：TATCCGTATTTGGTGGAGTAGG R：GGAGTCCGCAAGGTTTAGTT	588 – 691	*atpB* gene	103	51
2	F：CCAATGAAATCGAGTGCTTACT R：GCTGGATCCGAAGTAGTAGG	-113 – 33	Intergenic region	148	50

Oligonucleotides are oriented 5'–3', bp = base pairs.

Taq Master Mix. The reaction mixture contained 50 ng of genomic DNA, 2 µL LC Green (Idaho Technology, Inc, UT, USA) (Studer et al., 2009), and 0.5 mmol L^{-1} of each primer. Finally, 10 µL mineral oil (Bio-Rad) was added to avoid evaporation of the PCR mix during DNA amplification. PCRs were performed in a PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with a cycling program consisting of 5 min of initial denaturation at 95℃ and 30 cycles of 1 min at 94℃, 1 min at 54℃ and 1 min at 72℃ and a final extension of 10 min at 72℃.

HRM analysis

After amplification, the plates were quickly centrifuged and imaged in a 96-well LightScanner (Idaho Technology, Hi-Res Melting™ system 96-well plate format, Inc., Salt Lake City, Utah，USA). Melting curves were generated by ramping the temperature from 55℃ to 95℃ at 0.1℃/s using the "Scanning" mode and expert scanning analysis (Montgomery et al., 2007). The reference samples were set as a baseline. In the analysis of the 48 different samples, 5 samples including the *B. napus* sample Chikuzen, *Eruca* sample Cilezhahong, *B. rapa* samples Baichengbaiyoucai, Chuanlingyoucai, and Quxu, whose sequences were the same as GenBank GQ861354, were chosen as reference samples. In the 90 *B. napus*, the samples SavariA, Hongyou-3, and Bounty were used for reference, and their sequences were 100% identical to GenBank GQ861354.

Negative PCR products were verified and eliminated before normalisation was performed. The normalised melting curves were temperature-overlaid (to eliminate slight temperature errors between runs) by selecting a fluorescence range (low fluorescence, high temperature, typically 5 to10% fluorescence) and shifting each curve along the *x*-axis to better overlay a standard sample within this range. Then, the derivative ($-dF/dT$) of the fluorescence signal was plotted against the temperature to show the melting peak, and a difference plot was generated by subtracting the curves from a reference curve to group samples with similar melting curves (Muleo et al., 2009).

Sequence analyses

Genotype homogeneity within clusters was confirmed by sequencing any samples on the edge of apparent clusters. Sequencing was performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China). Alignment of the sequences with their DNA homologues and DNA phylogenic clustering were carried out using the Vector NTI suite 9.0 software package (Invitrogen, Carlsbad, CA, USA).

RESULTS

Selection of the scanning region and primer detection

The positions of the two regions detected are shown in Figure 1. The first fragment contained two SNPs at positions 619 bp and 651 bp, respectively (Figure 2a). The intergenic fragment contained one INDEL at -59 bp (Figure 2b). The GC content of the two fragments was 51 and 50%, respectively (Table 1). To verify the suitability of the primer for different tribe samples, we first detected the PCR product by agarose gel electrophoresis; all the *atpB* products were of the expected size, and no undesired products or dimer products were detected. However, two samples (one *B. napus* and one *Sinapis*) did not amplify any product within the intergenic fragment (data not shown).

HRM analysis of the cp gene *atpB* and the intergenic region at different taxonomic levels of Crucifer species

In order to perform a thorough analytical evaluation of various amplicons, we initially examined the two primer sets. We used HRM to detect variants among a panel of 48 materials from different taxonomic samples as listed in Electronic Supplementary Material S 1. All the accessions were plotted against temperature, and the reference samples (subtracted from themselves) were set at zero. Different melting profiles were distinguishable among the species based on the peak position and the melting temperature (Figure 3).

An *atpB* intergenic fragment 148 bp in length (Fragment 2 in Figure 1) was scanned by HRM to identify polymorphic variants in the seven different genera. The melting curves obtained clearly differentiated the 48 samples into 5 groups and indicated the presence of SNPs in the fragment (Figure 3). The *B. rapa* accession Jiningtianjinlv had an adenine (A) insertion at -59 bp and an A deletion at -68 bp (MC1 group), *A. thaliana* Tri had multiple SNPs, and the *B. rapa* sample Taicai had no variants that were grouped together (MC1 group). At

