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Sex determination in date palm (*Phoenix dactylifera* L.) by PCR based marker analysis

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ABSTRACT

Date palm (*Phoenix dactylifera* L.) is an economically important fruit crop in hot arid regions. It is cultivated in many regions of Thailand. However, during the establishment of plantations, male and female trees cannot be clearly distinguished until about five years after planting when the palms first flower. The objective of this research was to differentiate gender (male / female) at seedling stage in the Thai date palm cultivar KL1 by using a specially developed DNA marker technique. A PCR based method, which included the use of specific tetra-primers, was found to be successful using extracts from young leaf samples. With these markers, flowering male date palms were represented with two amplicons (430 bp and 320 bp) while the female date palms were represented with only one amplicon (430 bp). When 100 non-flowering seedling of cv. KL1 were screened, 50 were identified as male and 50 as being female. This ratio of 1:1 between male and female is consistent with the principle of gender distribution in dioecious plants. When the tetra-primer method was used with other cultivars, including Deglet nour, Barhi, Hayani, Medjool and Tunisia, the same DNA banding patterns were determined. This method has potential to be used for the differentiation of gender in other date palm cultivars at the seedling stage. It would reduce the time required for gender determination from five years to a few hours.

1. Introduction

Date palm (*Phoenix dactylifera* L.) is a heterozygous perennial monocot, belonging to the Arecaceae family. It is one of the most important horticultural crops in arid and semi-arid countries (Alami-Saeid et al., 2014), being used as food, in construction, for tools, and as an important part of religious ceremonies (Al-Mahmoud et al., 2012). Date palm is a long-living, dioecious evergreen fruit tree (Dhawan et al., 2013). The major date producers in the world are located in the Middle East and North Africa (Zaid, 2002), producing fruits with high nutritional value and helping to maintain life in fertile desert areas (Cherif et al., 2013). Thai date palm cv. KL1 was developed using Deglet Nour (Israel) and Barhi (Jordan) as the parents. It is cultivated mainly in the Chiangmai region and is well adapted to the environment in Thailand (Intha et al., 2015). It is unusual in that it produces fruit very early, only two years after planting. Like all other dioecious plants, plants propagated from seed have approximately equally numbers of males and females; however only females produce fruit and only a few male plants are need for pollination. Screening out male plants early is essential for efficient orchard establishment.

Current sex-determining mechanisms are uncertain and there are no reliable methods for determining the sex of date palm plants before they reach reproductive age (Cherif et al., 2013), which typically occurs between 5 and 8 years after planting (Aberlenc-Bertossi et al., 2011; Bendiab et al., 1993). Several researchers have identified molecular makers that could segregate sex in date palm and methods have been developed for identifying the sex at an early stage, including the use of isozymes (Torres and Tisserat, 1980), peroxydases (Majourhat et al., 2002), and random amplified polymorphic DNA (RAPD) (Moghaieb et al., 2010). However, a major limitation of all of these studies has been that none of this marker research has been shown to work across a broad range of date palm cultivars (Al-Mahmoud et al., 2012), although some markers could identify of sex in one or two cultivars (Younis et al., 2008). Investigation of the regions involved in sex determination have revealed that date palm employs a XX/XY (2n = 36) system with the male being heterogametic. The critical regions also showed significant polymorphism between the male and female alleles. This polymorphism can be used in the development of assays to distinguish sexes at an early stage (Al-Mahmoud et al., 2012).

In this study, we aimed to develop molecular markers for identification of sex in a range of date palm cultivars, by using PCR based assays. The main focus was to differentiate the sex of seedlings of the

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Table 1
Sequences and the corresponding references of the tetra-primers that were used in the amplified DNA fragment tests in date palm.

Number	Sequences	Reference
1F	GCATTAGCACCATAGTAAATTGT	–
1R	GTCCCAATCAGAGTGCACTCAA	Al-Mahmoud et al. (2012)
2F	GCAATAGCACCATAGTAAATTGCCTA	–
2R	CGCTAACTTGGTGCACGGATCTCT	–

cv. KL1 by using simplified laboratory-based methods. The universality of the technique was tested by evaluating the usefulness of the method to identify sex in other cultivars.

2. Materials and methods

2.1. Plant material

Young leaf samples were collected from fully mature known male and female trees of cv. KL1 for the initial technique development. Subsequently, young leaves from seedlings from recently germinated seed of cv. KL1 were used to identify sex at the early, juvenile phase. The applicability of the method was evaluated on mature trees of five other cultivars where the sex was known. All trees were grown at Ban-Rai Intaphalum farm in Kanchanaburi, Thailand.

2.2. Genomic DNA isolation

Genomic DNA was isolated from young leaf samples using an innuPREP Plant DNA Kit (Analytik Jena, Germany). The DNA quality/quantity was determined on 0.8% agarose gel made and run in 1X TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0). Analysis was carried out using a SmartView Pro 1200 Imager System (Scientific Biotech, Taiwan) and band intensity was compared with a 100 bp DNA Ladder RTU (Genedirex®, Taiwan). All DNA samples were diluted to a concentration of ~50 ng μL^{-1} using an elution buffer and stored at −20 °C.

2.3. Identification and development of markers

A range of combinations of twenty primers (data not shown) was used, together with PCR amplification, to distinguish between male and female genotypes in the mature leaf samples of KL1. The PCR-amplified DNA fragments were separated by electrophoresis in submerged horizontal agarose gels (1.5% w/v) mixed with 1 µL 100 mL^{-1} RedSafe™ (iNtRONBioteechnology, Korea) agarose gel in TBE buffer, visualized, and photographed using a SmartView Pro 1200 Image System (Major Science, Taiwan). Only primers that could generate DNA fragments in either male or female samples (as the dominant marker) were selected.

In the development of the specific markers for identifying male and female samples, one set of two primers (the dominant marker) was used to amplify the male sex-specific DNA target band, while a second primer set was selected from the range of primers tested to generate the homomorphic DNA band. This second primer set was used as a positive control in the reaction – it was selected as it was able to generate a DNA band that was different by more than 100–200 bp in size compared the first primer set. The marker, therefore, consisted of tetra-primers in the PCR reaction (Table 1).

In the PCR assays, 1 µL of genomic DNA was amplified using a One PCR™ Plus (Genedirex®, Taiwan) master mix in a total volume of 20 µL containing 5 pmol of each combination of the four primers. The reaction was activated at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 57.7 °C for 30 sec, 72 °C for 1 min and 72 °C for 5 min.

2.4. Data analysis

The number and size of DNA bands on the resultant gel were compared. Samples were identified that had a two-banding DNA pattern for male and a one-banding DNA pattern for female plants. Male and female seedlings were labelled for subsequent evaluation at the flowering stage.

3. Results

3.1. PCR amplifications

Each of the twenty primers showed some differences in banding patterns in the amplified fragments of the male and female genotypes. Almost all of the primers were homomorphic or provided patterns that were unrelated with sex. Only the primer combination 1F–1R, when amplified, was able to distinguish a male-specific fragment of approximately 320 bp (Fig. 1A). All other primers, when amplified with sex-specific fragments, could not provide clear identification of either male or female seedlings. Thus, a second combination of primers was needed to provide co-dominant markers for the identification of the both male and female individuals.

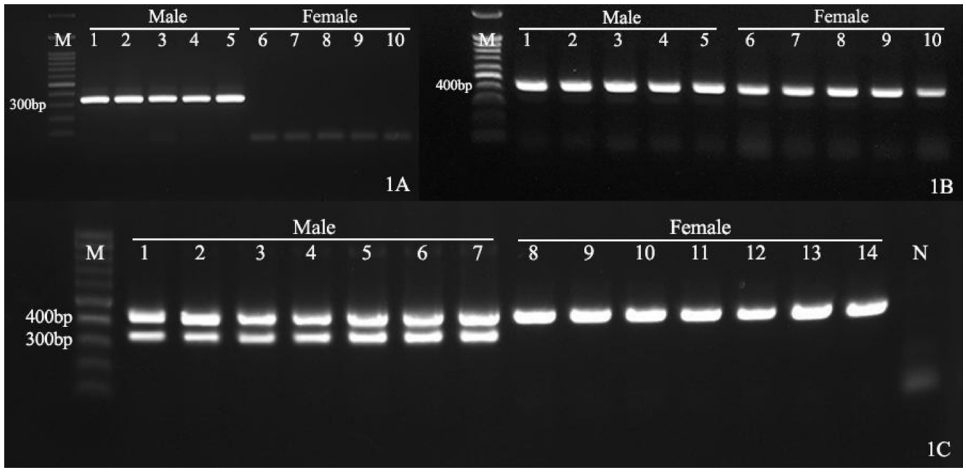

Fig. 1. PCR profiles of sex determination in date palm consists of: 1A; the first primer combination (1F–1R) used to differentiate male and female date palm genotypes of the cv. KL1. The length of the male-specific putative fragment was approximately 320 bp. There was no corresponding fragment in the female samples. 1B; PCR profiles of second primer combination 2F-2R used to differentiate male and female date palm genotypes. The length of the DNA band at approximately 430 bp could be used as a positive DNA banding pattern to identify both male and female genotypes. Lanes Male 1–5 were for known individual male genotypes while lanes Female 1–5 were for known individual female genotypes. Lane M represents a 1 Kp DNA ladder which was used as a marker. 1C; PCR profiles for seven mature male and seven mature female samples of cv. KL1 prepared using the four selected primers. The male sample showed the expected double-banding pattern while the female samples showed the expected single-banding pattern. Lanes Male 1–7 were for known individual male genotypes while lanes Female 8–14 were for known individual female genotypes. Lane M represents a 100 bp DNA ladder that was used as a marker and Lane N was a negative control without genomic DNA.