Chapter 3

Floral development and gene expression during flowering

3.1 Introduction

Flower development can be divided into four steps that occur in a temporal sequence. First, in response to both environmental and endogenous signals, the plant switches from vegetative growth to reproductive growth; this process is controlled by a large group of flowering time genes. Second, signals from the various flowering time pathways are integrated and lead to the activation of a small group of meristem identity genes that specify floral identity. Third, the meristem identity genes activate the floral organ identity genes in discrete regions of the flower. Fourth, the floral organ identity genes activate downstream "organ building" genes that specify the various cell types and tissues that constitute the four floral organs (Thomas, 2004).

Flowering involves the sequential action of two groups of genes: those that switch the fate of the meristem from vegetative to floral (floral meristem identity genes), and those that direct the formation of the various flower parts (organ identity genes) (Mandel and Yanofsky 1995). Therefore, genes that control flowering time can be expected to interact with floral meristem identity genes, which in Arabidopsis include LEAFY (*LFY*), APETALA1 (*AP1*), CAULIFLOWER (*CAL*), *AP2*, and UNUSUAL FLORAL ORGANS (*UFO*). The floral meristem identity genes are themselves capable of influencing flowering time. For example, overex-pression of *LFY* and *AP1* causes early formation of determinate floral meristems (Mandel and

Yanofsky 1995), whereas mutations in *TFL1* affect both flowering time and meristem identity (Shannon and Meeks-Wagner 1991).

The differential display (DD) is powerful procedure for quantitative detection and isolation of differentially expressed genes and was first reported by Liang and Pardee (1992). This method can be used to amplify low-abundance transcripts by polymerase chain reaction (PCR). It was statistically indicated that 80–120 primer combinations would be sufficient to cover all the transcript populations in cells. This technique possesses the following advantages over other similar techniques: it is based on simple and established methods, it does not require biochemical information about proteins, more than two samples can be compared simultaneously, and only a small amount of starting material is needed (Yamazaki and Saito, 2002).

The power of differential display lies in its simplicity. It is a combination of three frequently used molecular biology techniques that allow one to visualise and compare gene expression patterns between two or more samples. The principle of the method are using sets of anchored and arbitrary primers to generate complementary DNA (cDNA) fragments by reverse transcription of polyadenylated (poly-A) RNA, polymerase chain reaction (PCR) using special the differential display primers and polyacrylamide gel electrophoresis. First, high-quality total RNA is reverse transcribed using reverse transcriptase and one of three anchor primers designed to anneal to the 3’ poly-A tail of messenger RNA (mRNA). The resulting cDNA species are subsequently used as templates in a PCR, utilizing the same anchor primer from the reverse transcription reaction in combination with an arbitrary primer. Because nonspecific binding of the primers is maximized and small oligonucleotides

(10–13 bp) are used, an expression fingerprint with several cDNA species can be visualised after running the DD product on a polyacrylamide gel. DD cDNAs can be recovered, cloned and sequenced for further molecular characterization (Liang and Pardee, 1992). In the last 10 years, the differential display has been used to isolate genes from plants which are involved in physiological events, signal transduction, stress response and secondary metabolism. With the differential display, scientists have been able to isolate genes encoding membrane proteins and transcription factors; these genes occur in small amounts, thus are typically difficult to identify (Yamazaki and Saito, 2002). Differential display has also been used to inclusive profiling of genes expressed in particular plant cell types (Yamazaki and Saito, 2002). In the other plant, the differential display analyses of plant gene expression during morphological development such as embryogenesis (Momiyama *et al*., 1995; Heck *et al*., 1995). flower development (Nambara *et al*., 2000; Yu and Goh, 2000) were investigated. The objective of this study was to study gene expression during flowering of *Curcuma alismatifolia*.