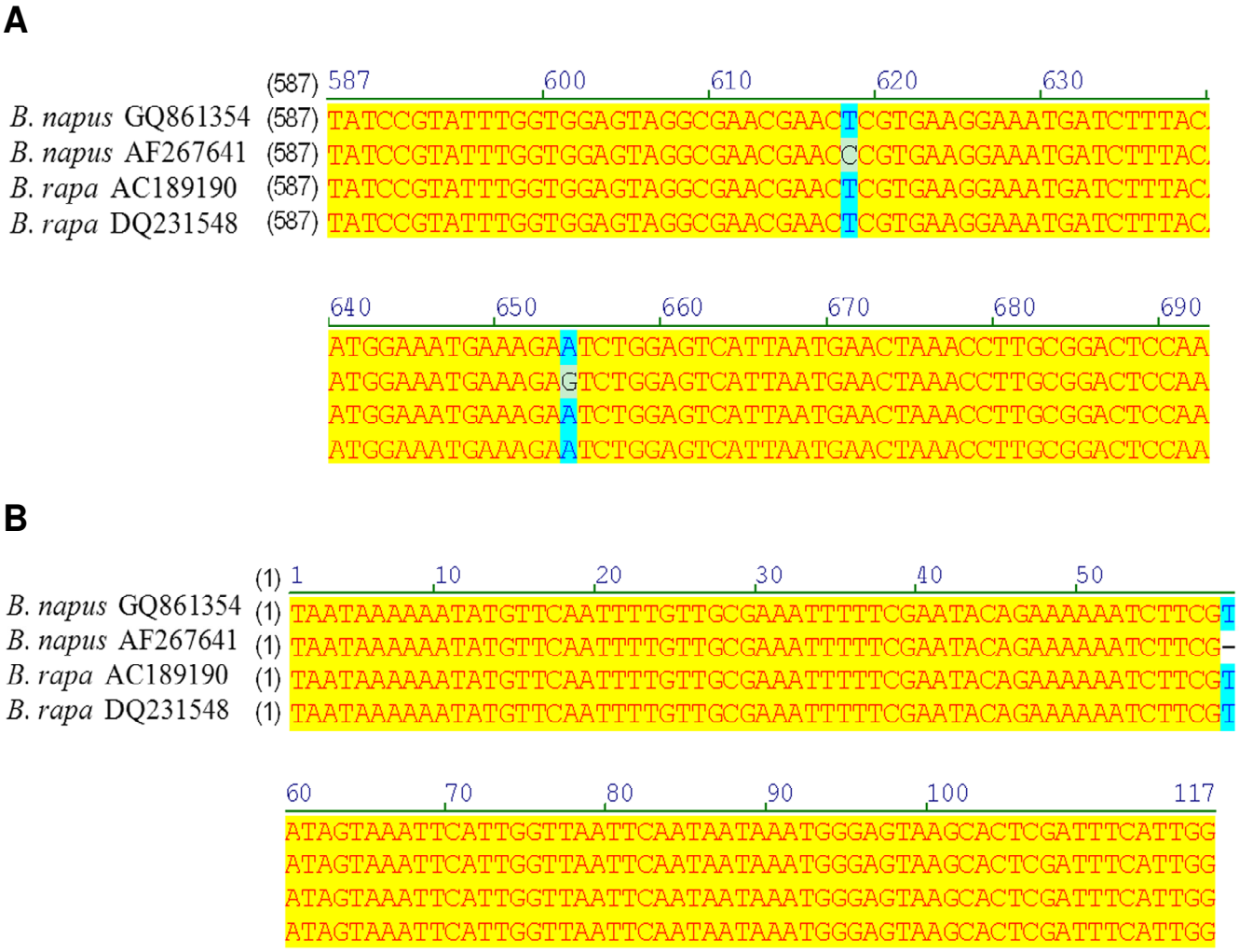

Figure 2. Alignment of the two fragments internal to the primers. (a) Alignment of the fragment of *atpB* gene. (b) Alignment of the intergenic fragment.

higher taxa, the Hancai species of the genus *Rorippa indica* had a guanine (G) to thymine (T) substitution at -16 bp and a T Insertion at -10 bp, and the *A. thaliana* sample Kas had 4 SNPs clustered in one group (MC2 group). *A. thaliana* had the most SNPs (4), indicating that the variation was more complex at higher taxonomic levels. The *B. nigra* sample Henjie with C to T substitution generated an MC3 curve, and the *B. napus* species Zhongshuang 4 with one SNP T to G substitution produced an MC4 curve. The results indicate that the intergenic region varied between the most closely related species, such as *B. napus* samples Zhongshuang 4 and H47 (accessions from Russia). At the same time, the results demonstrated that single base changes can be identified by high-resolution melting. The other samples were all grouped together with the reference samples (reference type, Figure 3a), indicating that there were no variants (Table 2, Figure 3a).

