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Research Article

Development of a sequence-characterized amplified region (SCAR) marker for female off-season flowering detection in date palm (*Phoenix dactylifera* L.)

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Abstract Date palm (*Phoenix dactylifera* L.: Arecaceae) is a dioecious species where only female trees bear fruits. In their natural state, date palms produce dates once a year. However, in Thailand, some trees were observed to produce dates during the off-season, despite no variations in morphology. The availability of such off-season fruits can significantly increase their market value. Interestingly, most female off-season date palms investigated in this study were obtained through micropropagation. Hence, there is an urgent need for genetic markers to distinguish female off-season flowering plantlets within tissue culture systems. In this study, we aimed to develop random amplification of polymorphic DNA-sequence characterized amplified region (RAPD-SCAR) markers for the identification of female off-season flowering date palms cultivated in Thailand. A total of 160 random decamer primers were employed to screen for specific RAPD markers in off-season flowering male and female populations. Out of these, only one primer, OPN-02, generated distinct genomic DNA patterns in female off-season flowering (FOFdp) individuals compared to female seasonal flowering genotypes. Based on the RAPD-specific sequence, specific SCAR primers denoted as FOFdpF and FOFdpR were developed. These SCAR primers amplified a single 517-bp DNA fragment, predominantly found in off-season flowering populations, with an accuracy rate of 60%. These findings underscore the potential of SCAR marker technology for tracking off-season flowering in date palms. Notably, a BLAST analysis revealed a substantial similarity between the SCAR marker sequence and the transcript variant mRNA from *Phoenix dactylifera* encoding the SET DOMAIN GROUP 40 protein. In Arabidopsis, this protein is involved in the epigenetic regulation of flowering time. The genetic potential of the off-season flowering traits warrants further elucidation.

Keywords Date palm, Dioecious species, Off-season flowering, *Phoenix dactylifera* L., RAPD-SCAR marker

Introduction

Date palm, *Phoenix dactylifera* L., is one of the most important members of the Arecaceae (previously Palmae) family. It is a dioecious plant with a long generation time that produces socioeconomically valuable edible fruits. It is cultivated in several regions worldwide, including arid and semiarid areas. Most date palm plantations are in the Middle East and North Africa (Al-Khayri et al. 2018; Chao and Krueger 2007; Ghnimi et al. 2017). Nowadays, it is cultivated in many regions of Thailand. Date fruit is a famous sweetened fruit of high nutritional value, containing rich dietary fiber, essential minerals, antioxidants, vitamins, amino acids, and phenolic compounds (Al-shahib and Marshall 2003; Chao and Krueger 2007; Ghnimi et al. 2017; Kamal-Eldin and Ghnimi 2018; Vayalil 2012). Due to the many potential health benefits of date fruit, it is considered an almost ideal food (Al-shahib and Marshall 2003). Therefore, demand for date fruits has continued to increase each year. Nowadays, fresh date fruits are popular in Thailand because of their taste and properties. Therefore, if commercial fresh date fruit could be harvested year-round, it would be beneficial to the farmers.

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Date palms are dioecious, which means they have distinct male and female plants. Given that only female date palms produce fruits (Al-Khayri et al. 2018; Chao and Krueger 2007; Dhawan et al. 2013; El Hadrami and Al-Khayri 2012), there is a significant interest in determining the sex of date palm plants at an early stage, well before they reach sexual maturity, which can take several years. This would save space, time, and resources. There are three techniques for date palm propagation: seed, offshoot, and tissue culture. The seed method by itself is not sufficient to predict the gender of date palm seedlings, which have a roughly equal chance of being male or female (Chao and Krueger 2007). Offshoots from the date palm trees are used to grow new trees, which will ensure identical trees and fruits to their parents. Six to eight years are needed before the pups can be transplanted to the land to form a new tree, and another six years will elapse before they are able to produce fruits. However, offshoot quantity is massively limited and the offshoot removal process can damage the mother plant (Al-shahib and Marshall 2003; Chao and Krueger 2007; Ghnimi et al. 2017). To overcome the limitation of female tree production from these traditional propagations, plant tissue culture is a recent micropropagation technique for rapid proliferation that can select the appropriate genotypes and genders (Abahmane 2013; Chao and Krueger 2007; Jain 2007; Mazri and Meziani 2015). Using tissue culture techniques in date palm cultivation has gained popularity for several reasons, including the propagation of elite varieties, disease elimination, and faster multiplication. However, the specific use of tissue culture for the selection of female date palm plants, which are the fruit-bearing trees, is a more niche aspect. Most female date palm trees cultivated in several orchards were derived from tissue culture. Generally, the date palm is a seasonally flowering tree whose flowers bloom once a year during the growing season (around January to March), depending on the cultivar and environmental conditions (Kanoeithip 2015). Interestingly, off-season flowering date palms have been discovered in Thailand. The morphological characteristics of these seasonal and off-season flowering date trees, especially the vegetative parts, are indistinguishable, except for the number of flowering times per year and the fact that flowers bloom outside of the typical season. The phenomenon of the off-season flowering trait may involve genetic variation from somaclonal variation in tissue culture, cross-pollination of different parent trees, or the effect of environmental factors.

