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ORIGINAL PAPER

High-resolution melting analysis of cDNA-derived PCR amplicons for rapid and cost-effective identification of novel alleles in barley

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Abstract An original method has been established for the identification of novel alleles of *eukaryotic translation initiation factor 4E* (*eIF4E*) gene, which is required for resistance to agronomically important bymoviruses, in barley germplasm. This method involves scanning for sequence variations in cDNA-derived PCR amplicons using High-resolution melting (HRM) followed by direct Sanger sequencing of only those amplicons which were predicted to carry nucleotide changes. HRM is a simple, cost-effective, rapid and high-throughput assay, which so far has only been widely used in clinical pathology for molecular diagnostic of diseases and patient genotyping. Application of HRM allowed significant reduction in the amount of expensive Sanger sequencing required for allele mining in plants. The method described here involved an investigation of total cDNA rather than genomic DNA, thus permitting the analyses of shorter (up to 300-bp) and fewer overlapping amplicons to cover the coding sequence. This strategy further reduced the allele mining costs. The sensitivity and accuracy of HRM for predicting genotypes carrying a wide range of nucleotide polymorphisms in *eIF4E* approached 100%. Results of the current study are promising and suggest that this method could also potentially be applied to the discovery of superior alleles controlling other important traits in barley as well in other model and crop plant species.

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Introduction

Large numbers of accessions of model plant species as well as all the key world crops and their wild relatives are held in seed stock centres and regional genetic resource collections. For example, the Arabidopsis Biological Resource Centre at Ohio State University, USA (http://www.arabidopsis.org/abrc/) and the Nottingham Arabidopsis Stock Centre (NASC) at Nottingham University, UK (http://arabidopsis.info/) hold >2,500 ecotypes of the model plant species *Arabidopsis thaliana*, whereas the US National Plant Germplasm System (http://www.ars-grin.gov/npgs/) holds >28,000 accessions of barley (*Hordeum vulgare* subsp. *vulgare*), >25,000 accessions of corn (*Zea mays* subsp. *mays*), >43,000 accessions of wheat (*Triticum aestivum* subsp. *aestivum*), >41,000 accessions of rice (*Oryza sativa*), and over 100,000 accessions of other common grain, vegetable and fruit crop species collected globally. These genetically diverse collections contain a wealth of undiscovered superior gene variants (i.e. alleles) controlling important traits such as resistance to pests and diseases, tolerance of abiotic stresses, enhanced nutrient use efficiency as well as improved yield, processing quality and nutritional value. Exploration of this allelic diversity at the molecular level and utilisation of novel superior alleles through development of improved crop varieties using targeted molecular breeding ('precision breeding'; McCouch 2004) is of prime importance in view of the increasing global human population and growing demand for food and environmental protection. The analysis of natural allelic variation in model plant species such as *A. thaliana* is also

very important and is increasingly being used in gene function studies as well as in research addressing questions in evolutionary and developmental biology, and ecology.

Finding the unknown valuable variant alleles at locus of known function is called 'allele mining' (Kaur et al. 2008). This is a relatively novel research field aiming to unlock the natural genetic diversity existing in world genetic resource collections by identifying allelic variation at genes and loci of potential agronomic relevance. Allele mining involves assembly of a reasonable sized core germplasm collection usually comprising of ~1,000 accessions representative of genetic diversity existing in the global collections. This is followed by careful selection of candidate genes, verifying the target gene model, and finally identification of novel alleles at candidate gene loci using molecular methods. For the latter step, the primers are designed to amplify the whole sequence of a target gene or only its exonic sequences from genomic DNA of each accession from the core collection. Often, the resulting PCR fragments are then sequenced using the Sanger method, and novel alleles are identified (Buckler et al. 2006; Kaur et al. 2008; Turuspekov et al. 2008; Varshney et al. 2007; Wang et al. 2008). This approach is very low throughput, laborious, expensive and time consuming.

Several methods for detecting DNA polymorphisms in plants have been described, e.g. denaturing high-performance liquid chromatography (dHPLC; Caldwell et al. 2004), single strand conformation polymorphism (SSCP; Martins-Lopes et al. 2001), denaturing gradient gel electrophoresis (DGGE; He et al. 1992), and Ecotilling (Comai et al. 2004). These methods could be used for pre-screening individuals in germplasm collections for possible sequence variations in the target genes, reducing the amount of Sanger sequencing required and thereby increasing the cost effectiveness of allele mining. However, some of these methods are either imprecise, lack the required sensitivity or quite expensive. Moreover, all these methods including Ecotilling, which is currently considered the most accurate, highest-throughput and least expensive pre-screening method, require an additional post PCR in-gel or column DNA strand separation step that makes them not very favourable for routine assays. Therefore, other rapid and inexpensive strategies are required to identify useful variant alleles of loci of known function from a wide range of crop plants.

Recently, a next generation mutation scanning technology called High-resolution melting™ (HRM) has been developed (Wittwer et al. 2003). HRM appears to offer considerable cost and especially time savings over other methods for detection of DNA polymorphisms. It is a simple closed-tube assay, which is performed on DNA amplicons post-PCR importantly without additional processing. The sensitivity and specificity of this method is reportedly better than those of dHPLC (Chou et al. 2005) and approaches 100% for PCR amplicons <400-bp (Reed and Wittwer 2004). HRM is also attractive because it is amendable to high-throughput delivery on 96- or 384-well PCR plates, and because it is non-destructive, thus permitting post-melting DNA sample recovery for downstream analyses by Sanger sequencing, gel electrophoresis, or cloning. Briefly, in this method, amplicons from genotypes carrying wild type and variant alleles are produced by PCR in the presence of a dsDNA-intercalating saturating fluorescent dye and are then transferred to a HRM instrument for melting analyses. The dye does not interact with ssDNA but intercalates with dsDNA and fluoresces brightly in this state. HRM instruments slowly heat the samples while simultaneously monitoring the progressive reduction in fluorescence caused by the release of a fluorescent dye from dsDNA amplicons as the molecules are denatured (Wittwer et al. 2003). The data output from a HRM instrument appears as so-called melting profiles or melting curves that plot the reduction in fluorescence against the increased temperature. Amplicons containing a sequence variant yield altered shapes of melting curves compared with wild-type control samples. The power of HRM depends directly on the resolution of the melting instrument and the choice of DNA binding dye. Cross-platform comparison of instruments and dyes has shown that the highest HRM resolution and throughput can be achieved when the LCGreen® Plus dye, which does not inhibit PCR at concentrations that saturate the PCR product, was used in combination with the Idaho Technology LightScanner® or instruments recently designed by other companies specifically for high-resolution melting (Herrmann et al. 2006, 2007).

Since its invention in 2003, HRM has been used widely in clinical chemistry and human pathology for efficient molecular diagnostic of diseases and patient genotyping (reviewed in Reed et al. 2007). However, until very recently, there have been no reports on using HRM in the plant sciences field. During the last 12 months, several groups more or less simultaneously reported the application of HRM for mapping single nucleotide polymorphism (SNP) or microsatellite markers in various crop species (Chagne et al. 2008; Croxford et al. 2008; Lehmensiek et al. 2008; Mackay et al. 2008; Mader et al. 2008; Wu et al. 2008) and one group reported the use of HRM for detection and quantification of RNA editing in *Arabidopsis thaliana* (Chateigner-Boutin and Small 2007). The main objective of our current study was to test whether HRM could be efficiently applied to the identification of novel alleles of a gene of interest in barley. In this proof of concept study, we targeted the *eukaryotic translation initiation 4E* (Hv*eIF4E*) gene. This gene was considered a good target for an allele mining study for four main reasons: first, eIF4E and/or its isoform eIF(iso)4E is known as an important host factor

required for infection by large number of potyvirus species (Robaglia and Caranta 2006). Second, two variant alleles of *eIF4E* gene in barley, *rym4* and *rym5*, are known to confer resistance to *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV) belonging to the genus *Bymovirus* in the family *Potyviridae*, which cause serious yellow mosaic disease (Kanyuka et al. 2005; Stein et al. 2005). Third, bioassays to identify resistance to a range of bymovirus isolates are inherently difficult and therefore a rapid molecular screening approach in the first instance to identify allele variants was considered desirable. Finally, a tremendous natural sequence variation exists in Hv*eIF4E* (Kanyuka et al., unpublished; Nils Stein, Frank Ordon and Andreas Graner, personal communications) and more than 30 variant alleles of this gene characterised using Sanger sequencing were available for this study. Average gene lengths in Arabidopsis and rice are ~2,200 and ~3,350 base pairs (bp), respectively, with each gene on average containing ~4.2 and ~5.3 introns, respectively (Satoh et al. 2007; Wortman et al. 2003). The average gene length in plants with more complex genomes (e.g. barley, wheat) is predicted to be even higher due to the presence of generally longer intronic sequences. Therefore, in allele mining, either using direct sequencing or using Ecotilling in combination with sequencing several primer pairs amplifying either overlapping gene segments or only the exonic sequences are generally required. This elevates the cost of allele mining. On the other hand, the average length of Arabidopsis and rice cDNA is ~1,500 nucleotides (nt) and the average length of open reading frame (ORF) in cDNA is only ~1,000-nt (Satoh et al. 2007). The length figures for cDNA and ORF in other grass species are expected to be similar to those in rice. For this reason, an additional objective of our study was to test the feasibility of using total plant RNA rather than genomic DNA for allele mining in barley.