A 103 bp fragment of *atpB* gene was analyzed, and the results showed that there were fewer variants within the gene than in the intergenic space. The variants were partitioned into 7 groups (reference type, H1, H2, H3, H4, H5, and H6 groups) and 8 accessions that potentially contained variations were selected by HRM analysis (Figure 3b). The sequencing results showed 5 groups containing SNPs except two groups containing 4 false positives (FPs) (Table 2, Figure 3b). That is to say, only four samples contained variation that is at a higher taxonomic level – *R. indica* Hancai, *A. thaliana* accessions Tri and Kas, and *E. sativa* Shanxiyunjie.

The above results demonstrated that we obtained six haplotypes (Table 2). The two *A. thaliana* materials belonged to Haplotype “TH 2”; “TH 6” contained one *E. sativa* sample; “TH 3” included one *Rorippa* sample; and the 3 out of 36 *Brassica* samples belonged to “TH 1”, “TH4”, and “TH 5” Haplotypes, respectively (Table 2).

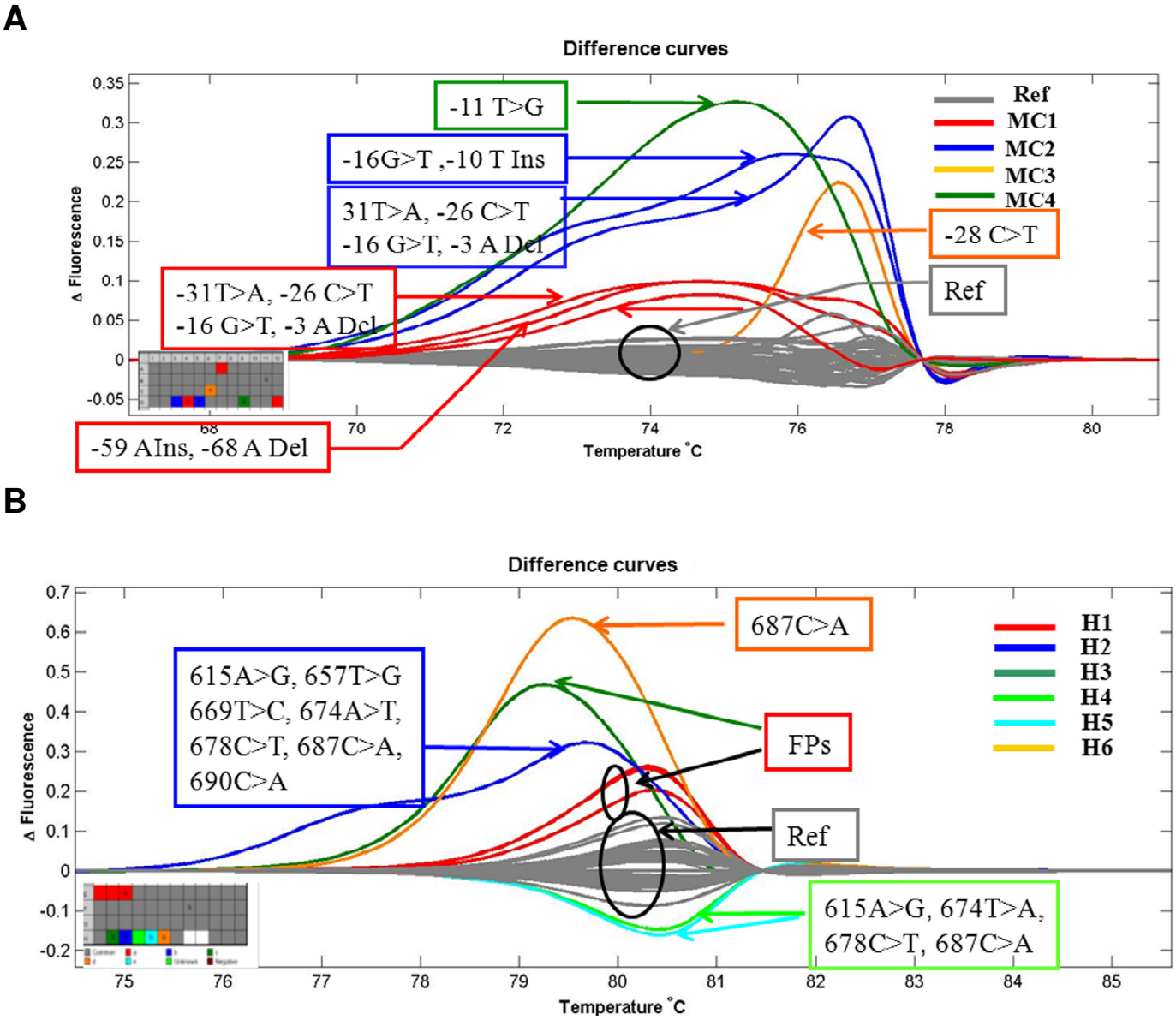

Figure 3. HRM analysis of the 48 samples from different taxonomic materials. (a) Difference curves resulting from the intergenic region. The samples that have no variations compared with the reference were referred to as "Ref" (grey curves). The samples were clustered to different groups. The MC3 and MC4 groups were differentiated successfully. However, the MC1 group contains one FP. The MC2 group showed resulted in misclassification two types of variations. The percent of FPs were 2.1% in the analysis. Ins: insertion; Del: deletion. (b) The difference curves resulting from the coding region of *atpB* gene. The sample Piaobai was used as reference and the data were analyzed at normal sensitivity level. By comparing to the wild-type reference sequence (grey curves), HRM analysis clustered these samples into six different groups. H2 with multiple SNPs and H6 with one SNP were accurately clustered, respectively. However, HRM curve analysis resulted in four FPs. In addition, the H4 and H5 shared the same variation type. The percent of FPs was 8.7% in the analysis. *: The samples that failed to amplify any products (coloured white) were filtered before data analysis.

HRM detection of *atpB* gene and the intergenic region polymorphisms within *B. napus*

To detect the intraspecific variations, the two sets of primers were applied to 90 *B. napus* samples from different countries. We found that five samples potentially contained variances in the intergenic region (Variation group, Figure 4a). One sample could not be assigned to any other group (Unknown group, Figure 4a). According to the sequencing results, the intergenic fragment had more variance, similar to the results shown in Table 2. Though there were variations among the most samples

