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Application of High Resolution Melting for Detection of Induced DNA Polymorphism in *Curcuma alismatifolia*

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Summary

In order to reveal the induced variations in genomic DNA of mutants, simple sequence repeats (SSR) are used in plant genetics and breeding programs, which are commonly analyzed by fragment size separation using gel electrophoresis. However, post-PCR handling processes are laborious and costly. In addition, gel electrophoresis based methods cannot detect SNPs present in the sequences flanking repeat motif. High resolution melting curve (HRM) analysis is a new technique, which is efficient, accurate, and cost-effective in detecting the sequence differences in polymerase chain reaction (PCR) amplicons, even single nucleotide polymorphisms (SNPs) or insertions or deletions (INDELs). In present study, we used the HRM followed by DNA sequencing to discriminate induced microsatellite polymorphism among irradiated and non-irradiated individuals of *Curcuma alismatifolia* in third generation (M_1V_1). The results showed that the combination of HRM with sequence confirmation is a powerful high-throughput, accurate, and reproducible approach to detect induced SSR polymorphism among mutants of *C. alismatifolia*. For the mutants with SNPs polymorphism present in the flanking region, HRM also gave distinct melting curves, which the gel electrophoresis was not able to detect. In conclusion, it has been approved that HRM as pre-sequencing, efficient, and cost-effective screening method enables rapid SSR polymorphism detection in mutant population of *C. alismatifolia*.

Key words. *Curcuma alismatifolia* – DNA polymorphism – Gamma rays – High resolution melting curve – SSR

Introduction

Curcuma alismatifolia is a monocotyledonous perennial with short fleshy rhizomes, tuberous roots, and often with a dormancy period, which is used as cut flowers, potted plants and garden plant have recently become popular in the ornamental flower market because of their colorful, long-lasting and showy inflorescences (Hagiladi et al. 1997). *C. alismatifolia* is common on the plateau of northeastern Thailand ranging from near sea level up to 790 m above sea level, which is the western part of the Indo-Chinese phytogeographical region. The distribution range extends to the lowland of southeastern coast of Thailand west of Kampuchea (Paisooksantivatana and Thepsen 2001).

Radiation breeding is exposing plants to radiation and is a sub class of mutation breeding, which is one method for obtaining new cultivars of ornamental plants (Broertjes et al. 1976; Zalewska and Jerzy 1997; Ahloowalia and Maluszynski 2001). Radiation breeding was discovered in the 1920 s when Lewis J. Stadler of the University of Missouri used X-rays on barley seeds. Extensive work has been carried out for the last 30 years for development of new and novel varieties of ornamental plants for floriculture trade. According to IAEA (International Atomic Energy Agency) Mutant Varieties Database (http://mvgs.iaea.org), 3200 mutant varieties of 170 different plant species have been officially released worldwide. Of these, 24 % represent ornamental and decorative plants. Gamma rays were employed to develop 64 % of the radiation-induced mutant varieties, followed by X-rays (22 %) (Ahloowalia et al. 2004).

The effect of mutagens on plants can be investigated by different methods. However, molecular markers allow a direct comparison of the effects of genotypes at the DNA level (Senthamizh Selvi et al. 2007). SSRs have been highly popular genetic markers for last two decades because of benefits such as hyper variability, co-dominance, multiallelic nature, high reproducibility, and high throughput genotyping (Agarwal et al. 2008; Kalia et al. 2011). SSRs, which were constantly isolated and characterized in a wide range of economically important plant species have been used extensively in plant genetics, biodiversity and cultivar identification. However, the routine protocols used for SSR employ PCR amplification of the DNA fragment containing nucleotide repeats, and separating of