Materials and methods

Plant materials and growth conditions

Seeds of 643 landraces and old cultivars of barley (*Hordeum vulgare* subsp. *vulgare*) of diverse geographical origin, obtained from the National Small Grains Collection (USDA-ARS, Aberdeen, Idaho, USA), were used in this study. The barley plants were cultivated in the glasshouse under natural light conditions at 18°C during the day and 14°C during the night.

Preparation of barley cDNA samples and RT-PCR

Six seedlings from each accession were harvested at the two-leaf stage, combined into a 2 ml Eppendorf tube, snap frozen in liquid nitrogen and freeze-dried. Three 3 mm chrome steel beads were placed into each tube containing the freeze-dried leaves (approximately 20 mg) and samples were ground using a TissueLyser (Qiagen). RNA was then extracted using TRIzol® reagent (Invitrogen) following the manufactures protocol. The cDNA was synthesised using oligo(dT)-primers and the SuperScript™ III reverse transcriptase (Invitrogen) according to the manufacturer's instructions, and the reactions were subsequently treated by RNaseH (New England Biolabs). A 620-bp fragment spanning most of the barley *eIF4E* ORF was amplified from the cDNA templates in 10-µl reaction volumes using the primers RESK-99 and RESK-102 (Table 1) essentially as described in Kanyuka et al. (2005). The relative concentration of these primary PCR products was assessed by agarose gel electrophoresis and each product was diluted with deionised water between 5× and 10× to equalise their concentration. This 620-bp fragment was not used for HRM analysis, but was used as the template for synthesis of the six smaller amplicons for the HRM optimisation analyses and the four amplicons selected for the main analysis.

Sequencing

The *eIF4E*-specific 620-bp RT-PCR products obtained from each of the 643 analysed barley accessions using the primers RESK-99 and RESK-102 (Table 1) were purified by precipitation in the presence of 1 volume of 5 M ammonium acetate, pH 7.0 and 3 volumes of absolute ethyl alcohol and then used as templates for the sequencing reactions. Sequencing was carried out using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions and primers RESK-99 and RESK-102 (Table 1). After the cycle sequencing, the reactions were analysed on a 3730xl® DNA Analyzer (Applied Biosystems) at the John Innes Centre Genome Laboratory (Norwich, UK). Sequences were analysed and alignments were carried out using the Vector NTI® Advance 10 software (Invitrogen).

Using the obtained sequence information, we selected 35 barley accessions representing all 32 identified novel *eIF4E* alleles as well as 12 accessions containing the *WT* allele indistinguishable from that of BaMMV and BaYMV susceptible Morex barley for High-resolution melting analyses.

High-resolution melting using the LightScanner® platform and data analysis

PCR amplifications were carried out in 10-µL volumes containing 5-µL HotShot™ Mastermix (Cadama Medical Ltd), 1-µL LCGreen® Plus Dye (Idaho Technology Inc.), 1-µl of each 10 µM primer and 2-µL of the 620-bp RT-PCR

Table 1 Barley *eIF4E*-specific primers used for HRM analyses

Amplicon size	Primer name	Sequence	Ta (°C)[a]	Position of amplicon (nt)[b]	
				Start	End
130-bp	LSF-455	5′-TTGGTGATGAAATTTGCGGAGCAG-3′	68	437	566
	LSR-560	5′-TCCAGAAACTCCTTCCACTGCTTACCG-3′			
165-bp	LSF-510	5′-TGCTGCCAATGAAACTGCTCAGA-3′	68	510	674
	LSR-670	5′-CGGGCCTGCCTTGGAAGCTC-3′			
281-bp	LSF-400	5′-TGGTTGCATACTTTGCTGGCATTGA-3′	68	394	674
	LSR-670	5′-CGGGCCTGCCTTGGAAGCTC-3′			
239-bp	LSF-190	5′-GCACCATCCACCCCATCCAC-3′	66	191	429
	LSR-430	5′-TTGTTCACCAATCAATGCCAGCAA-3′			
299-bp	LSF-130	5′-ACGCCTGGACCTTCTGGTTCGAC-3′	68	131	429
	LSR-430	5′-TTGTTCACCAATCAATGCCAGCAA-3′			
544-bp	LSF-130	5′-ACGCCTGGACCTTCTGGTTCGAC-3′	68	131	674
	LSR-670	5′-CGGGCCTGCCTTGGAAGCTC-3′			
620-bp[c]	RESK-99	5′-ACCCCTCTGGAGAACGCCTGGACCT-3′	62	119	738
	RESK-102	5′-ACAGCATCCACCCGCTACAAGCTA-3′			

[a] PCR annealing temperature

[b] Position of amplicon within the *eIF4E* mRNA of Morex barley relative to translation start site

[c] This amplicon, which spans most of the *eIF4E* ORF, was only used as a template in secondary PCRs yielding smaller amplicons amenable for HRM analyses (see "Materials and methods")

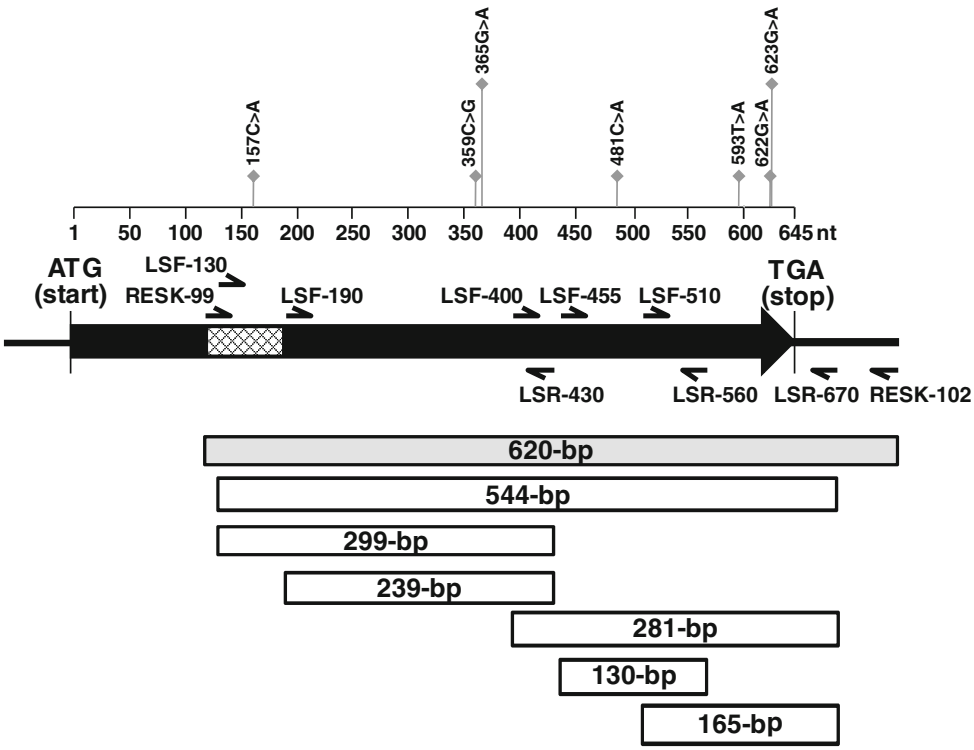


Fig. 1 Schematic representation of the barley *eIF4E* ORF (*thick solid black arrow*) showing the locations and sizes of PCR amplicons (*grey* and *white rectangles* below the ORF) and the corresponding amplicon primers (*one-sided arrows* immediately above or below the ORF) used in the HRM assays. The positions of seven SNPs used for optimisation of the HRM assay are indicated above the nucleotide 'ruler'. The hatched region in the 5′-half of ORF represents a very GC-rich sequence (70% G + C content) of 60-bp in length

products as the template. For all standard experiments, 1-µL of each diluted 620-bp RT-PCR product derived from a test genotype was mixed with 1-µL of diluted 620-bp RT-PCR product derived from the wild-type reference barley genotype Morex that is known to be susceptible to BaYMV and BaMMV (Kanyuka et al. 2005; Stein et al. 2005). All HRM assays were replicated two to three times for each sample. The primer pairs used for HRM analyses are

