MATERIALS AND METHODS

Materials

Plant material and conditions for the post-harvest vase life of *Dendrobium* flowers

The inflorescence of *Dendrobium* 'Miss Teen' or 'Kenny', *Dendrobium* 'Sakura' *Dendrobium* 'Pompadour' *Dendrobium* 'Willie' and *Dendrobium* 'Karen' were purchased from a grower for export in Bangkok suburban. Inflorescences were harvested in the morning with five to six open florets and three to five flower buds. They were brought to the laboratory within 2 h of harvest. The peduncle and attached buds were cut off and only the first five open flowers were used. Each inflorescence peduncle was cut in an angle, 12 cm from basal end of the first open flower, and held in a 15-ml centrifuge tube containing 10 mL of distilled water. Inflorescence was held under natural light condition at ambient temperature (average 25^{o}C) and relative humidity (average 70% RH) during the period of study.

Methods

Experiment 1 Identification of compatible and incompatible pollinia

Open flowers of *Dendrobium* 'Miss Teen' or 'Kenny' were used as a female parent. The opened flowers were hand cross pollinated by placing pollinia from 5 cultivars of *Dendrobium* namely 'Miss Teen', 'Karen, 'Pompadour', 'Sakura' and 'Willie' on the stigma without removing anther cap and pollinia. The experiment was arranged in 25 replications (one flower/replication) and six treatments as following:

1. Unpollinated *Dendrobium* 'Miss Teen' as a control
2. *Dendrobium* 'Miss Teen' x *Dendrobium* 'Miss Teen'
3. *Dendrobium* 'Miss Teen' x *Dendrobium* 'Karen'
4. *Dendrobium* 'Miss Teen' x *Dendrobium* 'Pompadour'

5. *Dendrobium* ‘Miss Teen’ x *Dendrobium* ‘Sakura’
6. *Dendrobium* ‘Miss Teen’ x *Dendrobium* ‘Willie’

Treatment 2 and 3 were incompatible pollination. Whereas treatment 4, 5, and 6 were compatible pollination.

Post-pollination developmental events were observed and recorded as following

1. Symptoms of senescence were monitored. The alteration of pollinated flowers and time to exhibit senescence symptoms (such as epinasty (the pollinated flowers turn upside down), droop, venation, fading, lip yellowing, and petal senescences) in individual open flower was determined by daily observation.
2. Distance between lip and peduncle (cm) (Ketsa and Rugkong, 1999)
3. The ovary diameter (mm) were determined at the proximal end, using caliper
4. Ethylene production

To measure ethylene production, two inflorescences bearing only 4 open flowers from the bottom were weighed and held in centrifuge tube containing 10 ml distilled water and placed into an empty, air-tight 4 l bottles with gas sampling ports. At various intervals the bottles were sealed for 2 h and 1 ml gas sample was withdrawn from the headspace for ethylene determination by a gas chromatograph equipped with flame ionization detector (Shimadzu GC-14A). After each determination, all inflorescences were removed from the bottles.

Experiment 2 Measurement of pollen tube growth, IAA and ACC content in pollinia

In this experiment, post-pollination development revealed that ‘Sakura’, ‘Pompadour’, and ‘Willie’ pollinia are compatible pollinia.

2.1 The length of pollen tube

Individual cultivar pollinia were placed on the stigma of *Dendrobium* 'Miss Teen' to allow pollen tube growth. Ten pollinated flowers of *Dendrobium* 'Miss Teen' were picked at various intervals ranging from 1 to 7 days and fixed immediately in 50% FAA solution. After over night fixation, pollinia of each cultivar were collected from pollinated stigma, placed, spread, and mounted on a glass slide to measure the length of the pollen tube under a light microscope.

2.2 IAA and ACC content in pollinia

To analyze IAA and ACC content, pollinia of five cultivars were collected from the open flowers for extraction and analysis. Each cultivar was done in 3 replications.