the PCR products using laborious gel electrophoresis based methods. In addition, the electrophoresis based methods are not able to detect nucleotide variations such as single nucleotide polymorphisms (SNPs) in the flanking sequences of repeat motif unless the genotypes have been differentiated with length polymorphisms (Distefano et al. 2012). Recently, high resolution melting analysis (HRM) has been identified as a relatively efficient, accurate, inexpensive, and powerful method that can be applied to analyse genetic variations including SNPs, insertions or deletions (INDELs), and methylations of DNA in PCR amplicons (Herrmann et al. 2007; White et al. 2007). It depends on fluorescence change along with the double strand DNA melting using a saturating DNA dye and a highly precise optical detection system, which are usually attached to real time PCR machines. Any soft variation in the DNA sequence leads to detectable change of melting curve, and thus the allelic differences among PCR amplicons are distinguished. HRM technique has already been used to study genetic variability of plant species, for instance, apple, barley, grapevine, olive, almond, pepper and sweet cherry (Wu et al. 2009; Chagne et al. 2008; Lehmensiek et al. 2008; MacKay et al. 2008; Muleo et al. 2009; Jeong et al. 2010; Ganopoulos et al. 2011). High-resolution melt analysis (HRM) has been established as an alternative screening platform for genes with multiple small exons separated by larger introns (Ririe et al. 1997). We suppose that HRM can efficiently and accurately distinguish the induced DNA variations mainly SNPs in the sequence flanking repeat motif of mutants of *C. alismatifolia*. The aim of this study was to use HRM as a screening, pre-sequencing, and alternative to electrophoresis-based methods to distinguish more polymorphisms in mutants of *C. alismatifolia*.

Material and Methods

Plant materials

The plant materials used in this study were the rhizomes of three *C. alismatifolia* varieties, namely 'Chiang Mai Red' (SK 2051/12), 'Sweet Pink' (SK 2052/12), 'Kimono Pink' (SK 2054/12), and one *Curcuma* hybrid, namely 'Doi Tung 554' (SK 2053/12) with parents of *C. alismatifolia* 'Chiang Mai Pink' (2n = 32) and *Curcuma sparganifolia* (2n = 32), measuring about 1.5–2.5 cm with 4–7 storage root. Rhizomes were provided from a *Curcuma* Nursery (Ubonrat) in Doisaket District, Chiang Mai 50220, Thailand.

Gamma irradiation of rhizomes of C. alismatifolia

Irradiation of rhizomes of *C. alismatifolia* varieties was conducted in the Faculty of Science and Technology, University Kebangsaan Malaysia (UKM) using a Gammacell 220 Excel Irradiator (MDS Nordion, Ottawa, ON, Canada). The source of gamma rays was Cobalt 60. One hundred and sixty sprouted rhizomes of four varieties were acutely irradiated at two optimum doses of 10 Gy (12.8 s), and 20 Gy (25.6 s). For each variety, twenty rhizomes were treated for each dose and 20 non-irradiated rhizomes were used as control (0 Gy). After irradiation, the rhizomes were planted in 25 cm pots in growing media consisting of topsoil: cocopeat: rice husk at a ratio of 1:2:1 and arranged in randomized complete block design (RCBD) with five replications and four samples in each. In order to determine the morphological and genetic variation stability, the plants were grown until third generation (March 2011–Oct 2013).

DNA extraction

DNA was isolated from the M_1V_3 fresh and young leaves using cetyl-trimethylammonium bromide (CTAB) extraction buffer. Briefly, fresh young leaves were ground to a fine powder in liquid nitrogen and incubated with modified CTAB extraction buffer (Taheri et al. 2012). The purity and concentration of isolated DNA was determined by measuring the absorbance at 260 and 280 nm using a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA). Purity of the DNA was determined by comparing the values recorded at 260 and 280 nm. The DNA samples were adjusted with TE buffer to approximately 70 ng μL^{-1}.

Primer Screening

A preliminary experiment using four DNA of non-treated samples was performed to select polymorphic primers out of 17 SSR primers, which were developed for *Curcuma longa* in previous studies (Sigrist et al. 2009). Primers were used to amplify DNA of *C. alismatifolia* in order to evaluate the size and the band pattern in agarose gel (1.5 %). Only the monolocus markers with fragment size shorter than 250 bp were used for further analysis.