The date palm 'Khonaizi' is one of the most valuable cultivars in Thailand due to the properties of its fruits. The featured characteristics of these fruits are a reddish-brown color, a pleasing taste, smooth edible parts without splinters, and a high tolerance for moisture during fruit product transportation. Remarkably, some 'Khonaizi' trees that were generated from tissue culture and produced off-season fruit were accidentally found in Thailand. However, there was no prior evidence of how off-season flowering occurred in date palms. Therefore, the genetic potential of this trait needs to be further characterized.

Previous studies of off-season flowering in other fruit trees have reported the use of chemical reagents to induce off-season flowering, such as potassium chlorate ($KClO_3$), hydrogen peroxide (H_2O_2), and nitric oxide (NO) in longan (Cutler et al. 2007; Manochai et al. 2005; Subhadrabandhu and Yapwattanaphun 2001; Zhang et al. 2016), and paclobutrazol in mango (Husen et al. 2021; Nartvaranant et al. 2000). Furthermore, the application of external hormones (Astiari et al. 2021), light intensity (Nguyen et al. 2020), or physical stresses (Kulkarni 1986) can stimulate off-season flowering as well. However, temperature-insensitive longan varieties can produce off-season fruits without any chemical treatments; Cutler et al. (2007) discovered an off-season flowering-specific band (275 bp) using molecular techniques. Ninety nucleotides from this gene matched the 12-oxophytodienoic acid (OPDA) reductase gene. OPDA is a direct precursor to jasmonic acid and functions as a master switch for lipid-derived environmental signaling in response to flowering or osmotic stress. These results suggest that there may be an off-season flowering specific gene involving the jasmonic acid signaling pathway.

Due to market interest, fresh off-season date fruits can experience a significant increase in value. Unfortunately, currently, the low number of female off-season date palm trees in orchards is insufficient to produce off-season fruits for commerce. The off-season date palm trees could not be identified from the morphological traits until the first time of flower blooming. Furthermore, the long period of juvenile development before the tissue culture-derived date palms flowering ($\geq$ 6 years) is an obstacle to date palm orchard management (Abahmane 2013; Abul-Soad and Mahdi 2010). Interestingly, the prompt detection of the female off-season date palm plantlets that regenerated from tissue culture can increase their price. To save time and enhance the value of products, female off-season date palm trees must be identified early for the benefit of orchard farmers and tissue culture producers.

Molecular markers are an effective technique that can be used to detect expected characters at the seedling stage (Al-Khalifah and Askari 2007; Al-Qurainy et al. 2018;

Awan et al. 2017). The Random Amplified Polymorphic DNA (RAPD) marker is one of the most widely used molecular markers to investigate the patterns and distribution of genetic variability using polymerase chain reaction (PCR) with short arbitrary primers. Due to the simplicity of the RAPD technique, the whole genome or random segments can be used for analysis. In addition, genetic traits of various varieties or species can be compared to determine differentiation. Importantly, no prior specific knowledge of the DNA/genome sequence of the target plant is necessary (Abass et al. 2017; Al-Khalifah and Askari 2007; Al-Khayri and Naik 2017; Al-Qurainy et al. 2018; Amiteye 2021; Aslam et al. 2015; Jain 2007; Kumar and Gurusubramanian 2011; Premkrishnan and Arunachalam 2012). For these reasons, the RAPD technique was applied in this study to detect a novel female off-season flowering gene in date palm. However, the major disadvantage of this methodology is its sensitivity to reaction conditions that reduce reproducibility. To solve this problem, replication of RAPD-PCR amplification (at least three times) helps to verify the reproducibility of the results (Amiteye 2021; Cutler et al. 2007).

Moreover, the improvement of a highly efficient marker, the Sequence Characterized Amplified Region (SCAR) marker, can be developed from an expected specific RAPD fragment (Amiteye 2021; Babu et al. 2021; Yang et al. 2014). Additionally, increasing annealing temperature during PCR helps to reduce template mismatches and enhance reproducibility (Amiteye 2021; Cutler et al. 2007). With these advantages, the RAPD-SCAR technique has already been effectively used for gender determination (Al-Qurainy et al. 2018; Awan et al. 2017; Dhawan et al. 2013; Younis et al. 2008) and for the identification of different genetic variations in date palms (Al-Khalifah and Askari 2007). There have been various research initiatives to determine the sex of date palms at the seedling or even tissue culture stage. Molecular markers and other genetic tools have been developed for early sex determination. If tissue culture techniques are combined with these molecular tools, then it can be possible to select female off-season plants *in vitro*.

In this study, applying RAPD-SCAR markers is an appealing technique to investigate the novel female off-season flowering specific gene in date palms, especially given the lack of previous information. The results are expected to provide early differentiation of female off-season flowering date palms using a molecular RAPD-SCAR marker.