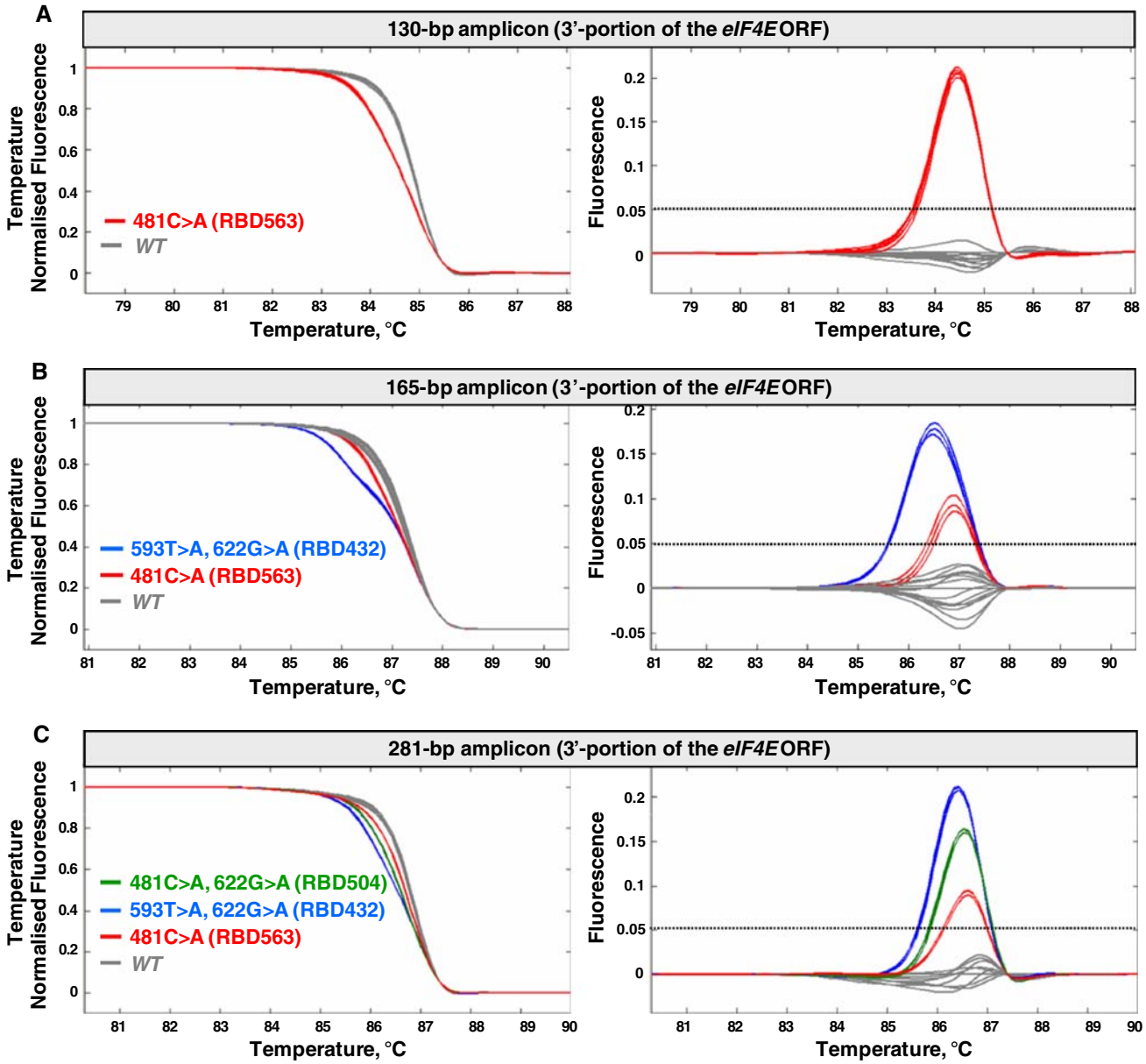
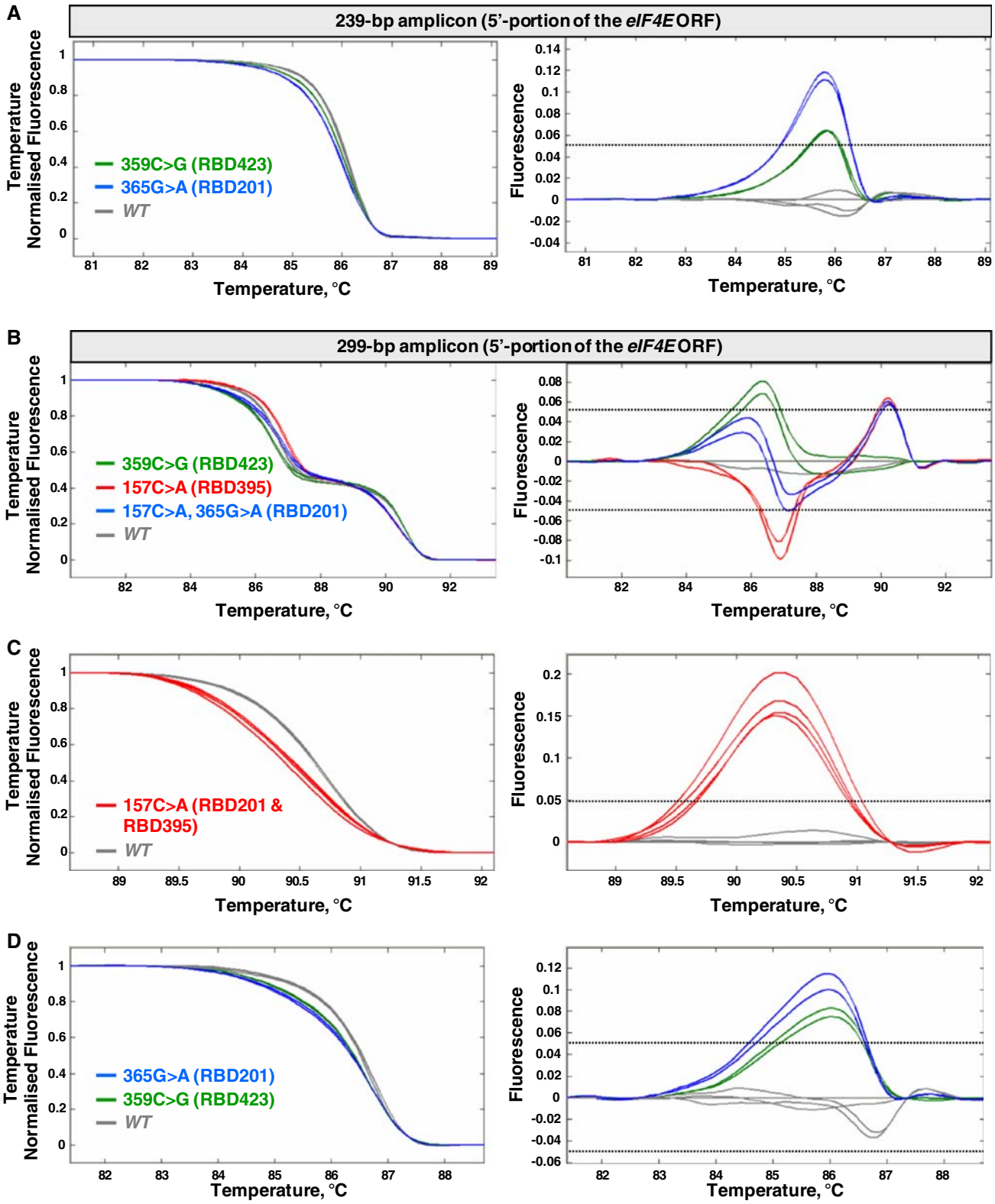