PCR amplification and HRM analysis

The optimized PCR amplification was performed in 10 μL (to save costs) reaction volumes containing 5 μL of 2x HRM PCR Master Mix (Type-it HRM PCR kit), which contained (HotStarTaq *Plus* DNA Polymerase, EvaGreen Dye, optimized concentration of Q-solution, dNTPs, and $MgCl_2$), 2.6 μL RNase-Free water, 0.7 μL of 5 μM of each forward and reverse SSR primers, and 1 μL of 70 ng μL^{-1}. DNA. The PCR amplification was carried out in touchdown PCR, what is often the best option for HRM PCR (Korbie and Mattick 2008). The PCR program was the following: initial denaturation of the template DNA at 94 °C for 3 min. This was followed by 10 cycles of 94 °C for 40 s, touch-down 1 °C decrement for annealing temperature started with 7 °C above Tm for each primer for 30 s and 72 °C for 1 min. Then, followed by 30 cycles of 95 °C for

40 s, last annealing temperature for 30 s and 72 °C for 1 min and final extension of 72 °C for 10 min. The HRM phase was started immediately after PCR. The HRM reaction procedure and melting analysis were performed as follows: temperature range was 55–95 °C with rising 0.1 degree per step. Cooling to 65 °C, and then the temperature was raised to 72 °C. Subsequently, the temperature was raised to 95 °C.

DNA sequencing

In order to confirm the HRM results, the PCR products of 24 selected irradiated and non- irradiated samples at M_1V_3 for screened primers were sent to DNA sequencing. 25 µl PCR reaction volumes were prepared with 1 × Pfu buffer with $MgSO_4$, 200 µM mixed dNTP, 0.75 unit Pfu DNA polymerase (all from Thermo Scientific, Pfu DNA polymerase Kit, Catalog no.#EP0579, approximately 70 ng DNA template, 0.4 µM of each primer set and 18.7 µL dionized water. The PCR amplification was performed with the same program as previously described. The provided samples were sequenced using the sequencing services provided by NHK Bioscience Solutions SDN. BHD, Malaysia. Each PCR product was sequenced in both strands (forward and reverse). In order to find DNA polymorphism, DNA sequences obtained from the PCR products of the two parents for the same primer were then aligned using Bioedit and CLC Sequence Viewer 6.8.2 software (Hall 1999). Then obtained contigs for each mutant were aligned with its related non-treated contig to find the DNA variation.

Results and Discussion

SSR primer screening

Amplifications were successful for all the 17 SSR markers assayed. This reflects a high level of homology between SSR flanking regions in *C. longa* and *C. alismatifolia*. Out of 17 primer pairs, eight primer pairs resulted in polymorphic PCR product amplifying fragments less than 250 bp (Table 1).

DNA polymorphism detection through HRM analysis in M_1V_3 generation

SSR allelic polymorphisms are routinely assessed by gel electrophoresis. These methods require time-consuming post-PCR sample handling and can only detect amplicons with different size. Many procedures of INDEL (insertion/deletion) detecting have been developed. The simplest of DNA polymorphism detection is by alignment of sequences from a particular region of genome of several individuals using software (Jones et al. 2009). However, the DNA sequencing cost is high, especially for high number of

Table 1. SSR primers.