3.2 Materials and methods

3.2.1 Morphological change of the terminal buds

Determination of the development stage of the terminal buds was done by using paraffin embedded technique. Ten shoot tips were sampling at 0-5, 6-10, 11-15, 16-20 and 21-25 cm. The shoot tip samples were stopped activity and fixed in fixative reagent. After 24 hrs of exposure to the fixative the vials containing the shoot tip sample were placed in a dessicator under vacuum, in order to remove any air remaining in the sample. The shoot tip sample were dehydrated by the tertiary butyl

alcohol procedure (Johansen, 1940). It is important to leave the shoot tip sample for an adequate time period for each step in the dehydration series; 50, 70, 85, 95, 100% tertiary butyl alcohol saturated with erythosine and pure tertiary butyl alcohol, followed by a 48-hrs exposure to a mixture of equal parts of tertiary butyl alcohol and paraffin oil. After that the sample were kept in paraplast solution in the hot oven (55-60 °C) 48-hrs and embedded in paraplast. The blocks of sample in paraplast are trimmed so as to provide a 2-3 mm margin of paraffin around the edge of the sample. Sections are cut on a rotary microtome to obtain 15 micrometer thick serial sections. These ribbons are arranged on black construction paper in necktie boxes and scanned under a dissecting microscope. Sections plant material are discarded and the remaining sections are cut to convenient lengths and are mounted onto glass slides. After gentle warming of the flooded slides so that wrinkles in the ribbons flatten out, they are drained and allowed to dry at least 24 hours. Staining and coverslipping were performed as described by Johansen (1940).

3.2.2 Gene expression by differential display

Shoot and flower bud were collected 0-5, 6-10, 11-15, 16-20 and 21-25 cm in height.

RNA isolation

1. Add 500 µl of CTAB solution to up to 0.1-0.5 gram of frozen, ground sample tissue. Mix by brief by vortexing until the sample is thoroughly resuspended.

2. Incubate the tube for 15 min at 65 °C.

3. Clarify the solution by centrifuging at 12,000 rpm in microcentrifuge for 2 min at 4 °C. Transfer the supernatant to a clean Rnase-free tube.

4. Add 0.1 ml of 5M NaCl to the clarified exract. Mix by tapping the tube.

5. Add 0.3 ml chloroform to the sample. Mix thoroughly by inversting the tube.

6. Centrifuge the sample at 12,000 rpm for 10 min at 4 °C to separate the phases. Transfer the upper, aqueous phase to a clean Rnase-free tube.

7. Add to the aqueous phase an equal volume isopropyl alcohol. Mix and let stand at -80 °C for 10 min.

8. Centrifuge the sample at 12,000 rpm for 10 min at 4 °C.

9. Decant the supernatant, taking care not to lose the pellet, and add 1 ml of 75% ethanol to the pellet.

10. Centrifuge the sample at 12,000 rpm for 1 min at 4 °C. Decant the supernatant carefully, taking care not to lose the pellet.

11. The pellet was air-dried and resuspened in 10-30 µl DEPC treated water.

12. To remove residual contaminating genomic DNA, the extracted RNAs were treated with 10 units of Rnase-free Dnase I in 30 µl reaction volume containing 1x Dnase buffer, 100 mM of DTT. Sample were incubated at 37 °C for 1 h, add EDTA 25 mM 10 µl, followed by heat inactivation of the enzyme at 65 °C for 10 min and then purified by using RNAspin Mini RNA Isolation Kit. RNA concentrations were measured spectrophotometrically at 260 nm and RNA purify was assessed by the ratio of absorbance at 260 nm and 280 nm, typical values being approximately 1.7 (pure RNA has ratio of 1.8). RNA samples were subjected to electrophoresis on 1.2% FA gel and visualized with ethidium bromide to examine the integrity. The purified RNAs were kept at -80 °C until analysis.

Differential display reverse transcription polymerase chain reaction (DDRT-PCR) assays

PCR differential display analysis was performed following the method described by Bauer *et al*., (1993) and Liang and Pradee (1992). First stand cDNA was synthesized using oligo(dT) primers that had to anchored nucleotides at 3' end, $(dT)_{12}$ VA, $(dT)_{12}$VC, $(dT)_{12}$VG and $(dT)_{12}$VT (V indicates a variable base either A, C or G).