Table 2. Derived haplotypes for *atpB* gene and the intergenic amplicons based on 16 SNP positions in a set of 48 samples from different tribes.

Haplotype [a]	Representative	Number	Base position [b]														
			-68	-59	-31	-28	-26	-16	-11	-10	-3	615	657	669	674	678	687	690
TH1	Jiningtianjinlv	1	— [c]	A	A	C	T	T	G	T	—	A	T	T	T	C	C	C
TH 2	Kas	2	A	—	T	C	T	T	T	—	—	G	T	T	A	T	A	C
TH 3	Hancai	1	A	—	T	C	C	T	T	T	A	G	G	C	A	T	C	A
TH 4	Heijie	1	A	—	T	T	C	G	T	—	A	A	T	T	T	C	C	C
TH 5	Zhongshuang 4	1	A	—	T	C	C	G	G	—	A	A	T	T	T	C	C	C
TH 6	Shanxiyunjie	1	A	—	T	C	C	G	T	—	A	A	T	T	T	C	A	C

[a]Haplotypes that were derived using Vector NTI 9.0 software and validated using available reference sequences.
[b]Base under the two amplicons.
[c]Deletion at this position

selected by HRM, the analysis failed to differentiate the variant type; for example, the samples in the variation group, H51, Shengli-qinggeng, Qingyou-6, 2000-5, and 05zaV2 had three variation types. The first type was an A deletion at -66 bp, the second was a T to G substitution at -11 bp, and the third was a T to G at -11bp and an A insertion at -58 bp (Table 3). The results showed that the accessions *B. napus* from China had more variations, and the genetic diversity was abundant.

Analysis by HRM indicated that variations in the *atpB* gene existed in only three samples. The sequence analysis of the amplification fragments showed that the *atpB* gene had no variation compared with the reference, and the accessions detected were FPs (Figure 4b).

DISCUSSION

The cp gene *atpB* is 1497 bp long and is located from positions 51361 bp to 52857 bp following the numbering system for the *B. napus* cpDNA (GenBank accession number GQ861354) (Hu et al., 2011). This gene is 788 bp downstream from *rbcL*, but it is transcribed in the opposite direction of *rbcL* (Hu et al., 2011). Gene *atpB* and the intergenic region were suitable for phylogenetic study. In addition, the speed of *atpB* evolution appeared to be slow, and *atpB* was easily amplified and sequenced with universal PCR primers. Recent investigations based on sequencing of the *atpB* gene have revealed relationships within families and between families in angiosperms (Hoot et al., 1999). Though the cost has decreased with the development of sequencing, sequencing large populations is still expensive. Therefore, we developed HRM analysis as a high-throughput, accurate, time-saving, and cost-effective approach for polymorphism detection (Wu et al., 2008).

