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Full Length Research Paper

Development of a single nucleotide polymorphism (SNP) marker for detection of the low phytic acid (*lpa1-1*) gene used during maize breeding

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Breeding for low phytic acid (LPA) in maize is hampered by a tedious and destructive colorimetric assay in mature dry grain. There are no molecular markers available for the single recessive gene of *lpa1-1*, restricting breeding programmes. The aim of this study is to develop a molecular marker to identify the *lpa1-1* gene at the early vegetative plant stage. The parental lines are temperate LPA line (CM 32) and tropical line (P 16). The *lpa1-1* allele is due to a single amino acid change from alanine to valine. The nature of the single nucleotide polymorphism (SNP) marker was validated by DNA sequencing of the parental PCR products. Using high resolution melt (HRM) profiles and normalised difference plots, we successfully differentiated the homozygous dominant (wild type), homozygous recessive (LPA) and heterozygous genotypes. The reduced phytate content of the LPA parental line was confirmed. The profiles from low cost crude and high quality DNA extraction were comparable when distinguishing between the parental lines. The cost of HRM analysis was 8% of the cost of PCR amplification and DNA sequencing. The development of the marker will make maize breeding for LPA efficient and fast, and it will enable the earlier release of *lpa1-1* varieties.

Key words: High resolution melt (HRM) analysis, low phytic acid, maize, single nucleotide polymorphism (SNP) marker.

INTRODUCTION

Phytic acid is an anti-nutritional component found in many cereals and legumes. In maize, 90% of total seed phosphorus (P) is in the form of phytic acid (O'Dell et al., 1972). Phytic acid chelates essential minerals such as iron, zinc, potassium, magnesium and calcium as it passes through the digestive system of humans and other monogastric animals. This leads to increasing iron and zinc deficiencies in communities that subsist on maize as their daily food requirements. There is a considerable amount of evidence to support the fact that dietary phytate has a negative effect on the bioavailability of dietary minerals in humans as the substitution of LPA grain in a maize based diet was associated with a substantial increase in zinc (Adams et al., 2002; Hambidge et al., 2004), iron (Mendoza et al., 1998, 2001; Hurrell et al., 2003), calcium (Hambidge et al., 2005) and magnesium (Bohn et al., 2004) absorption.

Monogastric animals have high levels of phytic acid which results in very low bioavailability of mineral nutrients. This is due to their lack of the enzyme phytase activity to digest phytic acid to release P and minerals in the gut. A result of high phosphorus excretion by monogastric animals is the environmental pollution of water and soils. A possible solution is the isolation of cereal mutants accumulating less phytic P and more free phosphate in the seed. In animal feeding studies using low phytate maize, there was an increase of two to five fold the amount of bioavailable phosphorus observed (Douglas et al., 2000; Li et al., 2000; Spencer et al.,

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2000a, b; Yan et al., 2000, 2003; Veum et al., 2001; Peter and Baker, 2002). Therefore, reduction of phytic acid levels in maize grain would be effective in improving human and animal nutrition and would benefit the environment through reduced eutrophication.

Since phytic acid is mainly found in the germ of the mature seed (O'Dell et al., 1972), a colorimetric assay of the crushed seed is usually used as the method of detection which effectively destroys the seed (Chen et al., 1956; Lucca et al., 2001; Chiera et al., 2004) and removes the opportunity for advancing the generation of that particular seed. This method is long and comprises an overnight incubation as well as requiring several hours of manual labour preparation for the assay (Chiera et al., 2004; Lorenz et al., 2007). Therefore, this method is detrimental in breeding programmes, especially in cases of segregating material when screening for the LPA trait once the seed has been crushed for the assay; though it cannot be used to generate a seedling. Consequently, testing and selection for LPA trait is delayed to advanced generations with many seeds, but with serious consequences for the costs of carrying forward many materials in the breeding program.

Maize inbred lines containing lower levels of phytic acid have been produced using ethylmethane sulphonate-induced (EMS) mutagenesis (Raboy, 2000). The maize *lpa1-1* mutants show a reduction in phytic acid accompanied by inorganic phosphate. The genes from *lpa1-1*, *lpa2* and *lpa3* have all been cloned (Shi et al., 2003, 2005, 2007). The *lpa1-1* gene encodes a multidrug resistance associated protein (MRP) ATP-binding cassette (ABC) transporter (Shi et al., 2007), while *lpa2* gene encodes an inositol phosphate kinase (IPK) belonging to the Ins (1, 3, 4) P3 5/6 kinase gene family (Shi et al., 2003), and *lpa3* gene encodes a myo-inositol kinase (MIK) gene (Shi et al., 2005).