Locus	Primer sequence (5 '–3')	Repeat motif	Range size (bp)	Tm (°C)	Ta (°C)
Clon01	F: ACTGGACTGTCCGAGAGCAT R: TCGTTTAGCGACAACGGATT	$(TA)_6TTG(TC)_{16}$	194–212	60.7	68–58
Clon04	F: TAAATTTGCGAAGGCAATCC R: CCGCAGAGGAATTTGAAGAG	$(TATAG)_2(AG)_{17}$	179–200	58.3	65–55
Clon08	F: CCGGTGAGGGTGATATCTTG R: AAGCTCAAGCTCAAGCCAAT	$(GT)_{10}$	245–274	60.7	68–58
Clon09	F: GGAGGAGGCAGTTGATTTGT R: GCTTTGGTGGCTAGAGATGC	$(AC)_{14}$	181–188	60.4	67–57
Clon10	F: GTGGGAATTGGATTGCTCTC R: GAGAACTCCCCATGCTTCAG	$(GT)_7$	204–232	60.7	68–58
Clon11	F: GGGCTTTGTTTAGTTGTCGTG R: CAGGAATGAAGTCGGCAAC	$(AGA)_8$	163–167	60.7	68–58
Clon12	F: GATTGGATCACATGGTGTGC R: TGGGTTGATGGTTTCTCTGTT	$(CT)_{20}$	195–231	59.0	66–76
Clon14	F: TCAGTCGAGGGGTTCCTACT R: GAGAGCTGATCGCAAAAACC	$(CTT)_7$	175–181	60.7	68–58

samples. HRM was found to be a sensitive, simple, reproducible, cost-effective and pre-sequencing method for mutation detection among treated and non-treated *C. alismatifolia* individuals. As was confirmed by Li et al. (2010), HRM detection using Evagreen dye led to a lower cost and a higher accuracy. In present experiment the HRM analysis was done for all 44 irradiated and non-irradiated individuals in M_1V_3 (third generation) with eight SSR primers. By HRM analysis, all studied irradiated individual plants produced melting curves with different melting temperature (T_m) from their control individual plants. Whereas the MetaPhor™Agarose gel was not able to detect small DNA polymorphism, the HRM technique could detect small variations in DNA sequence caused by gamma irradiation.

The melting temperature of non-treated Sweet Pink was 81.6 °C while the melting temperature of SP10-1 and SP20-1 was 82.5 and 82.7 °C, respectively (Fig. 1A). As illustrated, deviation of melting curves in 10 Gy irradiated Sweet Pink individuals compared to non-irradiated individuals can occur because of insertions of Guanin nucleotide, deletion or substitution of Adenine and Thymine nucleotides in mutant individuals. Mutation scanning by high resolution scanning depends on the melting of heteroduplexes that distort the shaping of the melting curve. This distortion can be seen by comparing the normalized melting curves of homozygous standard to heterozygous mutants (Fig. 1B). Since it is effective to distinguish single base differences, the technique is widely used in the detection of single nucleotide polymorphism (SNP) and INDEL (insertion/deletion) mutations of gene fragments. A homozygous sequence variant usually changes the Tm of the DNA duplex. Based on DNA sequencing alignment, individuals with C/T and G/A, or C/A and G/T base change showed a more Tm shift (0.8 > Tm < 1.4 °C) than C/G or A/T base changes (< 0.2) from untreated individuals (Liew et al. 2004). In this part the 20 Gy irradiated individual plants also showed more Tm deviation than 10 Gy individuals, which showed that higher dose of radiation probably caused greater damage and in some cases resulted in death of the cells. Gady et al. (2009) demonstrated that HRM are fast, affordable and sensitive tech-