Fig. 2 Optimisation of the HRM assay using the three 3′-portion of the *eIF4E* ORF-derived amplicons each containing one melting domain, and selected variant *eIF4E* alleles. The HRM data is presented as two different graphs. The *graph* on the left in each panel displays temperature normalised melting curves for the tested samples, whereas the graph on the right shows difference curves displaying the difference in fluorescence (ΔF) of the respective sample in relation to the wild-type (*WT*), barley Morex sample. **a** The 130-bp amplicon, **b** the 165-bp amplicon, and **c** the 281-bp amplicon. Samples containing specific nucleotide polymorphisms and the wild-type sample used in the assay are colour coded and are indicated inside the corresponding graph on the left in each panel. The curves are also colour coded according to the colour of the corresponding sample. Each sample was analysed in triplicate or duplicate

summarised in Table 1. The optimal annealing temperatures for each primer pair were determined by gradient PCR. PCR amplifications were done using 96-well FrameStar® PCR plates (4titude® Ltd, Surrey, UK), and the reactions were overlaid with mineral oil (Sigma-Aldrich). The PCR conditions were: 30 cycles at 95°C for 15 s, 66°–68°C for 30 s and 72°C for 1 min with an initial denaturation at 95°C for 2 min and a final extension step at 72°C for 10 min. After the final extension, an additional denaturation step at 95°C for 30 s was carried out. Subsequently, the plates were briefly centrifuged and were used directly for high-resolution melting using the LightScanner® instrument (Idaho Technology, Inc.). The data obtained were analysed using the LightScanner® analytical software with Call-IT™ 2.0 (Idaho Technology, Inc.). The normalisation and the temperature shifting of the melting curves were carried out according to the LightScanner® Operator's Manual (Idaho Technology Inc.). Two different ways to display the



results obtained by HRM were used. The temperature normalised melting curves displayed the change of the fluorescence value in each sample during melting of the PCR products as the temperature (°C) increased incrementally. The difference curves displayed the relative difference in fluorescence (Δ Fluorescence, ΔF) of a respective sample in relation to a reference sample. According to the manufacturer instructions, the difference curve peak with a ΔF

◀ **Fig. 3** Optimisation of the HRM assays using the two 5′-portion of the *eIF4E* ORF-derived amplicons, and selected variant *eIF4E* alleles. The HRM data is presented as two different graphs. The *graph* on the left in each panel displays temperature normalised melting curves for the tested samples, whereas the graph on the right shows difference curves displaying the difference in fluorescence (ΔF) of the respective sample in relation to the wild-type (*WT*), barley Morex sample. **a** The 239-bp amplicon containing one melting domain, and **b–d** the 299-bp amplicon containing two melting domains. **b** Analyse of both temperature melting domains together, whereas **c** and **d** display the separate analyses of the high melting temperature and the low temperature melting domain of the 299-bp amplicon, respectively. Samples containing specific DNA polymorphisms and the wild-type sample (*WT*) used in the assay are colour coded and are indicated inside the corresponding graph on the left in each panel. The curves are also colour coded according to the colour of the corresponding sample. Each sample was analysed in triplicate or duplicate

value of 0.05 was considered as significant. Following high-resolution melting, the amounts of the PCR products were verified by standard agarose gel electrophoresis.

The EMBL accession numbers for the sequences of 32 novel barley *eIF4E* alleles are FM244870 through to FM244901.

Results

Primary data output

At the end of a PCR run, the 96 wells plate was transferred without further processing to the HRM instrument (LightScanner®, Idaho Technology) for analysis. The raw data acquired during HRM are delivered to the user as a graph hereafter referred to as the melting curve, which plots the fluorescence versus temperature. Melting curves were then semi-automatically processed with the LightScanner® analytical software and presented as two different graphs. One output graph, hereafter referred to as the normalised melting curve, plotted the temperature-normalised fluorescence versus temperature. Briefly, for normalisation, two temperature regions of the melting curve on each side of the melting transition were selected defining 100% fluorescence and a 0% baseline. This step was necessary because raw data generally vary in the magnitude of the fluorescence for each sample owing to the differences in the starting template or optics. In the next temperature-shifting step, the melting curves of the samples were superimposed over the same temperature range to overcome the effect of absolute temperature variation from well to well across the PCR plate. The second output graph, hereafter referred to as the difference curve, plotted a subtractive difference of the fluorescence (ΔF) in comparison to a single reference sample versus temperature. Difference curves were generated by choosing a reference sample as the baseline and subtracting the fluorescence of each test sample relative to this baseline. This aided in the differentiation between wild-type reference and test samples by emphasising curve shape in conjunction with melting temperature. Melting curves for amplicons derived from barley genotypes homozygous for variant *eIF4E* alleles, for simplicity hereafter referred to as the 'variants', and a reference barley cultivar Morex, hereafter referred to as the 'wild-type' (*WT*), generally exhibited a simple melting temperature (T_m) shift (data not shown). Depending on the nature and position of the nucleotide polymorphism within the amplicon, it was rather difficult or impossible to differentiate between the homozygous wild-type reference and variant samples (data not shown). On the other hand, heterozygous variants samples were more easily differentiated from the homozygous *WT* based on a change in the shape of the melting curve. For this reason, in all HRM assays of the current study, amplicons derived from individual barley variants were each mixed in equal proportion with the corresponding amplicons derived from Morex barley (*WT*) unless otherwise stated.

Optimisation of the HRM assay for identification of variant *eIF4E* alleles in barley germplasm

The *eIF4E* gene in Morex barley is 4,571-bp long and consists of 5 exons (Stein et al. 2005), whereas the total length of *eIF4E* mRNA is ~1,040-nt with the open reading frame (ORF) length of 645-nt (Kanyuka et al. 2005; Stein et al. 2005; also see Fig. 1). To identify optimal HRM conditions, melting curves of six *eIF4E*-specific amplicons differing in size from 130 to 544-bp (Fig. 1; Table 1) derived from leaf mRNA of Morex barley and of several barley accessions homozygous for different variant alleles of *eIF4E* were compared.

The nucleotide polymorphism detection capability of three amplicons, a 130-bp, a 165-bp and a 281-bp, derived from the 3′-portion of the *eIF4E* ORF (Fig. 1), was tested. Normalised melting curves and the corresponding difference curves for these three amplicons derived from variant barley accessions RBD432, RBD504 and RBD563 were produced (Fig. 2). In all cases, the difference between *WT* and variant patterns was clear. The amplicons containing one (C > A) or two (T > A, G > A and C > A, G > A) single nucleotide polymorphisms (SNPs) were clearly distinguished from the *WT* amplicons at $\Delta F > 0.05$, the value which in HRM assays is considered to be significant. In addition, the difference curves for the amplicons containing two SNPs peaked noticeably higher than for those containing a single SNP.