The *lpa1-1* mutation shows a 66% reduction in phytic acid P (Raboy, 2000). The *lpa1-1* gene has been mapped onto the chromosome 1 distal region and reported to be due to a single recessive mutation (Raboy et al., 2000). The gene was completely sequenced (5149 bp) and the *lpa1-1* phenotype was reported to be probably due to an alanine to valine substitution (Shi et al., 2007). However, the exact position of the mutation or molecular marker information to identify *lpa1-1* gene was not available. Molecular markers can be used for detection of traits that are difficult to phenotype or for recessive genes. In the case of phytic acid, the trait is both difficult to phenotype as well as being a single recessive mutation; hence, molecular markers can be applied to identify the *lpa1-1* trait with increased reliability, accuracy and cost effectiveness.

SNP markers are stable; they have a high frequency in the genome and a higher inheritance than most other markers. It has been conservatively estimated that there are 20 million SNP polymorphisms in maize (Rafalski, 2002), thereby showing the potential value of using SNPs for marker-assisted selection (MAS). A limitation of SNP marker application to high-throughput is the method of detection of the sequence variation which usually involves sequencing the product, which is costly and time consuming. SNP markers have been used extensively both in animal and human genetics (Picoult-Newberg et al., 1999; Schork et al., 2000; Vignal et al., 2002) to study complex traits and diseases and in some studies in conjunction with HRM analysis (Vossen et al., 2009). In plants, there have been a few extensive studies with their uses being genome mapping, high resolution genetic mapping of traits and association studies. There was a comparison study on SNP markers over the major cereal crops of the world which included wheat, barley, maize, sorghum and rice (Barker and Edwards, 2009).

There are reports on SNP markers associated with economic value genes in rice (waxy gene controlling amylose content, dwarfing gene), onion (male sterility), soybean (cyst nematode resistance), and SNPs for cultivar identification and diversity evaluation in barley, tree species, wheat and maize (Gupta et al., 2001). There have been direct SNP markers (C/T transition type) used in rice studies to identify the fragrance gene (fgr) (Jin et al., 2003), semi-dwarfing gene (sd-1) (Sasaki et al., 2002) and blast resistance gene (Bryan et al., 2000). SNP markers have been used in maize studies to estimate genetic diversity, population structure and familial relatedness (Hamblin et al., 2007; Jones et al., 2007; Yan et al., 2009).

High resolution melt (HRM) analysis is a post-PCR technology which, under correct conditions, can distinguish PCR amplicons on the basis of their melt profiles in the presence of an appropriate dye or probe. The HRM analysis technology has the advantages of low cost, high throughput, repeatability and accuracy of markers. This technology is able to differentiate between alleles of homozygote recessive, homozygote dominant and heterozygotes. A major advantage of HRM over gel electrophoresis is the differentiation of PCR products of the same length on the basis of sequence-dependent alterations of melt curves. It eliminates the post-PCR analyses such as gel-based or sequencing analyses, thereby increasing efficiency of marker detection assay as well as reducing costs, time and labour. Detailed reviews of the HRM technology are given by Ririe et al. (1997), Liew et al. (2004), Graham et al. (2005) and Montgomery et al. (2007). The factors determining the melt curve profiles include GC content, length and sequence of the product (Ririe et al., 1997). The Rotor-Gene 6000 real time rotary analyser (Corbett Research, Australia) is able to perform the HRM analysis immediately after PCR amplification.

HRM has been used successfully to identify SNPs in various crops including potato (Yuan et al., 2008), almonds (Wu et al., 2008; 2009), apple (Chagne et al., 2008), barley (Lehmensiek et al., 2008), lupin (Croxford et al., 2008; Lopez et al., 2008), grapevine and olives

(Mackay et al., 2008), tomato (van Deynze et al., 2007) and capsicum/pepper (Park et al., 2009). Gady et al. (2009) used HRM to detect point mutations in EMS tomato population. While molecular markers have been used to detect LPA mutants in rice (Zhao et al., 2008), soybean (Saghai-Maroof et al., 2009; Scaboo et al., 2009) and barley (Larson et al., 1998; Roslinsky et al., 2007), there have been no molecular markers available for LPA mutants in maize to date. A survey of the literature indicates that although SNPs are widely used in plants, the HRM technology has been scarcely applied in maize breeding and never in developing LPA maize varieties.