Materials and Methods

Plant materials

Young folded leaves were harvested from fully mature 'Khonaizi' date palm trees of a total of 20 genotypes from the Therdthai date palm orchard, Bangkok, Thailand. Both off-season and seasonal-flowering female and male trees were selected for this study. Most female off-season trees were generated from tissue culture. Detailed information on each sample is listed in Table 1. The samples were stored at -20°C to preserve their genomic material.

Genomic DNA extraction

Genomic DNA was extracted from young leaves using modified protocols of the DNeasy® Plant Mini Kit (Qiagen, Germany) and Al-Qurainy et al. (2018). Date palm leaves (0.2 g) were ground into a fine powder with a mortar and pestle using liquid nitrogen. Frozen powdered tissues were transferred into a 1.5 ml microcentrifuge tube and treated with the extraction buffer (QIAGEN, Germany) following the manufacturer's protocol. Total genomic DNA was verified by the NanoDrop micro-UV/VIS spectrophotometer (Thermo Scientific, USA) for genomic qualitative and quantitative assessment. All DNA samples were diluted to a concentration of 50 ng/μl using AE buffer (QIAGEN, Germany) and kept at -20°C.

RAPD-PCR amplification

A total of 160 RAPD decamer primers of arbitrary sequences (Operon Technologies, USA), which comprised of OPA, OPB, OPC, OPD, OPAH, OPBD, OPN, and OPU series (Supplementary Table S1), were applied for polymorphism analysis. Each 25-μl RAPD-PCR reaction contained 1-μl DNA (50 ng/μl), 2.5-μl RAPD primer (10 μM), 2.5-μl 10x ViBuffer A, 1-μl 50 mM $MgCl_2$, 0.5-μl 10 mM dNTPs (Vivantis, Malaysia), 0.5-μl *Taq* DNA polymerase (Vivantis, Malaysia), and 17-μl sterile deionized water. PCR amplification was executed using a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, USA) with the following program: first denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, primer annealing at 46°C for 30 s, primer extension at 72°C for 45 s, and final extension at 72°C for 5 min (modified from Cutler et al. 2007). Amplification products were then analyzed on a 1.5% agarose gel in 0.5X TAE buffer and stained with DNA stain G (SERVA, Germany). DNA patterns were

Table 1 Information regarding the date palm samples ('Khonaizi' cultivar) tested in this study

Accession number	Gender	Type
1. KNZFBK 2102-2(T)	Female	Off-season flowering tree
2. KNZFBK 2904-2(T)	Female	Off-season flowering tree
3. KNZFBK 2904-3(T)	Female	Off-season flowering tree
4. KNZFBK 2904-4(T)	Female	Off-season flowering tree
5. KNZFBK 2701-23(T)	Female	Off-season flowering tree
6. KNZFBK 1910-4(N)	Female	Seasonal flowering tree
7. KNZFBK 2701-12(N)	Female	Seasonal flowering tree
8. KNZFBK 2904-6(N)	Female	Seasonal flowering tree
9. KNZFBK 2102-1(N)	Female	Seasonal flowering tree
10. KNZFBK 2701-26(N)	Female	Seasonal flowering tree
11. KNZMBK 1910-1(T)	Male	Off-season flowering tree
12. KNZMBK 1910-2(T)	Male	Off-season flowering tree
13. KNZMBK 3107-1(T)	Male	Off-season flowering tree
14. KNZMBK 3107-8(T)	Male	Off-season flowering tree
15. KNZMBK 2701-3(N)	Male	Seasonal flowering tree
16. KNZMBK 2701-7(N)	Male	Seasonal flowering tree
17. KNZMBK 2701-8(N)	Male	Seasonal flowering tree
18. KNZMBK 2701-10(N)	Male	Seasonal flowering tree
19. KNZMBK 2701-16(N)	Male	Seasonal flowering tree
20. KNZMBK 2701-18(N)	Male	Seasonal flowering tree

visualized and photographed using a gel documentation system (CHEMI HR16 BIOIMAGING SYSTEM, Syngene G Box, USA) with the GeneSnap program. The amplicons' sizes were estimated based on a VC 100 bp Plus ready-to-use DNA ladder (Vivantis, Malaysia). Each amplification reaction was resolved using a single primer and repeated in triplicate to verify the reproducibility of the results.