Two amplicons of 239 and 299-bp in length were selected to scan for nucleotide polymorphisms in the 5′-portion of the *eIF4E* ORF (Fig. 1) in variant barley accessions RBD201, RBD395 and RBD423. The only difference between these amplicons was that the 299-bp

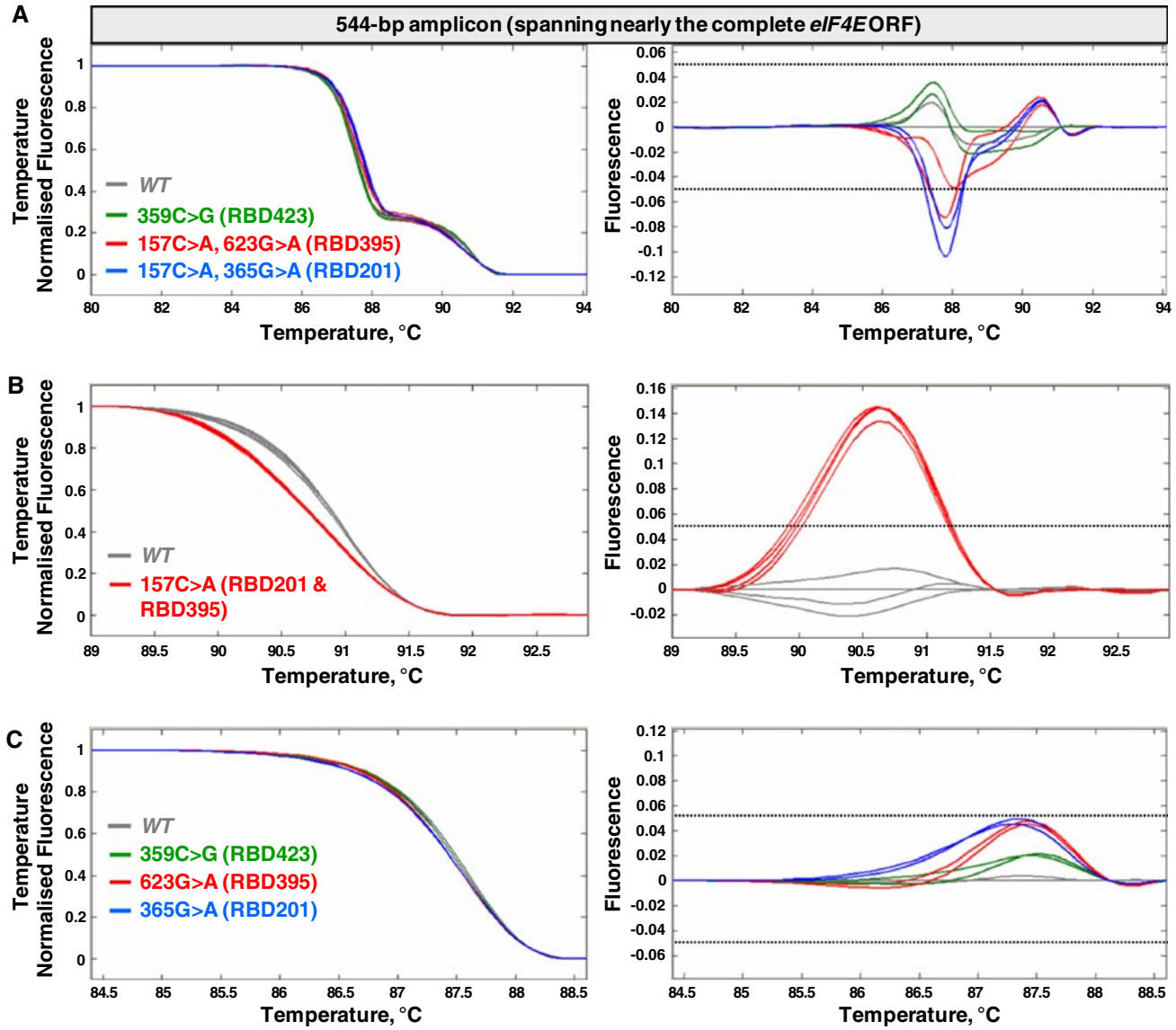


Fig. 4 Optimisation of the HRM assay using the large (544-bp) amplicon spanning a nearly complete *eIF4E* ORF and containing two melting domains, and selected variant *eIF4E* alleles. The HRM data are presented as two different graphs. The graph on the left in each panel displays temperature normalised melting curves for the tested samples, whereas the graph on the right shows difference curves displaying the difference in fluorescence (ΔF) of the respective sample in relation to the wild-type (*WT*), barley Morex sample. **a** Analyse of both temperature melting domains together, whereas **b** and **c** display the separate analyses of the high melting temperature and the low temperature melting domain, respectively. Samples containing specific nucleotide polymorphisms and the wild-type sample used in the assay are colour coded and are indicated inside the corresponding graph on the left in each panel. The curves are also colour coded according to the colour of the corresponding sample. Each sample was analysed in triplicate or duplicate

amplicon covered an additional 60 nt of GC-rich sequence (70% G + C content) at the 5′-end of the *eIF4E* ORF (Fig. 1, hatched region). All DNA polymorphisms were reliably predicted using these two amplicons with ΔF well above 0.05 cut off point (Fig. 3). Interestingly, the melting profile of the 299-bp amplicons revealed the presence of two melting domains, ~86 and ~90.5°C, and a rather complex difference curves pattern (Fig. 3b). The 239-bp amplicons had only a single ~86°C melting domain (Fig. 3a), suggesting that the second high-temperature melting domain of the 299-bp amplicon corresponds to the short GC-rich segment (nucleotide position 131–190). This was confirmed in two additional re-melt assays of the 299-bp amplicons using the temperature intervals of 78–88°C and 88–98°C, respectively, and subsequent analysing of each of the two melting domains separately. This time, the difference curves for each individual melting domain had simple patterns (Fig. 3c, d) and, importantly, the difference curves

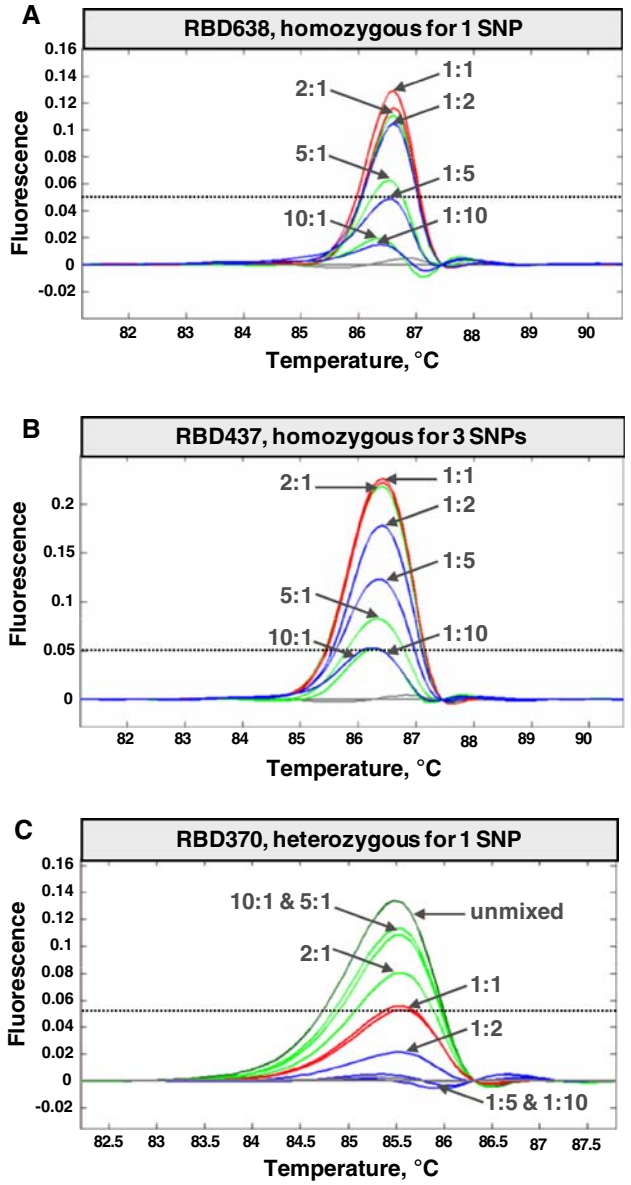


Fig. 5 HRM assays using different mixing ratios of variant and wild-type samples as the templates. The HRM data are presented as graphs displaying the difference in fluorescence (ΔF) of three different variant/wild-type sample mixtures in relation to the wild-type (*WT*), barley Morex sample. **a**, **b** The 281-bp amplicon, and **c** the 239-bp amplicon. Each variant sample was mixed with a *WT* sample at the following ratios: 10:1, 5:1, 2:1, 1:1, 1:2, 1:5 and 1:10

for the 239-bp amplicons were very similar to those for the ~86°C melting domain of the 299-bp amplicons (compare Fig. 3a and Fig. 3d). Moreover, aberrations in the difference curve pattern for the ~90.5°C melting domain of the 299-bp amplicons corresponded only to those genotypes that contained SNPs at the nucleotide position 157. Conversely, aberrations in the difference curve pattern for the ~86°C melting domain of the 299-bp amplicons corresponded to genotypes with SNPs outside the 131–190-nt region of the *eIF4E* ORF.