The objectives of this study are to develop a molecular marker/s for use at the early vegetative stages of the plant to detect the *lpa1-1* gene in maize genotypes during breeding; to compare the effect of high quality DNA extraction and crude extraction methods on the melt curves of the parental lines; and compare the cost of the HRM analysis to the conventional method of DNA sequencing for detection of the SNP marker. The sequence of the amplified PCR products was determined to validate the *lpa1-1* nucleotide change.

MATERIALS AND METHODS

Confirmation of phytate content of parental lines

Two inbred maize lines were used in this study. The normal (wild type) tropical locally adapted line was P 16 and the temperate *lpa1-1* source was CM 32. The F_1 cross between the parental lines was used as the heterozygote genotype. The phytic acid content of the two parental lines was determined according to the method of Wheeler and Ferrel (1971). This method involved the extraction of phytic acid with trichloroacetic acid from the parental seed material. The phytic acid was precipitated as a ferric salt due to its reaction with $FeCl_3$. The ferric salt produced was reacted with a HNO_3/KSCN reagent to produce the chromogenic Fe $(NO_3)_3$ solution whose absorbance was measured at 480 nm using a spectrophotometer. The Fe content of the Fe $(NO_3)_3$ produced from the sample extract was read off from the Fe $(NO_3)_3$ calibration curve. The phytic acid (phytic phosphorus) content of the sample was calculated from the Fe results assuming a constant of 4 : 6 for iron : phosphorus molecular ratio in the precipitate (Wheeler and Ferrel, 1971).

DNA extraction

Leaf material was sampled and ground in liquid nitrogen for high quality genomic DNA extraction using Wizard genomic DNA purification kit (Promega, Whitehead Scientific, Cape Town, South Africa). Various crude extraction methods were tested with a modification of the Edwards et al. (1991) method used in this study. In the crude extraction method, leaf samples for PCR analysis were collected using the lid of a sterile 1.5 ml microcentrifuge tube to punch out a disc of leaf material into the tube. The tissue was macerated with 400 µl extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % (w/v) SDS) and vortexed for a few seconds. The sample was heated for 10 min at 65°C and centrifuged for 2 min. The supernatant was removed, after which an equal volume of ice-cold isopropanol was added and mixed before incubation at -20°C for 30 min. The sample was centrifuged for 5 min at 4°C and the pellet was dried before re-suspending in 20 µl of TE (100 mM Tris-HCl, 1 mM EDTA, pH 8) buffer overnight. The DNA extracted using the genomic DNA extraction kit was visualised by screening on a 1% (w/v) agarose gel in TAE buffer.

Primer design

The wild type *lpa1-1* gene was sequenced by Shi et al. (2007) who stated that the *lpa1-1* mutation is a result of an alanine to valine amino acid change at position 1432 of the protein sequence. From this statement, it was inferred that this was a result of a C to T transition. The wild type sequence of *lpa1-1* is available on the National Centre for Biotechnology Information (NCBI) database (accession number: NM_001112590). Nine PCR primers were designed using the Primer3 programme (Rozen and Skaletsky, 2000) to flank the *lpa1-1* mutation position and had a predicted size range of 91 to 148 bp. The primers were purchased from Integrated DNA Technologies (Whitehead Scientific, Cape Town, South Africa), and were tested and optimized for annealing temperature.

PCR and HRM analysis

The Rotor-Gene 6000 real-time rotary analyser (Corbett Research, Australia) was used for PCR amplification and HRM analysis. PCR amplifications were performed in 20 µl reaction volumes consisting of 15 ng of genomic DNA template, Quantace SensiMixdT for the PCR reaction components (Celtic Diagnostics, Cape Town), 1 × SYBR Green I dye and 200 nM of forward and reverse primers. The PCR amplification was initiated for 10 min and held at 95°C as an initial denaturation and activation step, followed by 40 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 20 s. A negative control was added in each set of PCR reactions with no DNA included to ensure non-contamination of PCR reagents. HRM analysis was performed automatically after the PCR, and programmed to ramp temperature from 72 to 95°C, raised by 0.1 degree/step after the final extension step. The Rotor-Gene 6000 real time rotary analyser created the melt curves. The Rotor-Gene 6000 real time rotary analyser software version 1.7 was used to discriminate genotypes (Corbett Research, Australia). PCR products were screened initially for confirmation of successful amplification by electrophoresis in a 1.5% (m/v) agarose gel in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and visualized under UV light after staining with ethidium bromide (0.5 µg ml^{-1}). The optimization of the HRM method included the temperature range, checking for reliability between samples and between different runs and the ability to distinguish between homozygous dominant (WT), homozygous recessive (LPA) and the heterozygous genotypes.

