Faculty of Resource Science and Technology

PRIMER OPTIMISATION AND SCREENING FOR ELEMENTS THAT ARE INVOLVED IN THE CONTROL OF FLOWER FORMATION IN SAGO PALM

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SYAHRUL BARIYAH BINTI ANJI

This project is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

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Primer Optimisation and Screening for Elements that are involved In the Control of Flower formation in Sago Palm.

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ABSTRACT

Understanding of the function of flowering genes in sago palm is crucial to enable its manipulation in the future for a shorter flowering period. The objective of this study was to identify and clone of *CO*-like, *LFY*-like and *AP3*-like gene from sago palm as well as to test various sets of established primers and custom design primers for various genes that are involved in the flowering processes. DNA visualization using agarose gel electrophoresis indicated that CTAB method was successfully employed to extract genomic DNA from the shoot of the sago palm. When the extracted DNA was subjected for PCR with 5 set primers namely *LFY*, *Ath LFY*, *AP3*, *cctCO* and *haCO*, single band was detected for *LFY* primers whereas multiple bands were detected for *AP3*, *AthLFY* and *cctCO* primers. Multiple bands were detected due to the degeneracy of the primers used. PCR product for *LFY* and *AP3* genes were purified and introduced into the ligation reaction and subsequently transformed into *E.coli* JM109. After transformation, a few white colonies and blue colonies where observed on the Luria Broth agar plates containing ampicilin, X-Gal and IPTG. However, the PCR amplification of the isolated plasmids from the white colonies failed to detect the presence of inserted DNA fragments. The usage of restriction enzyme, *EcoRI* also failed to detect the presence of the inserted fragments. Yet, the DNA could not be cloned due to time constrain.

Keywords: Flowering genes, CTAB, PCR, restriction enzyme

ABSTRAK

Pengetahuan tentang fungsi gen-gen pembungaan dalam pokok sagu adalah penting untuk membolehkan penggunaannya pada masa yang akan datang bagi mempercepatkan proses pembungaan. Objektif kajian ini adalah untuk mengenal pasti dan mengklonkan gen seperti LFY, AP3 dan CO daripada pokok sagu dan juga untuk menguji beberapa set primer untuk pelbagai gen yang terlibat semasa proses pembungaan. Pengesanan DNA melalui teknik elektroforesis gel agarose menunujukkan penggunaan kaedah CTAB untuk mengekstrak DNA daripada pucuk pokok sagu telah berjaya. Apabila DNA yang telah diekstrak tersebut digunakan untuk proses PCR, satu fragmen dikesan untuk primer LFY manakala beberapa fragmen untuk primer AP3, AthLFY dan cctCO. Beberapa fragmen yang dikesan adalah akibat penggunaan primer yang tidak spesifik. Produk PCR bagi primer LFY dan AP3 yang telah ditulenkan kemudiannya digunakan untuk proses ligasi dan seterusnya ditransformasikan ke dalam E.coli JM109. Setelah proses transformasi, beberapa koloni biru dan putih telah dikesan di atas agar Luria yang mengandungi ampicilin, X-gal dan IPTG. Namun, setelah plasmid daripada koloni putih diekstrak dan diamplifikasikan, ianya gagal untuk mengesan kehadiran fragmen-fragmen yang telah diklonkan ke dalamnya. Penggunaan enzim pemisahan iaitu EcoRI juga gagal menunjukkan kehadiran fragmen-fragmen tersebut. Setakat ini, proses pengklonan masih gagal disempurnakan kerana kesuntukan masa.

Kata kunci: Gen pembungaan, CTAB, PCR, enzim pemisahan

CHAPTER 1

INTRODUCTION

1.1 Flower formation

Flower is a part of the plant that allows sexual processes. According to Weberling (1989), flower is "a section of a shoot, or a branch resembling a short shoot, which bears leaf organs which serves for sexual reproduction and which are transformed accordingly".

Studies from the model plant *Arabidopsis thaliana* show that flowering is regulated by three classes of genes acting in consecutive order: the flowering time genes, the floral meristem identity genes and the floral organ identity genes (Coupland and Piñeiro, 1998).