ACC content

ACC content was extracted and analyzed according to Lizada and Yang (1979) and modified by Hoffman and Yang (1982). Pollinia were weighed (0.03 g) and ground in 5 ml of 9% trichloroacetic acid (TCA) using a mortar and pestle. After holding for 12 h at 4°C, the extract was centrifuged at 12000g for 20 min to remove insoluble cellular debris. The supernatant volume was measured. ACC content in pollinia (or floral tissue) was measured according to the method described by Lizada and Yang (1979). In a 6 ml tube, 0.1 ml of $HgCl_2$ and 0.3 ml of distilled water was added in 0.5 ml pollinia extract, and the tube was sealed with the serum cap. Approximately 0.1 ml of an ice-cold solution of bleach base (two parts of 5.25% sodium hypochlorite and one part of a saturated solution of sodium hydroxide) was injected into the tube through the seal. The tube was vortexed briefly and placed on ice. After 5 min, ethylene levels were measured in 1.0 ml of the headspace. To measure the efficiency of conversion of ACC to ethylene in the extracts, 0.5 ml of extract was spiked with 0.4 nmol of ACC to give a volume 0.9 ml after the addition of the $HgCl_2$. The ethylene production was measured after the addition of the bleach-

base solution as described above. The conversion efficiency in freshly made floral extracts was found to be close to 100%. ACC content was expressed as nmol ACC formed per g FW.

IAA in pollinia

IAA of pollinia was extracted and analyzed according to method of Abdel-Rahman *et al.* (1975). Three hundred mg of pollinia were extracted in 15 ml cold methanol (95%). The homogenate was filtered through Whatman No.1 paper and the residue washed once by 10 ml of 95% methanol. The pooled filtrate was evaporated in a rotary evaporator at 35°C and the residue was washed with 15 ml of 0.5 M sodium phosphate buffer (pH 8). The extract was transferred to a separatory funnel and 20 ml ethyl acetate added. The separatory funnel was vigorously shaken to mix the layer and then the layers were subsequently allowed to separated, soluble IAA being in the upper (ethyl acetate) phase. The lower aqueous layer was withdrawn into another separatory funnel and pH adjusted to 2-2.5 with 6 N HCl, and extracted four times with 20 ml of ethyl acetate. Ethyl acetate extracts were combined and evaporated by a rotary evaporator at 35°C. The residue was dissolved in 1 ml of 100% methanol and IAA in the purified extract was determined by high pressure liquid chromatography (HPLC).

High-performance liquid chromatographic analysis of Indole-acetic acid

One milliliter of pollen extraction sample was filtered through Nylon Acrodisc 13 (0.45 μm). The HPLC analyses were carried out using a Shimadzu class LC-VP HPLC system withclass LC-VP software, a pump (LC-10AD VP), and a UV detector (SPD-10A VP) (Shimadzu, Kyoto, Japan). The seperation was carried out on a Alltima C18 column 5 μ (250 x 4.6 mm) Lot No. 2672 (Alltech, USA) with guard column (CLC-ODS(4), 10 x 4 mm I.D.), using 30% methanol and 0.8% acetic acid as a mobile phase at a flow rate 1.5 ml/min and monitored at 280 nm. The retention time for IAA peak is 12.8 min.

Experiment 3 Comparison of ACC content, ACC synthase activity, and ACC oxidase activity in *Dendrobium* 'Miss Teen' flowers following compatible and incompatible pollination

From Experiment 1, three treatments were selected to represent following:

1. Nonpollination or control (unpollinated 'Miss Teen' flower)
2. Compatible pollination ('Miss Teen' x 'Karen')
3. Incompatible pollination ('Miss Teen' x 'Sakura').

To analyze ACC content and activities of ACS and ACO in flowers, at various times after pollination, the tissue from 30 flowers of each treatment were separated into two parts (the first part is petal, sepal plus lip and the second part is column plus pedicle) and pooled, immediately frozen in liquid nitrogen and stored at –70^{o}C until use.

Extraction and assay of ACC synthase activity

For extraction and analysis of ACS activity, the floral tissue (5g) was rinsed twice with distilled water, blotted with a paper towel, and ground in 100 mM EPPS buffer (pH 8.5) containing 4 mM dithiothreol (DTT) and 0.5 mM pyridoxal phosphate (1:2 w/v), using mortar and pestle. The homogenate was centrifuged at 12,000 g for 20 min. The supernatant was contained in dialysis bag, soaked in dialysis buffer containing 2 mM EPPS buffer, 0.1 mM DTT and 0.2 μM pyridoxal phosphate (1: 10, v/v). The extract was dialyzed for 24 h. All steps were carried out on ice or at 4^{o}C. ACS activity was assayed according to method of Hoffman and Yang (1982) by incubation of 0.4 ml extraction in 6-ml tube with 50 μl of 0.5 mM SAM and 90 μl of distilled water at 30°C for 2 h. The ACC produced was determined as described above. ACS activity was expressed as nmol ACC formed per h per mg protein.