Validation of *lpa1-1* SNP marker

The presence and identity of the *lpa1-1* SNP was confirmed and validated by forward and reverse sequencing of the PCR products of the parental inbred lines (Inqaba Biotech, Pretoria, South Africa). Due to the small size of the product, the PCR product was first cloned into a pJET vector and then sequenced (Inqaba Biotech, Pretoria, South Africa) to ensure the sequencing of the entire product. Sequence alignment was performed using the programme ClustalX (Ramu et al., 2003).

Comparison of DNA extraction methods

Two DNA extraction methods were compared to determine the efficiency of the marker in detecting the different genotypes. The

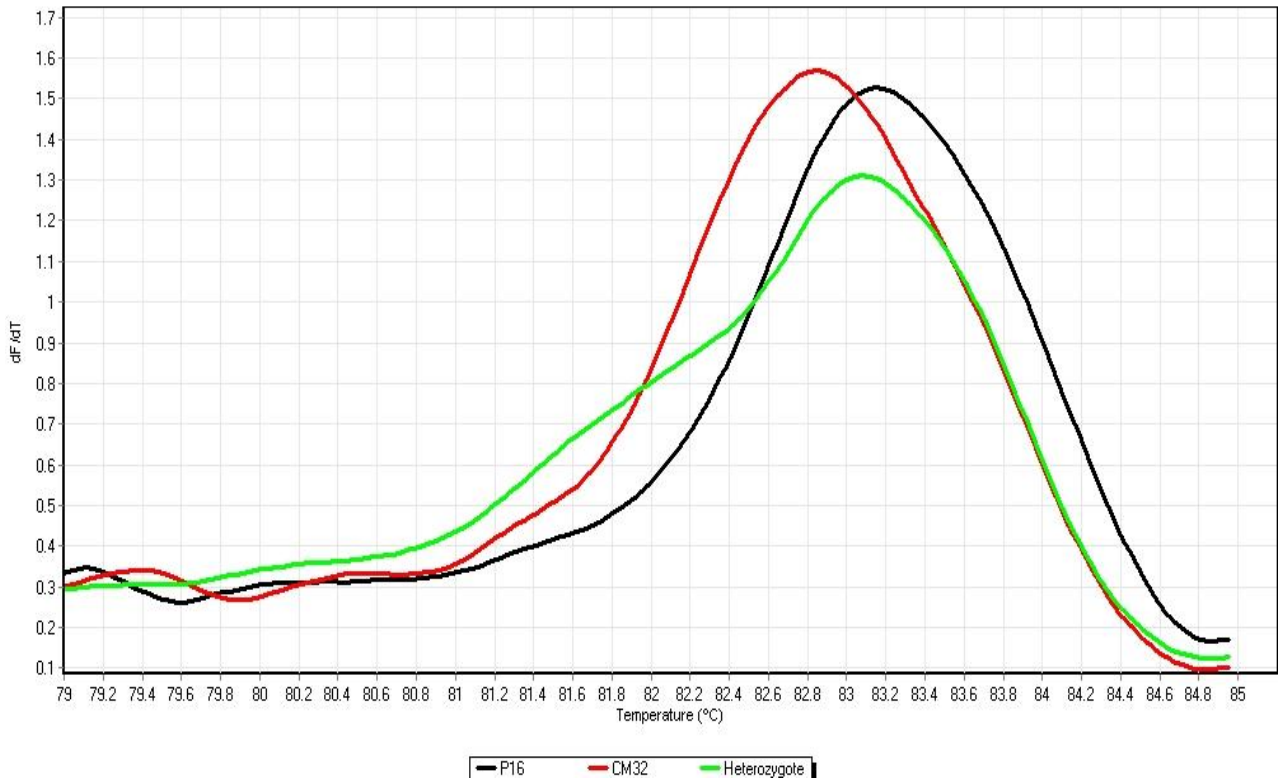

Figure 1. Melt profiles of P 16, CM 32 and heterozygote PCR amplification products from HRM analysis. The negative derivative of normalized fluorescence with respect to temperature was determined using the Rotor-Gene analysis software (version 1.7.75). Melt transitions appear as peaks with a 0.5°C difference observed between the parental inbred maize lines (P 16 and CM 32).

leaf material was ground in liquid nitrogen, and the Wizard genomic DNA purification kit (Promega, Whitehead Scientific, Cape Town, South Africa) was used for the high quality DNA extraction. The crude extraction method was a modification of the method of Edwards et al. (1991) using fresh leaf sample punch. The melt profiles of the parental lines were compared with high quality and crude extractions of genomic DNA to determine effectiveness of the *lpa1-1* SNP marker.