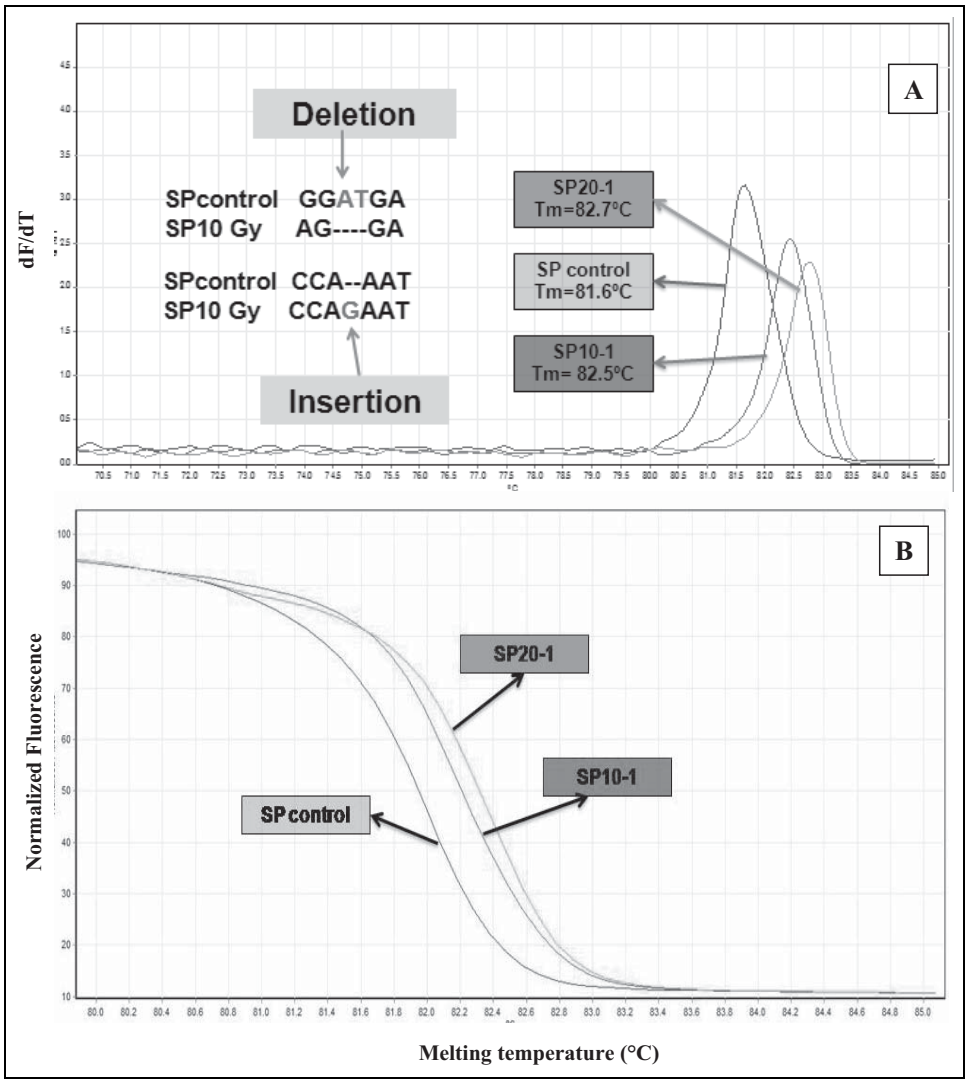


Fig. 1. Melting curves of irradiated and non-irradiated individuals of *C. alismatifolia* for clon01. Plot A represents melting curves, SNPs, and different melting temperature for control, 10, and 20 Gy 'Sweet pink', plot B represents normalized data for 10 and 20 Gy irradiated individuals of 'Sweet Pink' variety.

niques for mutation detection in DNA pools and therefore allows the rapid identification of new allelic variants in a mutant population.

DNA sequencing

As illustrated (Fig. 3A), by employing 4 % MetaPhor agarose gel (MAGE), we were successful to clearly separate alleles of microsatellite markers with a size difference of ≥ five bp while the small DNA polymorphism or SNPs (with same fragment size) could not be detected on the MetaPhor agarose gel. However, the HRM technique was able to detect these small changes. Sequence analysis is the most direct way of identifying DNA variation. This study used PCR-product sequencing approach to verify the HRM results (Yuan et al. 2009). Using HRM for pre-sequence screening, in particular for larger genes, may yield significant cost savings (Provaznikova et al. 2008). In present study, based on clustering of HRM (Fig. 2), 20 samples with more morphological variations (Taheri et al. 2014) were selected for DNA sequencing. Forward and reverse sequences of 20 irradiated samples (CMR10-2, CMR10-3, DT10-1, DT10-2, DT10-3, DT10-4, DT20-1, DT20-2, DT20-3, DT20-4, DT20-5, SP10-1, SP10-3, SP10-5, KP10-1, KP10-3, KP10-4, KP20-1, KP20-2, KP20-3) and four non-treated samples were aligned by BioEdit and CLC Sequence Viewer 6.8.2 software. On the other hand, 20 Gy irradiated individuals of 'Chiang Mai Red' and 'Sweet Pink' did not produce any inflorescence and were not selected for DNA sequencing. To compare the DNA sequence of treated individuals with their non-treated sequence, the obtained consensus sequence for each treated individual was aligned with its non-treated consensus sequence. The DNA sequencing confirmed the DNA polymorphisms resulted from gamma irradiation in 20 samples. Fig. 3B. is showing some insertions, deletions, and substitutions, which occurred in DNA sequence of 10 Gy 'Sweet Pink' mutant (SP10-1).