Extraction and assay of ACC oxidase activity

ACO from floral tissue was extracted and assayed according to the procedure of Kasai *et al*. (1998). The floral tissue was weighed for 3 g and homogenized with homogenizer in 10 ml of extraction buffer (0.1M Tris-HCl buffer, pH 7.2, containing 30% glycerol, 10 mM sodium ascorbate, and 5 mM dithiothretol). The homogenate was centrifuged at 14,000 g, 4^{o}C for 20 min. The supernatant was assayed for ACO activity in a reaction medium consisting of 0.1 M Tris-HCl buffer, pH 7.2, 30% glycerol, 1mM ACC, 10 mM sodium ascorbate, 50 μM $FeSO_4$ and 10 mM $NaHCO_3$ in total 1 ml in a sealed test tube with and atmospheric volume of 6 ml. The sealed test tube was incubated with gentle shaking at 37^{o}C. One ml of the headspace gas was withdrawn for ethylene determination by a gas chromatograph (Shimadzu GC-R14A) equipped with a flame ionization detector. ACO activity was expressed as amount of ethylene converted from ACC during the reaction period.

Protein content

Protein content was detected for calculating specific enzyme activity using the method described by Bradford (1976). Four ml of Coomassie blue were added to 1 ml of enzyme solution. The samples were measured at 595 nm and protein concentration determined for each sample using a bovine serum albumin as a standard.

Experiment 4 Role of signals and effectors on pollination

One of the major signals believed to move from the pollinia to the stigma is IAA. Therefore, the IAA in pollinia was depleted by soaking in water. In the experiment, the compatible pollinia (*Dendrobium* ‘Sakura’) were soaked in distilled water for various times (0, 1, 3, 5 and 10 min.) before placing on the stigma of *Dendrobium* ‘Miss Teen’. The following parameters: distance between lip and peduncle, ovary diameter, time to occur petal senescence symptoms were recorded

and IAA in soaking pollinia was analyzed. IAA was extracted and analyzed by HPLC, and compared with standard IAA.

Experiment 5 Effect of inhibitors on postpollination changes

5.1 Effect of inhibitors

Whole inflorescences of *Dendrobium* 'Miss Teen' bearing only open flowers were treated by applying a volume of either 50% ethanol as control or inhibitor for ethylene production; aminooxyacetic acid (AOA) 0.3 nmol per floret, anti-auxin transport; 2, 3, 5-triiodobenzoic acid (TIBA) 5 μg per floret, anti-auxin action; α-p-chlorophenoxy isobutyric acid (PCIB), 5 μg per flower on the stigma prior pollination by compatible pollinia (*Dendrobium* 'Sakura'). For experiments involving the use of inhibitors, flowers were pretreated with inhibitor for 18 h to ensure penetration of the inhibitor prior to pollination. After pollination the data were recorded the same as experiment 3.

5.2 Effect of synthetic auxin (2-napthoxyacetic acid: NOA)

The only open flowers of *Dendrobium* 'Miss Teen' were pollinated, or treated by applying a volume of either 50% ethanol as control or NOA (5 and 10 μg per flower to the stigma. The ovary diameter of the treated-flowers was measured.

5.3 Effect of shorts chain fatty acid (octanoic acid: OA)

Whole inflorescences of *Dendrobium* 'Miss Teen' bearing only open flowers were treated by applying a volume of either distilled water as control or 500 ng octanoic acid (OA) prior to treatment with pollinia from *Dendrobium* 'Karen' or 0.3 nmol ACC, 1 nl/l ethylene for 12 h, or a combination. Flowers were pretreated with OA for 18 h to ensure penetration of the OA prior pollination or application ACC or ethylene. The effect of OA on senescence was recorded.

5.4 Effect ethylene inhibitor on pollen tube length

Whole 'Miss Teen' inflorescence were exposed in 400 ppb 1-MCP for 4 h or application 0.3 nmol AOA to the stigma prior pollination by compatible pollinia (*Dendrobium* 'Pompadour'). Seven days after pollination, the pollinated flowers of *Dendrobium* 'Miss Teen' were picked and fixed immediately in 50% FAA solution. After over night fixation, pollinia of each cultivar were collected from pollinated stigma, then placed, spread, and mounted on a glass slide to measure the length of the pollen tube under a light microscope.

Experiment 6 Gene expression in *Dendrobium* 'Miss Teen' following compatible and incompatible pollination

ACO gene involved in compatible and incompatible pollinated flowers were analyzed by northern blot analysis and RT- PCR.