Comparison of costs of HRM analysis and DNA sequencing

The costs of DNA sequencing were compared in a price survey involving four different laboratories across South Africa to the cost of running the *lpa1-1* SNP marker on the Rotorgene 6000 with HRM analysis. The costing of the SNP marker with HRM analysis in a 20 µl PCR reaction included costs for DNA extraction, PCR reagents, SYBR green dye and PCR tube. The cost of a standard sequencing of one sample was used.

RESULTS AND DISCUSSION

The phytic acid content for the parental lines was found to be 77.6 mg g^{-1} of phytate for P 16 and the LPA line CM 32 had 27.2 mg g^{-1} of phytate. The LPA line showed a reduction of 65% which closely correlates with findings of Raboy (2000) who found a 66% reduction of phytic acid in *lpa1-1* mutant lines. These results confirmed the LPA and normal germplasm classification of the inbred maize lines used in this study.

The PCR optimisation used high quality genomic DNA of the inbred maize parental lines. There were nine PCR primer pairs tested in the initial optimisation for the *lpa1-1* SNP marker. The annealing temperature ranged from 50 to 65°C, with the optimum annealing temperature at 55°C selected for amplification of the PCR fragment. The selection of the most suitable forward and reverse primers for the *lpa1-1* SNP marker was based on the most stable and reliable melt profiles as well as the largest melt temperature differences between the parental lines. It was hypothesized that shorter PCR products would show larger melt temperature differences between the parental inbred lines; however, this was not observed as the shorter PCR products did not show significantly larger temperature shifts.

A 150 bp PCR product was amplified from the maize parental inbreds at an annealing temperature of 55°C (Forward 5'- ATA ACT GGA GCG TGG GAC AG-3' and reverse 5'-CTG CGG ATG ATC TTT TGG AT-3'). The raw data of the melt profiles were transformed (dF/dT) to give melt profiles of the parental lines and heterozygote PCR amplification products (Figure 1). Both parental lines

were unmistakably distinguished from each other in the melt profiles by 0.5°C melt temperature difference (Figure 1). The detection of the homozygous and heterozygous genotypes was completely consistent over all runs performed (in excess of 30 replicates). The wild type inbred maize line (P 16) had a melt temperature of 83.05°C, *lpa1-1* inbred maize line (CM 32) had a melt temperature of 83.53°C and the heterozygote maize line had a melt temperature of 82.80°C. The heterozygote profile was more clearly distinguishable in the difference plots and this was used to define the heterozygous genotypes.

Herrmann et al. (2006) found type I SNPs (C to T or G to A) to have melt differences around 1°C, while the Rotor-Gene protocols suggest a difference of >0.5°C (Corbett Life Science, 2006); however, this is amplicon-dependent. The melt difference found in this study is well within the discriminatory power of the Rotor-Gene 6000. Although there have been similar SNP markers identified in LPA rice mutants (Jin et al., 2003; Kim et al., 2008; Xu et al., 2009), HRM analysis was not used in the post-amplification detection; therefore those studies can not be compared to this study.

In this study, the 150 bp product from the maize parental lines was clearly differentiated with the HRM analysis. It has been shown with HRM analysis that there is 100% sensitivity for PCR products of 300 bp or less in identifying heterozygotes and homozygote genotypes. However, this has decreased to 96.1% sensitivity and 99.4% specificity for 400 to 1000 bp products (Reed and Wittwer, 2004). This is due to the fact that as the product length increases, the difference between the melt curves decreases, thereby leading to errors in detection of the particular allele.

In this study, the differences between the parental inbred PCR products can be more clearly visualised and quantified by using the difference plots of normalised than the melt curve analysis. A difference plot highlights the differences between individual curves relative to one of the sample melt curves plotted as a baseline. They can be used to differentiate between the homozygous and heterozygous genotypes more clearly than the melt curves, especially in cases where the melt differences are very small. The heterozygote curve which weaves into the LPA curve is seen clearly in Figure 2 where samples are normalised to LPA, and P 16 is positive with the heterozygote both positively and negatively. The distinctive shape of the heterozygote is used to differentiate heterozygous from homozygous genotypes.