SCAR marker development

The RAPD primer-generated banding profiles were compared to identify the high-intensity unique amplicons for off-season or seasonal flowering date palm trees. These unique bands were then excised from agarose gel and purified using the FavorPrep™ GEL/PCR Purification Kit (FAVORGEN BIOTECH CORP., Taiwan). After purification, the purified fragment was cloned into a T&A™ cloning vector (T&A™ Cloning Kit, Yeastern Biotech, Taiwan) using the manufacturer's recommended procedure. After bacterial transformation, the recombinant plasmids were then sequenced using Barcode-Tagged Sequencing (U2Bio, Korea). Similarity searches were conducted for the nucleotide sequences of RAPD amplicons by comparison against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

As an extension of the original RAPD primer, which has 10 arbitrary bases, the obtained sequences were also used to design specific SCAR primers using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The SCAR primers were then synthesized using U2Bio (Korea) and used for amplification of genomic DNA from all tested date palm samples in order to validate their specificity for off-season flowering in date palm. Each 25-µl SCAR-PCR reaction contained 1-µl DNA (50 ng/µl), 1-µl each of the SCAR forward and reverse primers (10 µM), 2.5-µl 10x ViBuffer A, 1-µl 50 mM $MgCl_2$, 0.5-µl 10 mM dNTPs (Vivantis, Malaysia), 0.5-µl *Taq* DNA polymerase (Vivantis, Malaysia), and 17.5-µl sterile deionized water. PCR amplification was executed on a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, USA) with the following program: first denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 60-70°C for 30 s, primer extension at 72°C for 45 s, and final extension at 72°C for 5 min. The annealing temperature of the new SCAR primers is 70°C for FOFdp-SCAR primers and 60°C for FSFdp-SCAR primers. Amplification products were then analyzed on 1.5% agarose gel, as described above. The PCR reactions were repeated at least 3 times to verify their reproducibility.

Results and Discussion

RAPD-SCAR markers for off-season flowering date palm

Early on, the morphological characteristics of seasonal and off-season flowering date palms are indistinguishable. A long development time of greater than or equal to six years is needed for tissue culture-derived date palm trees to reach the reproductive age, which reveals their specific off-season flowering characters (Abahmane 2013; Abul-Soad and Mahdi 2010).

Previously, several studies have explored sex-linked RAPD-SCAR markers in the date palm. According to Dhawan et al. (2013), the approach of RAPD-SCAR technology has been successfully applied to identify male genotypes. The SCAR primer pair amplified a common amplicon of 406 bp in both the male and female plants, whereas a different amplicon of 354 bp was amplified only in male plants. However, so far there have been no reports on the study of molecular markers for off-season flowering date palms.

In this study, 160 arbitrary decamer RAPD primers (OPA, OPB, OPC, OPD, OPAH, OPBD, OPN, and OPU series) were primarily screened to identify off-season flowering markers among male and female date palm trees of the Khonaizi cultivar (Table 1). The screening revealed a reproducible band ranging from 200 to 2,500 bp among the tested samples. From the 160 RAPD decamer primer screenings, the results revealed that only OPN-02 (5'-ACCAGGGGCA-3') produced a female off-season flowering specific band (FOFdp band) of 1,711 bp (Fig. 1). This prominent band was detected in 3 of 5 off-season flowering female trees but not in 5 seasonal flowering female trees. This band was also detected in 1 of 4 off-season flowering male tree and 2 of 6 seasonal flowering male trees. The genetic difference detected by RAPD analysis made it quite evident that this technique could be employed for the identification of related genotypes. Accordingly, this 1711-bp RAPD band, which was reproducibly found in 60% of off-season flowering female trees tested but was completely absent in seasonal flowering female trees, can be used to select the off-season flowering genotype of female date palms.

In order to create a reliable marker, the 1711-bp RAPD fragment was cloned and sequenced. The obtained nucleotide sequence was then subjected to a homology search by BLAST against the NCBI database. The BLAST results revealed that the sequence of the FOFdp fragment (1,711 bp) had an 84.89% similarity to the *Phoenix dactylifera* clone dpB2Y sex-determination region sequence (Accession no. MH681002.1). This finding suggests that the sequence found in this study might be related to the genetics of sex determination in the date palm. The sequence was then further used to design a pair of specific primers using Primer3Plus. Finally, a pair of FOFdp-SCAR primers, FOFdpF (5'-GGCTACGGTGATTAGCTGGA-3') and FOFdpR (5'-GGGAGGCAGGTCTACAAAGG-3'), was designed and synthesized by U2Bio (Korea) (Fig. 2). The FOFdp-SCAR primers were then applied for amplification of genomic DNA from all tested date palm samples (Table 1) to evaluate the specificity of amplification. The amplification profile of the primer pair is shown in Fig. 2. The results showed that a clear, specific amplicon of 517 bp was generated from only female off-season samples, but no amplicons were detected in the DNA samples from any of the female seasonal samples. Meanwhile, this band was also detected in one male off-season sample and 2 of 6 male seasonal samples (Fig. 3). The results of the FOFdp-SCAR marker matched the results of the previous 1711-bp RAPD marker.