To test the applicability of HRM for nucleotide polymorphism scanning in a larger segment of a cDNA, the 544-bp amplicon (Fig. 1) was selected and variant barley genotypes RBD201, RBD395 and RBD423, were tested. As expected, these amplicons also contained two separate melting domains, ~90.5 and ~87.5°C (Fig. 4a). However, the high-temperature melting domain was less pronounced than had been obtained for the 299-bp amplicons (compare Fig. 3b and Fig. 4a). The difference curves of the two samples with SNPs at the nucleotide position 157 of the *eIF4E* ORF displayed similar patterns to those observed in the analyses of the 299-bp amplicons, although the curve peaks were lower and reflected the significantly lower ΔF values. The 544-bp amplicon was re-melted using the temperature window of 88°–98°C and the ~90.5°C melting domain was analysed separately. In this analysis, two genotypes with the 157C > A SNP in the 5′ GC-rich *eIF4E* segment were clearly distinguished from the *WT* (Fig. 4b). However, similar analysis of the ~87.5°C melting domain revealed all three genotypes carrying SNPs outside the 5′ GC-rich region had $\Delta F \leq 0.05$ and were practically indistinguishable from the *WT* (Fig. 4c).

Testing the sensitivity of HRM for detecting variant alleles in genotype mixtures (pooled samples)

A pooling strategy is frequently used in mutation scanning studies to increase efficiency whilst reducing costs. To test for the detection efficiency of variant alleles in pooled samples, the 281-bp DNA amplicon derived from two defined homozygous variants RBD638 and RBD437 and the 239-bp amplicon derived from the heterozygous variant RBD370 were mixed in various proportions with the corresponding amplicon from the wild-type barley Morex. The difference curves for the 281-bp amplicon derived from barley accession RBD638 homozygous for the variant allele carrying a single SNP (584G > C) were obtained (Fig. 5a). As expected, a 1:1 (variant:*WT*) amplicon mixture resulted in the tallest difference curve peak. The presence of a variant allele was also reliably predicted when a 1:2 (variant:*WT*) amplicon mixture was used. A single SNP was also detectable when the variant-derived amplicon was mixed 1:5 with *WT* ($\Delta F \approx 0.05$). However, a presence of variant allele could not be reliably predicted in a 1:10 (variant:*WT*) amplicon mixture because the ΔF value for this sample was ≤ 0.05. Similar analyses using the 281-bp amplicon derived from barley accession RBD437 homozygous for the variant allele containing three SNPs (476A > G, 593T > A, 622G > A) demonstrated that this variant allele could be reliably predicted in a 1:1, a 1:2 and even a 1:5 (variant:*WT*) amplicon mixture (Fig. 5b). Moreover, difference curves for these samples had significantly higher ΔF values for each mixing ratio than the sample

Table 2 Summary of the results obtained for 32 variant alleles of *eIF4E* analysed by HRM and by Sanger sequencing from 35 barley accessions

ACCN	HRM predictions				Sequencing data		
	Amplicon size and spanned portion of the ORF (nt)				Determined nucleotide change	Predicted protein change	Accession status
	299-bp (131–429)	239-bp (191–429)	281-bp (394–674)	544-bp (131–674)			
RBD23	VAR	*WT*	VAR	VAR	157C > T, 483G > T	P53S, Q161H	Pure
RBD67	*WT*	*WT*	VAR	VAR	598Δ12	Δ200–203	Pure
RBD144	VAR	*WT*	*WT*	VAR	157C > A	P53T	Pure
RBD201	VAR	VAR	*WT*	VAR	157C > A, 365G > A	P53T, S122N	Pure
RBD252	VAR	*WT*	VAR	VAR	157C > T, 483G > T, 626C > G	P53S, Q161H, A209G	Pure
RBD350	*WT*	*WT*	VAR	?	483G > T	Q161H	Pure
RBD370	VAR	?	*WT*	?	358A > G	T120A	Mixed
RBD395	VAR	*WT*	VAR	VAR	157C > A, 623G > A	P53T, G208D	Pure
RBD397	VAR	*WT*	VAR	VAR	157C > A, 488G > A	P53T, R163K	Pure
RBD423	VAR	VAR	*WT*	*WT*	359C > G	T120S	Pure
RBD427	VAR	VAR	VAR	VAR	278T > A, 359C > G, 481C > A	V93D, T120S, Q161K	Pure
RBD429	*WT*	*WT*	VAR	VAR	617A > G, 622G > A	D206G, G208S	Pure
RBD430	VAR	VAR	VAR	VAR	359C > G, 477G > T, 481C > A	T120S, K159N, Q161K	Pure
RBD431	*WT*	*WT*	VAR	VAR	593T > A, 622G > A	V198D, G208S	Pure
RBD432*	nt	nt	VAR	nt	593T > A, 622G > A	V198D, G208S	Pure
RBD434	VAR	VAR	VAR	VAR	359C > G, 481C > A, 623G > A	T120S, Q161K, G208D	Pure
RBD437	*WT*	*WT*	VAR	VAR	476A > G, 593T > A, 622G > A	K159R, V198D, G208S	Pure
RBD439	VAR	VAR	VAR	VAR	359C > G, 481C > G	T120S, Q161E	Pure
RBD441	VAR	VAR	VAR	VAR	359C > G, 623G > A	T120S, G208D	Pure
RBD442	*WT*	*WT*	VAR	VAR	622G > A	G208S	Pure
RBD479	VAR	VAR	VAR	VAR	359C > G, 481C > A, 625∇9	T120S, Q161K, 208∇VDKG	Pure
RBD482	VAR	VAR	VAR	VAR	359C > G, 481C > A	T120S, Q161K	Pure
RBD483	VAR	VAR	VAR	VAR	267C > A, 584G > A	S89R, G195E	Pure
RBD497	*WT*	*WT*	VAR	?	623G > C	G208A	Pure
RBD500	*WT*	*WT*	VAR	VAR	481C > A, 623G > A	Q161K, G208D	Pure
RBD503	VAR	VAR	VAR	VAR	278T > A, 481C > A, 622G > A	V93D, Q161K, G208S	Pure
RBD504*	nt	nt	VAR	nt	481C > A, 622G > A	Q161E, G208S	Pure
RBD517	*WT*	*WT*	VAR	VAR	481C > G, 622G > A	Q161E, G208S	Pure
RBD544	*WT*	*WT*	VAR	VAR	541G > C	G181R	Pure
RBD547	*WT*	*WT*	VAR	VAR	481C > A	Q161K	Pure
RBD558	*WT*	*WT*	VAR	VAR	623G > A	G208D	Pure
RBD563*	nt	nt	VAR	nt	481C > A	Q161K	Pure
RBD625	?	?	*WT*	*WT*	383C > T	S128F	Pure
RBD638	*WT*	*WT*	VAR	VAR	584G > C	G195A	Pure
RBD641	*WT*	*WT*	VAR	VAR	625∇9	208∇VDKG	Pure

ACCN Rothamsted barley accession number, *VAR* variant, *WT* wild-type, *nt* not tested, ? inconclusive, *598Δ12* deletion of 12 nt after the nucleotide position 598, *625∇9* insertion of 9 nt after the nucleotide position 625, *Δ200–203* deletion of four amino acid residues at positions 200–203 in the predicted *WT* sequence, *208∇DKG* insertion of three amino acid residues after the amino acid position 208, *pure* a single *eIF4E* sequence is present within the accession, *mixed* two different *eIF4E* sequences are present within the accession, *these three accessions carrying were only used in the initial HRM assay optimisation experiments presented in Fig. 2

containing a single SNP. Similar data were obtained following analyses of the 281-bp amplicon derived from barley accessions homozygous for variant *eIF4E* alleles containing two SNPs, either a 9-nt insertion or a 12-nt deletion (see Fig. S1).

Interestingly, a completely different result was obtained for the genotype RBD370 heterozygous for a single SNP (358A > G) (Fig. 5c). The tallest difference curve peak was obtained for the 281-bp RBD370-derived amplicon undiluted with the *WT*-derived amplicon. A single SNP was also detectable when the amplicon derived from heterozygous variant was mixed 1:1 with the *WT*-derived amplicon ($\Delta F \approx 0.05$). However, higher dilutions (i.e. 1:2, 1:5 and 1:10) of the variant-derived amplicon with the *WT*-derived amplicon resulted in melting curves that were practically undistinguishable from those derived from the wild-type barley Morex alone.