HRM is based on high-resolution melting of DNA duplexes in the presence of saturating fluorescent dyes and appears to have an accuracy equivalent or superior to other heteroduplex scanning methods (Reed and Wittwer, 2004). Identifying common genetic variants by HRM has been used recently for gene scanning for disease-related gene mutations in humans (Kennerson et al., 2007), as well as identification of different microorganisms (Jeffery et al., 2007; Maeta et al., 2008), fish (Dalmasso et al., 2007), and mapping plant SNP markers in almond, potato, apple, olive, and oilseed (De Koeyer et al., 2010). HRM analysis of the two hypervariable regions HVI and HVII in mtDNA has also been suggested to be used as a rapid and inexpensive pre-screening method prior to DNA sequencing (Biss et al., 2003). In the cp genome of *Brassica*, this approach had been used to differentiate the typical *B. napus* chloroplasts from those of *B. oleracea* and *B. rapa* based on single-base SNPs in the cp gene *ycf2* (Allainguillaume et al., 2009).

In our study, we chose a fragment of the *atpB* gene and its upstream region for analysis. Melting temperature (Tm)-based differentiation is possible at the genus or species level. The variation in intergenic fragment was more abundant than in the *atpB* gene, and we were able to perfectly distinguish between the different variations using HRM analysis, with the exception of one FP and one misclassification (Figure 2a). Although, the FPs was increased in *atpB* gene, the samples containing multiple sequence variants were

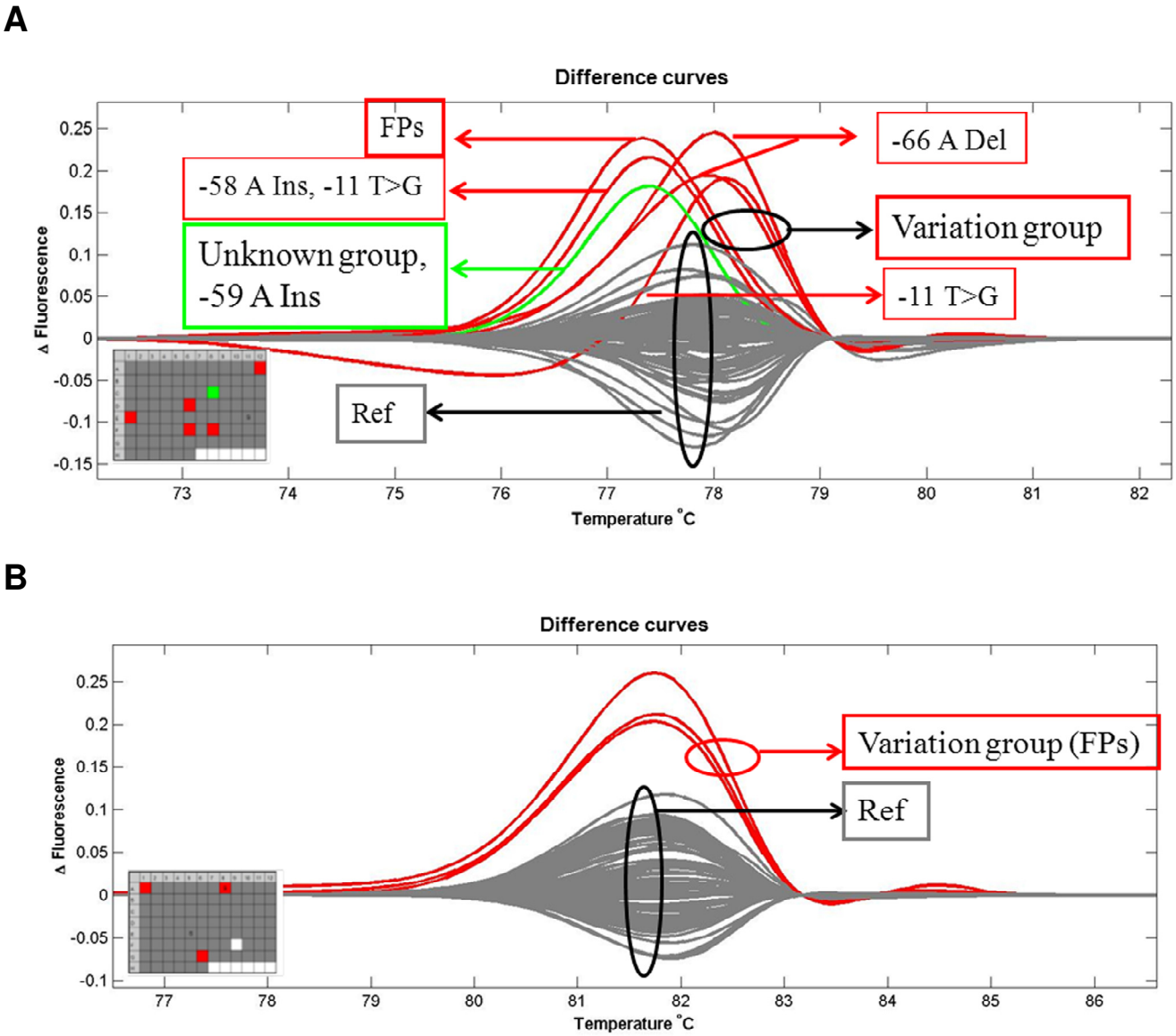

Figure 4. The HRM analysis of 90 *B. napus* entries by the two pairs of primers*. (a) The difference curves resulting from the intergenic fragment. The variation group differentiated the variations and "Ref" except one FP. However, the analysis resulted in misclassified the variances with INDELs and SNP into one group. The percent of FPs were 1.1% in the analysis. (b) The analysis result of the fragment of the *atpB* gene. There were no variation in the 90 samples and the three samples selected out were all FPs. The samples SavariA, Hongyou-3 and Bounty were used for reference, and their sequences were 100% identical to GenBank GQ861354.