To the best of our knowledge, this is the first report on the utilization of the HRM approach for mutation detection of *C. alismatifolia* mutants by employing SSR markers. In conclusion, we have demonstrated here that high resolution melting (HRM) analysis is a relatively efficient, accurate, and inexpensive method, which in combination with sequence confirmation could be a powerful method for induced mutation detection in *C. alismatifolia, which*

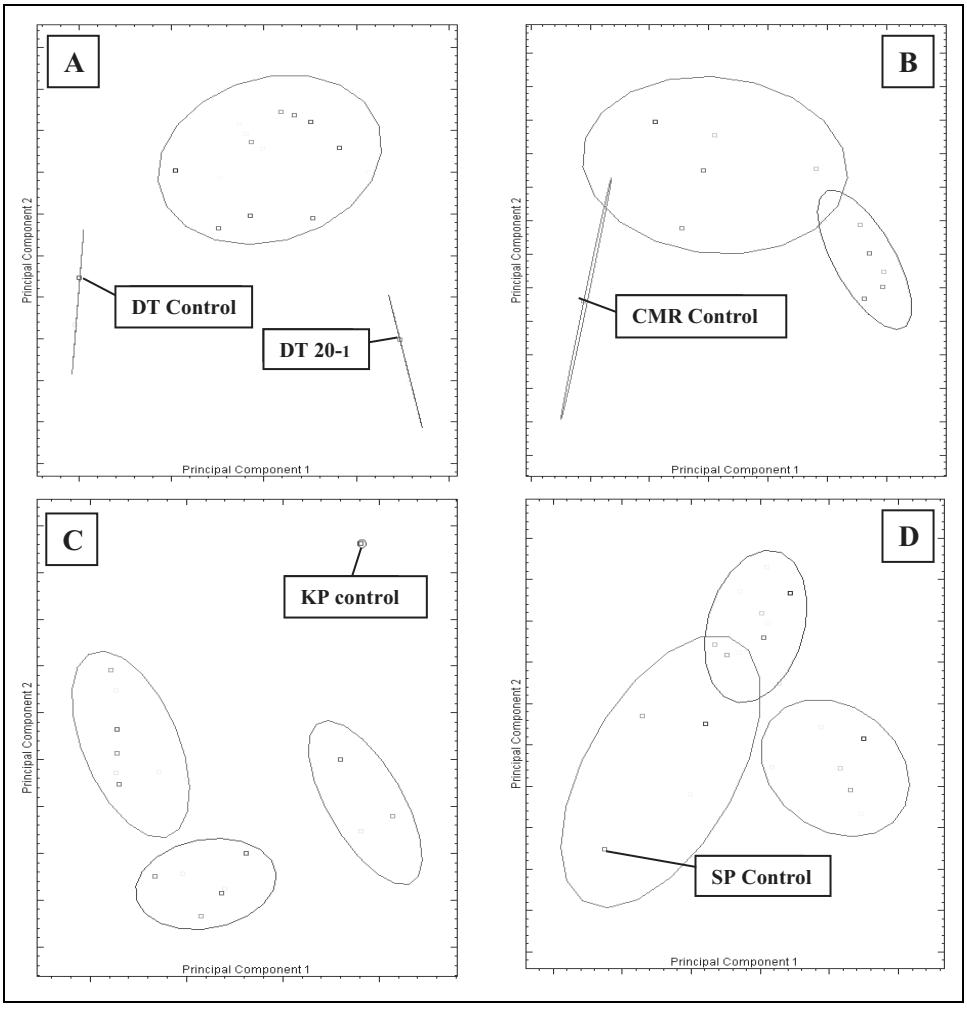