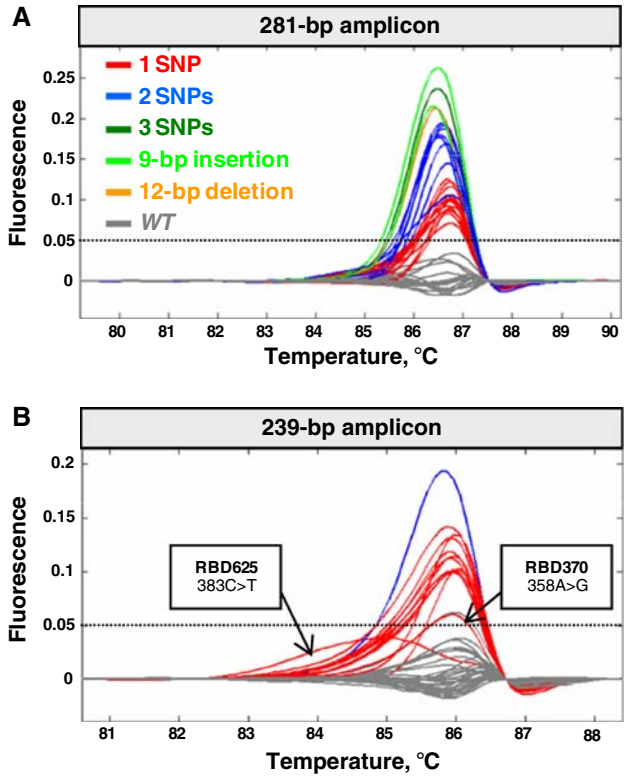


Fig. 6 HRM analysis of 32 different variant alleles of *eIF4E* using the two amplicons each containing one melting domain. **a**, **b** Analysis of 281 and 239-bp amplicons, respectively. Difference curves displaying the difference in fluorescence (ΔF) of the respective variant sample in relation to the wild-type, Morex barley sample are shown. The samples are colour coded according to the sequencing results. Two samples containing one SNP each and displaying aberrant difference curves but with low ΔF values, below 0.05, are indicated as boxed accession number/sequence variant in **b**

Evaluation of a panel of sequence characterised barley *eIF4E* alleles using HRM

For the main study, the sensitivity of the HRM assay for detection of different types of DNA polymorphisms was tested using 44 barley accessions from which the complete 620-bp cDNA-derived amplicon had been characterised by sequencing (Kanyuka et al., unpublished). Thirty-two of these accessions carried different variant alleles (Table 2), whereas the other 12 contained the *WT* allele indistinguishable from that of Morex barley. Each of the variant alleles contained between one and three SNPs, or a 9-nt insertion, or a 12-nt deletion compared to the *WT* (Table 2). All accessions were homozygous for a particular *eIF4E* allele, except for RBD370 that was heterozygous for the 358A > G SNP. Each accession was given a different anonymous number at the start of the experiment to ensure the data analysis was done without any knowledge of the sequencing results. Difference curves for four differently sized amplicons, which collectively span nearly the entire ORF, are presented in Figs. 6 and 7.

Analysis of the shapes and the amplitudes of the difference curves for the 281-bp amplicon, spanning the 3′-portion of ORF, predicted 27 genotypes as carriers of nucleotide polymorphisms and 17 genotypes as carriers of the *WT* sequence in this portion of the ORF. Sequencing data confirmed this prediction giving 100% sensitivity for HRM within this genotype set (Table 2). The genotypes containing two SNPs in the spanned region had taller difference curves peaks than those containing a single SNP (Fig. 6a). However, the tallest difference curve peaks were noticed for the genotypes containing three SNPs, a 9-nt insertion or a 12-nt deletion in this region (Fig. 6a).

Two amplicons of 239 and 299-bp in length were selected for HRM analyses of the 5′-portion of the ORF. As shown above, the 239-bp amplicon contained a single melting domain, whereas the 299-bp amplicon contains two melting domains due to the presence of an additional 60 bp GC-rich sequence at its 5′-end (Fig. 1). Melting profiles for both amplicons, clearly distinguished the ten genotypes, each contain a single SNP and one containing two SNPs, from those without any nucleotide polymorphism in this region of the ORF (Figs. 6b, 7c, d). In addition, the 299-bp amplicon was capable of detecting six additional genotypes each carrying a single SNP in the first 60-nt of sequence (i.e. the higher temperature melting domain) spanned by this amplicon (Fig. 7c). The status of one genotype (i.e. variant vs. *WT*), RBD625, based on HRM assay was uncertain. Although the 239 and 299-bp amplicons derived from this genotype had difference curves that were distinguished from the *WT* by shape, the ΔF value was below the 0.05 cut-off point (Figs. 6b, 7d). The 239-bp amplicon derived from another genotype, RBD370, had $\Delta F \approx 0.05$ (Fig. 6b). However, the 299-bp amplicon derived from this genotype had a ΔF clearly above the 0.05 cut-off point (Fig. 7d). Therefore, RBD370 was predicted to be a variant. Subsequent comparison with the sequencing data revealed that RBD370 was heterozygous for the 358A > G SNP, whereas RBD625 was homozygous for a single SNP (383C > T) located at less than 20 nt from the amplicon's reverse primer sequence.

Next, a large 544-bp amplicon, which covers most of the ORF except for the first 130-nt beyond the ORF's start codon, was tested in the HRM assay. As mentioned above, this amplicon also contains two melting domains (Fig. 7e), and therefore, in addition to the analysis of the complete amplicon (Fig. 7f) each of the melting domains was re-analysed separately (Fig. 7g, h). Based on these analyses, six genotypes were predicted to carry nucleotide polymorphisms in the 5′ 60-nt sequence (i.e. the higher temperature melting domain) spanned by this amplicon. This prediction