differentiated accurately, such as *A. thaliana*, *Eruca*, and *R. indica* (Figure 2b). The variation in *B.napus* was lower than in the higher taxon. There were only five types of variation in intergenic space and no variation in *atpB* gene, which indicated that *atpB* was more highly conserved than the intergenic region. How- ever, the variation of intergenic region also decreased within intraspecies samples (Table 3).

In summary, *atpB* gene was suitable for HRM analysis. HRM testing for a new gene or fragment is only valuable when the majority of the samples and amplicons generate wild-type sequences and/or harbour the repetitive detection of a common polymorphism. The fragment being detected should contain the following characteristics: (1) the sequence should be highly conserved, which is important for designing universal primers; and (2) there should be a few variations of the gene within a closely related population. Detection of multiple variations by HRM can be problematic (Tindall et al., 2009); (3) GC-rich regions have proven to be an obstacle for the

Table 3. Validation of the exact polymorphic loci in *atpB* gene and its intergenic region in *B. napus* by sequencing.

Name	Origin country	Base position [a]				
		-66	-59	-58	-54	-11
Midas	Canada	A	—[b]	—	—	T
Celebra	Canada	A	A	—	A	T
Shengliqinggeng	China	—	—	—	A	T
Qingyou-6	China	—	—	—	A	T
2000-5	China	A	—	—	A	G
05zaV2	China	A	—	A	A	G

[a]Base under the two amplicons.
[b]Deletion at this position.

screening methods. GC-rich regions should be avoided for HRM curve analysis (Tindall et al., 2009). Fragments with an average GC content ranging from 31 to 54% can be currently detected (Technology Assessment on HRM as reported by Helen White, National Genetics Reference Laboratory [NGRL], Wessex, United Kingdom; http:// www.ngrl.org.uk/Wessex/down loads_reports.htm). Gene *atpB* was more conservative (Figure 2), which confirmed the accuracy of HRM detection. The method at highly polymorphic cpDNA loci in particular populations is not recommended. We performed an assessment of the HRM analysis for the gene *accD* and found that amplicons (amplified from different tribe samples) with more INDELs demonstrate a high level of misclassification (has been submitted to another journal). The variation of the fragment within the population should be assessed based on the known sequence, which is a precondition for HRM analysis. The second factor that should be considered is the GC content. In our study, the GC content of the two fragments was 51 and 50% respectively (Table 1), which is considered an appropriate level of GC content. Hu et al. (2011) reported that most features of *B. napus* cpDNA are highly similar to those of *B. rapa* cpDNA. Alignment of the cpDNAs of *B. napus* and *B. rapa* showed that the total length of the gene coding regions and the intron region are highly similar between these species. A total of 1-3 SNPs were found in about 50% of the protein coding genes and *rrn* genes, and INDELs appeared in only three genes, including *accD*, *psbB*, and *rrn16*. Therefore, the cpDNA is conserved not only within species, but also among species. Therefore, most regions of cpDNA are suitable for HRM high-throughput detection for the rare polymorphisms among close relatives.

When using HRM analysis, the sensitivity level was very important for accurate detection. Sensitivity levels can also be selected by evaluating the degree of gene variation. In this study, the normal sensitivity levels for *atpB* gene varied between -1.43 and -2.66 according to the conservation of the gene and the known sequence. It was best to use several samples with known sequence to evaluate the sensitivity level. When no variants are available for evaluation, and variant curves located close to the wt curve can be detected; moreover, this circumvents the detection of frequent FP scores. We recommend re-evaluating the results again after performing the first series of diagnostic scanning tests. It should be considered that the software cannot always discriminate between different variants in the same amplicon. Therefore, common polymorphisms should never be judged only by their similarity in melt profiles; instead, they should always be confirmed by probe or sequence analysis to exclude the presence of a mutation with an identical melt profile. Also, in our study the overlap in melt profiles was not always simply explained by the similarity of the substitution and a short distance between the locations of the two variants. We do recommend taking note of the critical features mentioned in this study, which should be specifically addressed when applying HRM for mutation scanning analysis.