The 517-bp FOFdp-SCAR marker was cloned and sequenced. BLASTN of the sequence still showed an 86.15% match with the *Phoenix dactylifera* clone dpB2Y sex-determination region sequence (Sequence ID: MH681002.1). The date palm male allele sequence (dpB2Y) is a scaffold

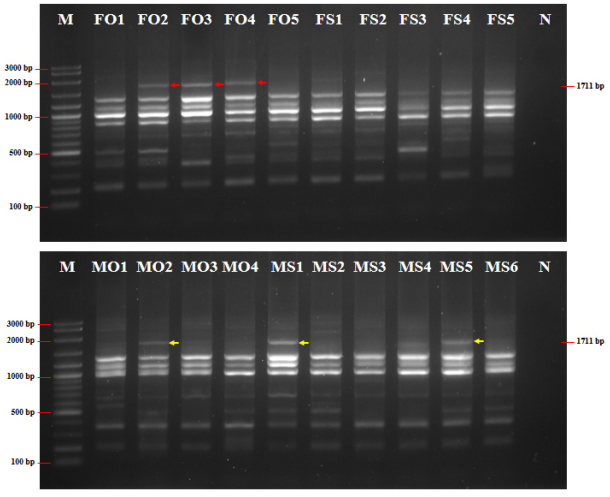

Fig. 1 Amplification profile generated using the OPN-02 RAPD primer, utilizing genomic DNA from date palm samples (Table 1). Lanes FO1-FO5: Female off-season samples (Accession number 1-5), Lanes FS1-FS5: Female seasonal samples (Accession number 6-10), Lanes MO1-MO4: Male off-season samples (Accession number 11-14), Lanes MS1-MS6: Male seasonal samples (Accession number 15-20), Lane N: Negative control, Lane M: 100 bp DNA ladder. Arrows indicate the presence of the female off-season flowering-specific (FOFdp) band at 1711 bp observed in female off-season samples (lanes FO2-FO4), a male off-season sample (lane MO2), and male seasonal samples (lanes MS1, MS5).

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CGAGCGGCTTGGATGGGCTTGTCCTATGTGCGGTGCATACTTCGAGCGAGTTCGGTTCTT 60
CTGGAGGGCGACTCGGCTACGGTGATTAGCTGGATTCGCAGGGGCCCGGTTGGTCACGGA 120
GATGCCCATCCTCTGCTTCGTGACATTTGGATGATGGTGAGAGAGGGTGTTACGCTACAG 180
GCTACGCATGTATATCGTGAGGCCAATGGGGCGGCCGATTGGGTGGCCCGCGTTTGTTGCG 240
AGCCACTCTGGAGGCACCCTATGGGCTGGGGAGAGGGAGATGCCCGGAGCACTTCGGGAC 300
ATTTTGTATTCTGACTCTTTAGGGTGTATTCGTACCCGTGATGTATGAAATGCCCGTTTA 360
AGCAAAAAAAAAAACAATTACCATACAAGAAGCATCTCTTTTTCAAGCATTTCCCCCTTA 420
ATATGCGTGACATATACAAAAATATCTGGAACAAATAGGAAAGAAACCCCAACGTGATTA 480
CTGTTCATAAACAGCCCATATTATGCTATAGGATTAATGAAAACAACATGTAACCACCTT 540
GTCACATAAATCTAACTACCAGACTGGTCAACCTTTGTAGACCTGCCTCCCCCACTCCCT 600
CAAAAATAAAAAACTCTCTCCCTTCTTTCTATCTCTATATCTGCAAGCTTGCATGTAGAA 660
TTGATGTTTAGCATTGATCTGGTTCATTTGCATGTACTGACCTAGTCATATGATTCTACT 720
TCTAGCATTGATGTAAGAAAACCACTTTATTGTTGGGTAGGAAAGTCATGTCTTTTTATT 780
TTGAGATGCACGTTATATGTTTATGGTTTGGTGCTCATGATTGGCTAATTTTTTCATTCA 840
TAGCAAACGGGAGAGCATATACCACCAGAAGACTCTTTTGAAGTAGCTCGCAAGGTGAAA 900
GAAATGTATTGTTACACTTGTTCAGACTTAGTCAAGGTAATTTTTCTTTTCATAAGGTTT 960
TTATTTGATAGCATTCTATTTTTAGTTCTGTAATTGAAATGTACTTTTCACTCTTATATT 1020
TTTGAGATTGTATCTGTCGAAGATAAATAATATTGTTTCTCGGTCAATATCACTATGGTT 1080
GCTGTAGAAATTGTAATTAGTCAATCTGACTTTTTACTAACTAAAGCAAATCTTACTGCT 1140
AGTGGTTTCAGTCTAACTTCTGATGATAATAATTAACTAGACAGAAGGTTATTGAAGTAT 1200
TTGAAATGTTTTTATTGATCGAGATGTCTTGAAGAAAGTAGTCTAGTATTACTGGTTAAA 1260
ATGTTACCTCATTGGTTTCAAACAAACCATAGAGTCAAGGATTGATGTCCCAGTGCATCT 1320
CAGTGCATCCTGCTGGTACTGTTCTGTTCCAACAAAAAAATGGTACTCAGGATGCCCCCC 1380
AAGATGCTAGATCGCTCATTTTTCGGGACGTCATACCGACTGGGATAAACCAGGACATTT 1440
ATGTCCCGACACTTGGGATGGTGGTGGGCCCCAAGATGCCCTTTTTTATTTCTGATTTTT 1500
CCCTCTTCATTCTTATTGTTAGTGGTTGTTCTGGTTTGTCCAGACCCGTCGCGGTACCAT 1560
ATCAACTTGGGACCAAACCAGGATGGGTATTGGCGTGGAGACTTCAATCCTTGCCATAGA 1620
TGATGGGCATAATTATTTATGCTTCTCCCGAATACTTGATATCAGCTTTACAGATTTTCT 1680
TTCACCAGTAGTTCCTACTAAGATTTGCCCC 1711
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Fig. 2 Nucleotide sequence (1711 bp) of the female off-season flowering-specific RAPD (OPN-02) fragment. Arrows indicate the positions of the primers for amplifying the sequence-characterized amplified region (SCAR) markers. A pair of FOFdp-SCAR primers were designed based on the forward primer. Highlighted (red, underlined, boldface, and italicized) nucleotides define the forward and reverse FOFdp-SCAR primers