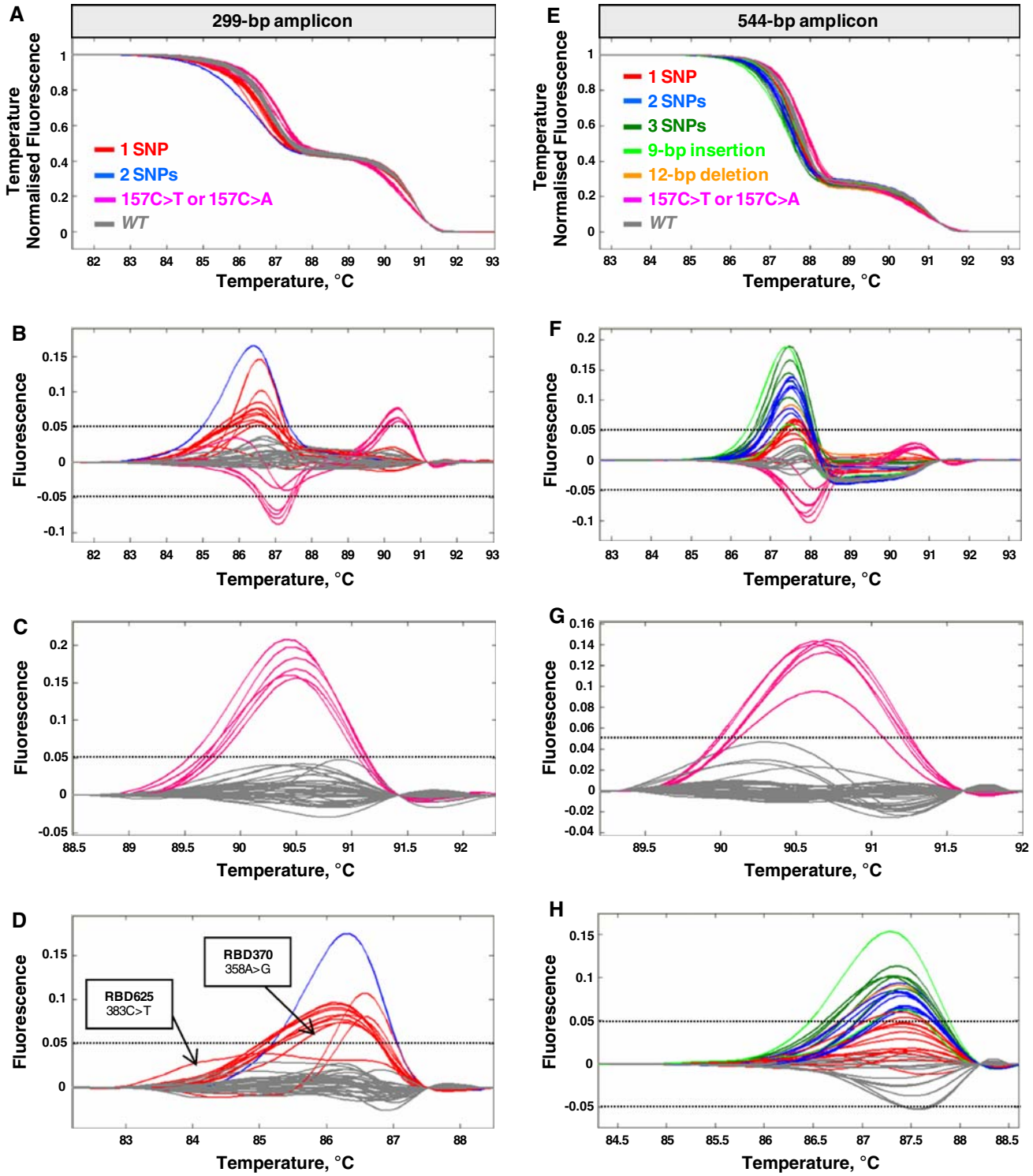


Fig. 7 HRM analysis of 32 different variant alleles of *eIF4E* using the two amplicons each containing two melting domains. The HRM data are presented as two different graphs. **a**, **e** Temperature normalised melting curves for the tested samples, **b–d, f–h** difference curves displaying the difference in fluorescence (ΔF) of the respective sample in relation to the wild-type, barley Morex sample. **b**, **f** analyses of both temperature melting domains together, whereas **c, d**, **g, h** analyses of high melting temperature and low temperature melting domains separately. The samples are colour coded according to the sequencing results. One sample containing a single SNP and displaying an aberrant difference curve shape but with low ΔF value, below 0.05 is indicated (*boxed* information in **d**)

was in complete agreement with the sequence data (Table 2). Analysis of the HRM data for the second, largest portion of a 544-bp amplicon (i.e. the lower temperature melting domain) predicted 21 genotypes as carriers of nucleotide polymorphisms in this region of the ORF. Sequencing data revealed that indeed, as predicted by HRM, all these genotypes carried DNA sequence polymorphisms, namely a 12-nt deletion (1 genotype), a 9-nt insertion (2 genotypes), three SNPs (5 genotypes), two SNPs (8 genotypes), and one SNP (5 genotypes) (Table 2). However, comparison of sequencing and the HRM data also revealed that HRM using the 544-bp amplicon failed to identify five genotypes carrying a single SNP. Moreover, the difference curves for the 544-bp amplicon derived from variant genotypes were all less pronounced and displayed significantly lower ΔF values than those for shorter amplicons. These data highlighted the limitations of the HRM assay for detecting single SNPs when using amplicons larger than 300-bp.

Discussion

The present study has demonstrated that the HRM analysis of cDNA-derived PCR amplicons is a rapid, simple and cost-effective method for identification of novel *eIF4E* gene alleles in barley. The advantage of using cDNA rather than genomic DNA for the present allele mining study was two-fold: it allowed identification of the most informative and useful mutations, i.e. those located in gene coding regions, and it required analysis of only two ~300-bp amplicons instead of at least 15 amplicons of the same size required to scan for mutations in the whole *eIF4E* gene sequence. In the blind study, we demonstrated that the sensitivity of the HRM assay was sufficient for detection of 32 different *eIF4E* alleles that have been recently sequence-characterised in our laboratory (Kanyuka et al., unpublished). The ability of these novel *eIF4E* alleles to control resistance to a range of European isolates of BaMMV and BaYMV is currently being tested in pathoassays and will be reported elsewhere.

Results of the current study suggest that the HRM analysis of cDNA-derived PCR amplicons is likely to be applicable for many other target genes in barley as well as in other crop and model plant species, and especially for genes either containing large introns or a large number of introns. The genomes of most crop species have not yet been sequenced, but for many of these species, comprehensive databases of tentative consensus full-length expressed transcripts are available (The Gene Index Project, http://compbio.dfci.harvard.edu/tgi/plant.html). Therefore, the allele mining strategy proposed here may also be applicable for many target genes in various crops for which currently only the transcript sequences are available. However, in certain cases, it may be advantageous to analyse genomic DNA-derived amplicons rather than cDNA-derived PCR amplicons; for example, when a target gene is known to be expressed at extremely low level or only at a specific developmental stage or only in specific tissues, or when alternative mRNA splice forms for a target gene are known to exist, or in studies requiring the identification of null mutations, i.e. those that lead to a target gene not being transcribed into mRNA.

The HRM analysis was very efficient in identifying a wide diversity of nucleotide polymorphism types (Table 2). These included SNPs, nucleotide transitions (C > T, G > A, and A > G) and nucleotide transversions (C > A, C > G, G > T, G > C, A > C, and T > A), as well as a short nucleotide insertion (9-nt) or a 12-nt deletion. The HRM assay based predictions approached 100% accuracy for amplicons containing either three or two or even a single nucleotide polymorphism. The A/T base changes have a slightly lower melting temperature than C/T, G/A and G/C base changes; however, in our study, all of these were reliably predicted based on a changes in the shape or the amplitude of the melting curves between the homozygous *WT* samples and samples carrying variant sequences in heterozygous conditions. One limitation of the HRM assay identified in this study was the inability to identify SNPs located very close to the amplicon's primers (<20-nt). Therefore, it is essential to design for each target gene or gene region amplicons that overlap by more than 20-bp. Another limitation of the HRM assay is the low accuracy in detecting single SNPs when using amplicons larger than 300-bp (this study) or 400-bp (Reed and Wittwer 2004).

In spite of the limitations indicated above, the HRM assay is still predicted to be advantageous for allele mining compared to other known methods for detecting DNA polymorphisms in plants including the standard Ecotilling. The advantages of HRM include (a) simplicity—the whole assay from PCR to generate the melting profiles is performed in a single tube and no additional post-PCR processing of DNA amplicons is required, (b) speed—only a maximum of 10 min post-PCR is required to generate the samples DNA melting profiles using the LightScanner® instrument, (c) cost effectiveness—although the cost of high-resolution melting instruments are comparable to those of either a 4300 DNA Analyser (LI-COR) or a dHPLC WAVE™ system (Transgenomic) commonly employed in Ecotilling, the HRM method offers great consumables savings because it does not require post-PCR in-gel or liquid chromatography analyses of DNA amplicons, and iv) non-destructive nature—the samples predicted to carry nucleotide polymorphism can be recovered post-melting and the nature and position of polymorphisms determined by direct Sanger sequencing.

Moreover, HRM is amenable to high-throughput delivery and potentially to robotisation. Our data suggest that the allele mining costs could be reduced even further by pooling up to five samples for HRM assays. For this reason, the HRM assay could also be potentially applied for TILLING (Targeting Induced Local Lesions IN Genomes; Till et al. 2003) studies, i.e. identification of mutations in target genes in large populations of chemically induced mutants. To detect mismatches in heteroduplex DNA that had been generated by PCR amplification from small pools of mutant and *WT* plants the TILLING technique uses either dHPLC, or standard or capillary gel electrophoresis following cleavage of the heteroduplexes with mismatch-specific endonucleases, e.g. CEL I and ENDO1 (Triques et al. 2008). The latter also requires the use of fluorescently labelled PCR primers, which are expensive. The application of the HRM assay for TILLING is predicted to be advantageous in terms of speed and simplicity. The costs are also likely to be reduced due to eliminating the need for dHPLC or gel-electrophoresis consumables, mismatch-specific endonucleases and fluorescently labelled primers. Therefore, the HRM assay would allow the TILLING procedure to become affordable even for laboratories operating on relatively low budgets.

It is envisaged that the application of the HRM assay to plant sciences will expand in the future to other genetic studies. For example, recent reports suggest that the HRM assay will prove to be useful for varietal certification (Mackay et al. 2008) and for developing and mapping of SNP and microsatellite markers (Chagne et al. 2008; Croxford et al. 2008; Lehmensiek et al. 2008; Mader et al. 2008; Wu et al. 2008).

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