We have summarized recommendations and guidelines that can be considered when setting up and performing HRM for other genes in the online supporting information. Finally, we supply a validated set of PCR primers for mutation scanning analysis of *atpB* gene on the Light Scanner using identical test conditions. The results indicated over 90% of the variations which included single or multiple variants and INDELs can be identified by HRM analysis. In conclusion, HRM analysis is an effective approach to identify variant of chloroplast genes. This study will facilitate further functional investigations into the role of chloroplast genes in photosynthesis, phylogeny, and molecular evolution.

ACKNOWLEDGEMENTS

We thank the Biotechnology Research Group, State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences (CAAS), Beijing, China, for providing LightScanner equipment. We thank Jie Zhang, Ph.D. in the Biotechnology Research Group for

assistance. We would also like to thank American Journal Experts for providing language help. This study was funded by the 973 Projects (2011CB109300 and 2006CB101600) and National Science and Technology Support Project (2011BAD35B09) of China.

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Electronic Supplementary Material

S 1. Forty-eight entries from the tribes Brassiceae and Arabideae assayed using HRM analysis

Genera	Name	Origin
Brassica carinata	Aijie	Ethiopia
B. carinata	Huangziaijie	Ethiopia
B. juncea	Nanfangren	Russia
B. juncea	Bangcai 02	China
B. juncea	Banyedatoucai	China
B. juncea	Donghaigaojiaofengweijie	China
B. juncea	Cv. Pusa Bold	India
B. juncea	CMS (Mri)	India
B. napus	Midas	Canada
B. napus	H47	Russia
B. napus	Chikuzen	Japan
B. napus	Zhongshuang 4	China
B. napus	Zhongshuang 4 NSA	China
B. napus	Luobozhicailiao	China
B. oleracea	Baoziganlan	China
B. oleracea	Ziganlan	China
B. oleracea	Shimianlianhuabai	China
B. oleracea	Baipilan	China
B. oleracea	Tuanyexiaohuacai	China
B. oleracea	Lilvqinghuacai	China
B. oleracea	Zhonghuajielan	China
B. oleracea	Yeshengganlan	China
B. oleracea	Yuyiganlan	China
B. rapa	Changningxiaoheiyoucai	China
B. rapa	Xishuibai	China
B. rapa	Guangfuqing	China
B. rapa	Baichengbaiyoucai	China
B. rapa	Chuanlingyoucai	China
B. rapa	Quxu	China
B. rapa	Jiningtianjinglv	China
B. rapa	Piaobai	China
B. rapa	Niuyezhongshucaixin	China
B. rapa	Wutacai	China
B. rapa	Taicai	China
B. nigra	Oiebra	Sweden
B. nigra	Heijie	Spain
Eruca sativa	Shanxiyunjie	China
E. sativa	Cilezhahong	China
Orychophragmus violaceus	Eryuelan	China
Raphanus sativus	Yuewangluobozi	China
R. sativus	Lanhuazi	China
Rorippa indica	Hancai	China
Sinapis alba	Baijie 01	China
S. alba	Baijie 02	China
S. arvensis	Xinjiangyeshengyoucai A	China
S. arvensis	Xinjiangyeshengyoucai B	China
Arabidopsis thaliana	Tri	Sweden
A. thaliana	Kas	Sweden

S 2. Ninety varieties in *B. napus* and its ancient parents used for intraspecies HRM analysis.