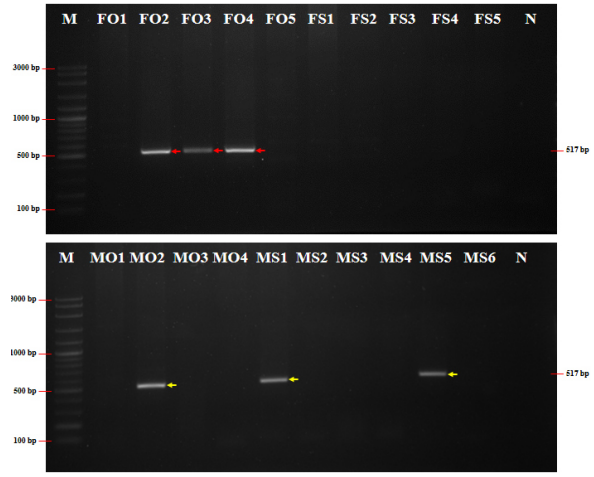

Fig. 3 FOFdp-SCAR primer-amplified region indicating bands for off-season flowering in date palm samples (Table 1). Lanes FO1-FO5: Female off-season samples (Accession number 1-5), Lanes FS1-FS5: Female seasonal samples (Accession number 6-10), Lanes MO1-MO4: Male off-season samples (Accession number 11-14), Lanes MS1-MS6: Male seasonal samples (Accession number 15-20), Lane N: Negative control, Lane M: 100 bp DNA ladder. The FOFdp-SCAR (517 bp) was detected in female off-season samples (lanes FO2-FO4), a male off-season sample (lane MO2), and male seasonal samples (lanes MS1, MS5)

sequence that contains male-specific k-mers conserved in only a few male date palms but is absent in females (Torres et al. 2018). Interestingly, the dpB2Y male-linked gene shared a high level of similarity to the FOFdp region that was observed in the female off-season date palms. This result prompted the hypothesis that mutation in the dpB2Y male allele sequence might be the causative factor for female off-season flowering in date palms.

In addition, the FOFdp region also showed 79.02% similarity to the *Phoenix dactylifera* transcript variant mRNA encoding protein SET DOMAIN GROUP 40 or SDG40 (Sequence ID: XM_008806822.4 and XM_008806821.4). It must be noted that the SET-domain protein methyl-transferase superfamily includes one of the proteins known to methylate histones on lysine, which then serves as a post-translational epigenetic modification that controls the expression of genes (Dillon et al. 2005). Interestingly, the expression of SDG40 could epigenetically regulate flowering time in Arabidopsis. Flowering time is controlled by a number of pathways that either repress or enhance expression of *FLOWERING LOCUS C* (*FLC*) (Thorstensen et al. 2011). Loss of function of SDG40 leads to the reduction of permissive H3K4me3 marks and to the increase of repressive H3K27me3 marks in *FLC* chromatin, leading to early flowering (Nasim et al. 2021).

As mentioned above, the off-season flowering phenomenon may involve genetic variation from more factors, including somaclonal variation from tissue culture. The somaclonal variation could unintentionally occur in the derived plants during *in vitro* culture (Al-Khayri et al. 2018; Bairu et al. 2011). There were several factors contributing to the somaclonal variation observed in plant micropropagation, such as the rapid multiplication of cells, a source of derived tissue (callus culture or protoplast cells), the age of the culture, the number of subculture cycles used, and media compositions (plant growth regulator concentrations, chemical mutagenesis, etc.) (Al-Khayri et al. 2018; Bairu et al. 2011; Biswas et al. 2009; El Hadrami et al. 2011; Ferreira et al. 2023). The instability of *in vitro* cultures caused by the above-mentioned factors may cause genetic and epigenetic changes in crops (Ferreira et al. 2023).