This first stand reaction was diluted 1: 20 for subsequent PCR reaction. The cDNA was amplified by PCR with anchored primers T_{12}VA in combination with 40 arbitrary 10 mer primers OPA 01-20 and OPF 01-20 (Table 3.1). The reaction mixture of 12 μl consisted of 2 μl cDNA, the conditions for amplification were initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 sec, annealing at 42 °C for 1 min and elongation at 72 °C for 1 min, followed by 5 min extension at 72 °C. To analyzed the differential gene expression pattern, the PCR products were denatured at 95 °C for 5 min and quickly cooled on ice. These denatured PCR produced were separated on 6% denaturing polyacrylamide gels in 1X TBE buffer and electrophoresis was performed at constant power (55 W) and temperature (50 °C) for 5 hrs. After electrophoresis, bands were visualized by silver staining by adding 10% acetic acid and 1% nitric acid for 20 and 20 min, respectively. The gels were washed 3 times with double distilled water (ddH_2O) and stain with 0.2% silver nitrate solution for 30 min. After washing with ddH_2O, the gels were developed with 3% sodium carbonate with was supplemented with 0.02% formaldehyde, until the cDNA-DDRT bands appeared. The reaction were stopped with 10% acetic acid for 2 min and washed again with ddH_2O. The gel dried on filter paper at 55 °C for 2 hrs under vacuum of the gel dryer.

3.3 Results and discussion

3.3.1 Flower bud development

The shoot apical meristem makes the transition to reproductive development and the production of flowers is controlled by environmental and endogenous signals (Bernier, 1988). In many species must reach a certain age or size before they can flower, the vegetative meristem is thought to first pass through a "juvenile" phase in which it is incompetent to respond to internal or external signals that would trigger flowering in an "adult" meristem. The acquisition of reproductive competence is often marked by changes in the morphology or physiology of vegetative structures leaf shape offers one example in a process known as vegetative phase change (Poethig, 1990). Other species are less sensitive to environmental variables and appear to flower in response to internal cues such as plant size or number of vegetative nodes (Levy and Dean, 1998). In *Curcuma alismatifolia* buds, the use of paraffin embedded technique showed the time frame for initiation of visual changes from a vegetative to a reproductive state. In this study, the vegetative growth (0-5 cm) the apical meristem flat and leaf primordial initiate from the periphery towards the centre (Fig. 3.1 A). On the transition of the apical meristem from vegetative to generative, the meristem swells to form a dome shape, a bract is formed in the apex and leaf initiation ceases. In this study, the first morphological indication of the transition from vegetative to reproductive development was the change in the shape of the apex from flattened to domed. When they were 6-10 cm. height, the plant started to initiate the inflorescence. The inflorescence apex is shallowly domed and produces bracts on the dome (Fig 3.1 B). The inflorescence apex appears larger than the vegetative apex because the youngest bracts do not enlarge as early or as rapidly as the vegetative leaves

(Kirchoff, 1988). When they were 11-15 cm. in height, the plant started to differentiate the inflorescence. Preceding and during bract formation the bud in axil of bract continues to enlarge. The first flower bud was initiated, the center of the flower primordium was slightly depressed with respect to the surrounding tissue (Fig. 3.1C). Floral organ formation is sequential, not generally simultaneous. However, in certain apices two or more organs may appear simultanously. The general sequence of organ formation between whorls is calyx, corolla and inner androecial whorl, outer androecial whorl, gynoecium (Kirchoff, 1988). In *C. alismatifolia*, the plant were 16-20 cm. in height, the plant started to floral organ formation in the first floral. (Fig. 3.1D).

The plant were 21-25 cm. in height, termination of the inflorescence formation stage. The inflorescence teminates by cessation of growth of the apex and precocious development of ther primary bracts. Floral organs are formed sequentially beginning with the calyx, and continuing with the corolla and inner androecial whorl, outer androecial whorl, and gynoecium. The inflorescence of *C. alismatiflolia* has 6-7 floret (Pubuopiend, 1992).

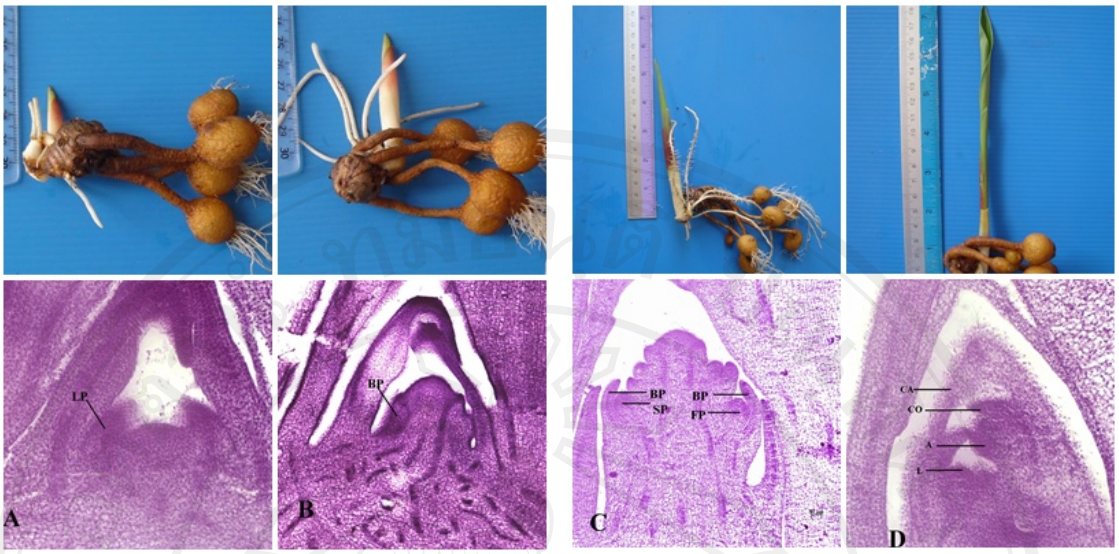


Figure 3.1. Flowering stage of *Curcuma alismatifolia* at different growth stage.

A : vegetative growth stage (0-5 cm in hight)

B : transitional stage (6-10 cm in hight)

C : inflorescence development stage (11-15 cm in hight)

D : floral organ formation stage (16-20 cm in hight)

LP : leaf primordium BP : bract Primordium

FP : first floral primordium SP : second floral primordium

CA : calyx CO : corolla

A : Anther L : Lip

Gene expression

The differential display has been used to isolate genes from plants which are involved in physiological events, signal transduction, stress response and secondary metabolism and have been able to isolate genes encoding membrane proteins and transcription factors (Yamasaki and Saito, 2002). Differential display has also been

used to inclusive profiling of gene expressed in particular plant cell types. In this research, A total of 1,146 cDNA fragments were amplified from shoot and inflorescence bud at 1-5, 6-10, 11-15, 16-20 and 21-25 cm growth stage of *Curcuma alismatifolia* using a combination primer of anchored ($dT_{12}VA$) and 40 random primers (Table 3.1). Comparing the banding patterns of the *Curcuma* four stages revealed 4 markers from 3 primers combinations (OPF10- $dT_{12}VA$, OPA03- $dT_{12}VA$ and OPF14- $dT_{12}VA$). Figure 3.2 shows the differential gene expression pattern in shoot of *Curcuma* of five stages. In this investigation, the profiling of differentially expressed cDNA band could be divided in to 3 catagories (Table 3.2): (i) band detected all stages but strong expression in 11-15, 16-20 and 21-25 cm (reproductive stage) (ii) bands only absent at 0-5 cm (vegetative stage) and (iii) bands only absent at 0-5 and 6-10 cm in height.

In this study, we have successfully applied DDRT-PCR to identify the differential gene expression profiling in shoot of *Curcuma* five stages.

Further characterization of the differentially expressed cDNA band (consist of reamplification, cloning and sequencing as well as quantitative PCR analysis) provides the important candidate genes for flowering of plant.

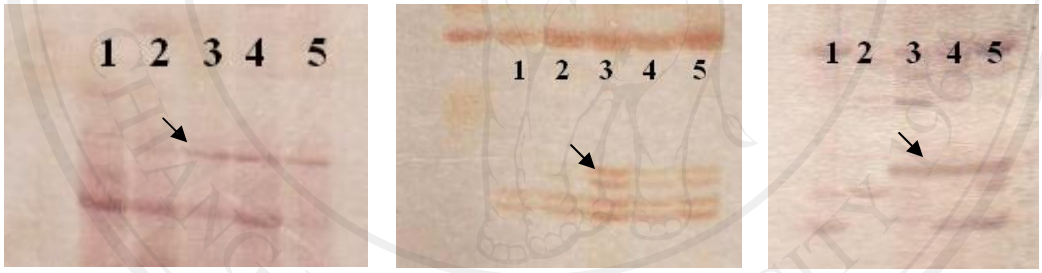
Table 3.1 Arbitrary 10 mer primers used for DDRT-PCR

Primer name	Sequence (5'→3')	Primer name	Sequence (5'→3')
OPA01	CAGGCCCTTC	OPF01	ACGGATCCTG
OPA02	TGCCGAGCTG	OPF02	GAGGATCCCT
OPA03	AGTCAGCCAC	OPF03	CCTGATCACC
OPA04	AATCGGGCTG	OPF04	GGTGATCAGG
OPA05	AGGGGTCTTG	OPF05	CCGAATTCCC
OPA06	GGTCCCTGAC	OPF06	GGGAATTCGG
OPA07	GAAACGGGTG	OPF07	CCGATATCCC
OPA08	GTGACGTAGG	OPF08	GGGATATCGG
OPA09	GGGTAACGCC	OPF09	CCAAGCTTCC
OPA10	GTGATCGCAG	OPF10	GGAAGCTTGG
OPA11	CAATCGCCGT	OPF11	TTGGTACCCC
OPA12	TCGGCGATAG	OPF12	ACGGTACCAG
OPA13	CAGCACCCAC	OPF13	GGCTGCAGAA
OPA14	TCTGTGCTGG	OPF14	TGCTGCAGGT
OPA15	TTCCGAACCC	OPF15	CCAGTACTCC
OPA16	AGCCAGCGAA	OPF16	GGAGTACTGG
OPA17	GACCGCTTGT	OPF17	AACCCGGGAA
OPA18	AGGTGACCGT	OPF18	TTCCCGGGTT
OPA19	CAAACGTCGG	OPF19	CCTCTAGACC
OPA20	GTTGCGATCC	OPF20	GGTCTAGAGG

Table 3.2 Categories of differential gene expression profiles in shoot and inflorescence bud at 1-5, 6-10, 11-15, 16-20 and 21-25 cm growth stage of *C. alismatifolia*.

Category	Growth stage					Markers
	1-5 cm	6-10 cm	11-15 cm	16-20 cm	21-25 cm	
i	+	+	+>	+>	+>	1
ii	-	+	+>	+>	+>	2
iii	-	-	+	+	+	1
Total						4

+ : present cDNA band, - : absent cDNA band, > : strong expression



a. OPA03 b. OPF10 c. OPF14

Figure 3.2. Differential display pattern from 5 stages were obtained from DDRT-PCR reaction (a. OPA03-$dT_{12}VA$, b. OPF10-$dT_{12}VA$, c. OPF14-$dT_{12}VA$). Arrow indicates differentially expressed cDNA band between 5 stages of plant.

3.4 Conclusion

C. alismatifolia changed from vegetative phase to reproductive phase at 11-15 cm in height and the banding patterns of the curcuma five stages revealed 4 markers from 3 primers combinations.