Code	Name	Ploidy	Genome	Origin
1	Mianza 03-33	4x	AACC (n =19)	China
2	Oro	4x	AACC (n=19)	Canada
3	Major	4x	AACC (n=19)	France
4	Primor	4x	AACC (n=19)	France
5	Yaojin Rape	4x	AACC (n=19)	Italy
6	Marnoo	4x	AACC (n=19)	Australia
7	Ujfertadi	4x	AACC (n=19)	Hungary
8	Savari A	4x	AACC (n=19)	Hungary
9	Expander	4x	AACC (n=19)	Germany
10	Ledos	4x	AACC (n=19)	Germany
11	Huyou 21	4x	AACC (n=19)	China
12	H51	4x	AACC (n=19)	Former Soviet Union
13	Janpol	4x	AACC (n=19)	Poland
14	Start	4x	AACC (n=19)	Poland
15	Mikado	4x	AACC (n=19)	United Kingdom
16	P20	4x	AACC (n=19)	United Kingdom
17	Lingot	4x	AACC (n=19)	United Kingdom
18	Wipot	4x	AACC (n=19)	Norway
19	Regent	4x	AACC (n=19)	Canada
20	Tower	4x	AACC (n=19)	Canada
21	Shiralee	4x	AACC (n=19)	Australia
22	Viking	4x	AACC (n=19)	Denmark
23	Cobra	4x	AACC (n=19)	Germany
24	Parter	4x	AACC (n=19)	Germany
25	Falcon	4x	AACC (n=19)	Germany
26	Nevin	4x	AACC (n=19)	France
27	Samouran	4x	AACC (n=19)	France
28	Roman-1	4x	AACC (n=19)	Netherlands
29	Tornado	4x	AACC (n=19)	Sweden
30	Legend	4x	AACC (n=19)	Sweden
31	Grant	4x	AACC (n=19)	Sweden
32	Celebra	4x	AACC (n=19)	Canada
33	Triton	4x	AACC (n=19)	Canada
34	Profit	4x	AACC (n=19)	Canada
35	Startigh	4x	AACC (n=19)	Sweden
36	Bounty	4x	AACC (n=19)	Sweden
37	Garrison	4x	AACC (n=19)	Sweden
38	Gcsunder	4x	AACC (n=19)	Germany
39	Disamant	4x	AACC (n=19)	Germany
40	Mar	4x	AACC (n=19)	Poland
41	Star	4x	AACC (n=19)	Denmark
42	Shengli Qinggen	4x	AACC (n=19)	Shanghai, China
43	Jiuer rape	4x	AACC (n=19)	Zhejiang, China
44	Hanfeng-1	4x	AACC (n=19)	Shanxi, China
45	Huayou-13	4x	AACC (n=19)	Wuhan, China
46	Aijiazao	4x	AACC (n=19)	Sichuan, China
47	Southeast-302	4x	AACC (n=19)	Sichuan, China
48	Yunyou-49	4x	AACC (n=19)	Yunnan, China
49	Qingyou-6	4x	AACC (n=19)	Qinghai, China

Supplementary 2. Contd.

50	Nonglin-18	4x	AACC (n=19)	Japan
51	F01*J6 1-1	4x	AACC (n=19)	Hubei, China
52	Ganyou-5	4x	AACC (n=19)	Wuhan, China
53	Zhongyou-821	4x	AACC (n=19)	Wuhan, China
54	Xiangyou-5	4x	AACC (n=19)	Hunan, China
55	Dong-Hae 23	4x	AACC (n=19)	Japan
56	Ganpol	4x	AACC (n=19)	Zhejiang, China
57	Norin 16	4x	AACC (n=19)	Japan
58	Zheyouyou-2	4x	AACC (n=19)	Zhejiang, China
59	Yuyou-2	4x	AACC (n=19)	Henan, China
60	Zhongyoudijie-1	4x	AACC (n=19)	Wuhan, China
61	Qingyou-12	4x	AACC (n=19)	Qinghai, China
62	Heyou 563	4x	AACC (n=19)	Japan
63	Zhongshuang 4	4x	AACC (n=19)	Wuhan, China
64	ISN-705	4x	AACC (n=19)	India
65	H0302	4x	AACC (n=19)	Hubei, China
66	2000-5	4x	AACC (n=19)	Hubei, China
67	H9944	4x	AACC (n=19)	Hubei, China
68	01 Za-654	4x	AACC (n=19)	Sichuan, China
69	05 Za-V2	4x	AACC (n=19)	Chongqing, China
70	HY8	4x	AACC (n=19)	Jiangsu, China
71	Youyan-10	4x	AACC (n=19)	Guizhou, China
72	Qianyou-20	4x	AACC (n=19)	Guizhou, China
73	6766	4x	AACC (n=19)	Hubei, China
74	H0202	4x	AACC (n=19)	Hubei, China
75	Zheyou-5002	4x	AACC (n=19)	Zhejiang, China
76	Zhongyouza-2	4x	AACC (n=19)	Hubei, China
77	Za-839	4x	AACC (n=19)	Hunan, China
78	Hongyou-3	4x	AACC (n=19)	Jiangsu, China
79	Zashuang-5	4x	AACC (n=19)	Henan, China
80	7633	4x	AACC (n=19)	Shanxi, China
81	Qinyou-7	4x	AACC (n=19)	Shanxi, China
82	Rape-23	4x	AACC (n=19)	Shanghai, China
83	Ganyou-4	4x	AACC (n=19)	Hubei, China
84	Huayou-3	4x	AACC (n=19)	Hubei, China
85	Huayou-8	4x	AACC (n=19)	Hubei, China
86	Chuannong Changjiao	4x	AACC (n=19)	Sichuan, China
87	Chuanyou-7	4x	AACC (n=19)	Sichuan, China
88	Luzhou-5	4x	AACC (n=19)	Sichuan, China
89	Nanyang-41	4x	AACC (n=19)	Henan, China
90	Hechengyoucai	4x	AACC (n=19)	Henan, China