In this study, most female date palm materials were derived from tissue culture. According to the nucleotide BLAST result of the 517-bp FOFdp region, there was a 79.02% similarity to the *Phoenix dactylifera* transcript variant mRNA encoding protein SET DOMAIN GROUP 40 (SDG40). The expression of SDG40 could epigenetically regulate flowering time in Arabidopsis (Nasim et al. 2021; Thorstensen et al. 2011). Interestingly, this finding suggested that off-season flowering traits in date palms may be related to epigenetic changes and their relationship to somaclonal variation. Furthermore, the effect of environmental factors probably causes the off-season flowering trait. To

reduce the effect of these factors, the date palm Khonaizi cultivar was only collected from the Therdthai date palm orchard, Bangkok. All plant materials were controlled under the same condition. However, the outcomes from this study cannot be absolutely assumed to show that the effect of environmental factors was not related to the off-season flowering phenomenon. Therefore, the factors that cause the off-season date palm occurrence should be further studied.

RAPD-SCAR markers for seasonal flowering date palm

Following the somewhat limited efficiency of the previously developed FOFdp-SCAR marker, which could also be amplified in some male off-season and male seasonal samples (Fig. 3), a SCAR marker for detection of female seasonal flowering date palms (FSFdp-SCAR marker) was subsequently developed.

Among 160 arbitrary decamer RAPD primers, OPAH-14 (5'-TGTGGCCGAA-3') could amplify a specific band (422 bp) in 4 of 5 female seasonal flowering samples. This band was also found in 2 of 4 off-season flowering male samples and one seasonal flowering male date palm (Fig. 4). This 422-bp RAPD marker was reproducibly present in 80% of seasonal flowering female trees but was completely absent in off-season flowering female trees. Therefore, this region can be used to identify the seasonal flowering genotype of female date palms and can confirm the off-season flowering of female date palms by the absence of this band.

Afterward, the 422-bp RAPD marker was developed into the reliable FSFdp-SCAR maker using similar methodology to the FOFdp-SCAR marker. A pair of FSFdp-SCAR primers (Fig. 5), FSFdpF (5'-ATGAAGCTGGGAG GAACGATG-3') and FSFdpR (5'-TGCATAGGCAACAC CTTCTCAT-3'), was developed and tested on the date palm samples (Table 1). The results exhibited a unique amplicon of female seasonal flowering, or FSFdp (357 bp), only in the seasonal samples within the female date palms, while this gene was found in 2 of 4 off-season flowering male samples and one seasonal flowering male date palm (Fig. 6).

Fig. 5 Nucleotide sequence (422 bp) of the female seasonal flowering-specific RAPD (OPAH-14) fragment. Arrows indicate the positions of the primers for amplifying the sequence-characterized amplified region (SCAR) markers. A pair of FSFdp-SCAR primers were designed based on the forward primer. Highlighted (red, underlined, boldface, italicized) nucleotides define the forward and reverse FSFdp-SCAR primers

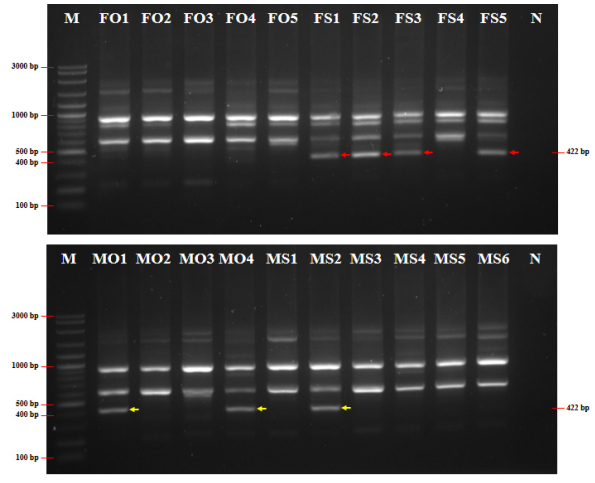

Fig. 4 Amplification profile generated using an OPAH-14 RAPD primer, utilizing genomic DNA from date palm samples (Table 1). Lanes FO1-FO5: Female off-season samples (Accession number 1-5), Lanes FS1-FS5: Female seasonal samples (Accession number 6-10), Lanes MO1-MO4: Male off-season samples (Accession number 11-14), Lanes MS1-MS6: Male seasonal samples (Accession number 15-20), Lane N: Negative control, Lane M: 100 bp DNA ladder. Arrows indicate the presence of the female seasonal flowering-specific (FSFdp) band at 422 bp observed in female seasonal samples (lanes FS1-FS3, FS5), male off-season samples (lanes MO1, MO4), and a male seasonal sample (lane MS2)