The flowering time genes including *CONSTANTS* (*CO*), *TERMINAL FLOWER 1* (*TFL1*), and *GIGANTEA* (*GI*) are those that display major effects on the duration of vegetative development (Tsaftaris *et al.*, 2004). Some of these kinds of genes promote flowering while others repress it; some appear to interact with environmental factors such as photoperiod and temperature while others appear to act in an autonomous way (Tasma and Shoemaker, 2003). Flowering time genes are often assumed to act before floral meristem identity genes and generally, to lead to their activation (Tsaftaris *et al.*, 2004).

Floral meristem identity genes are involved in switching the fate of meristems from vegetative to floral phase The best characterized of these genes are *APETALA1* (*AP1*), *APETALA2* (*AP2*), *CAULIFLOWER* (*CAL*) and *LEAFY* (*LFY*) (Tsaftaris *et al.*, 2004). Mutations in floral meristem identity genes cause primordia that develop in the

positions occupied by flowers to form organs with some of the characteristics of shoots (Howell, 1998).

A set of homeotic or floral organ identity genes, which fall into three classes: A, B and C class genes, determine the fate of floral organ primordia. These genes have been best characterized in *Arabidopsis*, snapdragon, and petunia (Zachgo *et al*, 1995). In all three species, flowers are composed of four concentric rings (whorls) of organs, with sepals in the first, outermost whorl, followed by petals, stamens, and carpels in whorls 2, 3, and 4, respectively. The ABC model explains how floral homeotic genes act combinatorially to specify each of the four organ identities (Bowman *et al*., 1991; and Coen and Meyerowitz, 1991). The class A genes lead to the formation of sepals in whorl 1, class A and B genes together lead to the formation of petals in whorl two, the class B and C genes specify the formation of stamens in whorl three, and the class C genes are required for the formation of carpels (Irish, 1999; Ma, 2000 and Theissen, 2001). Table 1 shows floral homeotic genes in *Arabidopsis* and *Antirrhinum*.

Table 1: Floral Homeotic Genes in *Arabidopsis* and *Antirrhinum*. Taken from Howell (1998). *Molecular genetics of plants Development*, p.197

Class	*Arabidopsis*	*Antirrhinum*
A	*APETALA 1* *APETALA 2*	*SQUAMOSA*
B	*APETALA 3* *PISTILLATA*	*DEFICIENS* *GLABOSA*
C	*AGAMOUS*	*PLENA*

Although there are numerous gene involved in the control of flower formation, this study was focus on *CO, LFY* and *AP3* gene which is the example of the flowering time genes, the floral meristem identity genes and the floral organ identity genes respectively.

The *Arabidopsis CO* gene is a key regulator of the long day-dependent flowering pathway. Molecular genetic analyses using *Arabidopsis* revealed many genes regulating flowering time upon long day (LD) induction. Among them, *CO*, that encodes a zinc finger protein, has been recognized as a genetic component of the LD-dependent flowering pathway (Putterill *et al.*, 1995). The *CO* mutant flowers later than the wild type under LD, but shows similar flowering time to the wild type under short day (SD). Consistently, the *CO* gene shows higher expression under LD than SD during the day and over expression of *CO* causes early flowering even under SD (Putterill *et al.*, 1995; Suarez-Lopez *et al.*, 2001). Therefore, the *CO* gene has been assigned to the photoperiod-dependent pathway (Putterill *et al.*, 1995).

The meristem identity genes, *LFY* are necessary for transition to reproductive growth and the concomitant formation of flowers. Loss of *LFY* function leads to leaves and shoots in place of flowers, while constitutive expression of *LFY* results in precocious floral development (Weigel *et al.*, 1992; Weigel and Nilsson, 1995). *LFY* is required for the transcription of representatives of all three classes of ABC genes (Weigel and Meyerowitz, 1993). *LFY* encodes a nuclear-localized product that can bind to DNA and so could act directly to regulate transcription of the floral homeotic genes (Parcy *et al.*, 1998).

AP3 which is confer B-class function in Arabidopsis is required for specifying petal and stamen identities, and is expressed in a spatially limited domain of cells in the floral meristem that will give rise to these organs. Loss-of-function mutations in this gene result in the conversion of petals to sepals and stamens to carpeloid organs (Bowman *et al.*, 1989; Hill and Lord, 1989; Jack *et al.*, 1992). Furthermore, in the presence of intact A and C function, their ectopic coexpression is sufficient to transform sepals to petals and carpels to stamens (Krizek and Meyerowitz, 1996). In accordance with their roles in specifying petal and stamen identity, *AP3* coincides largely with their functional domains and is maintained throughout petal and stamen development (Jack *et al.*, 1992; Goto and Meyerowitz, 1994).

1.2 Sago palm

The sago palm (*Metroxylon* sp.) which is belongs to the *Lepidocaryoid* subfamily of the *Arecaceae* (*Palmeae*) is the first plants used by man in South-east Asia and Oceania (Avě, 1977). These plants are once-flowering (hapaxantic) and tillering or suckering perennial. According to Flach (1984), the flowers of sago palm are borne spirally in pairs on the tertiary axis. Of each pairs of flower, one is male and the other complete but only functionally female. In its natural habitat the sago palm occurs in fresh water swamps on soil with more than 70% clay and up to 30% organic matter (Flach, 1984). There are many research on sago palm mainly the innumerable uses of the sago starch such as in the production of bread flour that has been reported by Dendy *et al.* (1970), and Clarke *et al.* (1980), and the production of high fructose syrup (Ito *et al.* 1979). Sim (1977) points out that sago starch has several advantages over other starches

such as it produces sizing pastes of lower viscosity at a given concentration than such pastes from maize and potato. In addition, it was easy to handle because of the sago pastes are less inclined to gelate under cooling than maize pastes. The sago pastes also show low retrogradation in which their stability in viscosity is high when kept for long periods at near boiling point, provided they are boiled for two hours before use. Figure 1 show the innumerable uses of the sago palm.

The major problem in commercializing the sago starch is the extremely long time of maturity of the crops. It takes a long time before harvesting in order to obtain the starch. The trunk of the sago palm is usually judged to be ready for starch processing by the stage of flowering. The reason is that the trunk is supposed to have reached its maximum starch content when the young fruits are developing (Flach, 1984). There are no exact measurements of the length of the growing cycle of the sago palm from seed to next generation of seeds. Reports on the length of the life cycle range from 8 to 17 years (Flach, 1984). Figure 2 show the relationships between palm age and starch accumulation.

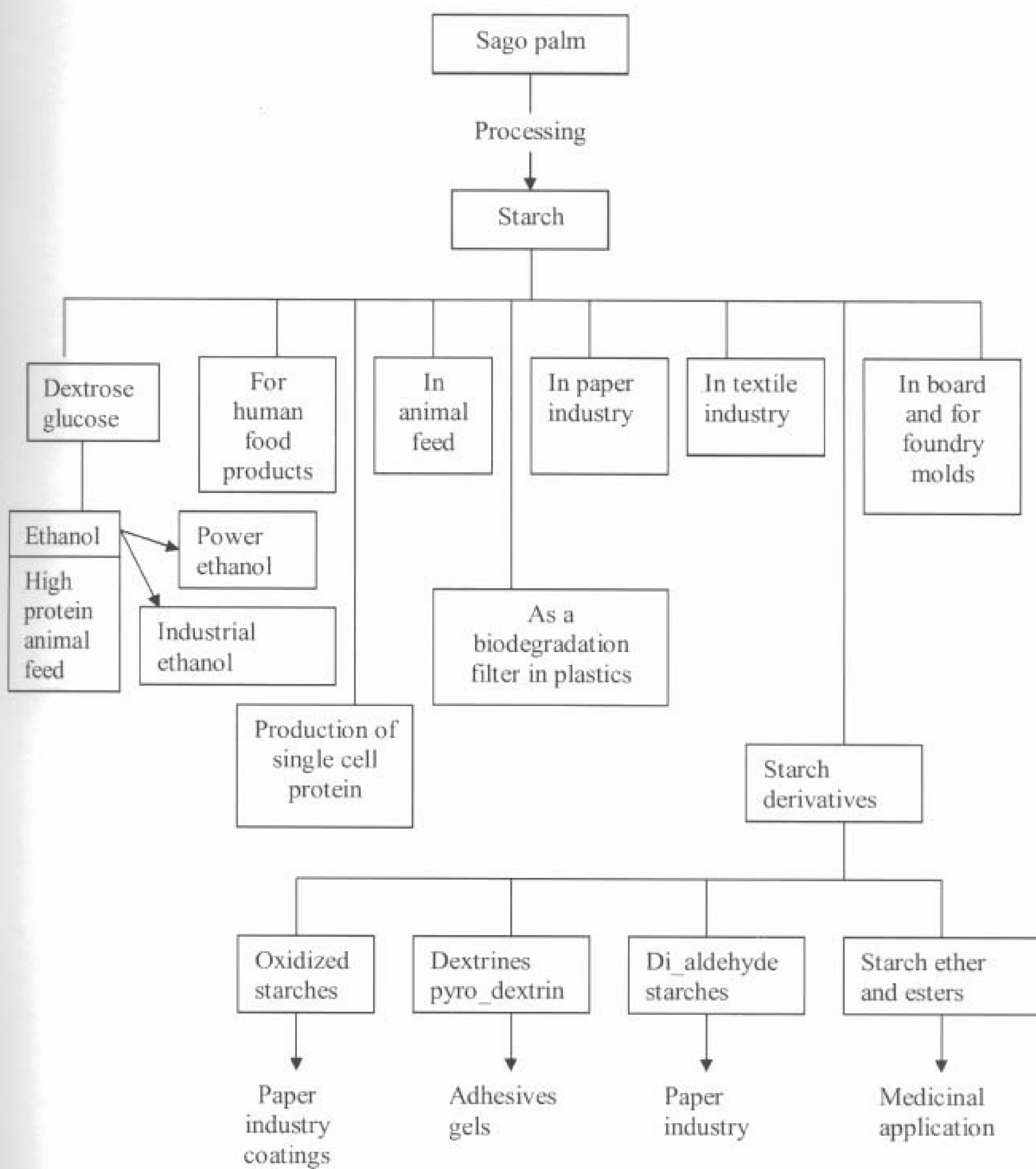

Figure 1: The innumerable uses of the sago palm. Adapted from Flach (1984). *The Sago Palm*, p.55.