Fig. 2. Clustering of irradiated and non-irradiated individuals of *C. alismatifolia* for clon04. The individuals inside the circles are mutants. A) 'Doi Tung554', B) 'Chiang Mai Red', C) 'Kimono Pink', D) 'Sweet Pink'.

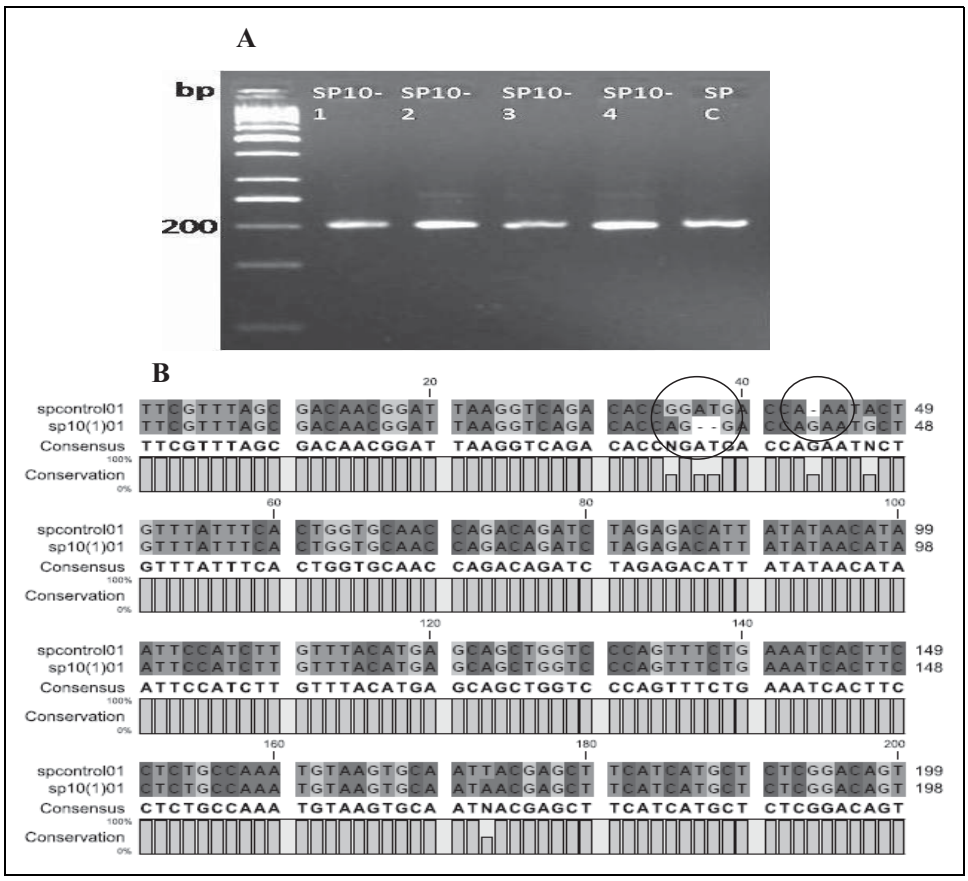


Fig. 3. A) Monomorphic bands on gel electrophoresis B) Sequence alignment of SP10-1 with non-irradiated amplicon. SP10 (1) 01 = 10 Gy irradiated 'Sweet Pink' (clon01).

could be extended to other crops or ornamental species. As well, using HRM for pre-sequence screening, in particular for larger genes, may yield significant cost savings.

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