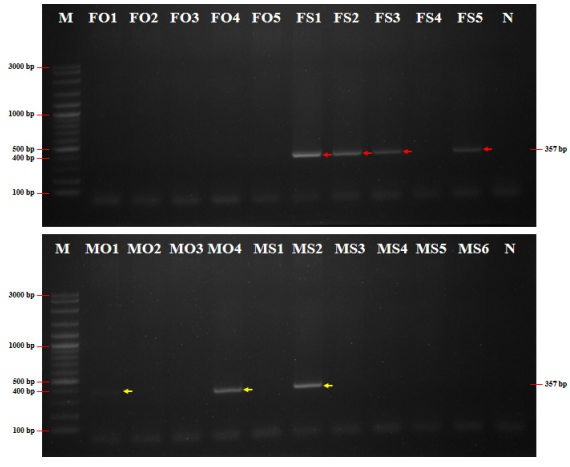

Fig. 6 FSFdp-SCAR primer-amplified region indicating bands for seasonal flowering in date palm samples (Table 1). Lanes FO1-FO5: Female off-season samples (Accession number 1-5), Lanes FS1-FS5: Female seasonal samples (Accession number 6-10), Lanes MO1-MO4: Male off-season samples (Accession number 11-14), Lanes MS1-MS6: Male seasonal samples (Accession number 15-20), Lane N: Negative control, Lane M: 100 bp DNA ladder. The FSFdp-SCAR (357 bp) was detected in female seasonal samples (lanes FS1-FS3, FS5), male off-season samples (lanes MO1, MO4), and a male seasonal sample (lane MS2)

The nucleotide sequences of FSFdp (357 bp) obtained in this study were blasted against sequences in the NCBI database, and results showed that the sequences had 56.41% identity to the predicted protein of *Phoenix dactylifera* (AEG74556.1). This result could not indicate the function of the FSFdp region. However, the FSFdp-SCAR marker can be used to support the application of the FOFdp-SCAR marker due to the absence of the FSFdp band in female off-season samples.

The combination of these two FOFdp and FSFdp-SCAR markers may be able to more accurately identify off-season flowering in female date palms, but not in males (Figs. 3, 6). Fortunately, it was no obstacle to applying these two SCAR markers for off-season flowering detection in female date palm plantlets regenerated from tissue culture. In terms of date palm orchard management, female trees are of economic importance as they bear the fruits, while male trees are only needed in the vicinity to pollinate the female trees to ensure fruit production.

However, the limitations of the 517-bp FOFdp-SCAR markers had 60% accuracy to identify female off-season date palm cv. Khonaizi. Moreover, none of the identified markers have been shown to work across a broad range of date palm cultivars. We have tried using the FOFdp-marker to identify off-season flowering female trees in other cultivars, such as Nawader, H1 and Zaghloul (Supplementary Table S2). Our preliminary results indicated that the FOFdp band (517 bp) was not specifically amplified in the samples tested (data not shown). Unfortunately, there were not so many samples of the other cultivars tested because off-season date palm trees were rarely found in general. Nevertheless, these results suggest that the markers developed in this study are only specific to the Khonaizi cultivar.

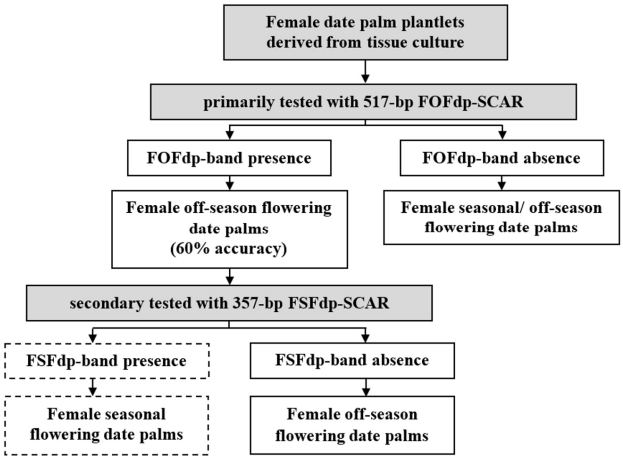

Fig. 7 Steps for off-season flowering trait detection in female date palm plantlets using the FOFdp and FSFdp-SCAR markers developed in this study. The 517-bp FOFdp-SCAR marker was used to primarily test for the presence of the FOFdp band, indicative of an off-season flowering trait. Subsequently, female off-season plantlets were subjected to a secondary test using the 357-bp FSFdp-SCAR markers to confirm the absence of the FSFdp band, verifying the off-season flowering feature. White boxes indicate the results for the female off-season and seasonal date palm plantlets tested with FOFdp and FSFdp-SCAR markers, respectively. White dashed boxes indicate the outcomes for female seasonal date palm plantlets tested with FSFdp-SCAR markers

Conclusion

This is the first report of RAPD-SCAR markers linked to off-season flowering female trees for the date palm cultivar Khonaizi. BLAST analysis showed that the amplified sequence had high similarity to the *Phoenix dactylifera* transcript variant mRNA encoding the protein SET DOMAIN GROUP 40, or SDG40. Further in-depth experiments analyzing the SDG40 gene will provide a better understanding of flowering regulation mechanisms in date palm.

The developed markers from this study provide a potential contribution to date palm orchard management by reducing time, labor, and production costs, as well as increasing the efficiency of plant screening, especially plantlets regenerated from tissue culture as shown in Fig. 7. There is still limited information about molecular markers for other date palm cultivars. An improved understanding of flowering regulation mechanisms and the identification of common markers for off-season flowering across diverse cultivars are still needed.

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