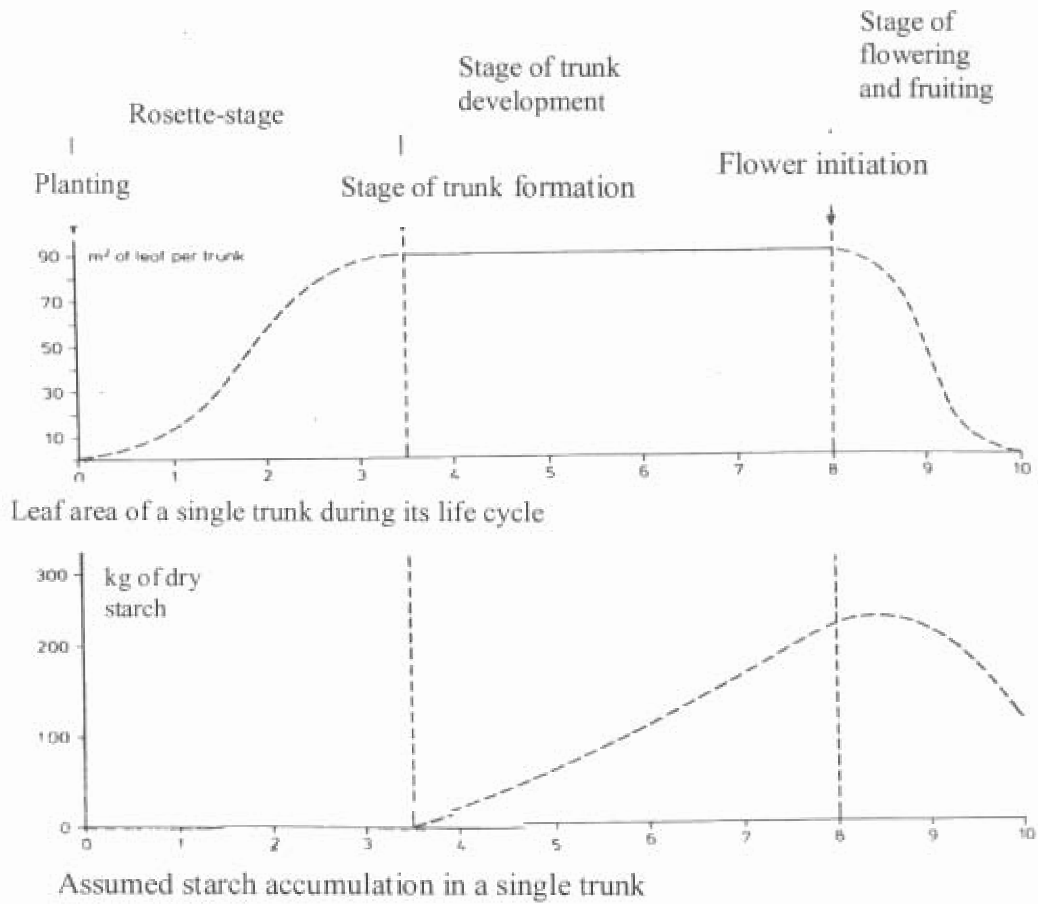

Figure 2: The relationships between palm age and starch accumulation.
Taken from Flach (1984). *The sago palm*, p.14

1.3 Polymerase Chain Reaction (PCR)

PCR is in vitro system for DNA amplification that employs the essential enzyme of cellular DNA replication, DNA polymerase, to selectively amplify a 'target' DNA region (Fox *et al.*, 1991). The key to this system is a pair of oligonucleotide primers which is single stranded DNA sequences of 20-30 nucleotides that serve as points of attachment for the polymerase (Kolmodin and Birch, 2002). The primer will bracket the region to be amplified: one primer is complementary to a sequence at the beginning of the target region, and the second is complementary to a sequence at the end of the target region on the anti parallel DNA strand. This PCR process requires a repetitive series of the three fundamental steps that defines one PCR cycle: double-stranded DNA template denaturation, annealing of two oligonucleotide primers to the single stranded template, and enzymatic extension of the primers to produce copies that can serve as templates in subsequent cycles (Fox *et al.*, 1991). As the cycles proceed, both the original template and the amplified targets serve as substrates for the denaturation, primer annealing and primer extension processes. Theoretically, every cycle doubles the amount of target copies. The advantages of PCR include it can allow one to detect (as opposed to characterize) the presence of particular gene sequences from extremely minute quantities of DNA (Fox *et al.*, 1991).

1.4 Research Objectives

1. To identify and clone the flowering genes of sago palm to enable its manipulation in the future for a shorter flowering period, thus for an earlier harvesting in order to extract the maximum starch content.
2. To identify and clone of *CO*-like, *LFY*-like and *AP3*-like gene from sago palm.
3. Test various set of established primers and custom design primers for various genes that are involved in the flowering processes.

CHAPTER 2

MATERIAL AND METHODS

2.1 Plant material

Samples of young sago shoots were obtained from sago seedlings grown in UNIMAS plant house.

2.2 Isolation of genomic DNA

Isolation of total genomic DNA was carried out using the DNA extraction protocols described by Doyle and Doyle (1990) with slight modification for miniprep extraction for PCR.

Approximately 0.1 g of shoot is grinded in the presence of liquid nitrogen and 1000µl of CTAB (hexadecyltrimethylammonium bromide) extraction buffer until a slurry is formed. The tissue with the extraction buffer was transferred into a 1.5 mL Eppendorf tube and incubated in a water bath at 65°C for one hour. The mixture was allowed to cool to room temperature for approximately five minutes prior to addition of 400 µl of CIA (chloroform: isoamylalcohol). The mixture was mixed gently to a single phase and centrifuged at 13,000 rpm for 5 minutes at 4°C. The upper (aqueous) layer was removed and transferred to a clean Eppendorf tube. 600 µl ice-cold propan-2-ol was added prior to left the tube to stand overnight at -20°C.

The next day, the tube was centrifuged at 13,000 rpm for 2 minutes at 4°C. The supernatant was discarded before the addition of 1 mL of wash buffer. The tube was agitated to dislodge the pellet and left to stand at room temperature for 30 minutes. The

supernatant was discarded after a second centrifugation at the same condition mentioned earlier. The pellet was air-dried, dissolved in 100 µl of TE buffer and stored at -20°C.

2.3 DNA visualization

Agarose gel electrophoresis was performed in order to check the presence of DNA after extraction protocol to determine whether DNA was successfully isolated and following a PCR amplification to determine whether the reactions were successful. The sample was mixed with the loading buffer to correct dilution and then pipette into the wells of the prepared gel (1% agarose containing 1 µg /mL of ethidium bromide) which is submerged in Tris-acetate (TAE) buffer in the electrophoresis tank.

2.4 DNA quantification

Genomic DNA was quantified using Ultraspec ® 1100 pro. 5 µl of genomic DNA was diluted in 495 µl of distilled deionized water in quartz cuvette. The concentration, absorbance of diluted DNA sample at the wavelength 260nm and 280nm (A_{260} and A_{280}) and the ratio of A_{260}: A_{280} was measured.

2.5 PCR

Five sets of primer were used to fish out the flowering genes from the sago palm. There are *LFY, Ath LFY, AP3, cctCO* and *haCO*. The nucleotide sequences of primer set *LFY, Ath LFY, AP3* and *cctCO* is determined based on literature review whereas primer set *haCO* is designed using computer program. The nucleotide sequences of *CO* gene from *A. thaliana* and *CO* like gene from three other species namely *Ipomoea nil, Brassica nigra* and *Malus domestica* which is retrieved from NCBI webpage have been aligned using DNASTAR computer program to find the most conserved regions prior to design the primer. The nucleotide sequences of all the primers used are showed in Table 2. PCR was carried out in a test tube by mixing genomic DNA from sago with a set of reagents and placing the tube in the Perkin Elmer (GeneAmp PCR System 2400). The parameter for PCR experiments are showed in the Table 3.

Table 2: The nucleotide sequences of *LFY*, *Ath LFY*, *AP3*, *cctCO* and *haCO* genes

Primer name	Sequences	Source	Reference
LFY (F/R)	Forward: 5' CGGAATTCATG(M)G(I)CA(Y)TA(Y) GT 3' Reverse: 5' CGGGATCCGG(Y)TT(R)CA(I)GC (Y)T 3'	*A. thaliana*	Southerton *et al.*, 1998
AthLFY (F/R)	Forward: 5' GACGCCGTCATTT(N)CT(N)CT 3' Reverse: 5' CCCGTCGTCATCCTC(N)CC(Y)TC 3'	*A. thaliana*	Brunel *et al.*, 1999
AP3 (F/R)	Forward: 5' AATGGTTTATTCAAGAA(R)GC(N) CA 3' Reverse: 5' CGAACGAGTTTGAAAGT(R)TT(Y)T 3'	*A. thaliana*	Brunel *et al.*, 1999
cctCO (F/R)	Forward: 5' CAGGGAGGCCAGGGTGCTCAG 3' Reverse: 5' CTCTTGGCGAAACGGCCCTTGA 3'	*A. thaliana*	Griffiths *et al.*, 2003
haCO (F/R)	Forward: 5' CCGCAAACCCTCTTGCTAGAC 3' Reverse: 5' CTTCTTCTCTCTGTATCTCAG 3'	*A.thaliana*, *Ipomoea nil*, *Brassica nigra*, *Malus domestica*	*www.ncbi.nlm.nih.gov*

Table 3: The parameters for PCR methods

Steps		Temperature (°C)	Time (min)
1	Initial denaturation	94	4
2	Denaturation	94	1
3	Annealing	50	1
4	Elongation	72	1 1/2
5	Step return to step 2 for 30 cycle		
6	Final extension	72	10

2.6 Purification of PCR products

A Qiagen purification kit was used for purification of PCR product of primer set *LFY* and *AP3*. The methods were carried out following manufacturer's recommendations.

2.7 Calcium Chloride ($CaCl_2$) Bacterial Competent Cell preparations

Esherichia coli JM109 was obtained from stock culture and inoculated into 5 ml of LB (Luria Broth) before incubated overnight at 37°C with shaking at 200 rpm until OD_{600} reading of about 0.5. The cells were then cooled on ice for approximately 20 minutes. The culture was transferred into 50 mL Falcon tubes and centrifuged at 3500 rpm for 5 minutes. After centrifugation, the supernatant was discarded and the cell pellets were resuspended in 25 mL of ice-cold 100 mM $CaCl_2$ for 10 minutes. The cells were further centrifuged at the same condition. Glycerol stocks were prepared by adding 200 μl of glycerol with 800 μl of $CaCl_2$ into each of the tube. 200 μl of aliquots from each tube were transferred into 1.5 mL Eppendorf tubes. The Eppendorf tubes were stored at - 80°C.

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2.8 Cloning of the PCR fragment

The purified PCR fragments of primer set *LFY* and *AP3* were ligated into the pGEM®-T Easy Vector supplied by Promega. The composition of ligation reaction is showed in Table 4. The reaction was incubated overnight at 4°C for the maximum number of transformants.

The next day, transformation was carried out using the *E .coli* JM109 competent cells. The transformation procedures were initiated by mixing 50 µl cold suspended *E. coli* JM109 competent cells with the entire ligation product. The mixture is incubated on ice for 30 minutes before a heat shock at 42°C for 50 seconds. Then the tubes were immediately returned into ice for 2 minutes. 950 µl of room temperature LB medium was added to the tubes and incubated for 1 hour 30 minutes. The cells were pelleted by centrifugation at 1000 rpm for 10 minutes prior to be resuspended in 200µl of LB medium. Finally, the cells were plated on pre-warmed LB Agar plates containing ampicilin (50 mg/µl), X-Gal (20 µg/µl) and 100 mM of IPTG and incubated overnight at 37°C.

Table 4: The composition of ligation reaction.

Reagents	**Volume** (μl)
2X Rapid ligation buffer	7.5
pGEM®-T (50ng)	1.0
PCR product	5.5
T4 DNA ligase (3 Weiss units/ μl)	1.0
Final volume	**15**

2.9 Plasmid isolation

The alkaline lysis method as suggested by Zyskind and Bernstein (1992) was employed for plasmid isolation of white colonies.

Seven white colonies after transformation (3 colonies for *LFY* PCR product and 4 colonies for *AP3* PCR product) was inoculated into 5 mL of LB and incubated overnight at 37°C. Following the incubation, the cells were centrifuge at 13,000 rpm for 10 minutes and the supernatant was discarded. The cells were resuspended in 200 μl of ice cold Solution I (GTE solution), 300 μl of freshly prepared Solution II (Lysis solution) and 300μl of Solution III (Potassium acetate, acetic acid and sterile water) accordingly and incubated on ice for 10 minutes for each step.

The supernatant were transferred to fresh 1.5 mL Eppendorf tubes after centrifugation at 13,000 rpm for 10 minutes. Equal volume of phenol:chloroform: isoamylalcohol was added and further centrifugation was done with the same condition as previous. The upper layer were transferred to new 1.5 mL Eppendorf tubes and 0.1