The PCR products of the heterozygote in this study were differentiated from the parental line PCR products due to the characteristic shape in the difference plots which was also found in other studies. The shape of the melt curve of the PCR amplicon for the heterozygotes is a characteristic “wave” shape in the difference plots, confirmed by the difference between genotypes visible in difference plots due to unique trace patterns (Wittwer et al., 2003). Difference plots were able to successfully distinguish between homozygotes and heterozygotes in a F_2 population in pepper using *Pp201* SNP marker (Park et al*.,* 2009) and they allowed more distinct separation of grapevine varieties for DNA fingerprinting (Mackay et al., 2008) than melt curve analysis alone. DNA melt analysis can also be used to detect heterozygotes as in the case of the PCR amplicon from the exon of the human N-methyl-D-aspartate receptor gene (NR1) that had an alanine to valine amino acid change due to C to T base change (Lipsky et al*.,* 2001).

The *lpa1-1* SNP marker was validated by forward and reverse DNA sequencing. The sequences were aligned using the programme ClustalX (Ramu et al*.,* 2003) and were identical with only the single base change from C (wild type) to T (LPA) (Figure 3). The effect of DNA purity was assessed by comparing melt profiles generated with high quality and crude genomic DNA extractions. The melt profiles of the parental lines obtained from amplification with high quality genomic DNA showed very little difference from the melt profiles amplified with genomic DNA isolated with the crude extraction (Figure 4). The differences between the crude and high quality DNA extraction parental profiles were consistent over all runs performed (in excess of 30 replicates). The melt curves were very similar and the melt temperatures of P 16 differed by 0.07°C and CM 32 by 0.04°C. This proved that the crude extraction of genomic DNA was just as effective as the high quality genomic DNA extraction. The crude extraction method was more cost-effective as well and especially in cases of large sample volumes. This is an added benefit as it reduces the time required to extract genomic DNA from leaf material.

The cost of the SNP marker with HRM analysis was approximately $0.79/sample with no additional post-PCR analysis costs as the Rotor-Gene 6000 is essentially maintenance-free. The cost of DNA sequencing, however, includes DNA extraction and PCR amplification as for the SNP marker with the additional cost of sequencing. The prices per sample for DNA sequencing of a PCR product varied in South African laboratories from $20.00 (Lab A), $17.86 (Lab B), $14.99 (Lab C) to $9.00 (Lab D) (prices accessed on the internet websites on 12 June 2011). Therefore, the cost of using the *lpa1-1* SNP marker with HRM ($0.79) is only 8% of the lowest cost of DNA sequencing ($0.79 + $9.00 – product amplification and detection through DNA sequencing). This is significantly lower than the DNA sequencing cost per sample even at the most cost effective laboratory.

The cost of the Rotor-Gene 6000 is considerably lower than any commonly used sequencing platform. Besides the HRM analysis capability, it can be used for both standard and quantitative PCR. Additional factors to be taken into account are the personnel labour and time required for “cleaning” the PCR product before DNA sequencing can occur (as experienced during cloning and sequencing process), with the HRM analysis

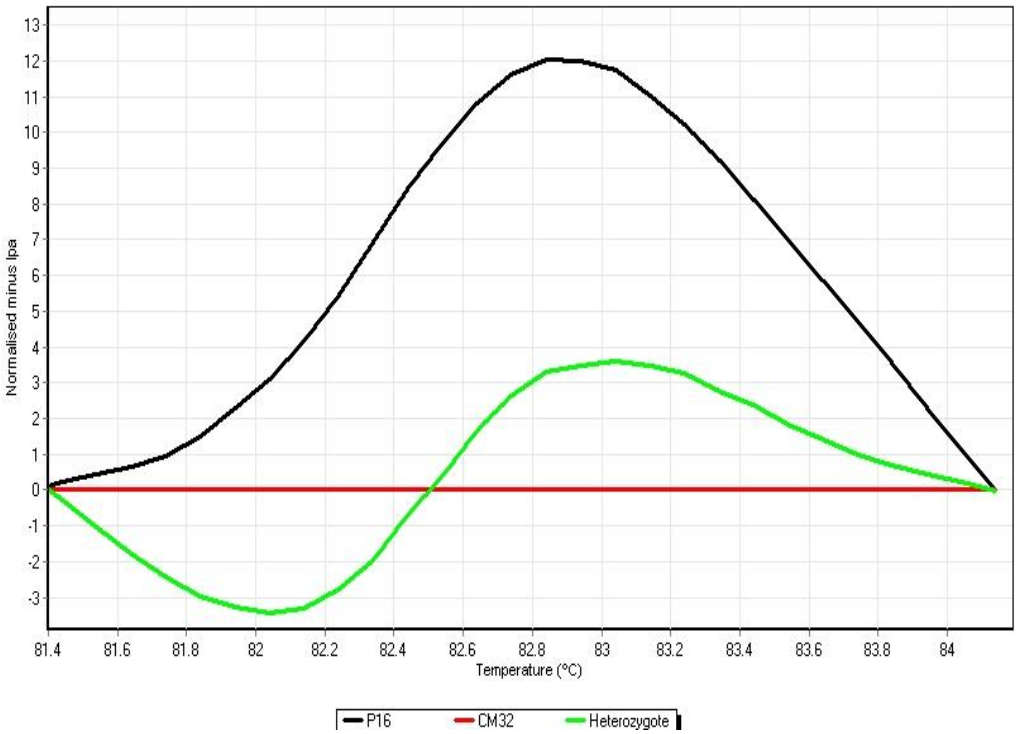

Figure 2. Difference plot of P 16 and heterozygote PCR amplification products with CM 32 normalized. Difference plots were generated using the Rotor-Gene analysis software (version 1.7.75). The heterozygote shows the characteristic “wave shape” used to distinguish heterozygous from homozygous genotypes.

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P 16            CAAAAATTTTGGTACTCGATGAGGCGACAGCATCTGTCGACACAGCAACAGACAATCTTA
Reference       CAAAAATTTTGGTACTCGATGAGGCGACAGCATCTGTCGACACAGCAACAGACAATCTTA
CM 32           CAAAAATTTTGGTACTCGATGAGGCGACAGTATCTGTCGACACAGCAACAGACAATCTTA
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Figure 3. Partial sequence alignment of P 16 (wild type), reference (wild type) and CM 32 (*lpa1-1*). This figure shows the C to T nucleotide base change. The reference sample was the wild type of the *lpa1-1* sequence published on NCBI (accession number of NM_001112590).

requiring much shorter time and less labour than DNA sequencing. All these factors validate the use of HRM analysis for *lpa1-1* SNP detection due to its lower cost (8%), the shorter time required for the HRM and less labour-intensive than DNA sequencing.

In plant breeding where there are large volumes of samples, especially during the early generations, screening methods that are quicker, less labour-intensive and more cost-effective than traditional screening methods are often sought. The characteristics that a molecular marker requires for use in plant breeding programmes include reliability, requiring low technical skill and short turn-around time with most markers meeting one or some of these criteria. This often poses a challenge for molecular biotechnologists and limits the application of molecular markers in plant breeding programmes. The *lpa1-1* SNP marker developed in this study meets all these requirements when used in conjunction with HRM analysis. However, in the case of SNP markers, this gap is slowly being bridged. These markers are widely distributed in the genomes and with the added technology of HRM analysis, SNPs can be used to successfully detect and distinguish between homozygous and heterozygous genotypes as well as being quick, reliable and very simple to use markers.

The use of HRM analysis has been very limited in plants, and in the case of the rice SNPs (*XS-lpa2-1* and *XS-lpa2-2*), phytic acid levels were detected with the colorimetric assay and the gene sequence was identified with PCR and RT-PCR (Xu et al., 2009). The SNP marker linked to the fragrance gene in rice also shows a C to T transition resulting in the change of phenotype; however, the SNP is detected by DNA sequencing of a 491 bp PCR product (Jin et al., 2003). In this study, the maize *lpa1-1* SNP is also due to a C to T transition which results in the change of the phenotype to LPA;

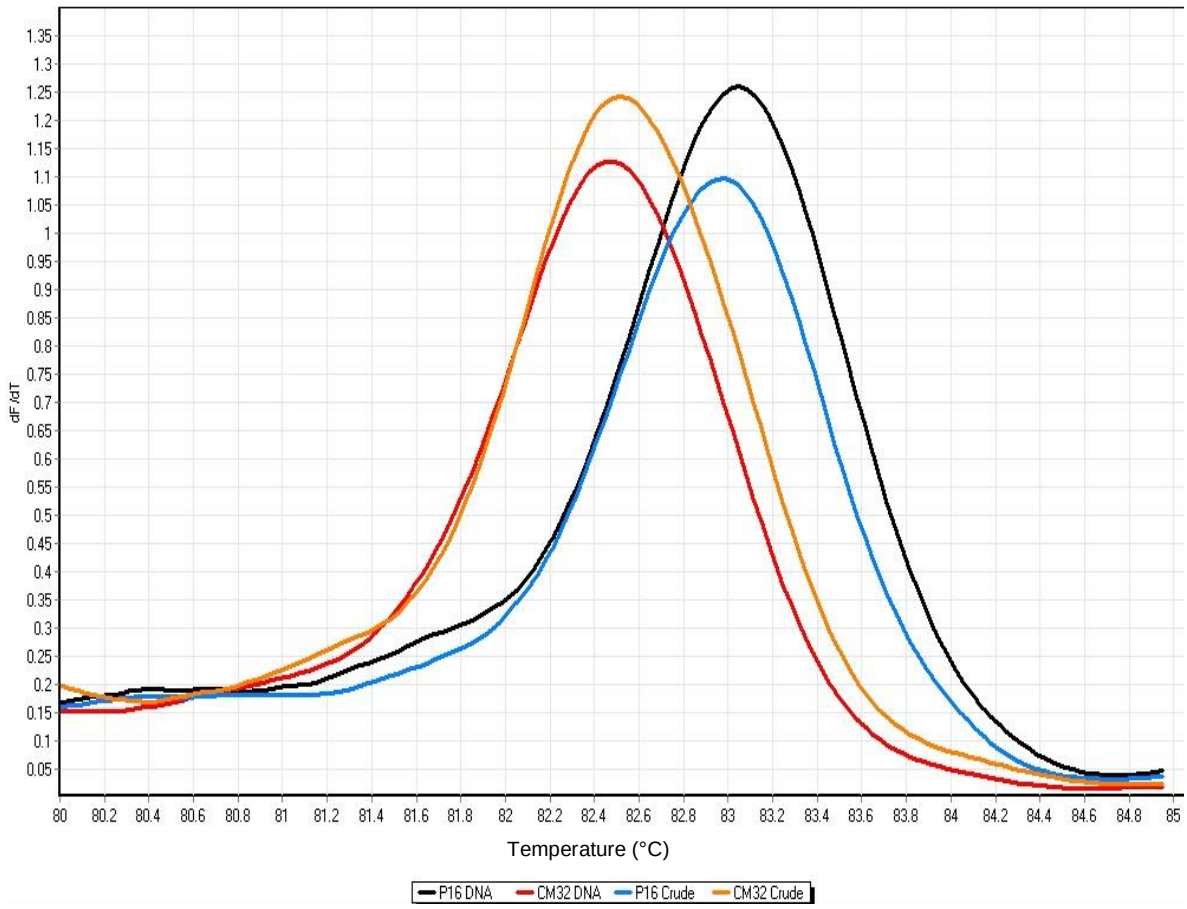

Figure 4. Melt profiles of high quality DNA extraction compared with crude DNA extraction of P 16 and CM 32. The negative derivative of normalized fluorescence with respect to temperature was determined using the Rotor-Gene analysis software (version 1.7.75). The DNA preparation procedure does not have a significant effect on the amplicon melt temperature.

nonetheless, the HRM analysis is able to successfully identify all the different forms of the alleles of *lpa1-1* gene. The potential to use HRM for the detection of maize SNP markers is clear. Therefore, this study is novel and unique in the use of HRM analysis to successfully detect the *lpa1-1* SNP maize genotypes without the use of the colorimetric assay and DNA sequencing of PCR products.

Conclusions

The following conclusions could be drawn from this study:

(1) The *lpa1-1* SNP marker using HRM analysis was able to successfully differentiate between the two inbred parental maize lines by the differences in melt profiles. The SNP marker was validated by DNA sequencing with the C to T transition clearly shown. The *lpa1-1* SNP marker is a co-dominant marker that is able to distinguish between homozygous dominant (wild type), homozygous recessive (LPA) and heterozygous alleles of the *lpa1-1* trait in maize.

(2) The inbred maize lines melt curves showed 0.5°C difference in melt temperature and the difference plots were effectively used to characterise the different genotypes, especially the heterozygotes.

(3) The crude DNA extraction was as effective as the high quality DNA extraction method for melt curve analysis.

(4) The *lpa1-1* SNP marker which is fast, stable and reliable can now be used to track the LPA trait in maize during the early vegetative phase instead of using the long conventional destructive colorimetric assay at the end of the growth cycle with consequences for time, breeding costs and loss of the actual seed germ which is supposed to be promoted to the next generation.

(5) In South Africa, for example, the cost of the *lpa1-1* SNP marker with HRM analysis was 8% of the cost of DNA sequencing which is the conventional method of detecting SNP genotypes.

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