6.1 Total RNA extraction

The protocol was modified from Chang *et al.* (1993) to make it suitable for extracting RNA from orchid floral tissue. Total RNA was isolated from frozen flowers (ca. 0.1 g). RNA extraction buffer consisting of 2% CTAB, 2% PVP, 100 mM Tris-HCl, pH. 8.0, 2M NaCl, 25 mM EDTA was preheated at 65^{o}C, and added with 2% β-mercaptoethanol prior to use. The extraction buffer was added to floral tissue ground to fine powder in liquid nitrogen. The powder was mixed in the 50 ml centrifugetube. After incubation at 65^{o}C for 10 min., 10 ml of chloroform/isoamyl alcohol (24:1, v/v) was added and then the mixture was vigorously mixed. After centrifugation at 8,000 rpm for 15 min, the aqueous phase was transferred to a new tube. Five ml of extraction buffer and 15 ml of chloroform/isoamyl alcohol were added, vigorously shaked and centrifuged at 8,000 rpm. The upper aqueous phase was transferred to a new tube and added with 1/3 volume of 8 M LiCl and kept at 4^{o}C overnight. After centrifugation at 12,000 rpm at 4^{o}C for 30 min., the pellet was kept and dissolved with 400 μl DEPC-treated water and then transfered to a new

microtube. The solution was precipitated by the addition with 0.1 volume of 2.5 M sodium acetate and 2.5 volume of absolute ethanol, then mixed and placed at –70 ^{o}C. After 1 h, the RNA pellet was precipitated and washed with 70% ethanol. After centrifugation at 12,000 at rpm at 4^{o}C for 10 min., the pellet was dried and dissolved with 50 μl DEPC-treated water and kept at –70 ^{o}C until use**.**

6.2 Amplification of *ACO* and *Actin* gene of *Dendrobium* 'Miss Teen'.by RT-PCR

The cDNA of *Dendrobium* 'Miss Teen' *ACO* was amplified by reverse transcriptation (RT) - polymerase chain reaction (PCR). For ACO, two oligonucleotide primers were designed for PCR (kindly provided from Miss Anjana Bhunchoth, National Center of Genetic Engineering and Biotechnology); *ACO-Den* F_1 primer, 5'- ATG GAG CTT CTT GAG GGT TC-3'; *ACO-Den* R primer, 5'-TCA AGC AGT AGG AAT CGG CTG –3'. The PCR mixture was initially denatured at 94 ^{o}C for 5 min and then subjected to 35 cycles at the following conditions: 94 ^{o}C for 30 sec, 55 ^{o}C for 30 sec, and 72 ^{o}C for 1 min with a final extension at 72^{o} C for 5 min. The cDNA was ligated into pGEM®-T Easy Vectors (Promega, USA) as described by the supplier's instruction. *E. coli* competent cells were transformed with recombinant plasmid. The sequences were determined by DNA Technology Laboratory, BIOTEC service. DNA sequence of the obtained product (approximately 950 bps) was found to be almost the same as *ACO* gene of *Dendrobium crumenatum* (91% identical at amino acid level, in Genbank accession number AF038840).

Actin gene was amplified using specific primers. The *Actin* primers were got from Miss Suganya Chidtrakul. The sequence of forward primer was 5' ATG TTT GAG ACC TCC AAT GTA CCT G 3' and the sequence of reverse primer was 5' GTT TCC ATA GAG ATC CTT CCT GAT A 3'. Following initial denaturation at 94^{o}C for 5 min, the PCR reaction was carried out for 35 cycles under the following conditions: denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 30 sec, and extension at 72^{o}C for 30 sec. The final extension was carried out at 72^{o}C for 5 min The cDNA product size approximately 520 bps was ligated, transformed and

sequenced in the same as *ACO* gene. The sequence of amplified *Actin* fragment was compared with the gene in the Genbank database using the BLAST program from NCBI (National Centre for Biotechnology Information). It was found to be almost the same as an actin-like protein (*ACT2*) of *Phalaenopsis* 'True Lady' (89% identical at nucleotide level, in Genbank accession number AF246715).

6.3 DIG labeling (DNA probe) using PCR DIG Probe Synthesis Kit (Roche, USA)

ACO probe

The competent cell contained inserted *ACO* gene was cultured in 3 ml LB medium overnight. The plasmid DNA was extracted and purified using QIAprep® spin Miniprep Kit (Qiagen, USA). Cells were centrifuged at 13,000 rpm for 3 min and collected only the pellet cells. The pellet cells were re-suspended in 250 μl of re-suspension buffer and transfer to a 1.5 ml microtube. Then, added 250 μl of lysis buffer and gently invert the tube to mix. Two hundred and fifty of neutralization buffer was added and inverted the tube immediately and centrifuged the tube at 13,000 rpm. The supernatant was taken to the spin column by pipetting. Centrifuged the tube for 1 min and the flow-through was discarded. The spin column was washed by adding 750 μl of washing buffer and centrifuged for 1 min, discarded the flow through and centrifuged again to remove the residual washing buffer. DNA was eluted by added 50 μl of elution buffer and centrifuged for 1 min. DNA was kept at -20^{o}C until used.

Plasmid DNA of *ACO* clone (10 pg) was used as template for amplification with the specific upstream and downstream primers. Template was used for the PCR reaction compared with DIG labeled probe and unlabeled DNA control. The PCR mixture was initially denatured at 95^{o}C for 5 min and then subjected to 35 cycles at the following conditions: 95^{o}C for 30 sec, 55^{o}C for 30 sec, and 72^{o}C for 1 min with a final extension at 72^{o}C for 5 min.

6.4 RNA blotting and hybridization

Ten μg of total RNA isolated from floral tissue were separated by electrophoresis on 1% agarose-denaturing formaldehyde gel containing 20 mM MOPS, pH 7.0, 0.5 mM sodium acetate, and 1 mM EDTA. After electrophoresis for 2 h, the RNA was visualized with ethidium bromide under UV light to confirm equal loading of RNA in each lane, and transferred to a positively-charged nylon membranes (Roche, Germany) by capillary action with 10X-SSC and then the membrane was baked for 2 h at 80^{o}C. The membranes were pre-hybridized in 10 ml of hybridization buffer (7% SDS, 50% formamide, deionized 5x-SSC, 0.1% N-lauroylsarcosine, 2% blocking solution, and 50 mM sodium phosphate, pH 7) at 65^{o}C for 1 h. Hybridization was performed overnight in the same buffer containing the labeled probe at 65^{o}C. The cDNA of *ACO* gene was labeled with DIG DNA labeling kit (Roche, Germany) following manufacturer's instructions. After hybridization, the membrane was washed with 2X-SSC containing 0.1% SDS for 15 min. at room temperature and then washed with 0.2X-SSC containing 0.1% SDS for 15 min at 50^{o}C. After the equilibration in buffer A (maleic acid and 0.15 mM NaCl, pH 7.5) for 5 min. at room temperature, the membrane was blocked with 1% blocking reagent in buffer A for 30 min. Subsequently, the membrane was incubated with anti-digoxigenin-alkaline phosphatase (Roche, Germany) in the 1X blocking buffer for 30 min at room temperature. After washed twice times for with 1x maleic acid washing buffer for 15 min at room temperature, the membrane was equilibrated in detection buffer (0.1 M Tris, pH 9.5 and 0.1 M NaCl) for 5 min, and then sealed the membrane in plastic bag and equilibrated in CDP-Star substrate (Roche, USA) for 1 min. The hybridization result was visualized by exposing membrane on the x-ray film (Kodak Medical X-Ray film).

6.3 RT-PCR analysis

Total RNA was extracted from column plus pedicel (or sepal plus petal plus lip) of *Dendrobium* 'Miss Teen' orchid. After the reverse transcription step, the total cDNA served as a template in the PCR amplification reaction using 5 μM ACO-

specific primers (*ACO-Den* F_1 primer, 5'- ATG GAG CTT CTT GAG GGT TC-3'; *ACO-Den* R primer, 5'-TCA AGC AGT AGG AAT CGG CTG –3'). A fragment of the *Actin* gene (176 bps) was used as an internal control. The cDNA was amplified with *ACO* and/or *Actin*-specific primers (*Den-Actin* F 5'- AAG CTG TTC TTT CCC TAT ATG CTA GTG G-3' and *Den-Actin* R 5'-CTT CTC CTT GAT GTC CCT GAC AAT TT-3') for PCR. The PCR products were separated on 1% agarose gel. After staining gel with ethidium bromide, DNA was visualized under UV light.

Statistical analysis

Five inflorescences of *Dendrobium* 'Miss Teen' were used in each treatment. The individual inflorescences bore only five open flowers and there were 25 open flowers per treatment. The mean comparisons were made using Duncan's new multiple range test (DMRT). The experiments were repeated twice.

The experimental place

Postharvest Research Unit, Central Laboratory and Greenhouse Complex, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom.