MORPHOLOGICAL, HORMONAL AND GENETICAL ANALYSES OF EARLY *IN VITRO* FLOWERING IN *DENDROBIUM* CHAO PRAYA SMILE

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2010

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(M. Sc.)

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHYLOSOPHY
DEPARTMENT OF BIOLOGICAL SCIENCES
NATIONAL UNIVERSITY OF SINGAPORE
2010

ACKNOWLEDGEMENT

I am heartily thankful to my supervisor, A/P Loh Chiang Shiong and A/P Yeoh Hock Hin, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject.

I am grateful to Mrs. Ang for her technical assistance; to Ping Lee and Madam Loy for their help and guidance on microtomy; to Kishor and Chye Fong for their precious advice on HPLC; to Say Tin for her technical assistance on ESI-MS/MS.

I owe my deepest gratitude to Sai Mun for his selfless sharing of knowledge and materials of molecular work.

My heart-felt thanks to my lab-mates and friends Wee Kee, Teng Seah, Carol, Daphne, Jacqueline, Baidah, Sean, Edwin and Reena for their help, advice, encouragement and moral support.

I would like to extend my thanks to my family for their continuous support; to my brother Gasi, especially, for his concern, understanding and continuous encouragement and motivation throughout the course of this project.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

CONTENT

SUMMARY

Dendrobium Chao Praya Smile was induced to flower in a two-layer (a Gelrite-solidified medium topped with a layer of liquid medium of the same composition and volume) medium within 6 months from seed germination using BA. The functionality of the *in vitro*-developed flowers was verified through sporad analysis and pollen grain germination tests. The *in vitro*-developed flowers were able to form seedpods and produce viable seeds upon self-pollination. With successful seed production in culture, the plantlets could complete a life cycle entirely *in vitro* in about 11 months, approximately one-third of the time in field-grown plants.

Histological analysis revealed that floral transition, as indicated by bolting, in *D.* Chao Praya Smile took place 54 days after growing in a BA-containing liquid medium. Subsequently, floral buds developed on the plantlets. During floral transition, the expression of *DCPSknox*, a gene involved in maintaining the indeterminacy of shoot apical meristem, was found to decrease. In *in vitro*-developed flowers, segregation of colors was observed - 4 types of flowers with different intensities of pink coloration were produced. It was possible that color segregation was naturally occurring as it was found that BA treatment did not affect the expression of *DCPSCHS*, a key gene involved in anthocyanin biosynthesis, in the plantlets. One-third of the flowers produced *in vitro* were found to be incomplete with missing or defective floral organs.

Using HPLC-ESI-MS/MS, changes in cytokinin and IAA contents were analyzed in flowering-induced *D.* Chao Praya Smile at different growth stages as well as in different tissues during floral transition. It was found that iPR significantly increased in the plantlet and shoot apex at floral transition. Higher cytokinin/IAA ratios were also

observed in the plantlet and shoot apex at floral transition. Hence, we propose that the endogenous cytokinin/IAA ratio, and not the absolute amount of cytokinins, which determines flowering in *D.* Chao Praya Smile. The inductive and inhibitory effects of iPR and IAA, respectively, on the flowering in *D.* Chao Praya Smile were also verified. A fragment of *DCPSCKX*, a gene involved in cytokinin homeostasis, was cloned and its expression was found to be strongly stimulated by BA treatment. Finally, a model of mechanisms underlying the BA-induction of flowering in *D.* Chao Praya Smile was proposed.

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LIST OF ABBREVIATIONS

BA	6-benzyladenine
CHS	Chalcone synthase
CKX	Cytokinin oxidase/dehydrogenase
CW	Coconut water
DHZ	Dihydrozeatin
DHZR	Dihydrozeatin riboside
DHZ9G	Dihydrozeatin-9-glucoside
DHZMP	Dihydrozeatin riboside-5'-monophosphate
DW	Dry weight
ESI	Electrospray ionization
FW	Fresh weight
GA	Gibberellic acid
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
iP	Isopentenyladenine
iPR	Isopentenyladenosine
iP9G	Isopentenyladenine-9-glucoside
iPMP	Isopentenyladenosine-5'-monophosphate
IPT	Adenosine phosphate-isopentenyltransferase
LD	Long-day
MS	Mass spectrometry
RT-PCR	Reverse transcription polymerase chain reaction
SAM	Shoot apical meristem
SD	Short-day
TIBA	2,3,5-triiodobenzoic acid
tZ	trans-zeatin
Z	Zeatin
ZR	Zeatin riboside
Z9G	Zeatin-9-glucoside
ZMP	Zeatin riboside-5'-monophosphate

Chapter 1

INTRODUCTION

Orchids are grown mainly for the beauty of their flowers. The plants have been cultivated and marketed globally as potted plants and cut flowers (Winkelmann *et al.*, 2006). Despite the increasing demand for these plants, it takes years before flowers can be produced in orchid plants, due to the presence of a long juvenile vegetative phase (Hew and Yong, 1994). For instance, the juvenile phase of *Dendrobium* hybrids before first flowering can range from 3.5 to 7.5 years (Wee, 1971). Therefore, various tissue culture methods have been developed to shorten the juvenile phase in orchids, and to induce flowering *in vitro* in order to achieve flowering in a shorter period of time. To date, flowering *in vitro* has been successfully induced in *Cymbidium* (Kostenyuk *et al.*, 1999; Chang and Chang, 2003), *Dendrobium* (de Melo Ferreira *et al*., Hee *et al.*, 2007; Sim *et al.*, 2007; Tee *et al.*, 2008; Wang *et al*., 2009), *Phalaenopsis* (Duan and Yazawa, 1995) and ×*Doriella* (Duan and Yazawa, 1994) orchids. Cytokinins, such as BA (6-benzyladenine) and thidiazuron, were used in the tissue culture methods for flowering induction.

Even with the successful induction of *in vitro* flowering in some orchid species, the mechanisms underlying the flowering induction process remained elusive. In other plant species such as *Arabidopsis thaliana*, *Nicotiana tabacum* and *Sinapis alba*, cytokinins have always been suggested and implicated as important factors relating to floral transition (Chaudhury *et al.*, 1993; Dewitte *et al.*, 1999; Bernier *et al.*, 2002). In these plant species, cytokinin content in the plant would be markedly elevated during floral transition (Chaudhury *et al.*, 1993; Dewitte *et al.*, 1999; Bernier *et al.*, 2002).

Moreover, cytokinins have been proposed as the mobile physiological signals that trigger the initiation of flowering in *S. alba* upon long-day induction (Bernier *et al*., 1993). In orchids, the physiological importance of cytokinins in flowering was mainly observed in field experiments involving foliar spray or injection of cytokinins (Sakai *et al.*, 2000; Blanchard and Runkle, 2008).

The objective of this project was to investigate the morphological, hormonal and genetical changes in the early *in vitro* flowering in *Dendrobium* Chao Praya Smile. *D.* Chao Praya Smile was induced to flower *in vitro* using BA. Viable orchid seeds were produced in culture by self-pollinating the *in vitro*-developed flowers. At different growth stages of flowering induction, morphological changes in the shoot apical meristem of *D.* Chao Praya Smile were studied to determine the timing of floral transition in the plantlets. The development of the flowers produced *in vitro* was observed for color segregation. The expression of *DCPSCHS* (anthocyanin biosynthetic gene) was investigated in order to find out if BA treatment has caused color segregation in the flowers developed *in vitro*. The *in vitro* flowering of *D.* Chao Praya Smile was then used as a model system to investigate the changes in cytokinin and indole-3-acetic acid (IAA) content as well as the expression of *DCPSCKX* (gene of cytokinin oxidase/dehydrogenase) at various growth stages, especially during floral transition. It was hoped that the information obtained from the study will contribute towards greater understanding of the involvement of cytokinins, IAA and *DCPSCKX* in the *in vitro* flowering in *D.* Chao Praya Smile.

Chapter 2

LITERATURE REVIEW

2.1 Phase change and flowering

Plants pass through a series of distinct developmental phases during their growth. In higher plants, these developmental phases take place in the shoot apex. The shoot apex undergoes three distinct phases during its post-embryonic development: a juvenile vegetative phase, an adult vegetative phase and a reproductive phase (Poethig, 1990). The transition from juvenile to adult vegetative phase usually occurs gradually and involves subtle changes in the morphology and physiology of the shoot apex. On the other hand, transition from vegetative to reproductive or flowering could be abrupt and noticeable changes would occur at the shoot apex (Poethig, 1990). Flowering transition is a major event in the life of a plant because the shoot apical meristem (SAM) will switch from leaf production to the initiation of floral organ. Flowering is a process whereby leaf development is suppressed and lateral buds differentiate as flowers of flower-bearing branches (Poethig, 2003). A combination of environmental, developmental, hormonal and genetic factors determines the eventual transition to flowering. To ensure reproductive success, flowering transition will only take place when these factors are most favorable. Since flowering leads to sexual reproduction, it is of paramount importance in agriculture, horticulture and plant breeding. A number of studies have been conducted to investigate factors that affect flowering transition in various plant species (de Bouillé *et al.*, 1989; Bernier *et al*., 1993).

Orchids have been marketed globally as cut flowers and potted flowering plants. Like many other flowering plants, a juvenile phase exists in orchids. Flowering and

reproduction can only take place when the orchids have reached a certain size, sufficient to maintain the energetic demands of flowering and seed production (Lopez and Runkle, 2005). When the plants have attained the competency to flower, environmental and cultural factors can be provided to induce flowering.

2.2 Factors regulating flowering

2.2.1 Plant growth regulators

Plant growth regulators could control the entire development of a plant and its interactions with external environment (Reski, 2006). Many studies have suggested that cytokinins were direct or indirect factors that led to floral transition. They were shown to increase progressively in the terminal buds of *Pinus pinea* from juvenile to adult phase (Valdés, *et al.*, 2004), indicating the importance of this plant growth regulator in promoting sexual maturation. Similarly, increased endogenous cytokinin levels have also been correlated to flowering in *Sinapis alba* (Bernier *et al.*, 2002). In *Arabidopsis thaliana*, cytokinin levels were found to increase in a mutant that flowered early (Chaudhury *et al.*, 1993). Furthermore, early flowering caused by the constitutive expression of pea ABA-responsive 17 (*ABR17*) in *Arabidopsis* (Srivastava *et al.*, 2006) and *Brassica napus* (Dunfield *et al.*, 2007) was attributed to increased cytokinin levels in the plants.

Cytokinins in SAM were crucial for floral transition. Higher cytokinin levels were detected in the apices of *B. napus* (de Bouillé *et al.*, 1989), *Chenopodium rubrum* and *Chenopodium murale* (Macháčková *et al.*, 1993) during floral transition. After long-day (LD) induction of flowering in *S. alba*, the phloem sap feeding the shoot apex was found

to be enriched with isopentenyladenine (iP)-type cytokinins (Lejeune *et al.*, 1994). The accumulation of iP in SAM tissue in *S. alba* during floral transition has also been demonstrated by Jacqmard *et al.* (2002). It was suggested that the increased iP in the SAM could be either transported from leaf into the phloem or locally synthesized because apical buds were capable of synthesizing cytokinins (Letham, 1994). Plasmodesmata are membrane-lined channels that connect higher plant cells to form a functional intercellular communication network of symplasm (Robards and Lucas, 1990). It was shown in *S. alba* that the number of plasmodesmata was dramatically increased in the SAM following LD induction of flowering (Ormenese *et al.*, 2000). A similar increase in plasmodesmata was observed when BA was applied to the plant (Ormenese *et al.*, 2006). Therefore, it was suggested that floral transition induced by LD was mediated by cytokinin. Although endogenous cytokinins were important for floral transition, exogenous cytokinin application did not cause flowering in *S. alba*, although it stimulated cell division (Jacqmard *et al.*, 1998) and transcription of the *SaMADS* gene (Bonhomme *et al.*, 2000), responses similar to those under LD induction. Therefore, it could be concluded that endogenous cytokinin mobilization or synthesis was crucial in floral transition in *S. alba*.

Plant growth regulators also appeared to be important in the flowering of *Dendrobium* orchids. It was postulated that photoperiod and low temperature that induced flowering in *Dendrobium* orchids could be associated with changes in the concentrations of endogenous plant growth regulators (Goh and Arditti, 1985). Furthermore, injection of cytokinin into *Dendrobium* Jaquelyn Thomas “Uniwai Princess” has been shown to increase the number of inflorescences (Sakai *et al.*, 2000). Cytokinins have also been shown to regulate inflorescence initiation of field grown *Doritaenopsis* and *Phalaenopsis*

orchids (Blanchard and Runkle, 2008). These orchids could be induced to flower earlier with more inflorescences and flowers per plant when treated with foliar sprays containing BA. Although BA promoted flowering in the orchids, it could not completely substitute for inductive low temperature. Therefore, it was suggested that cytokinins promoted flowering in orchids only when the environmental and cultural factors were in favor of flowering (Blanchard and Runkle, 2008).

It is well known that flowering in *Phalaenopsis hybrida* requires a period of low temperature (Hew and Yong, 1997). When subjected to high temperature, a condition not favoring for floral transition, total cytokinins were reduced and glucoside cytokinins were accumulated in the leaves of *Phalaenopsis* orchid (Chou *et al.*, 2000). In contrast, the levels of zeatin (Z), zeatin riboside (ZR) and dihydrozeatin (DHZ) were found to increase under low temperature (Chou *et al.*, 2000). This result might indicate that cytokinin metabolism could be affected by temperature and that free base and cytokinin ribosides might be related to floral transition (Chou *et al.*, 2000). Although many studies have indicated that cytokinins were important factors in flowering, the effect of cytokinins on flowering induction in field-grown orchids was not consistent. BA application to field-grown *Miltoniopsis* orchid hybrids was shown to promote the growth of new vegetative shoots and reduced the number of plants with inflorescence (Matsumoto, 2006). The reduction of flowering could be alleviated by the application of gibberellic acid (GA) in the BA treated plants.

Ascorbic acid-deficient mutants of *Arabidopsis* were shown to flower early irrespective of photoperiod when compared with the wild type (Kotchoni *et al.*, 2009). Conversely, flowering was delayed when the ascorbic acid content was artificially

increased. The effect of ascorbic acid on flowering could be related to plant growth regulator-mediated signaling processes that regulate floral transition because ascorbic acid could serve as cofactor for the synthesis of certain plant growth regulators (Barth *et al.*, 2006). Strigolactones, which are carotenoid-derived terpenoid lactones, were recently suggested to play a role in inflorescence development by regulating axillary bud outgrowth (Waldie *et al*., 2010).

It was difficult to draw a conclusion on which cytokinins were crucial in floral transition because different cytokinins were predominant in different plant species (Lejeune *et al.*, 1988). For example, a significant increase in the endogenous concentrations of isopentenyladenosine (iPR) was observed in the root and leaf tissues of *Arabidopsis* upon flowering induction using tricontanol (He and Loh, 2002). In addition, treating *Arabidopsis* plant with iPR was sufficient and effective to induce floral bud formation (He and Loh, 2002). The finding was in line with Lejeune *et al.* (1988) who reported that the root exudate of LD-induced *S. alba* was enriched with iPR. These findings appeared to indicate that iPR was involved in floral transition.

The interplay between cytokinins and IAA could be more important than cytokinins alone in regulating floral transition. A lower IAA/cytokinins ratio was observed at flowering stage in *T. recurvata* which was caused by the enhancement of cytokinins (Mercier and Endres, 1999). Similarly, flowering induction in longan (*Dimocarpus longan*, Lour.) was found to be associated with elevated Z and ZR in the buds and simultaneous decrease in the concentration of IAA, thereby creating a high cytokinins/IAA ratio at floral transition (Hegele *et al.*, 2008). *In vitro* flowering of *Dendrobium* Second Love induced by thidiazuron was associated with increased iPR, ZR

and IAA in the shoots (de Melo Ferreira *et al.*, 2006), creating a cytokinins/IAA ratio close to 1. All these results indicated that it was not cytokinins, but the ratio of cytokinins to IAA in the plant that was crucial in promoting floral transition.

2.2.2 Carbohydrates

Carbohydrates are important nutrients and energy sources in living organisms. During plant growth and development, photoassimilates produced in the leaf are translocated to different sinks for utilization or accumulation (Geiger, 1987). Sugars could help to regulate the timing of developmental phase change from juvenile to reproductive phases by ensuring an adequate supply of materials and energy for the successful completion of such transition. It was suggested that increased carbohydrate levels, especially sucrose, could promote flowering (Gibson, 2005).

It was shown in *Arabidopsis* that application of sucrose to the apical part of the plant induced flowering in complete darkness (Roldán *et al.*, 1999). In addition, late-flowering ecotypes flowered with similar number of leaves as early-flowering ecotypes in dark when treated with sucrose. It was suggested that rapid dark flowering of the late-flowering ecotype was the result of sucrose availability at the aerial part of the plant (Roldán *et al.*, 1999). By comparing the flowering induction in wild-type *Arabidopsis* and its starchless (*pgm*) and starch-in-excess (*sex1*) mutants, Corbesier *et al.* (1998) indicated that an early and transient increase in carbohydrate export from leaves to phloem was critical in floral transition. In *Spathiphyllum*, sucrose concentration was significantly decreased in leaves during floral induction, which was speculated to be transported from leaves to the SAM (Dewir *et al.*, 2008). In the process of carbohydrate

export, sucrose transporter1 (SUT1) was shown to be crucial for efficient phloem loading of sucrose in maize leaves (Slewinski *et al.*, 2009). In *sut1* mutants, phloem loading was impaired and carbohydrates were accumulated in mature leaves, which subsequently led to delayed flowering and stunted tassel development. The results therefore indicated that phloem loading and sucrose transport were important in regulating floral transition and reproductive development.

Photosynthetic activity increased in *Zantedeschia* leaves in response to GA-stimulated flowering (Kozłowska *et al.*, 2007), indicating a higher demand of carbohydrate at floral transition. The study of carbohydrate mobilization in the pseudobulb of *Oncidium* orchid has shown that mannan and pectin accumulated in the pseudobulb were converted to starch during the emergence of the inflorescence, which was subsequently degraded at floral development stage (Wang *et al.*, 2008a). The study also suggested that ascorbic acid, which was produced indirectly in the carbohydrate metabolic pathway, could solubilize pectin into oligogalacturonides, which could in turn function as signaling molecule in flowering induction (Wang *et al.*, 2008a).

The importance of carbohydrate in promoting flowering was further implicated by the involvement of carbohydrate metabolism enzymes during floral transition. Activity of glyceraldehyde 3-phosphate dehydrogenase, a key enzyme in glycolysis, was shown to fluctuate in shoot apical meristem of *Brassica campestris* during transition to flowering (Orr, 1987). Such phenomenon probably indicated that carbohydrate oxidation was involved during the transitional phase (Orr, 1987). In *Arabidopsis thaliana*, trehalose-6-phosphate synthase, the enzyme that catalyzes the first step in trehalose synthesis, was also found to be essential in floral transition (Van Dijken *et al.*, 2004). Cell wall

invertases are hydrolytic enzymes that cleave sucrose into the monosaccharide glucose and fructose. Their role in re-directing photoassimilates to storage organs of plants has been demonstrated in various species (Weschke *et al.*, 2003). Expression of cell wall invertase in the apical meristem of *Arabidopsis* has been shown to promote early flowering (Heyer *et al.*, 2004). The results therefore indicated the role of carbohydrate metabolism enzymes in regulating developmental process.

Although sugar has been suggested to promote floral transition in many plant species, high concentration of sucrose (5 %, w/v) was shown to significantly delay flowering time in *Arabidopsis* and increased the number of leaves at time of flowering (Ohto *et al.*, 2001). The effect of high concentrations of sucrose on flowering inhibition seemed to be metabolic than osmotic and it was suggested that sugar affected floral transition by activating or inhibiting genes controlling floral transition. Besides, although sucrose and cytokinins were shown to promote flowering in *S. alba*, they appeared to control different events of the floral transition in the SAM because changes caused by cytokinin application were different from those produced by extra-sucrose (Bernier *et al.*, 2002).

The ratios of carbohydrate to nitrogen (C:N) supplied to the apical meristem could be important at floral transition. It was shown in both *S. alba* and *Arabidopsis* that the C:N ratio of the phloem sap increased markedly after a single LD induction of flowering (Corbesier *et al.*, 2002). The importance of appropriate C:N ratio for flowering has also been demonstrated in *Torenia fournier*i (Tanimoto and Harada, 1981) and *Pharbitis nil* (Ishioka *et al.*, 1991).

2.2.3 Genetics

Several genes have been identified to regulate the transition from juvenile to reproductive phase in plants. In *Arabidopsis*, *HASTY* was found to lengthen the juvenile phase by reducing the competency of the shoot to respond to *LEAFY* and *APETALA1*, which regulated flowering time (Telfer and Poethig, 1998). In *Oryza sativa*, *plastochron1* regulated the duration of the vegetative phase by controlling the rate of leaf production in the meristem (Itoh *et al.*, 1998). On the other hand, *mori1* mutation lengthened the juvenile phase by suppressing the induction of the adult phase (Asai *et al.*, 2002). In *Lycopersicon esculentum* Mill., the *UNIFLORA* gene was found to play a role in the regulation of floral transition and maintenance of inflorescence meristem identity (Dielen *et al.*, 2001). In *Zea mays*, the *early phase change* (*epc*) gene has been shown to regulate shoot development in the juvenile phase, in which *epc* mutation shortened the duration of juvenile vegetative phase and caused early flowering (Vega *et al.*, 2002). Microarray analysis of vegetative phase change in maize also showed that genes involved in photosynthesis were largely up-regulated during the juvenile phase, suggesting that maize plants were primed for energy production in early vegetative growth (Strable *et al.*, 2008). In *Arabidopsis*, a Myb-like transcription factor, *REGULATOR OF AXILLARY MERISTEMS1* (*RAX1*), has been shown to play a role in the developmental transition from vegetative to reproductive phase (Keller *et al.*, 2006). The *rax1-2* mutant flowered earlier and contained more GA than the wild-type. RAX1 was therefore suggested to negatively regulate GA accumulation and inhibit differentiation of SAM. In *S. alba*, the activation of the MADS box gene, *SaMADS A*, was suggested as an intermediate event in

the cytokinin-triggered signal transduction pathway, which was involved in the regulation of floral transition (Bonhomme *et al.*, 2000).

Chromatin conformation controls gene expression both in undifferentiated and differentiated cells. It was reported that chromatin remodeling processes were involved in the negative control of flowering time genes including *FT* (*Flowering Locus T*), *SOC1* (*Suppressor of Overexpression of Constant 1*) or *AGL19* (*Agamous-Like 19*) during vegetative development and their expression upon flowering induction (Jarillo *et al*., 2009).

The knowledge of floral transition in orchids at the genetic level is limited. Yu and Goh (2000) showed that genes involved in transcriptional regulation, cell division and several other metabolic events were closely associated with the process of floral transition in *Dendrobium* grex Madame Thong-In. In addition, the *DOH1* gene, a class 1 *KNOX* gene, could interact with MADS box genes and the down-regulation of *DOH1* caused early flowering in the orchid (Yu *et al.*, 2000).

2.2.4 Florigen

Florigen refers to the flowering signal that can be transmitted from a flowering partner (donor) via a graft union to a non-flowering partner (receptor) (Zeevaart, 2008). Physiological approaches using photoperiodic species that can be induced to flowering by exposure to a single inductive photoperiod have led to the identification of several putative florigens such as sucrose, cytokinins, GAs and reduced N-compounds (Corbesier and Coupland, 2006). These compounds were found to be translocated from the leaves to the SAM in response to exposure to appropriate day lengths.

The roles of plant growth regulators and carbohydrates as florigens, or long-distance signaling molecules, in promoting floral transition have been reviewed (Bernier *et al.*, 2002; Suárez-López, 2005; Wilkie *et al.*, 2008; Mutasa-Göttgens and Hedden, 2009). GA was shown to promote flowering in *Arabidopsis* through the activation of genes encoding the floral integrators SOC1, LFY (LEAFY) and FT (Mutasa-Göttgens and Hedden, 2009). The roles of GAs and cytokinins in long-distance signaling are still questionable because different plant species respond in different ways to external application of GAs. Also, exogenous cytokinins could induce floral transition only when the treatment is combined with other factors slightly inductive for flowering (Suárez-López, 2005).

Recent progress towards the understanding of regulatory network of flowering in *Arabidopsis* has shown that FT protein is the main, if not the only, component of the universal florigen (Zeevaart, 2008). It was reported that CO (CONSTANS) protein accumulated in the leaves of *Arabidopsis* under LD and induced the expression of *FT* in the phloem companion cells. The FT protein was then transported in the sieve tubes to the shoot apex, in which it formed a heterodimer with FD (Flowering Locus D) protein. The FD/FT complex then activated expression of *SOC1* and *AP1* (*APETALA1*) leading to floral initiation (Turck *et al.*, 2008). A considerable increase in the number of plasmodesmata in the central zone of SAM was observed during floral transition, presumably to enhance intercellular exchange of these long-distance and short-distance signals (Milyaeva, 2007).

2.3 Shoot apical meristem (SAM) at floral transition

The SAM is a non-differentiated portion of the shoot apex located above the youngest leaf primordium. The SAM generates stems, leaves and lateral shoot meristems during the entire shoot ontogeny. Plant developmental stages determine morphogenesis of the SAM, which affects the identity of primordial produced at its periphery. SAM produces vegetative leaves in the vegetative phase. During the reproductive phase, SAM produces either bracts subtending lateral flower primordia, or perianth and reproductive organs (Kwiatkowska, 2008). SAM is organized into a central zone, a peripheral zone and a rib meristem based on cytological characteristics of the cells. The cells of SAM are heterotrophic as they do not contain chlorophylls (Fleming, 2006).

Temporal and spatial changes of growth and geometry take place at the SAM during the transition from vegetative to reproductive phase. The meristem growth switches from indeterminate to determinate at floral transition and the degree of determinacy depends on the floral architecture (Sablowski, 2007). In the vegetative phase, the central zone is the slowest growing region. Early during the floral transition, the cell division rate increases in this zone (Kwiatkowska, 2008). Simultaneously, the number of cells below the central zone increases, suggesting that portions other than the central zone contribute to reproductive organ formation. Besides, the sizes of cells in different zones change during floral transition. Cells of the central zone, which are larger during vegetative phase than cells of peripheral zone, become smaller at floral transition while the cells of the rib meristem increase in size (Kwiatkowska, 2008). In addition to changes in the growth and cell division rates of the SAM, floral transition is also characterized by the re-organization of symplasmic communication between meristem

cells in which the number of plasmodesmata dramatically increases (Ormenese *et al.*, 2000).

2.3.1 Hormonal and genetic regulation of shoot apical meristem (SAM)

The SAM is made up of undifferentiated cells that undergo cell division and differentiation during the course of plant development, undergo cell division and differentiation to produce various organs at different development stages. Therefore, cell division and differentiation are tightly controlled processes in plant development. Plant growth regulators could regulate growth and patterning of SAM. They have been found to be distributed heterogeneously across the SAM and this could be linked to the basic aspect of meristem behavior (Veit, 2009). It was suggested that high levels of auxin and GA were associated with the initiation of outgrowth of lateral organs. In contrast, high levels of cytokinin in the central zone could be linked to the maintenance of undifferentiated cells for indeterminate growth (Veit, 2009). Cytokinins have been shown to play a significant role in SAM function because reducing endogenous cytokinin content resulted in reduced meristem size and occasionally, meristem abortion in *Arabidopsis* (Werner *et al.*, 2003). Auxin, on the other hand, might play a key role in determining the site of leaf initiation in SAM. Formation of leaf primordia was blocked by mutations or chemical treatments that reduced polar auxin transport to the shoot apex, which could be overcome by exogenous auxin that induced leaf formation at site of application (Reinhardt *et al.*, 2000). Besides, auxin was able to activate ethylene dependent responses that limited growth of SAM (Woeste *et al.*, 1999). Brassinosteroids were reported to have additive or synergistic effects on auxin responses and could

therefore be potentially related to the dynamic behavior of SAM (Belkhadir and Chory, 2006). Spatial regulation of brassinosteroid activity had been shown to limit plant growth and differentiation (Savaldi-Goldstein *et al.*, 2007).

The interplay between transcription factors has been suggested to determine whether the cells within the SAM remain undifferentiated, differentiated into leaves or formed secondary meristem, which would subsequently develop into shoots and flowers (Long and Benfey, 2006). Among the transcription factors that have been shown to take part in the maintenance of SAM, class I *KNOTTED1*-like homeobox (*KNOX*) genes were proposed as central players in the control of SAM. They ensure the maintenance of SAM by repressing the differentiation of cells in the SAM (Hake *et al.*, 2004). *KNOX* genes, such as *KNOTTED1* (*KN1*) in maize and *SHOOTMERISTEMLESS* (*STM*) in *Arabidopsis*, were expressed throughout the SAM and down-regulated in the developing leaves (Jackson *et al.*, 1994), indicating the importance of these genes in maintaining determinacy in SAM. In addition, over-expression of *KNAT1*, a class 1 *KNOX* gene, in *Arabidopsis* led to the production of lobe leaves with ectopic meristem (Chuck *et al.*, 1996). Ectopic expression of *KNOX* genes in maize also resulted in abnormal cell divisions in leaf (Schneeberger *et al.*, 1995). These results indicated that mis-expression of *KNOX* genes was sufficient to induce abnormal cell division and meristem formation.

The control of cell division and differentiation by *KNOX* genes probably occur through modulation of the hormonal pathway. Over-expression of *KNOX* in tobacco resulted in delayed senescence, a phenomenon similar to plants with increased cytokinin levels (Kusaba *et al.*, 1998). Similarly, leaf senescence was delayed and cytokinin levels

were elevated in tobacco plants expressing the maize *KN1* gene (Ori *et al.*, 1999), probably indicating that *KNOX* genes acted through increasing cytokinin levels.

Transcription factors have been suggested to co-operate with plant growth regulators to balance meristem maintenance and organ production (Shani *et al.*, 2006; Long and Benfey, 2006). In *Arabidopsis*, two types of homeobox genes, *KNOX* and *WUSCHEL* (*WUS*), were reported to function in independent and complimentary pathways to establish and maintain shoot meristem (Long *et al.*, 1996; Mayer *et al.*, 1998). More importantly, the two pathways were found to have direct links with cytokinins. *WUS* expressed in SAM was found to repress the type-A *RESPONSE REGULATOR* (*ARR*) genes (Leibfried *et al.*, 2005), which were primary targets of cytokinin signal transduction (To *et al.*, 2004). On the other hand, KNOX proteins controlled the balance of cytokinins and GA to establish high cytokinins to GA ratio in the SAM, which was essential in maintaining the indeterminacy of SAM (Shani *et al.*, 2006). To achieve this, KNOX suppressed the GA biosynthetic gene (*GA20-ox*) and activated the cytokinin biosynthetic gene (*IPT*) (Jasinski *et al.*, 2005; Yanai *et al.*, 2005; Sakamoto *et al.*, 2006). Because *KNOX* expression was restricted in shoot meristem cells, this regulation effectively ensured a high cytokinins/GA condition in the SAM.

Another mechanism that regulated meristem activity, which involved fine-tuning of concentrations and spatial distribution of bioactive cytokinins by a cytokinin-activating enzyme, was proposed with the isolation of the cytokinin-deficient mutant, *lonely guy* (*log*), from rice (Kurakawa *et al.*, 2007). The LOG protein was shown to convert inactive cytokinin nucleotides directly to bioactive free base with the release of a ribose 5'-monophosphate. The *LOG* gene of rice was required to maintain meristem activity and its

loss of function caused premature termination of the shoot meristem, reduced panicle size, abnormal branching patterns and decreased floral organs (Kurakawa *et al.*, 2007). The results thus demonstrated that cytokinins were indeed required in the proliferation of undifferentiated meristematic cells in the SAM. It was suggested that the control of cytokinin levels by a single and final activation step could provide a powerful system in generating a cytokinin gradient which could work as local paracrine signal for the shoot meristem function (Kyozuka, 2007).

It was suggested that the undifferentiated cells in the SAM could autonomously produce cytokinins because both *KNOX* transcription factors and *LOG* could activate cytokinin biosynthesis and were found to be expressed in these meristematic cells (Jasinski *et al.*, 2005; Kurakawa *et al.*, 2007). This would be advantageous because it could provide a positive reinforcement of the functional identity of the SAM cells by generating a high cytokinin environment (Doerner, 2007). In addition, the meristem activity could be coupled directly to environmental cues that promoted growth.

Some novel molecules have also been identified to participate in SAM functioning. D class cyclins were shown to play important roles in maintaining cell proliferation and coordinating growth in SAM (Dewitte *et al.*, 2007), the activity of which could be promoted by cytokinins or sugars (Riou-Khamlichi *et al.*, 2000). *MAX* (*more axillary meristems*) was shown to suppress the outgrowth of axillary SAMs by modifying patterns of auxin transport (Bennett *et al.*, 2006). The expression patterns of CYP78A5 class cytochrome p450s in SAM and the abnormal growth phenotypes induced by their over-expression in *Arabidopsis* suggested the role of these molecules in SAM regulation (Zondlo and Irish, 1999).

2.3.2 *KNOX* homeobox gene

Plant homeodomain proteins participate as transcription factors in the regulation of a number of developmental processes by activating and/or repressing sets of target genes (Chan *et al.*, 1998). *KN1* was first identified from a maize mutant that produced outgrowth of indeterminate tissue, or "knots" on the leaf (Vollbrecht *et al.*, 1991). It also defined the first homeobox gene isolated in plants. *KNOX* genes can be divided into two classes (Kerstetter *et al.*, 1994): Class I genes share sequence similarity with *KN1* and are expressed in overlapping domains within the SAMs of both monocot and dicot plants. Class II genes share lower sequence similarity with *KN1* and are expressed in all tissues. In *Arabidopsis*, the *KNOX* gene family consists of eight *KN1* homologues, of which *STM*, *BREVIPEDICELLUS* (*BP*), *KN1*-like in *Arabidopsis Thaliana2* (*KNAT2*) and *KNAT6* are class I *KNOX* (*KNOXI*) genes, while *KN3*, *KN4*, *KN5* and *KN7* are class II *KNOX* (*KNOXII*) genes (Lincoln *et al.*, 1994; Long *et al.*, 1996). KNOX proteins were proposed to belong to the TALE superclass of homeodomain proteins (Burglin, 1997), which were capable to interact with a second group of TALE proteins, the BEL1 homeodomain (BLH) family (Bellaoui *et al.*, 2001). It was also suggested that different combinations of KNOX/BLH transcription factors might regulate different downstream genes.

KNOXI genes were mainly expressed in the SAM and loss of *STM* in *Arabidopsis* resulted in defects in SAM development or maintenance (Lincoln *et al.*, 1994; Long *et al.*, 1996). They were therefore required for SAM maintenance and establishment of shoot architecture. Conversely, transgenic plants over-producing KNOX proteins resulted in the formation of ectopic meristems on leaves (Matsuoka *et al.*, 1993). *KNOXI* genes could also be involved in leaf development because failure of down-regulation of its

expression in leaf primordia and mature leaves resulted in abnormal leaves (Ori *et al.*, 2000). It was also shown in *Arabidopsis* that leaf development required exclusion of *KNOX* expression from leaves because ectopic expression of *KNOX* caused dramatic change in leaf shape (Chuck *et al.*, 1996). There were also evidences indicating the involvement of *KNOXI* genes in defining inflorescence architecture (Douglas *et al.*, 2002; Venglat *et al.*, 2002) and lateral root initiation (Dean *et al.*, 2004).

Various studies have demonstrated the interactions between *KNOXI* genes and plant growth regulators on their coordinated involvement in SAM maintenance and organ production. KNOX proteins were suggested to inhibit auxin transport (Tsiantis *et al.*, 1999), probably indicating a feedback relationship between KNOX protein and auxin. *KNAT2* was also shown to interact antagonistically with ethylene in the regulation of leaf structure and SAM architecture (Hamant *et al.*, 2002). On the other hand, ectopic expression of *KNOXI* genes from rice could increase cytokinin levels in tobacco plants (Kusaba *et al.*, 1998). It was also found that *KNOXI* expression repressed GA activity and such interaction was a key component in maintaining SAM (Hay *et al.*, 2002).

In addition to maintaining the undifferentiated identity of meristem, *Helianthus tuberosus HtKNOT1* was suggested to play a role in initiating differentiation and/or conferring new cell identity because its expression was detected in differentiated floral organs such as floral bracts, petals, stamens and carpels (Michelotti *et al.*, 2007). Furthermore, its expression was detected in more differentiated flowers in the developing ovules and pollen mother cells. It was speculated that *HtKNOT1* cooperated with additional factors that specifically controlled floral organs and pollen development in *H. tuberosus* (Michelotti *et al.*, 2007). In orchid, *DOH1*, a class I *KNOX* gene, was required

in floral transition in addition to its role in maintaining plant architecture (Yu *et al.*, 2000). Transgenic orchid plants expressing antisense mRNA for *DOH1* was found to produce multiple SAM and caused early flowering (Yu *et al.*, 2000). In contrast to the role of *KNOX* in SAM maintenance, it was found in the moss *Physcomitrella patens* that class I *KNOX* genes were not involved in SAM maintenance but functioned in sporophyte development (Sakakibara *et al.*, 2008). Therefore, it was suggested that the genetic networks governing the indeterminate meristem in land plants could be variable.

2.4 Cytokinins and their functions

Cytokinins are adenine derivatives and can be classified by the configuration of their N^6-side chain as either isoprenoid or aromatic. They are a group of mobile plant growth regulators that play crucial roles in plant growth and development. Both isoprenoid and aromatic cytokinins are naturally occurring, with the former more frequently found and in greater abundance than the latter. Common natural isoprenoid cytokinins are trans-zeatin (tZ), isopentenyladenine (iP), dihydrozeatin (DHZ) and cis-zeatin. Among the four species, tZ and iP are most common in plants (Mok and Mok, 2001). As for the aromatic cytokinins, ortho-topolin, meta-topolin, their methoxy-derivatives, and BA are only found in some plant species such as poplar and *Arabidopsis* (Tarkowska *et al.*, 2003). Usually, all natural cytokinin nucleobases have the corresponding nucleosides, nucleotides and glycosides.

Cytokinins are involved in the regulation of apical dominance (Tanaka *et al.*, 2006), root proliferation (Werner *et al.*, 2001), leaf senescence (Kim *et al.*, 2006), phyllotaxis (Giulini *et al.*, 2004), reproductive competence (Ashikari *et al.*, 2005) and

nutritional signaling (Takei *et al.*, 2002). More importantly, cytokinins have been shown to participate in the maintenance of meristem function (Werner *et al.*, 2003; Kurakawa *et al.*, 2007). Tobacco mutants with elevated cytokinin oxidase/dehydrogenase (CKX) activity, in which cytokinin degradation was enhanced, showed retarded growth at the aerial parts of plants (Werner *et al.*, 2001). The internode length, leaf size and size of SAM were also decreased. The observed phenotypes were suggested as the result of the reduced rate of cell division, in which cell number decreased while cell size increased. By contrast, cytokinins were proposed as negative regulators of cell division in the root apical meristem because reducing cytokinins increased the total root mass, which resulted from the increased size of the cell division zone in root apical meristem (Werner *et al.*, 2001).

Apart from SAM maintenance, cytokinins could regulate carbon fixation, assimilation, partitioning of primary metabolites and cell cycle activity, which could all determine source or sink strength of the tissues. They were shown to stimulate chloroplast biogenesis, chlorophyll synthesis, photosynthetic rate and chloroplast development (Kusnetsov *et al.*, 1994; Reski, 1994; Polanská *et al.*, 2007). Various transcripts and proteins involved in photosynthetic reactions were shown to be affected by cytokinins (Lerbs *et al.*, 1984; Sugiharto *et al.*, 1992). Cytokinins were known to have regulatory roles on different cell cycle phases (Dewitte and Murray, 2003), which were important in determining sink strength. Werner *et al.* (2008) demonstrated that the capacity of the shoot sink to import and/or utilize carbohydrates was drastically reduced in cytokinin-deficient tobacco, which could in turn alter the shoot phenotype. The impaired carbohydrate metabolism was associated with reduced activities of invertase

enzymes. Cytokinins have been shown to up-regulate the activity of invertase which was involved in nutrient mobilization (Ehneβ and Roitsch, 1997).

The roles of cytokinins in floral transition and reproductive development have been investigated through the generation of cytokinin-overproducing (Catterou *et al.*, 2002) and cytokinin-deficient (Werner *et al.*, 2003) *Arabidopsis* mutant. The first cytokinin-overproducing *Arabidopsis* mutant, *hoc,* was capable of auto-regenerating shoots without exogenous growth regulators (Catterou *et al.*, 2002). Floral transition was delayed in the mutant with increased level of endogenous cytokinins, but the fertility and morphology of flowers were not affected. On the other hand, reduction in endogenous cytokinins in cytokinin-deficient mutant was associated with delayed flowering and reduced number of flowers (Werner *et al.*, 2003). Morphology and size of flowers of cytokinin-deficient mutant were similar to wild-type but the fertility was affected and very few seeds were produced.

2.4.1 Biosynthesis, translocation and perception of cytokinins

The initial step of cytokinin biosynthesis is catalyzed by adenosine phosphate-isopentenyltransferase (IPT) to produce iP nucleotides such as iP riboside 5'-triphosphate and iP riboside 5'-diphosphate because IPT predominantly uses dimethylallyl diphosphate and ATP or ADP as substrates (Kakimoto, 2001). In *Arabidopsis*, iP nucleotides are converted into tZ nucleotides by cytochrome P450 mono-oxygenases, encoded by *CYP735A1* and *CYP735A2* (Takei *et al.*, 2004). To become biologically active, iP- and tZ-nucleotides are converted to nucleobase forms through dephosphorylation (Chen and Kristopeit, 1981a) and deribosylation (Chen and Kristopeit,

1981b). Besides, active cytokinins could be released directly from the nucleotides via the reaction catalyzed by cytokinin 5'-monophosphate phosphoribohydrolase (Kurakawa *et al.*, 2007). Cytokinins can be inactivated by *O*-glycosylation at the terminal hydroxyl group of the Z-type cytokinins or by *N*-glycosylation at the N^3 or N^7 positions of the adenine ring (Sakakibara, 2006). *O*-glycosylation is reversible and therefore *O*-glycosylated cytokinins are regarded as a storage form. The cytokinin ribosides, which are also found in abundance in plants, may also be important as stored or transportable form (Sakakibara, 2006). By comparing the distribution of cytokinin in *Arabidopsis* plants grown under wind-protected and wind-exposed conditions, Aloni *et al.* (2005) concluded that the bulk of cytokinins was synthesized in the root tips, and exported through the xylem to the shoot by transpiration stream.

Cytokinin biosynthesis was found to be regulated by the spatial expression of cytokinin biosynthetic genes *IPTs* (*AtIPTs*) in *Arabidopsis* (Miyawaki *et al.*, 2004). The expressions of various genes involved in the synthesis of cytokinins were also found to be regulated by plant growth regulators including cytokinins, auxin and abscisic acid (ABA). In *Arabidopsis*, expressions of *AtIPT5 and AtIPT7* were promoted by auxin in root whereas the expression of *AtIPT1*, *AtIPT3*, *AtIPT5* and *AtIPT7* were negatively regulated by cytokinins (Miyawaki *et al.*, 2004). The expressions of *CYP735A1* and *CYP735A2* in roots were up-regulated by cytokinins, but down-regulated by auxin or ABA (Takei *et al.*, 2004). On the other hand, *CKX*, which encodes protein for cytokinin degradation, was up-regulated by cytokinins and ABA in maize (Brugiere *et al.*, 2003).

Translocation of cytokinins was suggested to be mediated by purine permeases and nucleoside transporters by sharing the purine and sugar conjugate transport systems,

respectively (Bürkle *et al.*, 2003; Hirose *et al.*, 2005). Cedzich *et al.* (2008) demonstrated the presence of both low- and high-affinity transport systems for the uptake of tZ in *Arabidopsis* cell culture. Through the study of kinetic properties and substrate specificity of adenine transport, the authors also implied a plant-specific purine permease that played a role in adenine transport for scavenging extracellular adenine and possibly in cytokinin uptake.

Cytokinins were perceived and transduced by a two-component signaling system (Kakimoto, 2003a). The two-component system consists of two proteins, histidine kinase and the response regulator (RR). A model of two-component signal transduction system involving type-A *Arabidopsis* RR and type-B *Arabidopsis* RR was proposed to elucidate the mechanisms of cytokinins in regulating meristem function in *Arabidopsis* (To *et al.*, 2008). Cytokinin-mediated nitrogen signaling in maize was suggested to involve Z-type cytokinins which triggered the induction of *Zea mays RR1* (*ZmRR1*) in response to nitrogen availability (Takei *et al.*, 2001).

2.4.2 Cytokinins as long-distance signals

In a plant, the shoot and the roots could control each other's growth by exchanging long-distance signals. The most common of these signals were suggested to be nutrient or plant growth regulators, which were transported in the xylem and phloem sap (Havelange *et al.*, 2000). It has been shown that the amount of cytokinins exported from the roots in the xylem sap could determine the degree of shoot branching (Letham, 1994). In turn, the genotype of the shoot could determine the concentration of cytokinins exported from the roots (Beveridge *et al.*, 1997), possibly via a signal originated from

shoot. It was found in *Pinus* seedlings that the shoot-to-root auxin could be stimulated by the application of cytokinins to the tap root (Atzmon *et al.*, 1996). Roots experiencing stress could also produce and export a chemical signal in the form of plant growth regulators to shoot so that growth could be adjusted at adverse conditions (Jackson, 1997). For example, export of cytokinins from roots to xylem sap increased during water stress (Bano *et al.*, 1993). A shoot-to-root-to-shoot physiological loop involving sucrose and cytokinins have also been shown in *S. alba* in the regulation of floral transition (Havelange *et al.*, 2000).

Cytokinins could act as long-distance signaling molecules because they were found in the xylem sap (Yong *et al.*, 2000; Kuroha *et al.*, 2002; Kudoyarova *et al.*, 2007). The major forms of cytokinins found in the xylem sap were tZ-type cytokinins, such as tZ riboside. It therefore appeared that roots were the major site for tZ production and tZ riboside could play a role in root-to-shoot acropetal signal. These root-derived signals could control the uptake and utilization of assimilates, and biomass distribution in response to nitrogen (Sakakibara *et al.* 2006). Cytokinins were shown to be transported across the roots to shoots in maize in response to nitrogen availability (Takei *et al.*, 2001). Cytokinins were also shown to mediate a signal transduction pathway to communicate the nitrogen nutrient status from root-to-shoot via the xylem vessel (Takei *et al.*, 2002). This response system could be important for plants to cope with unpredicted nitrogen availability in the environment. In contrast to xylem sap, phloem sap was found to contain predominantly of iP-type cytokinins (Lejeune *et al.* 1994; Corbesier *et al.*, 2003) which might function as basipetal signal. By using *S. alba* as a model, Kinet *et al.* (1993) suggested that there were shoot-to-root interactions during floral transition and

cytokinins played a critical role in this long-distance interaction in the control of flowering.

2.4.3 Cytokinins and auxin interactions

The interaction of cytokinin and auxin was first identified by their ability to stimulate cell division when applied to cultured plant cells simultaneously (Miller *et al*., 1956). The ratio of cytokinin to auxin was known to determine the type of organs regenerated from undifferentiated callus tissue *in vitro*: high cytokinin to auxin ratio in culture media stimulated shoot production, whereas the reverse produced mainly roots (Skoog and Miller, 1957). Since then, cytokinin and auxin interactions have been shown to take place in several physiological and developmental processes including apical dominance, control of cell cycle, lateral root initiation and the regulation of senescence (Coenen and Lomax, 1997; Swarup *et al.*, 2002). The interaction could be synergistic, as in the case of the regulation of cell cycle, or antagonistic, as in the case of the regulation of axillary bud meristems and the formation of the lateral roots.

The effects of cytokinins on auxin levels appeared to be variable. Exogenous application of cytokinins to maize (Bourquin and Pilet, 1990) and pea (Bertell and Eliasson, 1992) roots was found to increase the IAA content. Similarly, auxin level decreased with increased degradation of cytokinins in *Arabidopsis* (Werner *et al.*, 2001). In contrast, cytokinin was found to have a negative effect on auxin in tobacco, in which elevated cytokinin by over-expressing cytokinin biosynthetic gene *IPT* resulted in lower level of IAA (Eklöf *et al.*, 1997).

The effect of auxin on cytokinins could be species specific. In tobacco, cytokinin levels were reduced in transgenic tobacco that over-produced auxin (Eklöf *et al.*, 1997). Auxin increased the breakdown of cytokinins by stimulating CKX, the cytokinin degradation enzyme (Zhang *et al.*, 1995). In *Arabidopsis*, auxin treatment, however, increased the expression of cytokinin biosynthesis genes (Miyawaki *et al.*, 2004).

The interaction between cytokinins and auxin has been shown in some mutant studies in which mutation in the metabolism of either plant growth regulator would affect the sensitivity of both plant growth regulators. A mutation in the auxin influx carrier, *aux1*, was shown to alter the sensitivity of roots to both cytokinin and auxin (Coenen and Lomax, 1997). Similarly, sensitivity to cytokinins and auxin were both affected in *polaris* (*pls)* mutant that showed defects in root growth (Casson *et al.*, 2002).

The interactions of cytokinins and auxin have been further studied by investigating the overlapping gene expression profiles induced by these two plant growth regulators. It was shown that certain genes that were up-regulated upon cytokinin treatment were also up-regulated by auxin (Rashotte *et al.*, 2003). However, in a separate but similar study using Affimetrix GeneChip, it was found that the majority of the genes that were specially induced by cytokinin or auxin were unaffected by the simultaneous addition of the second plant growth regulator (Rashotte *et al.*, 2005). The study of cytokinin- and auxin-induced gene expression profiles also revealed that the majority of the genes under study were regulated independently or in an additive manner.

2.4.4 BA and its metabolism

BA is generally viewed as a synthetic compound. However, BA and its ribosides and corresponding nucleotide have been identified as naturally occurring plant products, and they formed the major endogenous cytokinin compounds in primary crown gall tumor of tomato (Nandi *et al.*, 1989a, b). The detection of BA and its riboside in *Pisum sativum* (Gaudinová *et al.*, 2005) and *P. patens* (Von Schwartzenberg *et al.*, 2007) further indicated that BA was indeed naturally occurring. BA was the most frequently and most successfully used cytokinin in micropropagation. Its application has been shown to promote chlorophyll retention and formation, increase shoot-to-root ratio, increase production of ethylene, lower stomatal resistance, increase leaf expansion and stimulate protein synthesis (Van Staden and Couch, 1996). In addition, BA was considered as the most active cytokinin in the class of ring-substituted aminopurine (Matsubara, 1990). Van Staden (1973) compared the activities of BA, BA riboside and BA nucleotide and concluded that BA appeared to be the most active, followed by its ribosides and nucleotide.

It was suggested that plant tissues converted exogenous BA into a great diversity of metabolites which included products of ring substitution (ribosides, nucleotides and *N*-glucosides), and products of side-chain cleavage (adenine, adenosine and adenosine 5'-monophosphate) (Letham and Palni, 1983). In *Spathiphyllum floribundum*, BA uptake could be metabolized into BA riboside and BA glucoside (Werbrouck *et al.*, 1995). BA and its glucoside were found to be exclusively located in the basal part of the plant while BA riboside was located in the basal part, petioles and leaf blade.

The uptake and metabolism of BA were studied during shoot organogenesis in *Petunia* leaf explants (Auer *et al.*, 1992). BA was only detected early in the treatment before shoot induction period. Instead, BA ribotide formed the major pool of cytokinins throughout the shoot induction and organogenesis periods. It was therefore suggested that BA ribotide, and not BA itself, was active or acted as a short-term storage form for the active cytokinin in petunia shoot organogenesis (Auer *et al.*, 1992). In another study on shoot organogenesis of *Petunia*, exogenously applied BA caused an increase in the endogenous cytokinin content, especially the concentrations of iP and iPR (Auer *et al.*, 1999). Other cytokinins such as Z, ZR and DHZ remained at consistently low levels. The activity of CKX also continuously increased upon BA application. These results suggested that BA regulated shoot organogenesis in *Petunia* indirectly by stimulating the production of other cytokinins. However, BA was suggested to directly regulate morphogenesis in *C. rubrum in vitro* because no significant changes in endogenous levels of isoprenoid cytokinins were observed upon BA application (Balžková *et al.*, 2001).

2.4.5 Cytokinin oxidase/dehydrogenase, CKX (EC 1.5.99.12)

The biological activity of cytokinins in plants was proposed to be controlled by a balance between biosynthesis, interconversion among distinct forms, and transient activation by conjugation and catabolic reactions (Sakakibara and Takei, 2002). CKX catalyzes the irreversible degradation of cytokinins by N^6-side-chain cleavage and in many plant species is responsible for the majority of metabolic cytokinin inactivation (Mok and Mok, 2001). The enzyme was first discovered in the crude extract of cultured tobacco tissue (Pačes *et al.*, 1971) and has since been identified in a number of higher

plants. The enzyme is a flavin adenine dinucleotide-containing oxidoreductase and has a preferred specificity for the substrates iP and its ribosylated form (iPR) yielding adenine or adenosine, respectively, and 3-methyl-2-butenal as reaction products (McGaw and Horgan, 1983). By contrast, cytokinin nucleotides, *O*-glucosides and cytokinins with saturated side chains are not CKX substrates (Armstrong, 1984). Wheat CKX was found to degrade BA at 40-fold less efficiency compared to iPR (Laloue and Fox. 1989). The recombinant enzyme AtCKX2 produced in *Saccharomyces cerevisiae* was also shown to react with aromatic cytokinins at a rate of two to three orders lower than using isoprenoid cytokinins as substrate (Frébortová *et al.*, 2007). Although CKX displayed low degradation activity towards aromatic cytokinins, these cytokinins could induce the accumulation of endogenous isoprenoid cytokinins which subsequently became substrates of CKX (Kamínek *et al.*, 1997). Exogenous application of cytokinins was found to stimulate the level of CKX in tobacco cells (Terrine and Laloue, 1980).

CKX was suggested to display a dual catalytic mode for cytokinin degradation: a low-rate and low-substrate specificity reaction with oxygen as electron acceptor, and a high activity and strict specificity for iP and analogous cytokinins with some specific electron acceptors (Frébortová *et al.*, 2004). The natural electron acceptor of CKX was suggested to be a *p*-quinone or similar compound. It was found that the enzyme acted as a dehydrogenase rather than an oxidase because re-oxidation of the reduced enzyme by molecular oxygen was too slow to be of physiological relevance (Frébortová *et al.*, 2004). Several potent inhibitors of CKX have been identified, they include N^6-but-2,3-dienyl-aminopurine (Suttle and Mornet, 2005), 2-chloro-6-(3-methoxyphenyl)aminopurine and 2-fluoro-6-(3-methoxyphenyl)aminopurine (Zatloukal *et al.*, 2008).

CKX genes have been isolated from maize (Bilyeu *et al.*, 2001), orchids (Yang *et al.*, 2003; Wang *et al.*, 2008b), barley and wheat (Galuszka *et al.*, 2004). In *Arabidopsis*, seven distinct CKX-encoding genes have been identified (*AtCKX1-AtCKX7*) (Werner *et al.*, 2001). The predicted proteins are similar in size (~ 60 kD) and have a conserved binding site for the cofactor flavin adenine dinucleotide and small highly conserved domains possibly involved in substrate recognition and electron transport. In addition, individual N-terminal signal peptides indicated different subcellular localizations of the AtCKX proteins (Schmülling *et al.*, 2003). The biochemical properties of *Arabidopsis CKX* genes were characterized using transgenic tobacco that over-expressed these genes individually (Galuszka *et al.*, 2007). The results showed that *Arabidopsis* CKXs exhibited different activity, pH optima and substrate preferences. It was also suggested that the differences between these CKX isoforms could possibly be related to the tertiary structures of the enzymes (Galuszka *et al.*, 2007). An overview of *Arabidopsis CKX* gene-expression patterns suggested that the functional specification of the individual *CKX* genes was significantly attributed to their differential regulation by biotic and abiotic factors (Werner *et al.*, 2006). Besides, their expression in vascular tissue also suggested a function in regulating cytokinin transport.

CKXs were recently shown to be tightly related to reproduction. Quantitative trait loci that increased grain productivity in rice have been identified as *OsCKX*s (Ashikari *et al.*, 2005). Reduced expression of *OsCKX2* caused cytokinin accumulation in inflorescence meristems and increased the number of reproductive organs, resulting in enhanced grain yield.

2.5 Flower development in plant

The process of flower development is controlled by genes that affect development of floral primordial, genes that alter floral symmetry and genes that specify organ identity (Schwarz-Sommer *et al.*, 1990). Genetic studies on *Arabidopsis* indicated that genes which regulated cell number in the meristem included *CLAVATA* (*CLV*) and *WUS* while genes that affected the number of floral organs included *PERIANTH* (*PAN*), *ETTIN* (*ETT*), *WIGGUM* (*WIG*) and *SUPERMAN* (Weiss *et al.*, 2005). In addition, most of the floral homeotic genes that were involved in the establishment of floral organ identity were suggested to exert some control over the growth of flowers. Mutation in *FLOOZY* (*FZY*) gene, which played a major role in floral architecture in petunia produced flowers lacking three outer whorls (Tobeňa-Santamaria *et al.*, 2002) which could be due to the failure of different primordial to grow.

Apart from genetic control, plant growth regulator like auxin was suggested to play an important role in early flower development (Bennett *et al.*, 1995). Disruption of auxin transport by mutation or inhibitors resulted in the production of flowers with decreased number of sepals and stamens (Bennett *et al.*, 1995). Although cytokinin metabolism was reported to cause the development of abnormal inflorescence in oil palms regenerated from tissue culture (Jones *et al.*, 1995), BA was shown to prevent the production of deformed flowers in *Phalaenopsis* orchid when applied on the flowering shoots (Chen *et al.*, 1997). Therefore, the effect of cytokinins on floral development could be dependent on plant species and the developmental stages of the plant.

2.5.1 Control of flower size and color

The final size of each plant is determined by intrinsic growth rate modulated by nutrient availability. It is a modular process that happens throughout the entire lifespan of the plant in response to internal developmental patterns and external conditions (Doonan, 2000). Floral size is one of the traits that play a role in pollination in a number of plant species especially when the reproduction of the plant relies on outcrossing with other members of the species (Clegg and Durbin, 2003). The control of floral size could be separated into two different aspects; one was the control of the number of organs in a whorl and the other one in the control of the size of each organ formed within a flower (Weiss *et al.*, 2005). Like the rest of the organs in higher organisms, the flower has a certain normal size in a species. During floral transition, the SAM produced flowers instead of leaves and there were different sets of genes that regulated the cellular mechanisms for each developmental step (Weiss *et al.*, 2005).

The anthocyanin biosynthetic pathway is responsible for the production of pigments in plant tissues that give rise to flowers of various colors. The pigments form the basis for nearly all pink, red, orange, scarlet, purple, blue and blue-black flower colors (Strack and Wray, 1994). The presence of these color pigments makes flower color one of the most important characteristics in ornamental plants in view of the fact that plant breeding has been directed towards creating new flower colors. The six core enzymes involved in this pathway are chalcone synthase (CSH), chalcone-flavanone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS) and UDP glucose flavonoid 3-oxy-glucosyltransferase (UF3GT) (Rausher *et al.*, 1999). Chalcone synthase is a key enzyme in the biosynthesis

of anthocyanins because it catalyzes the first committed step of the pathway, which is the condensation of three acetate residues from malonyl-CoA with p-coumaroyl-CoA to form naringenin chalcone (Springob *et al.*, 2003).

2.5.2 Chalcone synthase, CHS (EC 2.3.1.74)

Like most of the enzymes in plant secondary metabolism, CHS are encoded by small family of genes that originated through duplication over evolutionary time (Durbin *et al.*, 2000). This recurrent gene duplication and subsequent differentiation of *CHS* genes were suggested as major adaptive strategies in plant genome evolution. *CHS* have been cloned and characterized from a number of non-orchidaceous plants such as *Oryza sativa* (Shih *et al.*, 2008), *Physcomitrella patens* (Jiang *et al.*, 2006), *Citrus sin*ensis (Lu *et al.*, 2009), *Saccharum officinarum* (Contessotto *et al.*, 2001), *Cassia alata* (Samappito *et al.*, 2002) and *Psilotum nudum* (Yamazaki *et al.*, 2001). The *CHS* genes identified showed a high degree of similarity in their sequence as well as kinetic properties and substrate preference (Jiang *et al.*, 2006; Lu *et al.*, 2009). CHS of *C. alata* was found to accumulate predominantly in roots and was therefore suggested to function specifically in the biosynthesis of root flavonoids in this Thai medicinal plant (Samappito *et al.*, 2002). *P. nudum* represented a plant with most diverse CHS-superfamily enzymes and it was suggested this ability to diversify could provide a survival edge during evolution (Yamazaki *et al.*, 2001). Structural analysis revealed that CHS functioned as a homodimer of two 42 kD polypeptides (Ferrer *et al.*, 1999). In addition, CHS in rice was reported to interact with other enzymes in anthocyanin biosynthesis to form a macromolecular complex (Shih *et al.*, 2008).

Changes in *CHS* expression would markedly affect pigment production. A white-flower mutant of Chinese cabbage-pak-choi was produced by a mutational event in the *CHS* gene (Jiang and Cao, 2008). The unpigmented flower sectors in *Petunia hybrida* 'Red Star', which exhibited a star-type red and white bicolor pattern, was a result of sequence-specific degradation of *CHS* RNA (Koseki *et al.*, 2005). In view of this, genetic transformation of *CHS* gene has been employed as a tool to modify flower colors in a variety of plants. Transformation of antisense *CHS* gene in purple *Eustoma grandiflorum* produced flowers with colors ranging from small streaks of white to completely white (Deroles *et al.*, 1995). It was shown in *Torenia fournieri* that transformation of *CHS* gene in antisense orientation produced flowers with uniformly light color corolla while transformation of the gene in sense orientation produced flowers with whiter tube (Aida *et al.*, 2000). The original blue color of *Torenia fournieri* flowers was also modulated to white and pale colors by RNA interference against *CHS* gene (Fukusaki *et al.*, 2007).

Orchid is a large plant family with tremendous variations in flower color. These plants could have specific color patterns in the sepals, petals and lips that come in blotches, streaks, spots, flushes and shade of different intensity. *CHS* have been cloned from orchids like *Bromheadia finlaysoniana* (Liew *et al.*, 1998), *Dendrobium* hybrid (Mudalige-Jayawickrama *et al.*, 2005), *Phalaenopsis hybrid* cv. Formosa rose (Han *et al.*, 2006) and *Oncidium* Gower Ramsey (Chiou and Yeh, 2008). Southern-blot analysis of orchids indicated the presence of a small multigene *CHS* family that encoded polypeptides of 390 to 394 amino acids with predicted molecular weight of 42 to 43 kD. In addition, nucleotide sequence analyses of orchid *CHS* genes showed 59-68 % homology with other plant species (Liew *et al.*, 1998; Han *et al.*, 2006). The expression

of *CHS* in orchids varied from species to species. *CHS* of *B. finlaysoniana* was expressed in all floral organs whereas *CHS* of *Phalaenopsis hybrida* was expressed in petals at early flower development and in lip tissue when the flower just opened (Liew *et al.*, 1998; Han *et al.*, 2006). *Dendrobium CHS* was shown to express in floral and vegetative tissues but not in pseudobulbs (Mudalige-Jayawickrama *et al.*, 2005).

2.6 *In vitro* flowering

The study of *in vitro* flowering could provide a model system for investigation of flower induction and development and a means for conducting microbreeding. *In vitro* flowering has been studied in various orchidaceous and non-orchidaceous plants in which cytokinins, alone or coupled with other plant growth regulators, were most prominent to induce flowering *in vitro*.

2.6.1 *In vitro* flowering in non-orchidaceous plants

Cytokinins have been shown to induce *in vitro* flowering in *Murraya paniculata* (Jumin and Ahmad, 1999), *Pharbitis nil* (Galoch *et al.*, 2002), *Bambusa edulis* (Lin *et al.*, 2003), *Kniphofia leucocephala* (Taylor *et al.*, 2005) and rose cv. "First Prize" (Nguyen *et al.*, 2006). It was shown in *M. paniculata* that 95 % of the shoots could be induced to flower *in vitro* using BA and only the flower buds produced by plantlets grown from seeds *in vitro* developed into normal flowers and produced zygotic seeds (Jumin and Ahmad, 1999). BA was also found to be more effective than Z and iP to induce *in vitro* flowering in *K. leucocephala* (Taylor *et al.*, 2005). Auxin, which acted antagonistically with cytokinins, could completely nullify the flowering inductive effect of cytokinins in

P. nil (Galoch *et al.*, 2002) and bamboo (Lin *et al.*, 2003). Cytokinin was also shown to increase the flowering percentage and help in the normal development of floral bud *in vitro* in rose cv. "First Prize" (Nguyen *et al.*, 2006). It was suggested that a high cytokinin/auxin ratio was beneficial to induce *in vitro* flowering in *Crocus sativus* apart from factors such as explants age and culture conditions (Jun *et al.*, 2007).

Besides cytokinins, carbohydrates were also shown to promote *in vitro* flowering in some plant species. Sucrose was indispensable for *in vitro* flowering of *Spathiphyllum cannifolium* in solid culture but high sucrose levels were shown to inhibit flowering (Dewir *et al.*, 2006). In addition, sucrose accumulation in the leaves could significantly affect the inflorescence size and time to flowering. Carbohydrate was essential for the *in vitro* induction of inflorescence in *K. leucocephala* in that both the type and concentration of sugar influenced the percentage of flowering (Taylor *et al.*, 2007).

Plant growth regulators other than cytokinins have also been shown to induce flowering *in vitro*. Growth retardants such as paclobutrazol or ethephon could promote *in vitro* flowering in *Saposhnikovia divaricata* but inhibited flowering and caused the death of plantlets at high concentrations (Qiao *et al.*, 2009). Paclobutrazol was shown to promote flowering of *Euphorbia millii in vitro* (Dewir *et al.*, 2007a). GA induction of *in vitro* flowering in *S. cannifolium* could be stress-related because it was associated with an increase in antioxidative enzyme (Dewir *et al.*, 2007b). Silver nitrate and cobalt chloride were shown to induce flowering of *Capsicum frutescens in vitro* (Sharma *et al.*, 2008). It was speculated that both chemicals could promote flowering through their roles as inhibitors of ethylene biosynthesis and action, respectively.

2.6.2 *In vitro* flowering in orchids

The Orchidaceae is a large plant family with representatives found in almost all parts of the world. It comprises of more than 80 genera with approximately 25,000 species. Orchid plants generally have a long juvenile phase before they can flower. The duration of the juvenile phase in orchids can vary depending on genus, species or hybrid. Method to induce *in vitro* flowering in orchids would have significant impact on the orchid industry. A system of early *in vitro* flowering may allow early assessment of certain desired characteristics of the flowers before the clone is mass propagated through tissue culture. Furthermore, a method to shorten juvenile phase in orchids could provide a model system to study flowering initiation and development. BA represented the most promising plant growth regulator to induce *in vitro* flowering in *Cymbidium* (Kostenyuk *et al.*, 1999; Chang and Chang, 2003), *Dendrobium* (Hee *et al.*, 2007; Sim *et al.*, 2007; Tee *et al.*, 2008), *Phalaenopsis* (Duan and Yazawa, 1995) and ×*Doriella* (Duan and Yazawa, 1994), regardless of the starting material of culture.

It was reported that BA induced around 40 % of the rhizome-derived shoots of *C. niveo-marginatum* Mak to flowering *in vitro* (Kostenyuk *et al.*, 1999). The percentage of flowering could be markedly increased to nearly 100 % when BA treatment was coupled with root excision and a medium of restricted nitrogen supply and phosphorus enrichment, although the latter two conditions alone did not induce flowering. In addition, GA was found to markedly delay flowering in the *Cymbidium* under flowering-promoting conditions. Cytokinins were required for *in vitro* flowering induction in *C. ensifolium* var. *misericors* in the presence of naphthalene acetic acid (Chang and Chang,

2003). Unlike *C. niveo-marginatum* Mak, thidiazuron and iP were shown to be more effective than BA in flowering induction in *C. ensifolium*.

BA was used to induce *in vitro* flowering in *D.* Madame Thong-In in a two-layered medium (Sim *et al.*, 2007). It was reported that coconut water was required to trigger the transitional shoot apical meristem whereas BA enhanced inflorescence stalk initiation and flower buds formation in the *Dendrobium* orchid. Accordingly, the two-layered medium allowed more than 90 % of flowering *in vitro* to occur within five months from seed germination. The two-layered medium was adopted and successfully induced flowering in *Dendrobium* Chao Praya Smile (Hee *et al.*, 2007). Plantlets of *Dendrobium* Sonia 17 was induced to flower *in vitro* on half-strength MS solid medium supplemented with 20 µM BA (Tee *et al.*, 2008). However, the percentage of flowering was low (5 %) on the solid medium.

Adventitious shoots derived from the nodal sections of floral stalks of *Phalaenopsis* Pink Leopard "Petra" propagated in culture were induced to flowering *in vitro* using BA (Duan and Yazawa, 1995). Flowers produced *in vitro* were undersized and malformed. High concentration of nitrogen was found to inhibit flower development. On the other hand, low-temperature treatment, which was required for flowering in field-grown *Phalaenopsis*, did not induce flowering in the adventitious shoots, indicating that flowering induction in *Phalaenopsis in vitro* might be different from that *in vivo*. Floral bud formation was initiated in ×*Doriella* Tiny in medium with BA and appropriate content of sucrose and nitrogen (Duan and Yazawa, 1994). Subsequent floral development took place in BA-free medium. It was also found that kinetin, iP or coconut

water alone was insufficient to induce the formation of floral buds in culture in the orchids.

Certain orchid species have been shown to flower *in vitro* without supplementing plant growth regulators in the culture medium. *Oncidium varisocum* derived from micro-inflorescences were shown to flower *in vitro* after 8-9 months of culture in media containing no plant growth regulator (Kerbauy, 1984). In these plantlets, incomplete terminal flower was formed at the shoot tip. It was speculated that the origin of the explants, kind of light and photoperiod could have triggered the flowering in the orchid (Kerbauy, 1984). Seedlings of *Psygmorchis pusilla* could be induced to form floral spike *in vitro* under LD (20 h) and appropriate temperature on medium without plant growth regulator (Vaz *et al.*, 2004). The LD treatment however adversely affected floral bud development, inhibited anthesis and reduced flower longevity.

The objective of the present project is to develop a method to induce early *in vitro* flowering in *D.* Chao Praya Smile, in view of the fact that the system of *in vi*tro flowering could serve as a model for the investigation of flowering induction and flower development. Following that, the expression of genes (*knox*, *CHS* and *CKX*) isolated from *D.* Chao Praya Smile will be investigated in relation to flowering *in vitro*. In addition, changes in cytokinins and auxin at different growth stages during floral induction, especially at floral transition, in *D.* Chao Praya Smile will also be studied.

Chapter 3

Early *In vitro* flowering and seed production in culture for *Dendrobium* Chao Praya Smile

3.1 Introduction

The increase in popularity of orchids in Asia, Europe and the United States has led to continued increase in worldwide orchid production (Winkelmann *et al.*, 2006). Also, with increasing demand for orchid cut-flowers and potted plants, the need to generate new commercial cultivars is constantly expanding. Conventional orchid breeding is time consuming, irrespective of the demand for new clones, because orchid propagation requires a long period of *in vitro* culture. Orchid breeding involves pollination, seedpod maturation, protocorm development, *in vitro* growth of seedlings and subsequent *ex vitro* establishment of seedlings. The entire breeding cycle could be more than 3 - 5 years depending on the genotypes involved (Kamemoto *et al.*, 1999). For instance, it has been shown that breeding *Dendrobium* hybrids could take up to 5 years (Fadelah, 2006). This is primarily due to the long juvenility of these orchids which can span up to 30 months. During the juvenile phase, flowering normally does not occur under natural conditions (Hew and Yong, 1997).

To keep in pace with the increasing demand, methods for rapid *in vitro* propagation of orchids have been developed (Nayak *et al.*, 2002; Park *et al.*, 2002; Kuo *et al.*, 2005; Martin and Madassery, 2006). To overcome the long juvenile phase of orchid cultures, protocols to induce early *in vitro* flowering have been developed in several *Dendrobium* orchids (Wang *et al.*, 1997; de Melo Ferreira *et al.*, 2006; Sim *et al.*, 2007). These early *in vitro* flowering protocols could shorten the time required for flowering, which could be used to get an early indication of floral characteristics. But more

importantly, *in vitro* flowering could be used to fast-track breeding, provided viable seed production can be realized with such a system. Production of viable orchid seeds in culture following crossing has not been reported to date. The objectives of this chapter were: (1) to induce *in vitro* flowering in *D.* Chao Praya Smile; (2) to produce seedpods in culture with viable seeds by self-pollinating the *in vitro*-developed flowers; and (3) to examine and compare pollen and ovule development in flowers developing *in vitro* and in field.

3.2 Materials and methods

3.2.1 Plant materials, culture media and culture conditions

Flowers of *D.* Chao Praya Smile (*Dendrobium* Pinky × *Dendrobium* Kiyomi Beauty) were self-pollinated. The seedpods were harvested 120 days after pollination. The seeds obtained from the seedpods were germinated aseptically in 90 mm Petri dishes with 25 ml of modified Knudson C medium (KC) (Knudson, 1946) supplemented with 2 % (w/v) sucrose, 15 % (v/v) coconut water and 0.3 % (w/v) Gelrite. All media were adjusted to pH 5.3 before autoclaving at 121 ˚C for 20 min.

Eight-week-old protocorms were transferred to 50 ml of modified KC liquid culture medium containing (mg l^{-1}): $MgSO_4.7H_2O$ (250), KH_2PO_4 (500), $(NH_4)_2SO_4$ (250), $Ca(NO_3)_2.4H_2O$ (500), $MnSO_4.H_2O$ (5.68) and EDTA-Fe (28) supplemented with 2 % (w/v) sucrose and 15 % (v/v) coconut water in 100 ml Erlenmeyer flasks on rotary shakers at 120 rpm for proliferation. The liquid media were also supplemented with 6-benzyladenine (BA) at 0 to 22.2 µM. After three rounds of sub-culturing in the liquid medium at 3-week intervals, plantlets with 3 to 4 expanded leaves were transferred to two-layer (Sim *et al.*, 2007) modified KC medium (containing the same composition as

the modified KC liquid culture medium) in Magenta GA7^{TM} containers. The two-layer culture media consisted of 50 ml of Gelrite-solidified medium topped with a layer of liquid medium of the same volume and composition. All cultures were incubated at 25 ± 2 ˚C and a 16 h photoperiod of 40 µmol m^{-2} s^{-1} from daylight fluorescent lamps.

3.2.2 Effects of coconut water and sucrose on flowering induction

Protocorms were grown in modified KC liquid media followed by plantlets transferring to two-layer modified KC media, both supplemented with 11.1 µM BA and varying concentrations of coconut water (CW, 0 – 45 %, v/v) or sucrose (0 – 6 %, w/v). Cultures grown in media of varying CW or sucrose concentrations without BA were used as controls. The experiments were carried out in triplicates with 20 plantlets in each replicate.

3.2.3 Sporad analysis of pollinia

Pollinia were transferred from *in vitro*-developed flowers and flowers of field-grown plants onto a slide using a pair of fine forceps after removing the operculum. The pollinia were mounted in a drop of water and teased apart with a scalpel. One drop of acetocarmine (1 %, w/v) was added to the pollen grains and observed under the microscope.

3.2.4 Germination of pollen grains *in vitro*

Three *in vitro*-developed flowers that were open for 3 or 4 days were chosen. Four halves of pollinia from each flower were transferred, respectively, in a laminar flow hood onto 5 ml of solidified modified-Knops' medium in 35 mm Petri dishes. The

modified-Knops' medium consisted of (mg l^{-1}) H_3BO_3 (100), $Ca(NO_3)_2.H_2O$ (300), $MgSO_4.7H_2O$ (200), KNO_3 (100), sucrose (5 %, w/v) and Gelrite (0.3 %, w/v). Observation for germination of pollen grains was carried out after 2, 4, 8 and 12 days of incubation at 28 ˚C. For observation under microscope, the germinated pollen grains were transferred from the solidified modified-Knops' medium onto a glass slide with a drop of water. The pollen grains were separated with the aid of a needle and a blade. One drop of acetocarmine (1 %, w/v) was then added to the pollen grains. For each pollinium, 250 to 300 pollen grains were examined for germination.

3.2.5 *In vitro* pollination and seed production in culture

Plantlets that bore freshly-opened flowers were transferred to fresh two-layer KC medium. These *in vitro*-developed flowers were self-pollinated in a laminar flow hood using a pair of forceps. Upon pollination, the plantlets were observed for seedpod formation. At 120 days after pollination, the seedpods were harvested and cut open. Seeds from these *in vitro*-developed seedpods were germinated on modified KC medium. Plantlets grown from these seeds were further induced to flowering. All statistical analyses were carried out using One-Way ANOVA Tukey's test at 95 % confidence level.

3.3 Results

3.3.1 Inflorescence induction *in vitro*

D. Chao Praya Smile was induced to flower within 6 months from seed germination using BA in two-layer culture (Fig. 3.1a). The highest percent of flowering

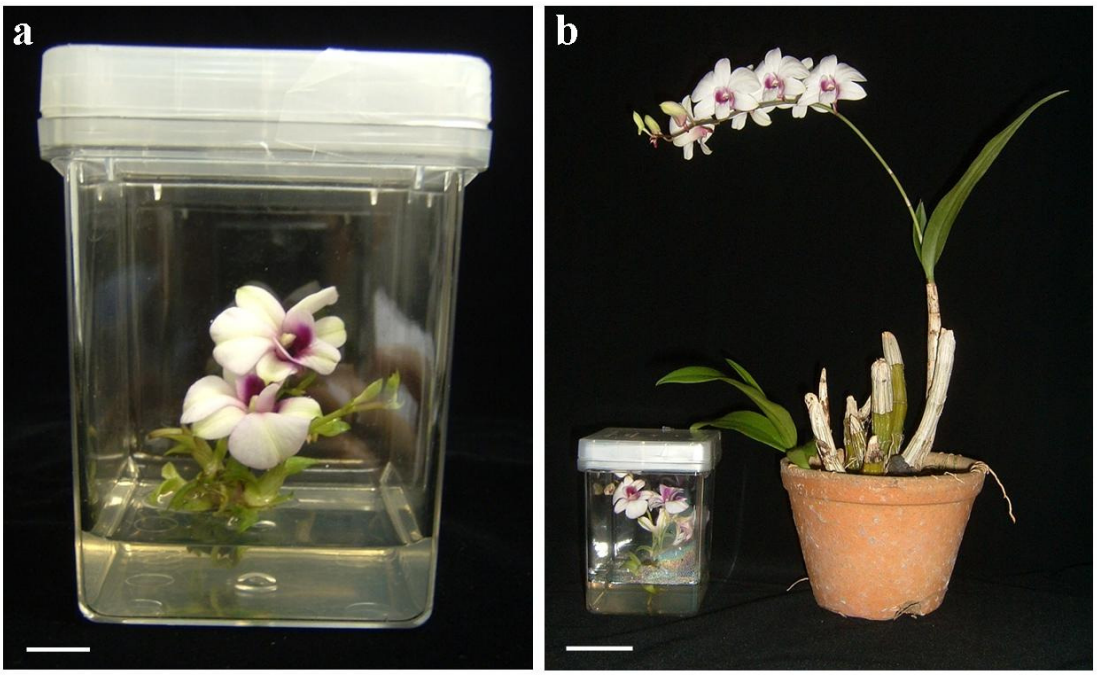

Fig. 3.1 *In vitro* flowering in *D.* Chao Praya Smile. a) Flowering in *D.* Chao Praya Smile in GA7^{TM} container. Bar = 1 cm. b) Comparison of *in vitro* flowering and flowering in field-grown plant of *D.* Chao Praya Smile. Bar = 4 cm.

(45 %) was induced in plantlets at 11.1 µM BA (Table 3.1). Plantlets grown in BA-free medium did not produce inflorescence. Each flowering plantlet produced one inflorescence stalk with an average of 3 to 4 flower buds. As for the duration of induction, inflorescences were produced earliest at 4 weeks upon transfer to two-layer culture at 4.4 and 11.1 µM of BA (Table 3.2), with the highest inflorescence induction rate at 11.1 µM of BA after 8 weeks. It was also observed that both complete and incomplete flowers were produced in the plantlets. Complete flowers had all floral organs. In incomplete flowers, some of the floral organs were absent. About 50 % of the flowering plantlets produced only complete flowers while another 44 % of the flowering plantlets produced both complete and incomplete flowers on the same inflorescence (Table 3.1).

Some plantlets died in the liquid medium containing BA. Plantlet mortality increased with increasing BA concentration. Thus about 34 % of the plantlets (n = 100) were dead at 22.2 µM of BA after 9 weeks of culture in liquid medium, compared to 5 % mortality in BA-free liquid medium. In order to secure more plantlets, they were treated with BA only in the two-layer medium. BA treatment at 11.1 µM in two-layer medium was sufficient to induce inflorescence production in 42 % of the plantlets (Table 3.3). In addition, nearly all inflorescences induced in this late-BA-treatment bore flower buds, although the number was lower than those in consecutive BA treatments in both liquid and two-layer cultures. To further improve inflorescence induction, morphologically normal plantlets in the liquid medium were selected prior to transfer to two-layer medium. This screening process increased the inflorescence induction from 45 % to 72 % at 11.1 µM BA (Table 3.4). The pre-selection method was useful as none of the morphologically abnormal plantlets produced inflorescence.

Table 3.1 Inflorescence induction and flower development in *D.* Chao Praya Smile.

BA (μM)	No. of plantlets	% plantlet with		% flowering plantlet producing		
		Inflorescence stalk[#]	Flower bud[†]	Both complete and incomplete flowers[††]	Complete flowers only[††]	Incomplete flowers only[††]
0	22	0	0	0	0	0
4.4	36	14 (5)	8 (3 ± 1)	67 (2)	33 (1)	0
11.1	20	45 (9)	45 (3 ± 1)	33 (3)	56 (5)	11 (1)
22.2	15	27 (4)	27 (5 ± 1)	50 (2)	50 (2)	0

The plantlets were grown in liquid medium for 9 weeks followed by two-layer medium, both of which contained the same concentrations of BA. Scoring of inflorescence production and flower bud formation were made at 10 weeks in two-layer culture medium when maximum number of flower bud had been formed. Assessment of flower development was made for individual flower bud at bloom.

[#]Numbers in the parentheses indicate the number of plantlets with inflorescence stalk.

[†]Numbers in parentheses indicate average number of flower bud per inflorescence ± SE. Same letters following the parentheses indicate no significant difference among the numbers of flower bud (One-Way ANOVA Tukey's test at 95 % confidence level).

[††]Numbers in the parentheses indicate the number of flowering plantlets.

Table 3.2 Effects of BA on early inflorescence induction in *D.* Chao Praya Smile.

BA	No. of	% plantlet with inflorescence	
(μM)	plantlets	4 weeks[#]	8 weeks[#]
0	32	0	0
4.4	30	7 (2)	17 (5)
11.1	26	8 (2)	39 (10)
22.2	18	0	22 (4)

The plantlets were grown in liquid medium for 9 weeks followed by two-layer medium, both of which contained the same concentrations of BA. Scoring of inflorescence production was made at 4 and 8 weeks in the two-layer cultures medium.
[#]Numbers in the parentheses indicate the number of plantlets with inflorescence stalk.

Table 3.3 Effects of BA on flowering induction in *D.* Chao Praya Smile.

BA in two-layer culture (μM)	No. of plantlets	% plantlet with	
		Inflorescence stalk[#]	Flower buds[†]
0	30	0	0
4.4	29	34 (10)	34 (3 ± 0)a
11.1	24	42 (10)	38 (3 ± 1)a
22.2	22	36 (8)	36 (4 ± 1)a

Plantlets were grown in BA-free liquid medium for 9 weeks followed by treatment with BA of various concentrations in two-layer medium. Scoring of inflorescence production and flower bud formation were made at 10 weeks in two-layer medium when maximum number of flower bud had been formed.

[#]Numbers in the parentheses indicate the number of plantlets with inflorescence stalk.

[†]Numbers in parentheses indicate average number of flower bud per inflorescence ± SE. Same letters following the parentheses indicate no significant difference among the numbers of flower bud (One-Way ANOVA Tukey's test at 95 % confidence level).

Table 3.4 Effects of plantlet selection on the percentage of inflorescence induction in *D.* Chao Praya Smile.

BA (μM)	% of inflorescence production in	
	Morphologically normal plantlets[#]	Morphologically abnormal plantlets[#]
0	0	0
4.4	53 (19)	0
11.1	72 (26)	6 (2)
22.2	22 (8)	0

Selections of 36 morphologically normal and abnormal plantlets, respectively, for each treatment were carried out prior to transfer to two-layer medium.
[#]Numbers in the parentheses indicate the number of plantlets with inflorescence.

The *in vitro* plantlets were approximately a quarter of the height of the field-grown plants at flowering and were 40 times lighter in terms of fresh weight (Table 3.5, Fig. 3.1b). They produced about the same number of leaves at flowering but the longest leaves of *in vitro* plantlets were 7.5 times shorter than those of field-grown plants. *In vitro* plantlets produced less and smaller flowers than field-grown plants. An average of 4 flower buds was produced in each *in vitro* plantlet, with a flower diameter of 2 to 2.5 cm, whereas field-grown plants could produce an average of 12 flowers of 4 cm in diameter (Table 3.5, Fig. 3.2a and b). Despite the smaller and lower number of flowers produced, the flowers produced *in vitro* could last for up to three weeks after anthesis, compared to 4 weeks in field-grown plants. The length of stomata on the lower epidermis of leaves of the *in vitro* plantlets and field-grown plants were 30.9 ± 2.0 µm and 38.5 ± 0.4 µm, respectively (Fig. 3.2c and 3.2d). Conversely, *in vitro* plantlets had higher stomatal density than field-grown plants, 38 ± 0 and 23 ± 1 per mm^2, respectively.

3.3.2 Effects of coconut water and sucrose on flowering induction

CW alone, at concentrations of 15 to 45 % (v/v), did not induce flowering in *D.* Chao Praya Smile (Table 3.6). In the presence of 11.1 µM of BA, CW at 15 % (v/v) induced the highest percentage of flowering (40 %) in the plantlets without pre-selection of plantlets. The percentage of flowering was reduced with increasing CW concentration beyond 15 % (v/v) and dropped to 5 % of flowering at 30 % (v/v) of CW. Flowering induction was inhibited at 45 % (v/v) of CW. Growth of the plantlets was retarded in culture media without CW because their fresh weight, dry weight, plant height, number of leaf and leaf size were all reduced, even in the presence of BA (Fig. 3.3, Table 3.7 and 3.8). On the other hand, the growth of both BA-induced and non-induced plantlets,

Table 3.5 Characteristic of field-grown and *in vitro D.* Chao Praya Smile plants at flowering.

Characteristics	Field-grown plants	*in vitro* plantlets
Average height at bolting of one shoot (cm)	12.1 ± 0.8	2.9 ± 0.3
Average fresh weight of one shoot (g)	28.8 ± 1.3	0.7 ± 0
Duration of flowering period (day)	29 ± 0.3	22 ± 0.4
Size of flowers (diameter, cm)	4.0 ± 0.04	2.3 ± 0.05
Number of leaves at bolting	5 ± 0	5 ± 0
Range of leaf length (shortest, longest) (cm)	(7.8 ± 0.3, 12.5 ± 0.3)	(0.6 ± 0, 1.7 ± 0)
Average number of flower buds per plant	12 ± 0	4 ± 0

The measurements were made on 10 field-grown plants and 10 *in vitro* plantlets of *D.* Chao Praya Smile.

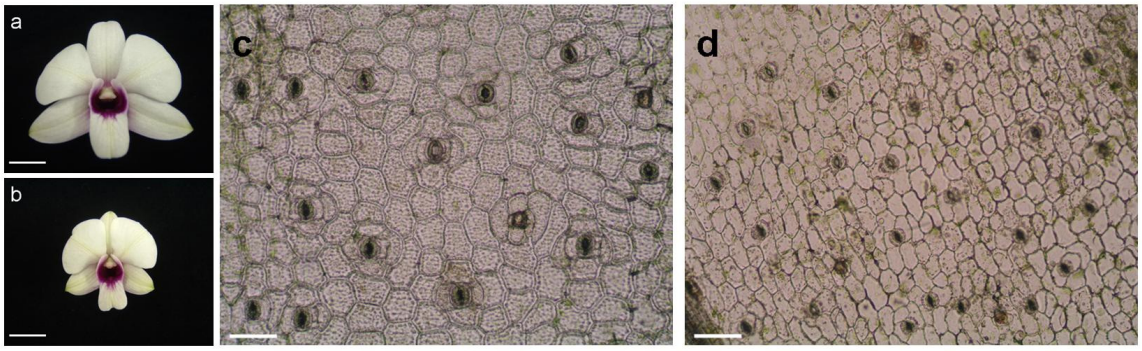

Fig. 3.2 Comparison of flowers and leaf epidermal peels of *D.* Chao Praya Smile grown in field and in culture. a, b) Flower of field-grown plant and *in vitro*-developed flower, respectively. Bar = 1 cm. c, d) Leaf epidermal peels of *D.* Chao Praya Smile grown in field and in culture, respectively. Bar = 100 µm.

Table 3.6 Effects of coconut water (CW) in the culture medium on flowering induction in *D.* Chao Praya Smile.

	% of flowering				
	CW (%, v/v)				
	0	15	22.5	30	45
Without BA	0	0	0	0	0
With 11.1 µM BA[#]	0	40.0 ± 2.9a	28.3 ± 5.8a	5.0 ± 2.9b	0

Plantlets were grown in liquid medium containing different concentrations of CW with or without BA for 9 weeks followed by two-layer medium. Scoring of inflorescence production was made at 10 weeks in two-layer medium.

[#]Same letters following the numbers indicate no significant difference among the percentages of flowering (One-Way ANOVA Tukey's test at 95 % confidence level).

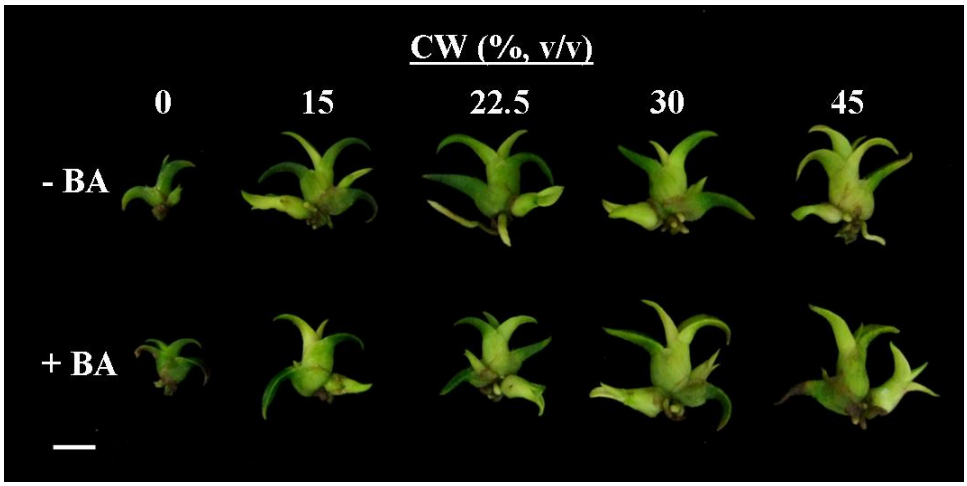

Fig. 3.3 Morphology of *D.* Chao Praya Smile plantlets after 9 weeks of growth in liquid media containing various concentrations of CW with or without BA (11.1 µM). Bar = 1 cm.

Table 3.7 Characteristics of *D.* Chao Praya Smile plantlets after 9 weeks of growth in BA-free liquid media containing various concentrations of CW.

	CW (%, (v/v)				
Characteristics	0	15	22.5	30	45
Fresh weight[#] (g)	0.17 ± 0.01a	0.56 ± 0.05b	0.76 ± 0.09bc	0.83 ± 0.06c	0.78 ± 0.06bc
Dry weight[#] (g)	0.015 ± 0.002a	0.044 ± 0.005b	0.059 ± 0.006bc	0.068 ± 0.005c	0.068 ± 0.006c
Water content[#] (%)	91.0 ± 1.7a	92.2 ± 0.5a	91.3 ± 1.3a	91.6 ± 0.8a	91.1 ± 0.6a
Plant height[#] (mm)	9.1 ± 0.5a	14.2 ± 0.4bc	16.3 ± 0.7b	13.5 ± 0.5c	14.3 ± 0.6bc
Leaf (fully expanded)					
Number	3 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0
Length[#] (mm)	6.7 ± 0.3a	8.9 ± 0.5b	9.4 ± 0.5b	9.8 ± 0.7b	9.0 ± 0.3b
Width[#] (mm)	2.4 ± 0.1a	3.9 ± 0.1b	4.6 ± 0.2bc	4.7 ± 0.2bc	5.2 ± 0.4c
Roots					
Number[#]	2 ± 0a	6 ± 1b	5 ± 1b	3 ± 1ab	4 ± 1ab
Length[#] (mm)	2.3 ± 0.2a	3.0 ± 0.3a	4.3 ± 0.4b	2.0 ± 0.2a	2.2 ± 0.2a
Number of lateral shoot	1 ± 0	2 ± 0	2 ± 0	2 ± 0	2 ± 0

[#]Same letters following the numbers of each characteristic indicate no significant difference among the characteristic (One-Way ANOVA Tukey's test at 95 % confidence level). n = 10.

Table 3.8 Characteristics of *D.* Chao Praya Smile plantlets after 9 weeks of growth in liquid media containing various concentrations of CW and 11.1 µM of BA.

	CW (%, v/v)				
Characteristics	0	15	22.5	30	45
Fresh weight[#] (g)	0.18 ± 0.02a	0.56 ± 0.06b	0.78 ± 0.08bc	0.77 ± 0.06bc	0.86 ± 0.07c
Dry weight[#] (g)	0.015 ± 0.001a	0.046 ± 0.005b	0.067 ± 0.010bc	0.072 ± 0.004c	0.072 ± 0.006c
Water content[#] (%)	90.4 ± 1.4a	91.4 ± 0.8a	90.4 ± 1.9a	90.4 ± 0.8a	91.3 ± 0.9a
Plant height[#] (mm)	8 ± 0.3a	14.2 ± 0.5b	15.6 ± 0.7b	15.4 ± 0.6b	14.9 ± 0.6b
Leaf (fully expanded)					
Number	4 ± 0	4 ± 0	4 ± 0	4 ± 0	4 ± 0
Length[#] (mm)	7.6 ± 0.6a	7.5 ± 0.3a	9.8 ± 0.8b	9.2 ± 0.5ab	8.8 ± 0.3ab
Width[#] (mm)	3.1 ± 0.2a	4.0 ± 0.2ab	4.6 ± 0.3b	4.6 ± 0.2b	4.7 ± 0.3b
Roots					
Number[#]	2 ± 0a	3 ± 1a	4 ± 1a	3 ± 0a	2 ± 0a
Length[#] (mm)	1.7 ± 0.2a	2.2 ± 0.1ab	2.7 ± 0.2b	2.4 ± 0.2ab	1.8 ± 0.2ab
Number of lateral shoot	1 ± 0	2 ± 0	2 ± 0	2 ± 0	2 ± 0

[#]Same letters following the numbers of each characteristic indicate no significant difference among the characteristic (One-Way ANOVA Tukey's test at 95 % confidence level). n = 10.

defined by the gain in fresh and dry weight, were enhanced with increasing CW concentration.

Similar to CW, addition of sucrose in the culture medium was not sufficient to induce flowering in *D.* Chao Praya Smile. In the presence of BA, flowering was induced at 1 and 2 % (w/v) of sucrose (11.7 ± 4.4 and 36.7 ± 4.4 %, respectively). The plantlets thrived well in media without sucrose, in the presence or absence of BA, and contained higher fresh weight than those grown in media supplemented with sucrose (Fig. 3.4, Table 3.9 and 3.10). In addition, plantlets in media without sucrose appeared greener (Fig. 3.4). In contrast, growth of plantlets was retarded with increasing sucrose concentration as indicated by the reduction of their fresh weights, plant heights and leaf sizes (Fig. 3.4, Table 3.9 and 3.10). Interestingly, water content was the highest in plantlets grown in media without sucrose, which was reduced in plantlets grown in increasing sucrose concentrations. This was apparent because the dry matter increased in plantlets grown in increasing sucrose concentration (Table 3.9 and 3.10).

3.3.3 Pollen and female reproductive organs

Three *in vitro*-developed complete flowers produced by plantlets in culture were examined for their pollen grains and female reproductive organs, in comparison to flowers of field-grown plants. Pollinia derived from the *in vitro*-developed flowers were green and consisted of four halves. They were waxy, 1.8 mm in length and half the thickness of the pollinia derived from flowers of field-grown plants (Fig. 3.5a and 3.5b). Stigma of the *in vitro*-developed flower was clear and sticky. Column and ovary of the *in vitro*-developed flower were clearly visible when the flower was dissected along the axis

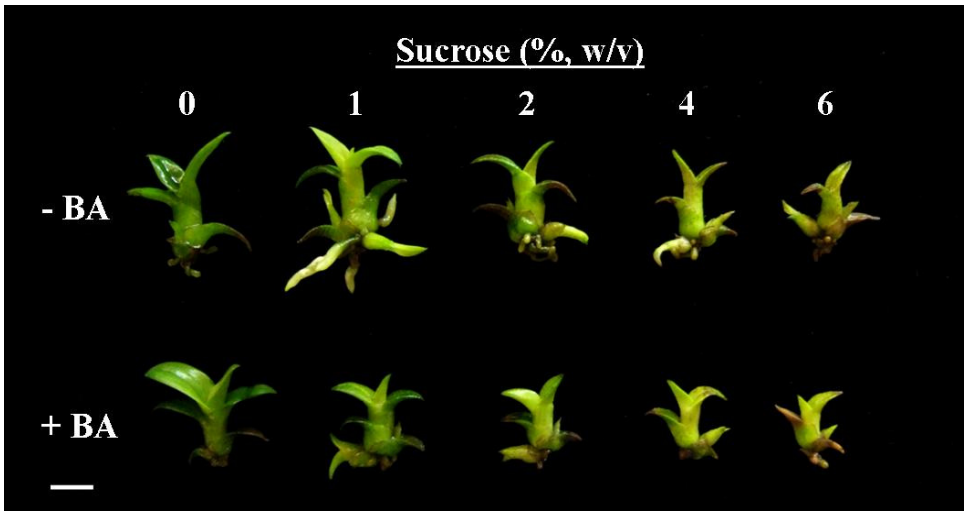

Fig. 3.4 Morphology of *D.* Chao Praya Smile plantlets after 9 weeks of growth in liquid media containing various concentrations of sucrose with or without BA (11.1 µM). Bar = 1 cm.

Table 3.9 Characteristics of *D.* Chao Praya Smile plantlets after 9 weeks of growth in BA-free liquid media containing various concentrations of sucrose.

	Sucrose (%, w/v)				
Characteristics	0	1	2	4	6
Fresh weight[#] (g)	0.37 ± 0.03ab	0.45 ± 0.03a	0.44 ± 0.04a	0.28 ± 0.02bc	0.23 ± 0.02c
Dry weight[#] (g)	0.018 ± 0.001a	0.030 ± 0.003b	0.043 ± 0.003c	0.035 ± 0.003bc	0.037 ± 0.003bc
Water content[#] (%)	95.3 ± 0.1a	93.3 ± 0.2b	90.1 ± 0.1c	87.3 ± 0.3d	83.9 ± 0.7e
Plant height[#] (mm)	13.0 ± 0.4a	15.4 ± 0.3b	14.9 ± 0.4b	11.3 ± 0.3c	10.8 ± 0.3c
Leaf (fully expanded)					
Number	4± 0	4 ± 0	4 ± 0	4 ± 0	3 ± 0
Length[#] (mm)	13.5 ± 0.5a	10.9 ± 0.7b	8.7 ± 0.4c	6.3 ± 0.3d	5.9 ± 0.3d
Width[#] (mm)	5.8 ± 0.2a	5.3 ± 0.2ab	4.8 ± 0.2b	3.7 ± 0.2c	3.5 ± 0.2c
Roots					
Number[#]	3 ± 0a	6 ± 1b	6 ± 1bc	4 ± 0ac	3 ± 1a
Length[#] (mm)	2.9 ± 0.3abc	6.2 ± 0.4d	3.9 ± 0.3a	2.8 ± 0.3ac	2.2 ± 0.2bc
Number of lateral shoot	1 ± 0	2 ± 0	2 ± 0	2 ± 0	1 ± 0

[#]Same letters following the numbers of each characteristic indicate no significant difference among the characteristic (One-Way ANOVA Tukey's test at 95 % confidence level). n = 10.

Table 3.10 Characteristics of *D.* Chao Praya Smile plantlets after 9 weeks of growth in liquid media containing various concentrations of sucrose and 11.1 µM of BA.

	Sucrose % (w/v)				
Characteristics	0	1	2	4	6
Fresh weight[#] (g)	0.38 ± 0.02a	0.38 ± 0.04a	0.30 ± 0.081ab	0.25 ± 0.02b	0.21 ± 0.01b
Dry weight[#] (g)	0.017 ± 0.001a	0.030 ± 0.004b	0.029 ± 0.001b	0.036 ± 0.003b	0.036 ± 0.002b
Water content[#] (%)	95.5 ± 0.1a	92.1 ± 0.3b	90.5 ± 0.2c	85.1 ± 0.4d	83.1 ± 0.4e
Plant height[#] (mm)	13.5 ± 0.5a	12.2 ± 0.5ab	10.8 ± 0.3bc	10.2 ± 0.2c	9.6 ± 0.2c
Leaf (fully expanded)					
Number	5 ± 0	4 ± 0	4 ± 0	3 ± 0	3 ± 0
Length[#] (mm)	13.8 ± 0.5a	8.1 ± 0.3b	6.9 ± 0.5bc	7.3 ± 0.4bc	6.1 ± 0.3c
Width[#] (mm)	6.2 ± 0.3a	5.3 ± 0.3ab	4.7 ± 0.2b	4.3 ± 0.2bc	3.5 ± 0.32c
Roots					
Number[#]	1 ± 0ab	2 ± 0b	1± 0bc	1 ± 0ac	0 ± 0ac
Length[#] (mm)	1.6 ± 0.3a	1.7 ± 0.2a	1.5 ± 0.3a	2.7 ± 0.4a	3 ± 1.0a
Number of lateral shoot	1 ± 0	2 ± 0	2 ± 0	1 ± 0	2 ± 0

[#]Same letters following the numbers of each characteristic indicate no significant difference among the characteristic (One-Way ANOVA Tukey's test at 95 % confidence level). n = 10.

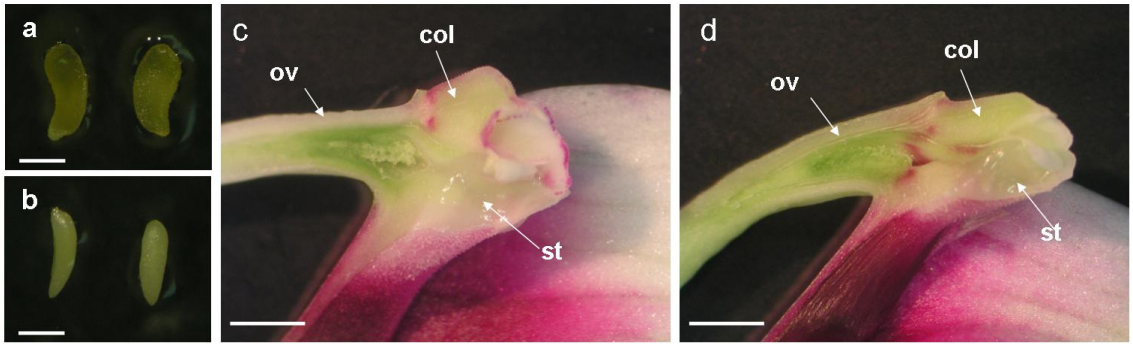

Fig. 3.5 Comparison of pollinia and female reproductive organs of *D.* Chao Praya Smile grown in field and in culture. a, b) Pollinia derived from flower of field-grown plant and *in vitro*-developed flower, respectively. Bar 1 = mm. c, d) Female reproductive organs in flower of field-grown plant and *in vitro*-developed flower, respectively. col, ov and st refer to column, ovary and stigma, respectively. Bar = 5 mm.

of symmetry (Fig. 3.5d). These female reproductive organs appeared to be anatomically similar to those in flowers of field-grown plants (Fig. 3.5c), albeit smaller. The ovary of the *in vitro*-developed flower was found to be approximately 1cm in length, compared to 1.5 cm in flowers of field-grown plants.

3.3.4 Sporad analysis germination of pollen grains

Observation on the pollen grains derived from *in vitro*-developed flowers showed 65 % normal tetrad and 35 % triad (Table 3.11). This was similarly observed in the pollen grains derived from flowers of field-grown plants in which 79 % of the sporads were tetrads. The tetrad pollen grains derived from both *in vitro*-developed flowers and flowers of field-grown plants were about 30-40 µm long (Fig. 3.6a and 3.6b). Monad and dyad, which resulted from irregular meiosis, were not observed in both cases. The pollen grains derived from *in vitro*-developed flowers and flowers of field-grown plants germinated on modified-Knops' medium after 2 days of incubation. After 8 days of incubation, 18.2 % and 52.8 % of pollen grains derived from *in vitro*-developed flowers and flowers of field-grown plants, respectively, germinated (Table 3.11, Fig. 3.6c).

3.3.5 Seed production in culture

Three out of four *in vitro* pollinations were successful and led to seedpod development (Fig. 3.7a). At the time of maturation, the seedpods were 1.5-1.8 cm in length, compared to 2.7-3.0 cm of the seedpods developed in field-grown plants (Fig. 3.7b and 3.7c). The seedpods developed *in vitro* were harvested 120 days after pollination when they turned slightly yellowish. These seedpods contained yellowish and dust-like

Table 3.11 Sporad formation and *in vitro* germination of pollen grains derived from flowers of field-grown plants and *in vitro*-developed flowers.

Pollen grains derived from	Sporad formation			% of pollen grains germination		
	Total sporad observed	(% of total sporad observed)		Incubation (days)		
		Triad	Tetrad	2	4	8
Flowers of field-grown plant	214	21	79	15.5	29.8	52.8
In vitro-developed flowers	280	35	65	3.0	5.5	18.2

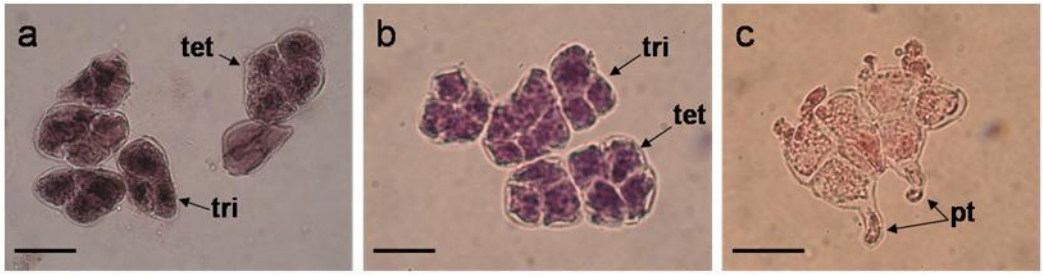

Fig. 3.6 Sporads and *in vitro* pollen grain germination. a, b) Sporads derived from flower of field-grown plant and *in vitro*-developed flower, respectively. tet and tri refer to tetrad and triad, respectively. Bar = 30 µm. c) Germination of pollen grains derived from *in vitro*-developed flower on modified-Knops' medium. pt refers to pollen tube. Bar = 30 µm.

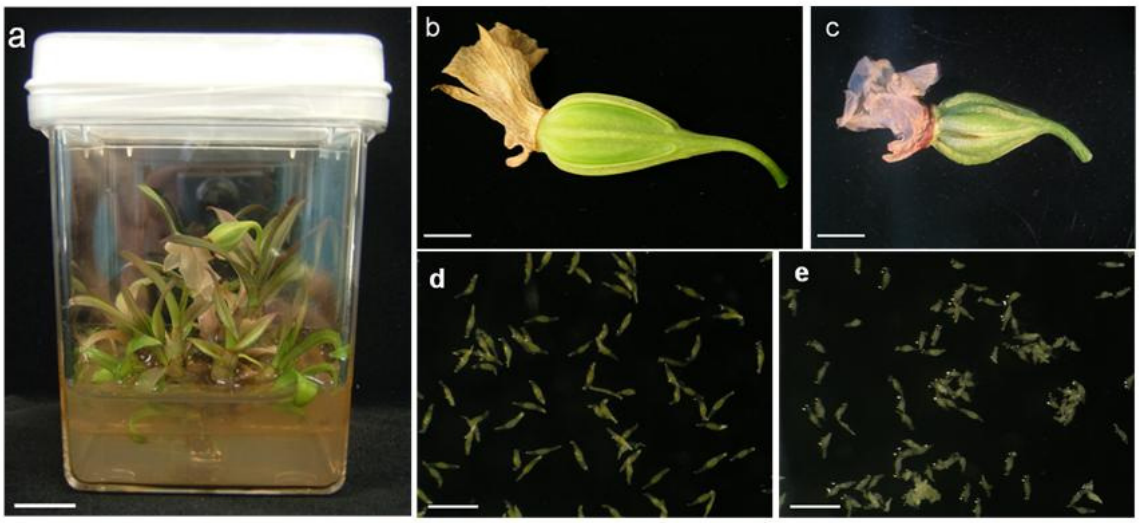

Fig. 3.7 Seedpod development and seed production in culture. a) Formation of seedpod in a plantlet upon self-pollination of an *in vitro*-developed flower. Bar = 1 cm. b, c) Seedpods developed in field and in culture, respectively. Bar = 1 cm. d, e) Seeds produced by field-grown plant and plantlet in culture, respectively. Bar = 1 mm.

seeds. The seeds were 428 ± 10 µm in length, shorter than those obtained from seedpods developed in field (684 ± 13 µm; Fig. 3.7d and 3.7e). The seeds produced in the *in vitro*-developed seedpods were fertile with more than 90 % developing into protocorms on modified KC agar medium after 8 weeks. Each *in vitro*-developed seedpod produced 500 to 1000 plantlets. These plantlets produced inflorescences upon induction using BA as described earlier (section 3.2.1).

3.4 Discussion

Reproduction is an important stage of plant development. In orchids, sexual reproduction can be effected through flowering resulting in the production of seedpod and seeds (Hew and Yong, 1997). In this study, *D.* Chao Praya Smile was shown to flower and produce seeds in culture (Fig. 3.1, Fig. 3.7). Optimal BA concentration was required to induce maximum inflorescence production in the plantlets (Table 3.1). The two-layer culture system was adopted in *D.* Chao Praya Smile because it was reported to promote normal development of flower buds in orchid (Sim *et al.*, 2007). In these experiments, it was observed that plantlets of *D.* Chao Praya Smile were unable to produce inflorescence when they were cultured on Gelrite-solidified medium.

Plantlets of *D.* Chao Praya Smile produced complete and incomplete flowers concurrently in culture (Table 3.1). As the aim of this study was to produce seeds in culture, the production of complete flowers that resembled the flowers of field-grown plants was desired. BA was required for normal development of floral buds in roses (Nguyen *et al.*, 2006), which possibly regulated floral development through genes controlling shoot apical meristem activity (Lindsay *et al.*, 2006). On the other hand, less

flowers were produced in the *in vitro* plantlets compared to field-grown plants. This could be due to the smaller size of *in vitro* plantlets as the number of flowers produced could be affected by plant size (Sletvold, 2002). However, considering the fresh weight of the *in vitro* plantlets (Table 3.5), they actually produced 6 flowers g^{-1} FW compared to 1 flower g^{-1} FW in the field-grown plants. Moreover, the *in vitro* plantlets produced flowers with diameters similar in value to their shoot heights, compared to flowers of field-grown plants which had an average diameter approximately one-third of the shoot height (Fig 3.1b, Table 3.5). Thus, the *in vitro* plantlets seemed to have produced relatively more and larger flowers than the field-grown plants despite their smaller size. Despite the production of a lower number of flowers in the *in vitro* plantlets, breeding success would not be hindered because numerous seeds could be produced in one seedpod and would be sufficient for breeding purpose. In this study, plantlets with abnormal leaf arrangement or non-expanding leaves were not selected for inflorescence induction. These abnormal plantlets could not produce any inflorescence upon BA treatment (Table 3.4). Morphological abnormalities in the plantlets could be the result of cytokinin activity because cytokinins have been reported to affect the morphogenesis of early seedlings (Nikolić *et al.*, 2006). Selection of morphologically normal plantlets for BA treatment would therefore ensure a higher percentage of inflorescence induction.

CW is the semi-clear liquid endosperm obtained from immature coconuts. It has been widely used in plant tissue culture, dueto its unique composition of sugars, vitamins, minerals, proteins, amino acids and growth-promoting factors (Arditti and Ernst, 1993). However, the precise composition of growth-promoting factors which makes CW an efficient nutrient medium remains unknown. The results of this study showed that CW

was essential to promote growth *D.* Chao Praya Smile, without which growth was severely stunted (Fig. 3.3, Table 3.7, Table 3.8). Using various sensitive analytical methods, cytokinins including iP, DHZ, Z, kinetin, *ortho*-topolin, DHZ-*O*-glucoside, Z-*O*-glucoside, ZR and kinetin riboside, and other plant growth regulators such as IAA and abscisic acid have been identified in CW (Ge *et al.*, 2004; Ge *et al.*, 2005; Ge *et al.*, 2006; Ma *et al.*, 2008). Even with such a rich content containing cytokinins and other plant growth regulators, CW could not induce flowering in the absence of BA, indicating the central role of BA in flowering induction. There is no report of the identification of BA in CW and it was suggested that cytokinin compositions in CW could vary depending on the origins of the coconuts (Ma *et al.*, 2008). It seems that there is an optimal concentration of CW for flowering induction, probably due to the presence of other plant growth regulators, such as IAA, in CW which could antagonize the effect of BA.

Sucrose is commonly used in tissue culture as the carbon source for plant growth. Flowering can only take place when there is an adequate supply of sugar to meet the energy demand of flowering process. Therefore, increased sugar supply, especially sucrose, was suggested to promote flowering (Gibson, 2005). Sucrose has also been shown to play a role in the flowering of orchids (Chen *et al.*, 1994). Similar to CW, sucrose did not induce flowering in the absence of BA. Besides, flowering did not occur in media with BA but containing no sucrose, despite normal growth of plantlets (Fig. 3.4, Table 3.9, Table 3.10). Therefore, sucrose was required for flowering but its role was secondary to BA. Although sucrose was required for flowering induction, its concentration beyond 2 % (w/v) completely inhibited flowering. A similar effect of sucrose on flowering was observed in *Arabidopsis* in which application of the sucrose to

apical part of the plant induced flowering (Roldán *et al.*, 1999) but a high concentration of sucrose (5 %, w/v) significantly delayed flowering time (Ohto *et al.*, 2001). Sucrose at high concentrations adversely affected the growth of *D.* Chao Praya Smile plantlets, which could also be a reason for flowering inhibition.

Morphology of pollens and female organs could be correlated to breeding and hybridization success (Fratini *et al.*, 2006). Morphological and anatomical examination of the pollinia and female organs of *in vitro*-developed flowers revealed that they were similar to flowers of field-grown plants (Fig. 3.5) and were therefore probably functional. In the female organs, the column connects stigma to ovary and allows the growth of pollen tubes towards the ovule during fertilization. Thus, the production of normal flowers with functional reproductive organs is imperative for successful breeding attempts using *in vitro* flowering technology.

Pollen quality of the *in vitro*-developed flowers was assessed by sporad analysis and *in vitro* pollen grain germination because it also determines the breeding success of the species. Meiotic behavior and sporads formation have been studied in orchids in relation to their fertility (Lee, 1987; Lee, 1988; McConnell and Kamemoto, 1993; Lee, 1994). In orchid microsporogenesis, regular meiosis results in four microspores grouped together, called a tetrad. When meiosis is irregular, polyploid spores in the form of monads, dyads or triads will be formed. Pollination of polyploid gametes could result in the formation of sexually sub-fertile or infertile progenies (Teoh, 1984). Therefore, a high percentage of tetrad formation in the pollen grains derived from *in vitro*-developed flower indicated regular meiosis and pollen fertility of *in vitro* plantlets. *In vitro* pollen grain germination is regarded as a reliable test of fertility with the assumption that pollen grains

capable of germination would be fertile pollen grains (Montaner *et al.*, 2003). However, the rate of pollen grain germination *in vitro* largely depends on the optimization of the medium (Heslop-Harrison *et al.*, 1984) and this factor has to be taken into consideration while counting germination as an indication of pollen quality. In the present study, modified-Knops' medium promoted the germination of the pollen grains derived from *in vitro*-developed flowers and flowers of field-grown plants (Table 3.11, Fig. 3.6) but germination of pollen grains on this medium was slow and the maximum germination was observed after 8 days of incubation (Table 3.11). However, the percentage of germination of the pollen grains derived from in *vitro*-developed flowers was lower than that derived from flowers of field-grown plants on this medium (Table 3.11).

In this study, it was shown that *D.* Chao Praya Smile could be induced to flower early and to produce seeds in culture. *In vitro* fruit development and fertile seed production have been reported in *Lycopersicon esculentum* (Rao *et al.*, 2005) and *Pisum sativum* L. (Franklin *et al.*, 2000). Despite the low percentage of germination of the pollen grains derived from *in vitro*-developed flowers, pollination of *in vitro*-developed flowers and subsequent seedpod formation produced a large number of seeds sufficient for breeding purposes. In the present protocol, the process from seed germination to production of the next generation seeds in culture was shortened from over 35 months to only about 11 months (Fig. 3.8). The method of seed production in culture would have produced 6 generations of progenies with the time that is required for 2 generations in conventional orchid breeding (Fig. 3.8). Therefore, seed production in culture would have tremendous application in orchid breeding in view of the fact that viable seed production is crucial in producing homozygous plants and new hybrids. The breeding period would

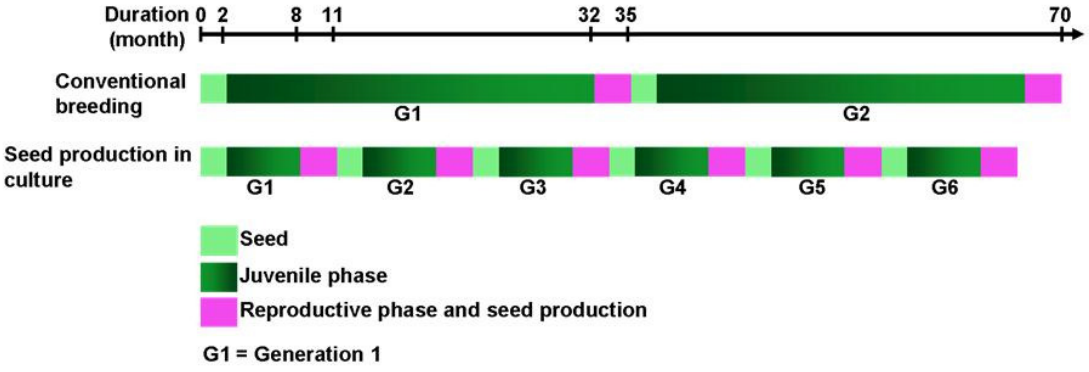

Fig. 3.8 Comparison of durations between conventional orchid breeding and method of seed production in culture.

be shortened by using the technology of seed production in culture which would in turn significantly decrease the cost of producing new orchid hybrids.

3.5 Concluding remarks

In this study, *D.* Chao Praya Smile was successfully induced to flower using BA in a two-layer medium within 6 months from seed germination. The percentage of flowering could be increased to more than 70 % with careful selection of morphologically normal plantlets prior to transfer to the two-layer medium. BA was essential in the flowering induction in *D.* Chao Praya Smile as CW or sucrose alone failed to induce flowering in the absence of BA. Despite the smaller and lower number of flowers produced in the *in vitro* plantlets as compared to the field-grown plants, morphological examination, sporad analysis and pollen grain germination tests revealed that the *in vitro*-developed flowers were functional and were able to produce viable seeds upon self pollination. Indeed, seeds were successfully produced in culture upon self-pollination of *in vitro*-developed flowers. Thus, the entire breeding cycle from seed germination to next generation seed production could be shortened to 11 months, which was shorter than one-third of the time required for breeding in field-grown plants. This method of early *in vitro* flowering and seed production in culture would undoubtedly benefit the orchid industry especially the orchid breeding and hybridization programs.

Chapter 4

Morphological changes in *Dendrobium* Chao Praya Smile during induction of flowering and development of *in vitro* flowers

4.1 Introduction

Plants produce different types of organs at different times during the course of development. The identity and morphology of the organs produced at a particular position on the shoot is determined by interactions between independently regulated and temporally coordinated processes (Poethig, 2003). It was suggested that in various species, the juvenile and adult phases of development could be morphologically distinguished by certain characteristics such as their phyllotaxis, leaf retention, growth habit of lateral branches, thorniness and adventitious root production (Poethig, 1990). For example, leaves produced at the juvenile phase are usually smaller and simpler in structure than those produced at adult phase. Therefore, one could investigate the morphogenetic switch in a higher plant through observation of morphological changes along plant development from juvenile, adult to flowering phases. All organs of upper-ground part in a plant are derived from the apical meristem. Therefore, histological analysis of the shoot apex could accurately determine the temporal sequence of each developmental phase, especially the floral transition which would be associated with more abrupt changes at the shoot apical meristem (SAM) (Kwiatkowska, 2008).

Segregation of flower colors is common in orchid progenies produced through orchid breeding (Kamemoto *et al.*, 1999), which represents a way to produce new orchid hybrids. Moreover, each seed derived from a single seedpod could have different genetic makeup and therefore different flower traits. In view of this, early *in vitro* flowering of

orchids could serve as a system for an early assessment of the desired characteristics of the flowers before the clone is mass-propagated through tissue culture. Up to this date, segregation of flower colors *in vitro* has only been observed in *Dendrobium* Madame Thong-In (Sim *et al.*, 2007). However, it was not known whether color segregation observed in the *in vitro*-developed flowers was the consequence of cytokinin treatment incorporated in the culture medium or that it was naturally occurring. The objectives of this chapter were: (1) to observe the morphological changes of *D.* Chao Praya Smile cultures and shoot apex during the course of flowering induction; (2) to investigate the expression of *D.* Chao Praya Smile *knox* (*DCPSknox*) gene in the plantlets in relation to flowering induction; (3) to examine the development and color segregation in flowers developed *in vitro*; and (4) to clone and analyze the expression of *D.* Chao Praya Smile chalcone synthase (*DCPSCHS*) gene in *D.* Chao Praya Smile plantlets.

4.2 Materials and methods

4.2.1 Plant materials, culture media and culture conditions

Flowers of *D.* Chao Praya Smile were self-pollinated and the seeds obtained from the seedpods were germinated aseptically in 90 mm Petri dishes with 25 ml of modified Knudson C medium (KC) (Knudson, 1946) supplemented with 2 % (w/v) sucrose, 15 % (v/v) CW and 0.3 % (w/v) Gelrite. All media were adjusted to pH 5.3 before autoclaving at 121 ˚C for 20 min. Eight weeks later, the protocorms were transferred to 50 ml of modified KC (Knudson, 1946) liquid culture medium supplemented with 2 % (w/v) sucrose and 15 % (v/v) CW in 100 ml Erlenmeyer flasks on rotary shakers at 120 rpm for proliferation. The liquid media were also supplemented with BA at 0, 4.4, 11.1 or 22.2

μM. Plantlets were sub-cultured in liquid medium on day 20 and 38. Subsequently, the plantlets were transferred to two-layer modified KC medium in Magenta GA7^{TM} containers on day 54. All cultures were incubated at 25 ± 2 ˚C and a 16 h photoperiod of 40 μmol m^{-2} s^{-1} from daylight fluorescent lamps.

4.2.2 Histological analysis

Apices of non-induced and BA-induced (11.1 μM) plantlets harvested 0, 20 38, 54 and 80 days after culture were fixed in 2 % (v/v) paraformaldehyde and 0.5 % (v/v) glutaraldehyde in phosphate buffer saline (PBS, containing 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8 mM K_2HPO_4, pH 7.2) under vacuum for 30 min and then at 4 °C overnight. The samples were then dehydrated in an ethanol series (60, 70, 85, 100 %, v/v) and embedded in paraffin wax. Transverse sections of 10 μm were cut with a rotary retracting microtome (Model 5030, Bright, Huntingdon, England) and stained with toluidine blue-O. Sections were observed under Olympus BH-2 light microscope and images were taken using an Olympus digital camera C-5050.

4.2.3 Morphological measurement

Morphological measurements were carried out on sections observed under Olympus BH-2 light microscope. Height of the SAM (h_1) and stem axis (h_2) were measured in the apex median longitudinal sections from the top of the SAM to, respectively, the base of the rib meristem, or the insertion level of the youngest expanded leaves on the stem (Fig. 4.1). SAM width (w) was the distance separating the outer border of the peripheral zone (Fig. 4.1). Measurements were carried out in triplicates.

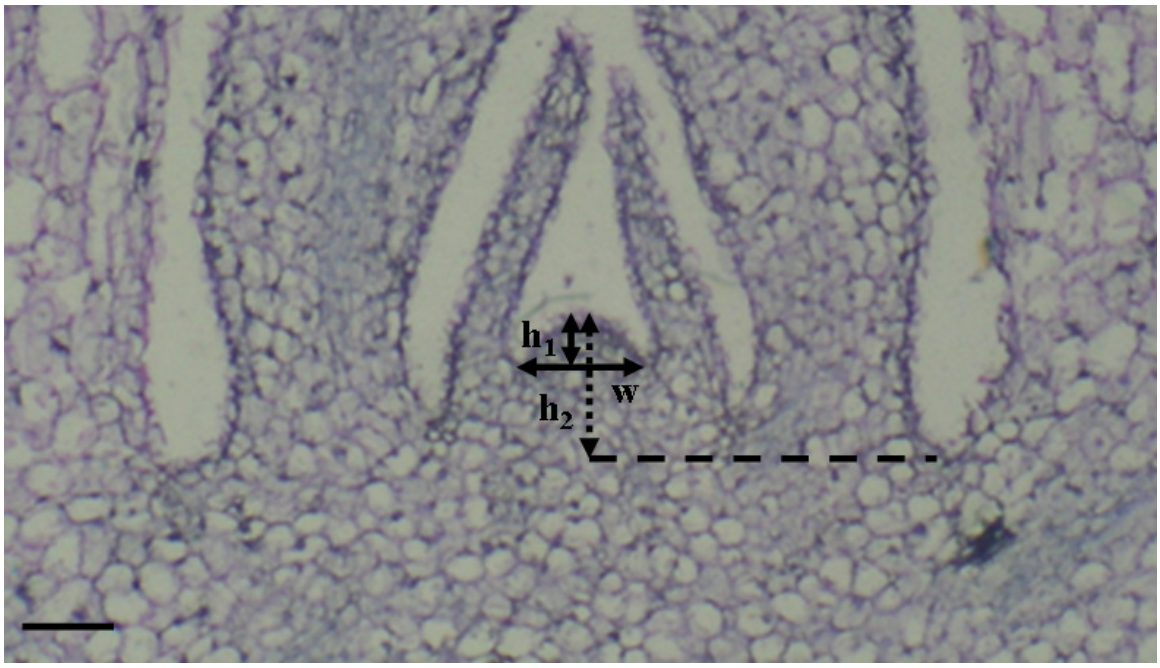

Fig. 4.1 Median longitudinal section through the apex of a *D.* Chao Praya Smile plantlet. Section was stained with toluidine blue-O. h_1, h_2 and w refer to height of the SAM, height of the stem axis and width of the SAM, respectively. Height of the SAM and the stem axis were measured in the apex median longitudinal section from the top of the SAM to, respectively, the base of the rib meristem (as indicated by solid vertical arrow), or the insertion level of expanded leaves on the stem (as indicated by broken vertical arrow). Measurement of w was indicated by solid horizontal arrow. Bar = 1 µm.

4.2.4 Analysis of development and color segregation of *in-vitro* developed flowers

Sixty flower buds produced *in vitro* in 60 individual plantlets grown from seeds derived from a seedpod were randomly selected. At anthesis, the flowers were analyzed for their overall morphology and the development of sepals, petals, lip and reproductive organs. For the developmentally complete flowers, color intensity and patterns were determined in the lip, petals and sepals.

4.2.5 Cloning of *D.* Chao Praya Smile *knox* (*DCPSknox*) and *CHS* (*DCPSCHS*) genes

RNA from shoot apices of BA-induced plantlets was isolated using the Plant RNeasy extraction kit (Qiagen, Hielden, Germany), followed by cDNA synthesis from 1 µg of total RNA using random hexamers and ThermoScript™ Reverse Transcriptase (Invitrogen). One twentieth volume of each cDNA was used as a template for PCR amplification using the following primers for target genes: *DCPSknox*: 5'-GGACCTTACGTCTCCGATGA-3' (forward), 5'-TTGATCTAGCCCTGTTGCCT-3' (reverse); *DCPSCHS*: 5'-CTCGTCTCAGCTTCCCAGAC-3' (forward), 5'-TATTCCCATACTCCGCAAGC-3' (reverse). The PCR products were isolated from 1 % agarose gels following electrophoresis and cloned into pGEM-T Easy (Promega, Madison, WI) and sequenced. Nucleotides sequences were analyzed using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI 3730*xl* DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Nucleotide and amino acid sequence alignments were generated using Clustal W (Thompson *et al.*, 1994).

4.2.6 Gene expression analysis by semi-quantitative RT-PCR

Total RNA was isolated using the Plant RNeasy extraction kit (Qiagen, Hielden, Germany). For time course analyses of *DCPSknox* expression, non-induced and BA-induced (11.1 µM) cultures were harvested 0, 20, 38, 54 and 80 days after culture and total RNA was extracted. For tissue-specific *DCPSknox* expression analysis, total RNA was isolated from shoot apices (sa), leaves (l), stems and leaf bases (s + lb) and stem bases (sb) from non-induced and BA-induced plantlets 54 days after culture (Fig. 4.2). Total RNA was also isolated from the roots of non-induced plantlets. For analysis of *DCPSCHS* expression, total RNA was extracted from five non-induced and five BA-induced (11.1µM) plantlets 54 days after culture. The total RNA extracted was reverse-transcribed for RT-PCR using the same sets of primers for the cloning of *DCPSknox* and *DCPSCHS*. PCR amplifications began with a 2 min denaturation at 94 ˚C and continued for 40 cycles of 94 ˚C for 40 s, 58 ˚C for 1 min, and 72 ˚C for 1 min, followed by a 7 min extension at 72 ˚C. *Actin* gene was used as control and PCR was carried out with the primers 5'- GCTGCTCGTGACCTGACTGA-3' (forward) and 5'-ACGGAACCTCTCAGCTCCAA-3' (reverse), using the same amplification protocol. Each PCR product was resolved on 1 % agarose gels and stained with SYBR® Safe (Invitrogen). Bands were visualized and analyzed using ImageJ software (v1.42, Wayne Rasband, NIH). To minimize sample variations, mRNA expression of the target gene was normalized relative to the expression of the house keeping gene *Actin*. All experiments were repeated three times for cDNA prepared for each batch of plantlets or tissues.

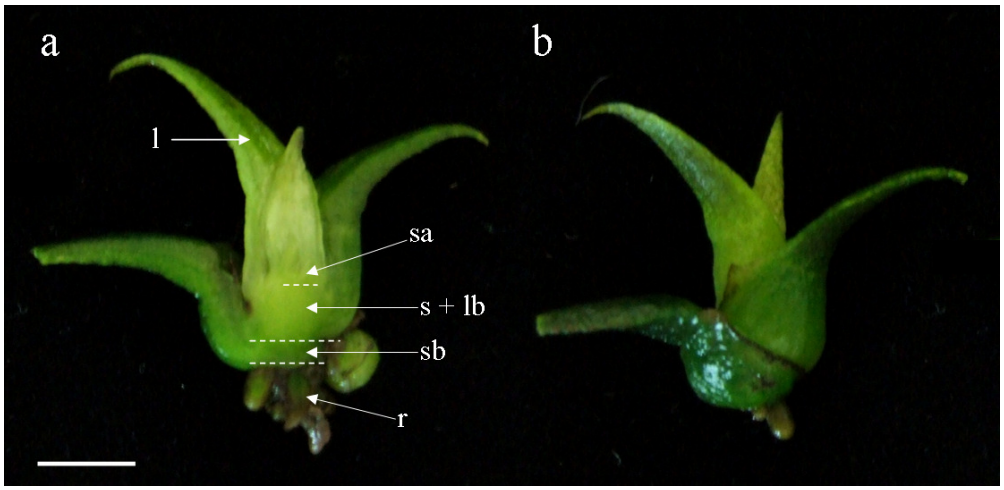

Fig. 4.2 Various tissues of a *D.* Chao Praya Smile plantlet. a) Median cross section of a non-induced plantlet after 54 days of culture. b) The other side of the same plantlet. BA-induced plantlet was similar to non-induced plantlet in morphology, but roots were not produced. Shoot apex and stem base of 1 – 1.5 mm were isolated from the tip and base of the pseudobulb, respectively. Tissue sandwiched between the shoot apex and stem base was regarded as stem and leaf base. sa, s + lb, sb, r and l refer to shoot apex, stem + leaf base, stem base, root and leaf, respectively. Bar = 5 mm.

4.3 Results

4.3.1 Morphological changes in *D.* Chao Praya Smile cultures at various stages of flowering induction

Growth of the plantlets was observed to increase rapidly from day 0 to day 54 in liquid medium containing 11.1 μM BA (Fig. 4.3). BA induction was initiated on protocorms with two leaves. After 20 and 30 days of culture, the third and fourth leaves were produced, respectively. The leaves produced were more expanded 54 days after culture before the plantlets were transferred to the two-layer medium. A similar growth pattern was observed in the non-induced plantlets. Growth of BA-induced plantlets was not affected by BA treatment because they showed similar fresh and dry weights as non-induced plantlets after growing in the liquid medium for 54 days; however, BA-induced plantlets were noticeably shorter (Fig. 4.4, Table 4.1). Both types of plantlets produced two lateral shoots before they were transferred to the two-layer medium. They also had similar number of leaves but the leaves in BA-induced plantlets seemed to be wider. The most prominent difference between BA-induced and non-induced plantlets was root production (Fig. 4.4, Table 4.1). After 54 days of culture, each non-induced plantlet produced 6 roots of approximately 2.7 mm in length, whereas each BA-induced plantlet produced 1 or no root at all (Table 4.1). Flower buds were formed in BA-induced plantlets about 4 weeks after they were transferred to the two-layer medium (80 days after culture) in which anthesis took place in 3 weeks (Fig. 4.3). During this time, non-induced plantlets remained vegetative. It was noticed that BA-induced plantlets started to produce root in the two-layer medium and the root system was as extensive as that in the non-induced plantlets when flowering took place.

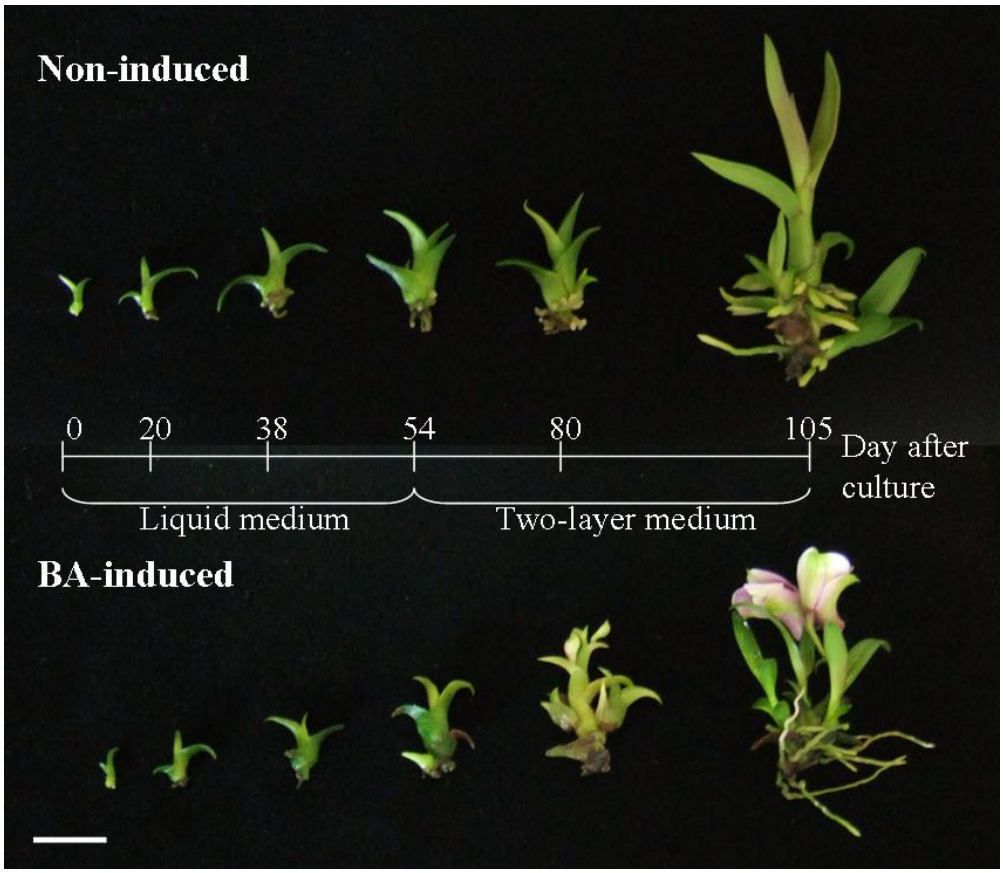

Fig. 4.3 Morphology of non-induced and BA-induced (11.1 μM) *D.* Chao Praya Smile plantlets at different days after culture. The plantlets were transferred to the two-layer medium after 54 days of culture in liquid medium. Bar = 1 cm.

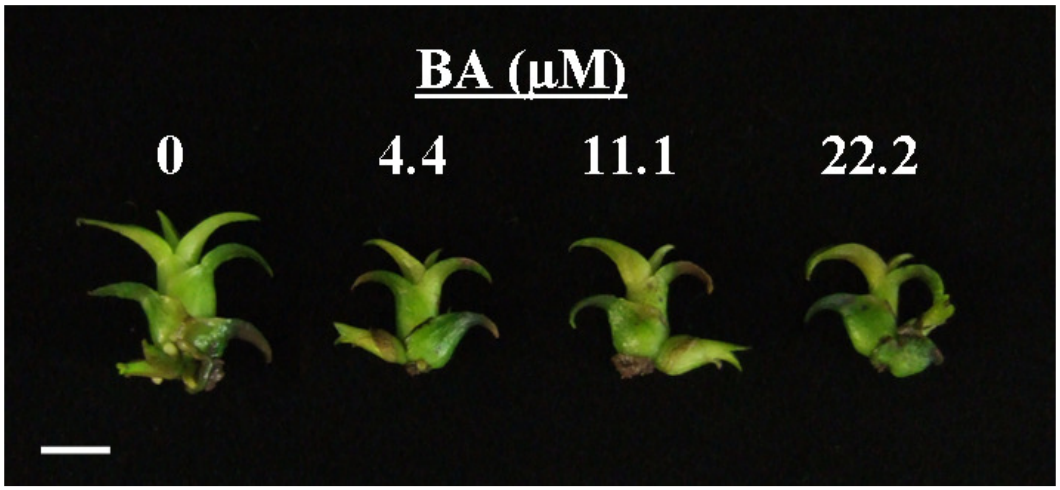

Fig. 4.4 Morphology of *D.* Chao Praya Smile plantlets grown in liquid media containing various concentrations of BA for 54 days. Bar = 8 mm.

Table 4.1 Characteristics of *D.* Chao Praya Smile plantlets cultured in liquid KC medium with various concentrations of BA for 54 days.

	BA (μM)			
Characteristics	0	4.4	11.1	22.2
Fresh weight[#] (g)	0.42 ± 0.04a	0.48 ± 0.03a	0.48 ± 0.04a	0.41 ± 0.03a
Dry weight[#] (g)	0.034 ± 0.004a	0.047 ± 0.004a	0.045 ± 0.004a	0.037± 0.004a
Water content[#] (%)	91.9 ± 0.3a	90.2 ± 0.4a	90.7 ± 0.5a	91.2 ± 0.6a
Plant height[#] (mm)	13.5 ± 0.4a	12.9 ± 0.3a	12.6 ± 0.4ab	11.3 ± 0.4b
Leaf (fully expanded)				
Number	5 ± 0	4 ± 0	4 ± 0	4 ± 0
Length[#] (mm)	8.9 ± 0.4a	9.6 ± 0.4a	9.7 ± 0.3a	8.7 ± 0.4a
Width[#] (mm)	3.4 ± 0.2a	4.6 ± 0.3a	4.1 ± 0.2ab	3.4 ± 0.3a
Roots				
Number[#]	6 ± 1a	1 ± 0b	1 ± 0b	0
Length[#] (mm)	2.7 ± 0.3a	2.3 ± 0.3a	1.9 ± 0.1a	0
Number of lateral shoot	2 ± 0	2 ± 0	2 ± 0	2 ± 0

[#]Same letters following the numbers of each characteristic indicate no significant difference among the characteristic (One-Way ANOVA Tukey's test at 95 % confidence level). n = 10.

4.3.2 Morphological changes in the shoot apex

The SAM of the plantlets contained cells that were smaller than those in the stem and expanded leave (Fig. 4.5). Similarly, cells in the newly emerged and non-expanded young leaves were distinguishably smaller than those in mature and expanded leaves. It was also observed that SAM and non-expanded young leaves were more densely stained than the stem and mature leaves. Anatomical changes in the shoot apices of non-induced and BA-induced cultures from 0 to 80 days after culture were as shown in Fig. 4.5. It was observed that SAM height, width and axis height (measurement as illustrated in Fig. 4.1) increased with the growth of plantlets (Fig. 4.6). After 38 days of culture, SAM of BA-induced plantlets started to expand both in height and width. Noticeably, the stem axis of BA-induced plantlets increased dramatically after 54 days of culture (Fig. 4.5h, Fig. 4.6c), probably indicating bolting in plants. Following the bolting of plants, flower buds were produced along the stem axis (Fig. 4.5j). During this period, the shoot apices of non-induced plantlets remained non-expanded (Fig. 4.5i). Therefore, it appeared that bolting of the plant and floral transition started as early as 54 days after BA treatment when the plantlets were grown in liquid medium. Subsequently, flower buds developed in the plantlets grown in the two-layer medium.

4.3.3 Cloning and expression of *DCPSknox* in *D.* Chao Praya Smile

Using oligodeoxynucleotide primers that were designed based on the conserved regions in *Dendrobium* orchids *knox* genes, RT-PCR was carried out using *D.* Chao Praya Smile shoot apex total RNA as template. A PCR product band of approximately the correct size (297 bp) was resolved on agarose gel electrophoresis and subsequently

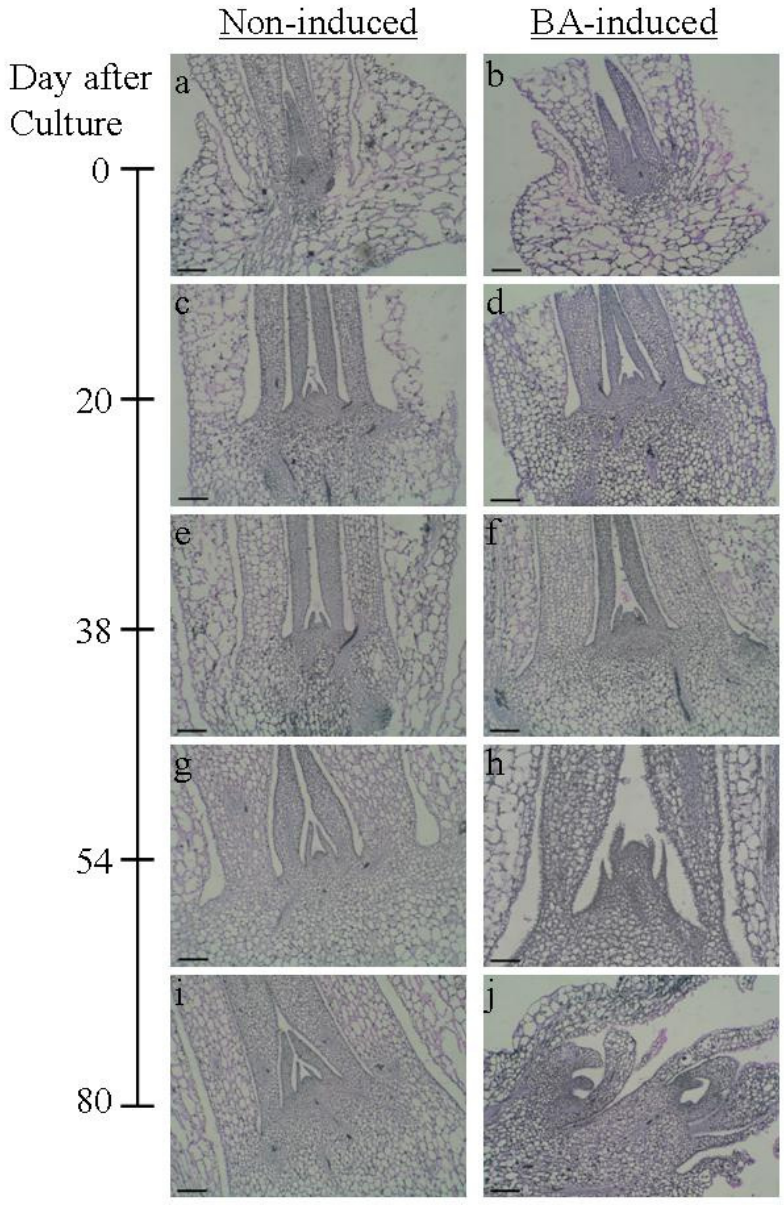

Fig. 4.5 Median longitudinal sections through apices of non-induced and BA-induced *D.* Chao Praya Smile plantlets at different days after culture. a, c, e, g and i are sections of apices of non-induced plantlets 0, 20, 38, 54 and 80 days after culture, respectively. b, d, f, h and j are sections of apices of BA-induced plantlets 0, 20, 38, 54 and 80 days after culture, respectively. Developing flower buds were shown in the section of BA-induced plantlet after 80 days of culture (j). Bar = 5 µm.

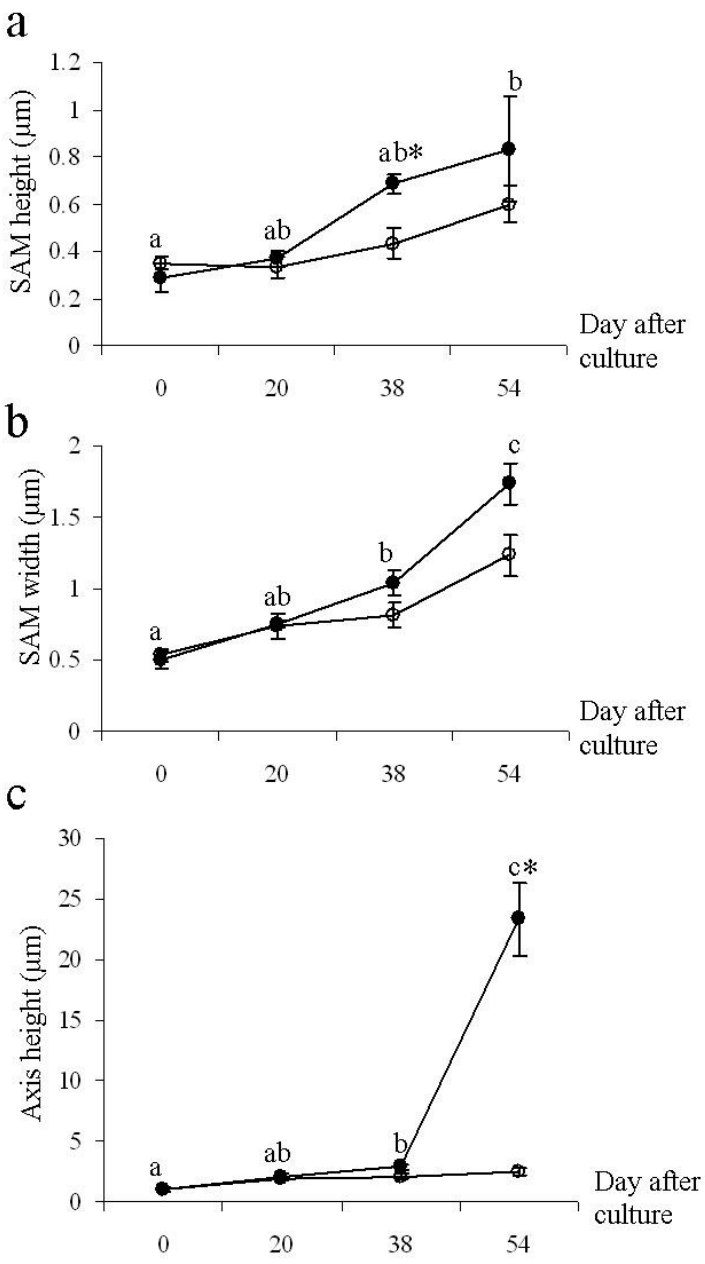

Fig. 4.6 SAM height (a), width (b) and stem axis (c) of non-induced (open circles) and BA-induced (closed circles) *D.* Chao Praya Smile cultures at different days after culture. Same letters above the closed circles indicate no significant difference among the SAM height, width and axis height in (a), (b) and (c), respectively, in the BA-induced cultures (One-Way ANOVA Tukey's test at 95 % confidence level). Asterisks following the letters in (a) and (c) indicate significant difference in the SAM height and axis height, respectively, between the BA-induced and non-induced cultures at the specific day after culture (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

purified. Nucleotide sequence analysis revealed that the isolated fragment represented the partial sequence of *DCPSknox* which showed more than 97 % sequence similarity to the corresponding regions of *KNOX*s from *D.* Madame Thong-In and *Dendrobium* nobile (Fig. 4.7). The fragment also showed 100 % sequence similarity to the corresponding regions of KNOX proteins from the *Dendrobium* orchids (Fig. 4.8). The cloned fragment also included the characteristic ELK domain and part of the homeodomain of *knox* gene. The isolated fragment was therefore used to analyze *DCPSknox* expression in *D.* Chao Praya Smile.

DCPSknox expression was analyzed at various stages of flowering induction. Compared to non-induced plantlets, the expression of *DCPSknox* was higher in BA-induced plantlets during the juvenile phase, which was subsequently reduced at floral transition and during floral bud development (Fig. 4.9). Besides, *DCPSknox* expression was also analyzed in various tissues of the plantlets after 54 days of culture (Fig. 4.10). *DCPSknox* was found to be highly expressed in the shoot apices of BA-induced and non-induced plantlets (Fig. 4.10). Below the shoot apex, the stem and leaf base, and the stem base also had similar levels of expression of *DCPSknox*. Expression of *DCPSknox* was low in the leaves of BA-induced plantlet, which was approximately one-tenth of that in the shoot apex (Fig. 4.10). Besides, *DCPSknox* was also expressed in the roots of non-induced plantlets, at a level close to that in the leaves.

4.3.4 Analyses of development and color segregation of *in vitro*-developed flowers

Two thirds of the 60 flowers produced *in vitro* were developmentally complete; the remaining 20 flowers were developmentally abnormal (Fig. 4.11). Analyses of color

```
DCPSknox        ------GGACCTTACGTCTCCGATGAGGCTGTGGGATCTTCGGACGAGGAACTTAGTGGA 54
DOH1            GCGCTTGGACCTTACGTCTCCGATGAGGCTGTGGGATCTTCGGATGAGGAACTTAGTGGA 60
Dnknox          GCGCTTGGACCTTACGTCTCCGATGAGGCTGTGGGATCTTCGGATGAGGAACTCAGTGGA 60
                      ************************************ ******** ******

DCPSknox        G---AAGGAGAGGCTCCAGAATCACATTTAAAAGGTGAAGAAAGAGATCTCAAAGAGAAA 111
DOH1            GGAGAAGGAGAGGCTCCAGAATCACATTTAAAAGGTGAAGAAAGAGATCTTAAAGAGAAA 120
Dnknox          GGAGAAGGAGAGGCTCCGGAATCACATCTAAAAGGTGAAGAAAGAGATCTTAAAGAGAAA 120
                *   ************* ********* ********************** *********

DCPSknox        CTCCTCCGAAAATATAGCGGTTATTTGAGTAGCTTGAAGCAGGAATTTTCAAAGAAAAAG 171
DOH1            CTCCTCCGAAAATATAGCGGTTATTTGAGTAGCTTGAAGCAGGAATTTTCAAAGAAAAAG 180
Dnknox          CTCCTCCGAAAATATAGCGGTTATTTGAGTAGCTTGAAGCAGGAATTTTCAAAGAAAAAG 180
                ************************************************************

DCPSknox        AAGAAAGGGAAGCTCCCCAAAGAAGCACGACAGATACTCTTTGAATGGTGGACAGCTCAT 231
DOH1            AAGAAAGGGAAGCTCCCCAAAGAAGCACGACAGATACTCTTTGAATGGTGGACAGCTCAT 240
Dnknox          AAGAAAGGGAAGCTCCCCAAAGAAGCACGACAGATACTTTTTGAATGGTGGACAGCTCAC 240
                ********************************** ********************

DCPSknox        TACAAGTGGCCCTACCCTACAGAAGCAGACAAGATCGCACTGGCTGAGGCAACAGGGCTA 291
DOH1            TACAAGTGGCCCTACCCTACAGAAGCAGACAAGATCGCACTGGCTGAGGCAACAGGGCTA 300
Dnknox          TACAAGTGGCCCTACCCTACAGAAGCAGACAAGATCGCACTGGCCGAGGCAACAGGGCTA 300
                ****************************************** ***************

DCPSknox        GATCAA------ 297
DOH1            GATCAAAAGCAA 312
Dnknox          GATCAAAAGCAA 312
                ******
```

Fig. 4.7 Nucleotide alignment of partial *DCPSknox* with *knox*s from *Dendrobium* grex Madame Thong-In (*DOH1*; AJ276389) and *Dendrobium* nobile (*Dnknox*; AY608889). Sequences were aligned using the CLUSTAL W program (Thompson *et al*., 1994). The positions of nucleotides are given on the right. The stars below each line of alignment indicate conserved sites.

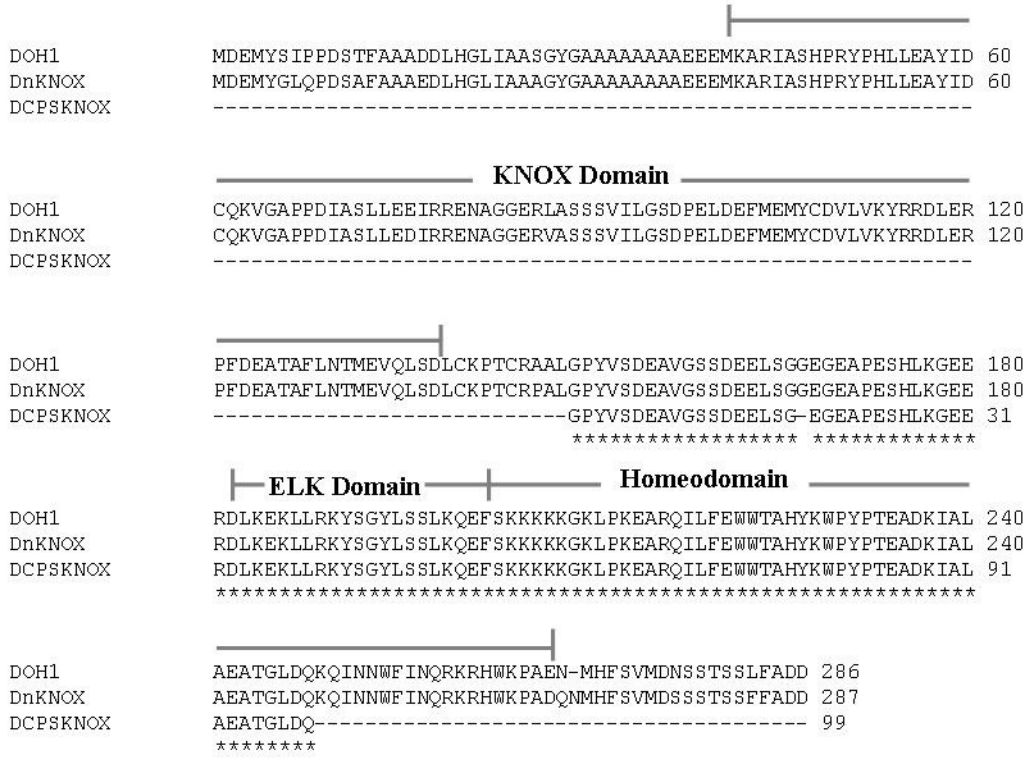

Fig. 4.8 Amino acid alignment of partial DCPSKNOX with KNOXs from *Dendrobium* grex Madame Thong-IN (DOH1; CAB88029) and *Dendrobium* nobile (Dnknox; AAT72917). Sequences were aligned using the CLUSTAL W program (Thompson *et al*., 1994). The KNOX domain, ELK domain and the homeodomain are indicated. The positions of amino acids are given on the right. The stars below each line of alignment indicate conserved sites.

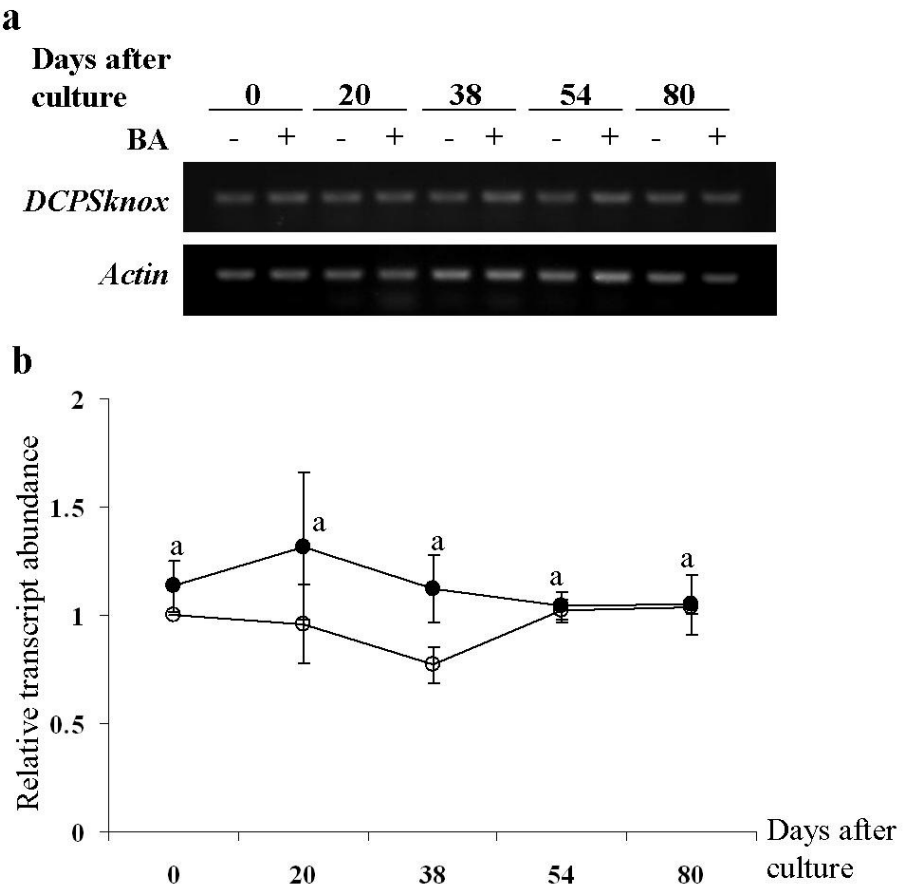

Fig. 4.9 Expression of *DCPSknox* in *D.* Chao Praya Smile cultures at juvenile (0, 20 and 38 days after culture), floral transition (54 days after culture) and flowering (80 days after culture) stages. (a) Semi-quantitative RT-PCR analysis of *DCPSknox*. Flowering was induced using 11.1 µM of BA and non-induced plantlets were used as controls. Total RNA was extracted and reverse transcribed for the analysis. Expression of *Actin* gene was used as control. (b) Relative levels of *DCPSknox* transcripts for non-induced (open circles) and BA-induced (closed circles) cultures at various stages. Transcript level of non-induced cultures at day 0 was arbitrarily set as 1. Transcript levels for *DCPSknox* products were determined by intensity-based quantification of each product using ImageJ software. Same letters above the closed circles indicate no significant difference among the relative transcript abundances (One-Way ANOVA Tukey's test at 95 % confidence level). Vertical bars denote SE. n = 3.

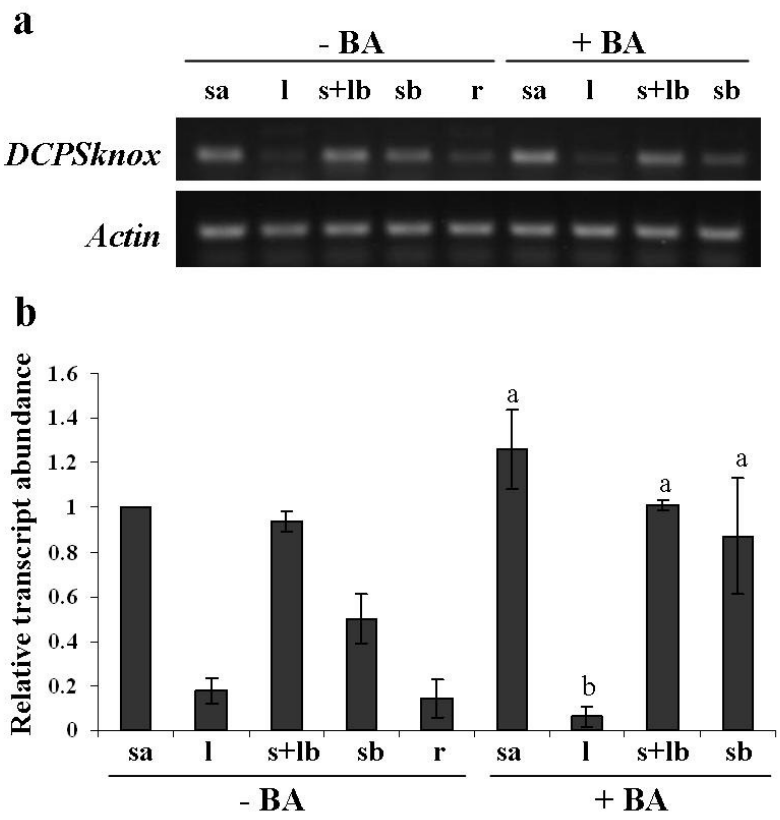

Fig. 4.10 Expression of *DCPSknox* in different tissues of non-induced and BA-induced plantlets after 54 days of culture. (a) Semi-quantitative RT-PCR analysis of *DCPSknox* in different tissues. sa, l, s+lb, sb and r refer to shoot apex, leaf, stem and leaf base, stem base and root, respectively. Total RNA was extracted from the respective tissues and reverse transcribed for the analysis. Expression of *Actin* gene was used as control. (b) Relative levels of *DCPSknox* transcripts in different tissues of non-induced and BA-induced plantlet. Transcript levels of *DCPSknox* in the shoot apex of non-induced plantlet were arbitrarily set as 1. Transcript levels for *DCPSknox* products were determined by intensity-based quantification of each product using ImageJ software. Same letters above the bars indicate no significant difference among the relative transcript abundances One-Way ANOVA Tukey's test at 95 % confidence level). Vertical bars denote SE. n = 3.

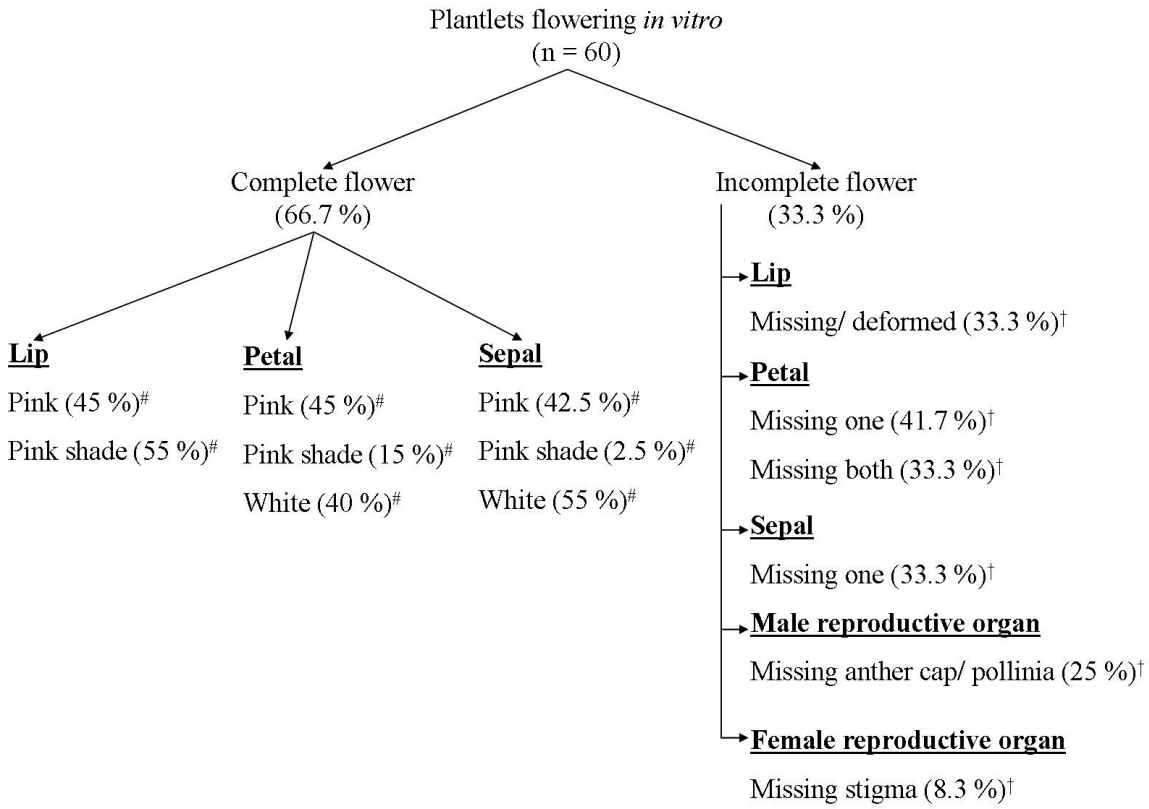

Fig. 4.11 Analyses of development and color segregation of *in vitro*-developed flowers of *D.* Chao Praya Smile. The colors of lip, petal and sepal (pink and pink shade) are illustrated in Fig. 4.12. Scoring of characteristics was made on 60 flowers developed *in vitro*.
#Numbers in the parentheses indicate the percentage of complete flowers.
†Numbers in the parentheses indicate the percentage of incomplete flowers.

and pattern of floral organs in these complete flowers showed that the lips could be completely pink or in pink shade, while the petals and sepals could be completely pink, in pink shade or completely white. Therefore, color segregation was manifested in flowers produced *in vitro*. Of all the color combinations, flowers with all pink floral organs (Fig. 4.12d, type-4 flower) and those with pink-shade lips, white sepals and white petals (Fig. 4.12a, type-1 flower) represented the two major flower types produced *in vitro*, which made up 42.5 and 40 %, respectively, of the complete flowers. The other two flower types were those with pink lips, pink-shade petals and white sepals (Fig. 4.12b, type-2 flower) and those with pink lips, pink petals and pink-shade sepals (Fig. 4.12c, type-3 flower), which made up 15 and 2.5 %, respectively, of the complete flowers.

Among the incomplete flowers developed *in vitro*, about 41.7 % lacked one petal and 33.3 % lacked two petals (Fig. 4.11, Fig. 4.13a). One third of the incomplete flowers were found to have one sepal missing or with deformed lip (Fig. 4.13b). Under-developed male or female reproductive organs were also occasionally observed (Fig. 4.13c and d).

4.3.5 Cloning and expression of *DCPSCHS* in *D.* Choa Praya Smile

RT-PCR was carried out using total RNA from shoot apex as template and primers that were designed based upon the conserved regions in *Dendrobium* orchid *CHS*s. A PCR product band resolved on agarose gel electrophoresis of approximately the correct size (295 bp) was purified. Nucleotide sequence analysis revealed that the isolated fragment represented the partial sequence of *DCPSCHS*, showing more than 94 % sequence similarity to the corresponding regions of *CHS*s from *Dendrobium* orchids (Fig. 4.14). The fragment also showed 80 and 70 % sequence similarity, respectively, to the

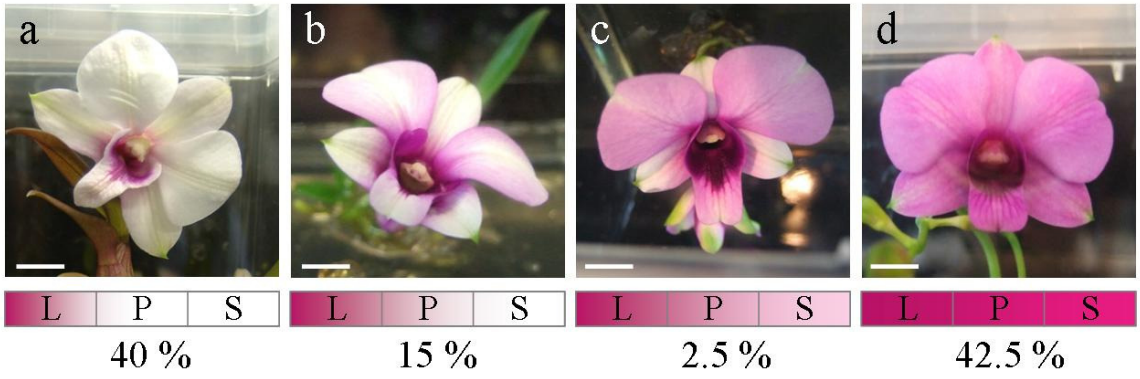

Fig. 4.12 Color segregation of *D.* Chao Praya Smile flowers developed *in vitro*. Four color types were found: a) Pink-shade lip with white petals and sepals (type-1), b) Pink-shade lip with pink-shade petals and white sepals (type-2), c) Pink lip with pink sepals and pink-shade sepals (type-3), and d) Lip, sepals and petals all in pink (type-4). Color bars below the pictures indicate the colors of the respective floral organs. L, P and S refer to lip, petal and sepal, respectively. The percentage of each type of flowers was also indicated below the pictures (n = 40). Bar = 5 mm.

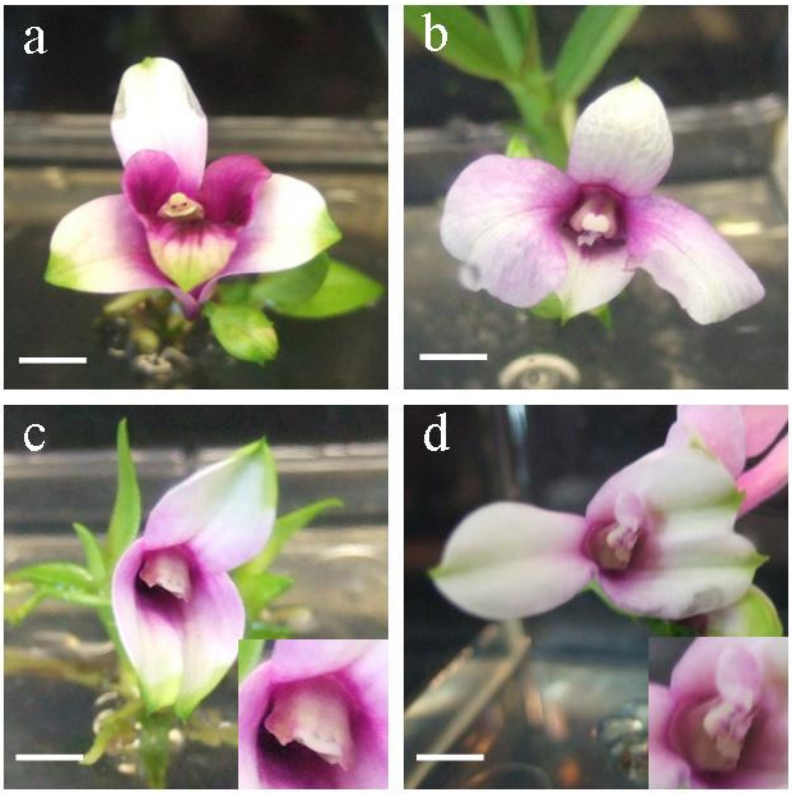

Fig. 4.13 Morphology of incomplete flowers produced *in vitro*. a) Both petals were not formed; b) The lip and a sepal were not developed; c) A severely malformed *in vitro*-developed flower without lip and petals. The under-developed male reproductive organ without the formation of anther cap and pollinia was shown in the inset; d) Incomplete flower with severely distorted and unrecognizable male and female reproductive organs (inset). Bar = 5 mm.

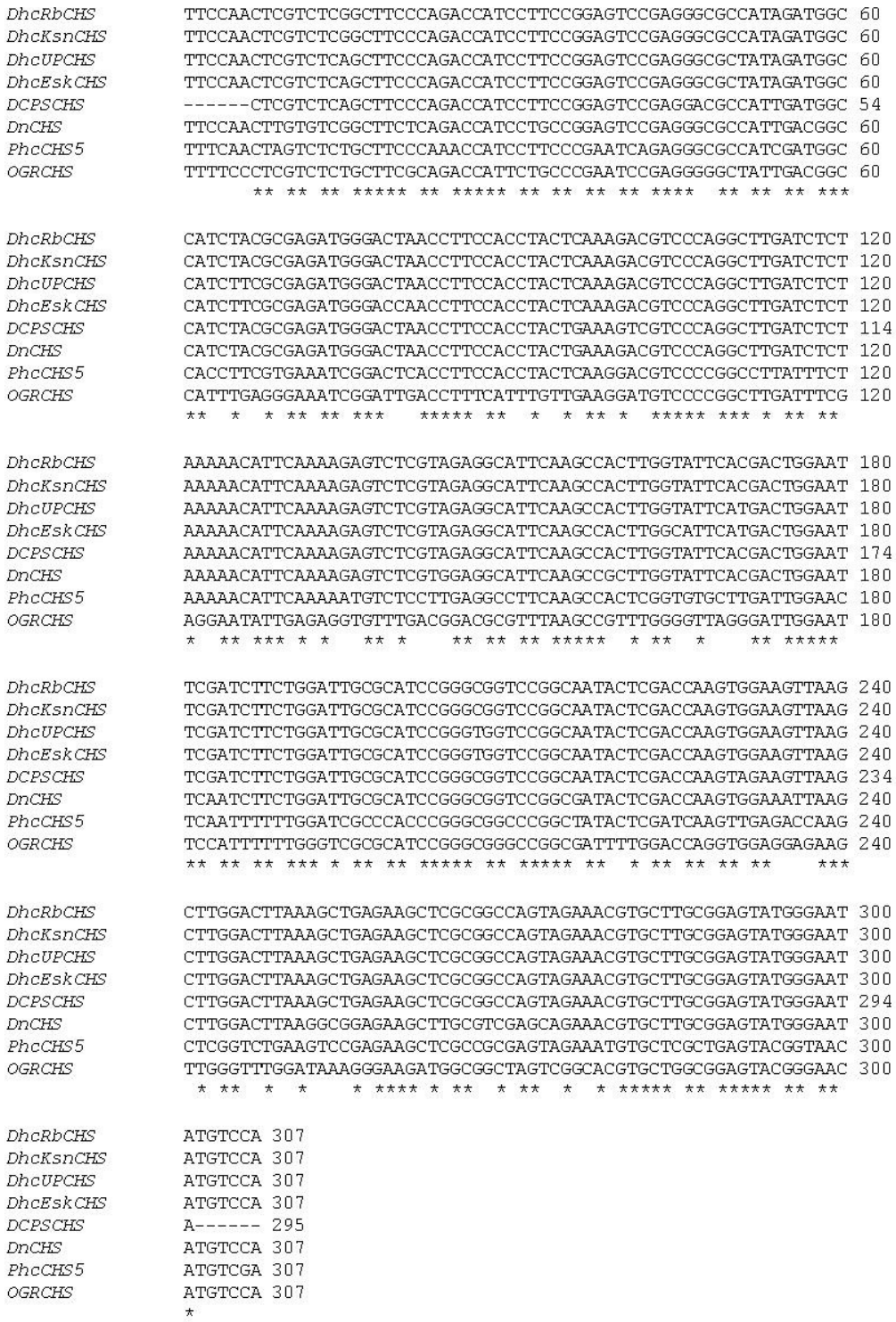

Fig. 4.14 Nucleotide alignment of partial *DCPSCHS* with *CHS*s from other orchid species: *Dendrobium* hybrid cultivar "Red bull" (*DhcRbCHS*; FM209430), *Dendrobium* hybrid cultivar "Kao sa nan" (*DhcKsnCHS*; FM209429), *Dendrobium* hybrid cultivar "Uniwai Prince" (*DhcUPCHS*; AY741319), *Dendrobium* hybrid cultivar "Ear sa kul" (*DhcEskCHS*; AM490639), *Dendrobium* nobile (*DnCHS*; DQ462460), *Phalaenopsis* hybrid cultivar (*PhcCHS5*; DQ089652) and *Oncidium* Gower Ramsey (*OGRCHS*; EF570111). Sequences were aligned using the CLUSTAL W program (Thompson *et al*., 1994). The positions of nucleotides are given on the right. The stars below each line of alignment indicate conserved sites.

corresponding regions of *CHS*s from *Phalaenopsis* and *Oncidium* orchids. At the protein level, the deduced amino acid sequence of the isolated fragment showed more than 95 %, 90 % and 80 % identity, respectively, to the corresponding peptide sequences from *Dendrobium*, *Phalaenopsis* and *Oncidium* orchids (Fig. 4.15). In view of the high sequence similarity at nucleotide and protein levels, the isolated fragment was used to analyze *DCPSCHS* expression in *D.* Chao Praya Smile.

DCPSCHS was expressed at different levels in 5 non-induced and 5 BA-induced plantlets after 54 days of culture (Fig. 4.16). The highest and the lowest expression levels in the plantlets were within 50 % from the average expression level. *DCPSCHS* expression was generally lower in BA-induced plantlets as compared to that in non-induced plantlets.

4.4 Discussion

During plant growth and development, the SAM produces leaves at the vegetative phase and floral organs at the reproductive phase. Observation of morphological changes of BA-induced and non-induced plantlets indicated that they exhibited similar growth pattern in liquid media during the first 54 days of culture, other than the fact that more roots were formed in plantlets grown in medium without BA (Fig. 4.3, Fig 4.4). Subsequently, flowering was induced in BA-induced plantlets while the non-induced plantlets remained vegetative. It was also observed that flowering plantlets produced extensive roots just as the non-induced vegetative plantlets (Fig 4.3), and the number of leaves produced at flowering (Table 4.1) was similar to that in field-grown plants (Table 3.5). Therefore, the morphological observation (Fig. 4.3, Fig 4.4, Table 4.1) might imply

```
DCPSCHS         --LVSASQTILPESEDAIDGHLREMGLTFHLLKVVPGLISKNIQKSLVEAFKPLGIHDWN 58
DhcUPCHS        FQLVSASQTILPESEGAIDGHLREMGLTFHLLKDVPGLISKNIQKSLVEAFKPLGIHDWN 60
DhcRbCHS        FQLVSASQTILPESEGAIDGHLREMGLTFHLLKDVPGLISKNIQKSLVEAFKPLGIHDWN 60
DhcKsnCHS       FQLVSASQTILPESEGAIDGHLREMGLTFHLLKDVPGLISKNIQKSLVEAFKPLGIHDWN 60
DhcEskCHS       FQLVSASQTILPESEGAIDGHLREMGPTFHLLKDVPGLISKNIQKSLVEAFKPLGIHDWN 60
DnCHS           FQLVSASQTILPESEGAIDGHLREMGLTFHLLKDVPGLISKNIQKSLVEAFKPLGIHDWN 60
PhcCHS5         FQLVSASQTILPESEGAIDGHLREIGLTFHLLKDVPGLISKNIQKCLLEAFKPLGVLDWN 60
OGRCHS          FSLVSASQTILPESEGAIDGHLREIGLTFHLLKDVPGLISRNIERCLTDAFKPFGVRDWN 60
                  *************.********:* ****** ******:**::.* :****:*: ***


DCPSCHS         SIFWIAHPGGPAILDQVEVKLGLKAEKLAASRNVLAEYGN-- 98
DhcUPCHS        SIFWIAHPGGPAILDQVEVKLGLKAEKLAASRNVLAEYGNMS 102
DhcRbCHS        SIFWIAHPGGPAILDQVEVKLGLKAEKLAASRNVLAEYGNMS 102
DhcKsnCHS       SIFWIAHPGGPAILDQVEVKLGLKAEKLAASRNVLAEYGNMS 102
DhcEskCHS       SIFWIAHPGGPAILDQVEVKLGLKAEKLAASRNVLAEYGNMS 102
DnCHS           SIFWIAHPGGPAILDQVEIKLGLKAEKLASSRNVLAEYGNMS 102
PhcCHS5         SIFWIAHPGGPAILDQVETKLGLKSEKLAASRNVLAEYGNMS 102
OGRCHS          SIFWVAHPGGPAILDQVEEKLGLDKGKMAASRHVLAEYGNMS 102
                ****:************* ****.  *:*:**:*******
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Fig. 4.15 Amino acid alignment of partial DCPSCHS with CHSs from other orchid species: *Dendrobium* hybrid cultivar "Uniwai Prince" (DhcUPCHS; AAU93767), *Dendrobium* hybrid cultivar "Red bull" (DhcRbCHS; CAR64528), *Dendrobium* hybrid cultivar "Kao sa nan" (DhcKsnCHS; CAR64527), *Dendrobium* hybrid cultivar "Ear sa kul" (DhcEskCHS; CAM32716), *Dendrobium* nobile (DnCHS; ABE77392), *Phalaenopsis* hybrid cultivar (PhcCHS5; AAY83389) and *Oncidium* Gower Ramsey (OGRCHS; ABS58499). Sequences were aligned using the CLUSTAL W program (Thompson *et al*., 1994). The positions of amino acids are given on the right. The stars below each line of alignment indicate conserved sites.

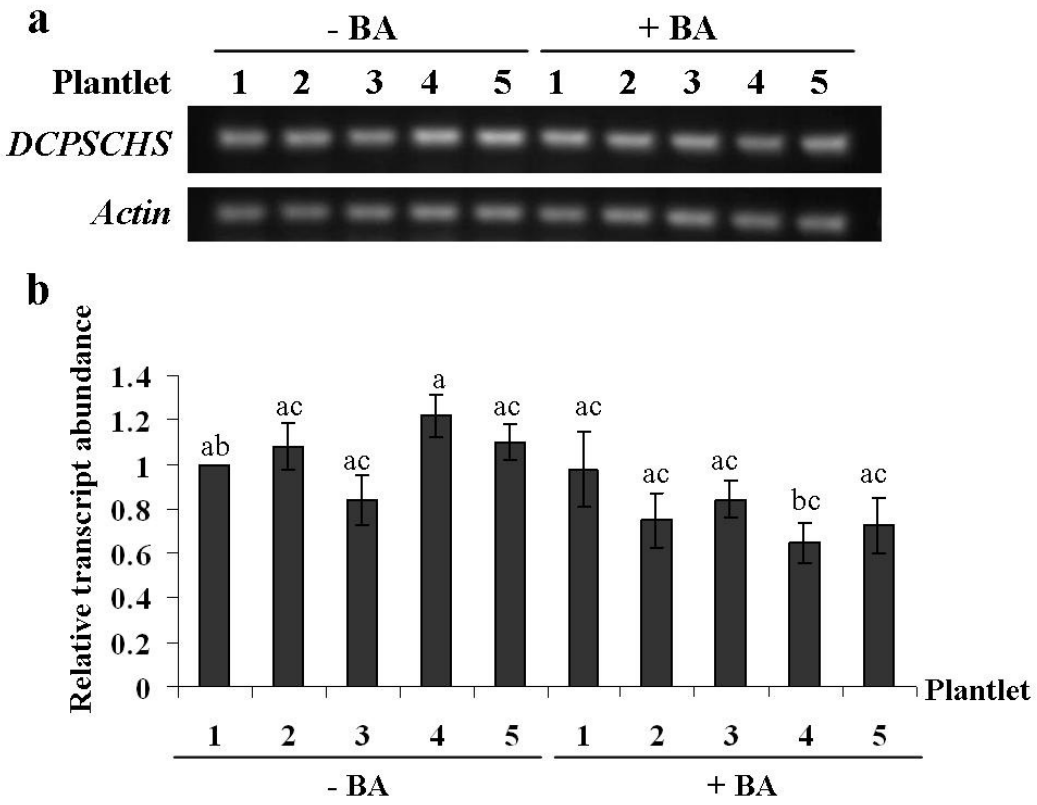

Fig. 4.16 Expression of *DCPSCHS* in non-induced and BA-induced plantlets after 54 days of culture. (a) Semi-quantitative RT-PCR analysis of *DCPSCHS*. Total RNA were extracted from the plantlets and reverse transcribed for the analysis. Expression of *Actin* gene was used as control. (b) Relative levels of *DCPSCHS* transcripts in five non-induced and five BA-induced plantlets. Transcript level of *DCPSCHS* in non-induced plantlet numbered 1 was arbitrarily set as 1. Transcript levels for *DCPSCHS* products were determined by intensity-based quantification of each product using ImageJ software. Same letters above the bars indicate no significant difference among the relative transcript abundances (One-Way ANOVA Tukey's test at 95 % confidence level). Vertical bars denote SE. n = 3.

that BA induced flowering in *D.* Chao Praya Smile not by accelerating growth or promoting maturation. On the other hand, BA might promote flowering by inhibiting root production during early plantlet growth and thereby limited the accumulation of root-produced substances that inhibited flowering. Apparently, root production did not affect flower development once the SAM was committed to flowering. The effect of root production on flowering inhibition has been shown in *Cymbidium niveo-marginatum* Mak in which the removal of roots greatly enhanced flowering in the orchid in *vitro* (Kostenyuk *et al.*, 1999). It was also reported in tobacco that a specific signal produced in the root could prevent flowering (McDaniel, 1996).

Temporal and spatial changes of growth would take place at the SAM during floral transition. In the development of an inflorescence, leaves would be replaced by bracts while flowers would be formed in the bract axils. The SAM growth would remain indeterminate for a considerable number of plastochrons until eventually the terminal flower was formed (Kwiatkowska, 2008). The histological analyses (Fig 4.5, Fig 4.6) successfully identified the juvenile, floral transition and floral bud development phases, phases that were associated with different morphological changes in the SAM in BA-induced plantlets. The timing of floral transition in the system of early *in vitro* flowering in *D.* Chao Praya Smile was also determined (Fig 4.5, Fig 4.6). In *Anagallis arvensis*, similar quantitative and geometrical analyses were conducted with the shoot apex for the comparative investigation of morphogenesis during reproductive and vegetative phases (Kwiatkowska and Routier-Kierzkowska, 2009). The SAM of the BA-induced plantlets was relatively flat in the juvenile phase. After 54 days of culture, the BA-induced plantlets proceeded to floral transition which was characterized by bolting as well as the

expansion of SAM both in height and width (Fig 4.5, Fig 4.6). An increase in both the meristem volume and apical height was observed in the shoot apex of *S. alba* at floral transition (Bernier, 1997). In addition, these morphological changes preceded the initiation of floral primordia, as was observed in the shoot apex of *D.* Chao Praya Smile (Fig 4.5). Bolting or elongation of the stem axis was also observed in shoot apex of *Arabidopsis* (Jacqmard *et al.*, 2003) and *Fragaria sp.* (Kurokura *et al.*, 2006) at floral transition, which was a result of rapid cell division in the central zone of SAM that also gave rise to subsequent floral organ formation (Kwiatkowska, 2008). The process of flowering in orchids can be separated into two processes: flower initiation and floral development (Hew and Yong, 1997). Following initiation, the flower bud will grow and its subsequent growth depends on the supply of photoassimilates from leaves and from its own photosynthesis. Histological observation (Fig 4.5) showed that floral initiation was induced when the plantlets were grown in liquid medium, and floral development took place in the two-layer medium.

knox genes encoded the homeodomain-containing transcription factors, which were required for the maintenance of the meristem and proper patterning of organ initiation (Hake *et al.*, 2004). *knox* genes were also found to regulate morphological events in *Arabidopsis*, in which ectopic expression of these genes resulted in reductions in the sizes of leaves, reductions in the size of sepals and petals, the formation of a less prominent midvein, the repression of adventitious root formation and late flowering (Ikezaki *et al*., 2010). During floral organ differentiation and development, the expression of *knox* genes in *Arabidopsis* was repressed by *Auxin Response Factor6* (*ARF6*) and *ARF8*, which regulated jasmonic acid biosynthesis (Tabata *et al*., 2010). KNOX proteins

were suggested to act as general orchestrators of growth regulator homeostasis in the maintenance of the SAM by simultaneously activating cytokinins and repressing gibberellic acid biosyntheses (Jasinski *et al.*, 2005; Yanai *et al.*, 2005). Over-expression of *knox* genes would lead to abnormal cell division and the formation of ectopic meristems (Schneeberger *et al.*, 1995; Chuck *et al.*, 1996). It was also shown that constitutive expression of *knox1* in *Taraxacum officinale* could change the leaf morphology from simple to compound (Müller *et al.*, 2006). In *D.* Madame Thong-In, *DOH1*, a class I *knox* gene, was required to maintain the basic plant architecture, in which multiple shoots were produced simultaneously when its expression was reduced (Yu *et al.*, 2000). The partial sequence of *DCPSknox* was highly similar with the corresponding sequences from *D.* Madame Thong-In and *D.* nobile, indicating that *knox* gene was conserved among the *Dendrobium* orchids (Fig. 4.7, Fig 4.8). The expression of *DCPSknox* was studied at different growth stages and in various tissues of *D.* Chao Praya Smile in relation to BA induction of flowering (Fig 4.9). The expression of *DCPSknox* in BA-induced plantlets was marginally but insignificantly higher than that in the non-induced plantlets during early vegetative development (Fig 4.9). The expression of *DCPSknox* could be affected by BA application because it was reported that cytokinins could stimulate *KNOX* gene expression and vice versa (Kusaba *et al.*, 1998). Although *knox* gene was reportedly involved in the early seedling development of *Arabidopsi*s, its expression did not increase with cytokinin treatment (Souček *et al.*, 2007). Similarly, expression of *BP* and *STM* were not significantly increased in transgenic *Arabidopsis* over-expressing a cytokinin biosynthesis gene (Craft *et al.*, 2005) or in young seedlings treated with exogenous cytokinins (Rashotte *et al.*, 2003). Nevertheless, an early and

transient increase in the transcript of *BP* and *KNAT4* was observed in *Arabidopsis* seedlings ectopically expressed a cytokinin biosynthesis gene (Hoth *et al.*, 2003). Expression of *DCPSknox* in BA-induced *D.* Chao Praya Smile plantlets was reduced at floral transition and during floral bud development (Fig. 4.9). The expression of *kn1* and *DOH1* in the SAM of *Arabidopsis* and *D.* Madame Thong-In, respectively, were also found to decrease during floral transition (Lincoln *et al.*, 1994; Yu *et al.*, 2000). The reduction in *DCPSknox* expression at floral transition probably indicated the commitment of SAM to floral organ production at these stages considering the function of *knox* gene in maintaining the indeterminacy of SAM.

knox was expressed in the shoot meristem at all growth stages and played a role in morphogenesis (Lincoln *et al.*, 1994). At bolting, the expression of *DCPSknox* in the shoot apex of BA-induced plantlet was not significantly different from that in vegetative shoot apex (Fig. 4.10). Yu *et al.* (2000) reported that the expression of *DOH1* would remain in the outer cell layers of the inflorescence meristem and throughout the floral primordia. *DCPSknox* was also found to be expressed at a low level in the roots of non-induced plantlet (Fig 4.10). In contrast, the expression of *DOH1* was not detected in the roots of *D.* Madame Thong-In (Yu *et al.*, 2000). In other plant species such as *Arabidopsis*, *KNAT6* was expressed in the roots for correct lateral root formation (Dean *et al.*, 2004). Moreover, *GmKNT1* was found to be strongly expressed in the roots of soybean (Liu *et al.*, 2008). Other homologues of *KNOX* genes in *D.* Chao Praya Smile could be cloned and investigated to better understand the function of these genes in the plantlets.

Segregation of flower colors is commonly observed in progenies of orchid hybridization. For example, the cross between *Dendrobium* Peewee and *Dendrobium* Chao Praya Gem produced progenies with variations in flower colors (Fadelah, 2006). The cross between *Dendrobium dicuphum* (semi-alba, flower with white sepals and petals, and a purple lip) and *Dendrobium Phalaenopsis* var. *compactum* 'Mauna Kea' (alba, flower with all white floral organs) produced uniformly purple offsprings and the F_2 progenies segregated into three discreet groups: purple, semi-alba and alba (Kamemoto *et al.*, 1999). Segregation of flower colors was also observed in *D.* Madame Thong-In plantlets that flowered *in vitro* in which four distinct flower types were produced (Sim *et al.*, 2007). Color segregation was observed in flowers of *D.* Chao Praya Smile produced *in vitro* with 40 % of them resembling the flowers of the parent plant (Fig.4.12, type-1 flower), from which the seeds were derived upon self-pollination. An almost equal proportion of the flowers produced were densely pigmented with all pink floral organs (Fig 4.12, type-4 flower). The remaining flowers represented the intermediate phenotypes between the type-1 and type-4 flowers. Interestingly, in the flowers produced *in vitro*, the lip of the flower appeared to be the center of pink coloration that radiated or flushed out to the petals and sepals to give rise to flowers of 4 color types (Fig 4.12). The gradient flushing of pink coloration to the lips, petals or sepals at different intensities would give rise to flowers of type-1, -2 and -3, respectively (Fig 4.12). Type-4 flowers were formed when the pink coloration from lips was flushed out thoroughly to all floral organs. *D.* Chao Praya Smile is semi-alba because it produces flowers with white sepals and petals, and a pink-shade lip, similar to *D. dicuphum*. It was therefore possible that the self-pollination of semi-alba plantlets could produce progenies

that segregated into semi-alba, pink and intermediate phenotypes as observed in the *in vitro* flowering of *D.* Chao Praya Smile. However, a larger sample size would be required to obtain an accurate ratio of segregation.

One-third of the flowers produced *in vitro* were incomplete due to missing floral organs such as sepals, petals, lips or reproductive organs (Fig 4.11, Fig 4.13). Similar incomplete or malformed flowers were also produced in *Dendrobium* "Sonia" (Tee *et al.*, 2008) and *Phalaenopsis* Pink Leopard "Petra" (Duan and Yazawa, 1995) plantlets induced to flower *in vitro*. The production of abnormal flowers could be due to the metabolism of cytokinins in tissue culture (Jones *et al.*, 1995) in view of the fact that BA was used to induce flowering. The absence of floral organs could possibly be due to the effects on genes controlling floral organ number such as *PAN*, *ETT*, *WIG* and *SUPERMAN* (Weiss *et al.*, 2005) but there was no evidence indicating the effect of cytokinins on these genes up to this date. The observed incomplete flowers could also be due to the failure of different floral primordia to grow during flower development.

Chalcone synthase (CHS) is a key enzyme in the biosynthesis of anthocyanins that give rise to color pigments in flowers (Springob *et al.*, 2003). It has been shown in *Torenia fournieri* that the degree of blue coloration in the corolla was affected by the expression of *CHS* gene (Aida *et al.*, 2000; Fukusaki *et al.*, 2007). Therefore, it was speculated that the different intensity of pink coloration in the floral organs of *in vitro*-developed flowers was attributed to different degrees of *CHS* expression in *D.* Chao Praya Smile. The gene encoding CHS (*DCPSCHS*) was cloned from *D.* Chao Praya Smile and its expression was analyzed in BA-induced and non-induced plantlets in order to find out whether BA treatment affected *CHS* expression, which in turn gave rise to

flowers of different color intensity (Fig. 4.12, type-1 to type-4 flowers). Partial sequence of *DCPSCHS* showed high similarity at nucleotide and peptide levels with the corresponding sequences from *Dendrobium*, *Phalaenopsis* and *Oncidium* orchids (Fig. 4.14, Fig. 4.15), indicating that *CHS* was highly conserved among the different orchid species. *DCPSCHS* was found to be expressed differently among the non-induced and BA-induced *D.* Chao Praya Smile plantlets (Fig 4.16), probably indicating that both types of plantlets had different degrees of anthocyanin biosynthesis in nature and hence pigment production. It could further imply that color segregation observed in the *in vitro*-produced flowers was naturally occurring and was not caused by the BA treatment in the culture media. Therefore, the *in vitro* flowering system was suitable for early evaluation of flower colors in orchid hybridization as natural flower colors were exhibited in the *in vitro*-developed flowers. Nonetheless, *DCPSCHS* expression was investigated in the vegetative tissues in this study and the gene might not have the same role in the flowers. Moreover, other isoforms of *CHS* gene could be present in *D.* Chao Praya Smile and yet to be investigated. Color segregation in different parts of floral organs could also be caused by physiological variation induced by tissue culture. Similarly, the possibility that BA treatment could have affected other genes involved in orchid color pigmentation such as *dihydroflavonol 4-reductase*, *flavanone 3-hydroxylase* and *Phenylalanine ammonia-lyase* could not rule out (Yu and Goh, 2001).

4.5 Concluding remarks

Plantlets of *D.* Chao Praya Smile grew rapidly in modified KC liquid medium before they were transferred to the two-layer medium after 54 days of culture, when 4 to 5 expanded leaves were already produced. Subsequently, flower buds were developed in BA-induced plantlets and flowering took place in 105 days after BA treatment. Histological analysis revealed the different phases of growth in BA-induced plantlets. Most importantly, the BA-induced plantlets were shown to proceed to the flowering phase, as indicated by the bolting of the shoot apices, after growing in liquid medium for 54 days. Therefore, flowering was initiated in the liquid medium while subsequent flower development took place in the two-layer medium. A partial sequence of *knox* (*DCPSknox*) gene was cloned from *D.* Chao Praya Smile and its expression decreased at stages corresponding to floral transition and floral bud development. On the other hand, color segregation was observed in the *in vitro*-developed flowers of *D.* Chao Praya Smile with 4 different color types. The segregation of colors was most likely naturally occurring because BA treatment did not affect the expression of *DCPSCHS*, the gene encoding the key enzyme in anthocyanin biosynthesis.

Chapter 5

Changes in cytokinins and IAA contents in flowering-induced *Dendrobium* Chao Praya Smile

5.1 Introduction

Cytokinins and IAA are plant growth regulators that control various aspects of plant development as well as the interaction of plant with the external environment (Reski, 2006). The involvement of cytokinins in flowering has been demonstrated in various plant species. Bernier *et al.* (1993) proposed that cytokinins could serve as flowering signals that were translocated from the root to the shoot in *Sinapis alba* upon flowering induction. In addition, their concentrations were shown to increase during floral transition in this plant species (Bernier *et al.*, 2002). Similarly, mutant and transgenic *Arabidopsis* and *Brassica napus* that flowered early were found to have elevated levels of cytokinins (Chaudhury *et al.*, 1993; Srivastava *et al.*, 2006; Dunfield *et al.*, 2007). Besides, shoot apices of *B. napus, Chenopodium rubrum* and *C. murale* were also found to be enriched with cytokinins during floral transition (de Bouillé *et al.*, 1989; Macháckova *et al.*, 1993). In orchids, the role of cytokinins in promoting flowering has been demonstrated in various foliar spray or cytokinin injection experiments (Sakai *et al.*, 2000; Blanchard and Runkle, 2008).

There was no consensus on the type of cytokinins that caused flowering in plants. It was found that iPR content in *S. alba* (Lejeune *et al.*, 1988) was significantly increased when grown under flowering-promoting conditions. Similarly in some *in vitro* studies, flowering induction in *Arabidopsis* (He and Loh, 2002) and *Dendrobium* (Sim *et al.*, 2007) have been correlated with increased iPR content in the plants. On the other hand, there were reports indicating that the ratio of cytokinin to auxin was closely associated with the developmental phase change to flowering (Mercier and

Endres, 1999; de Melo Ferreira *et al.*, 2006; Hegele *et al.*, 2008). All these studies across different plant species have indicated that endogenous cytokinins might be crucial in the induction of flowering. Also, a sensitive method for the determination of cytokinins is essential to reveal the specific roles of cytokinins in flowering induction. Therefore, the objectives of this chapter were: (1) to develop a sensitive and efficient HPLC-ESI-MS/MS method for the determination and quantification of cytokinins and IAA; (2) to analyze the cytokinin and IAA contents in *D.* Chao Praya Smile at different growth stages upon flowering induction; (3) to analyze the cytokinin and IAA contents in the shoot apices and other tissues in the plantlets at floral transition; and (4) to clone and analyze the expression of cytokinin oxidase/dehydrogenase gene (*DCPSCKX*) in relation to flowering.

5.2 Materials and methods

5.2.1 Plant materials for the analyses of cytokinins and IAA

Eight-week-old protocorms of *D.* Chao Praya Smile germinated from seeds were cultured in modified KC liquid culture medium containing (mg l^{-1}): $MgSO_4.7H_2O$ (250), KH_2PO_4 (500), $(NH_4)_2SO_4$ (250), $Ca(NO_3)_2.4H_2O$ (500), $MnSO_4.H_2O$ (5.68) and EDTA-Fe (28) supplemented with 2 % (w/v) sucrose, 15 % (v/v) coconut water and 11.1 µM BA in 100 ml Erlenmeyer flasks on rotary shakers at 120 rpm. These BA-induced plantlets were sub-cultured in BA-containing liquid media after 20 and 38 days of culture. BA-treatment was continued in two-layer modified KC media after 54 days of culture at which the plantlets were allowed to grow until flowering (observed after 80 days of culture). BA-induced plantlets were harvested 0, 20, 38, 54 and 80 days after culture for the analyses of cytokinins and IAA. For tissue-specific analyses of cytokinins and IAA, (a) shoot apices, (b) stems

and leaf bases, (c) stem bases and (d) leaves (Fig. 4.2) were isolated from BA-induced plantlets after 54 days of culture. Plantlets cultured in media without BA (non-induced plantlets) were harvested at the same times and used as control. In addition, roots were isolated from non-induced plantlets for analysis.

5.2.2 Cytokinin and IAA extraction and separation by high performance liquid chromatography (HPLC)

Samples were ground in Bieleski solution [chloroform: methanol: formic acid: water (25: 60: 5: 10, v/v) at 1 ml per 0.1 g of plant material] (Bieleski, 1964) containing deuterium-labeled internal standards for the various compounds (0.5 mM for cytokinins and 3.5 mM for IAA), and left to extract overnight at -20 ˚C in the dark. The deuterium-labeled compounds were used as internal standards for the quantification of each compound. Each sample was then centrifuged at 10,000 *g* for 10 min at 4 ˚C and the supernatant was transferred to a clean tube. The pellet was re-extracted in 4 ml of 80 % methanol for 1 h at 4 ˚ C on rotary shaker, and re-centrifuged. The supernatants were pooled and loaded on an Oasis HLB cartridge (Waters, 60 mg of solid phase), which had been equilibrated with 1 ml of 100 % methanol followed by 1 ml of 80 % methanol. The cartridge was rinsed with 500 µl of 80 % methanol. The purified extract was dried in a vacuum evaporator and reconstituted with 400 µl of Milli-Q water acidified with formic acid (at a final concentration of 15 mM and pH adjusted to 4.0 using ammonium hydroxide). Prior to HPLC separation, the samples were transferred to 1.5 ml Eppendorf tubes and centrifuged at 16,000 *g* for 5 min to remove any particulate matter that might have carried over through the purification process. To separate the cytokinins and IAA in the sample, 100 µl of the sample were injected into an Äkta^{TM} purifier (Amersham

Pharmacia Biotech) equipped with a 100 mm × 4.6 mm × 3.5 µm XBridge™ C18 column (Waters) and connected to a UV detector monitored at 268 nm. A binary solvent system was used; it was comprised of (A) 70 % methanol and (B) Milli-Q water acidified with formic acid (at a final concentration of 15 mM and pH adjusted to 4.0 using ammonium hydroxide). Separations were performed using segmented gradients of methanol from 0 to 20 % in 0.1 column volume (cv) followed by 20 % to 70 % in 12 cv and finally 70 % to 100 % in 0.1 cv with the flow rate maintained at 0.5 ml min^{-1}. For elution, fractions of 0.3 ml were collected using a fraction collector. Fractions containing the individual cytokinin or IAA, and each of the corresponding deuterium-labeled internal standard were pooled, dried in a vacuum evaporator and reconstituted in 200 µl of mass spectrometry buffer [50 % (v/v) methanol containing 15 mM formic acid]. All HPLC data were processed by the Unicorn v4.0 software.

5.2.3 Quantification of cytokinins and IAA by electrospray ionization mass spectrometry (ESI-MS/MS)

Standard solutions (10 µM) for each of the labeled and unlabeled cytokinins and IAA were prepared in 50 % methanol (v/v) containing 15 mM formic acid. For the selection of diagnostic precursor-to-product ion transitions, 20 µl of 10 µM standards for each labeled and unlabeled cytokinins and IAA were injected into the API 300™ triple quadrupole mass spectrometer (Applied Biosystems) outfitted with an electrospray (ES) ion source. ES capillary and cone voltage were optimized (source temperature 100 °C, capillary voltage +2.0 kV, cone voltage 20 V) for the production of the requisite molecular (precursor) ions in positive ionization mode. The collision energy was then optimized at 20 eV for the dissociation of molecular ions into diagnostic fragment (product) ions for each compound. The flow rate of the

mobile phase [50 % (v/v) methanol containing 15 mM formic acid] was maintained at 20 µl min^{-1}. Once the characteristic precursor-to-product ion transitions had been determined, calibration curves were generated by preparing serial concentrations of each unlabeled compound (0 to 1000 pmol) and analyzed for the product ion peak area. Quantification of cytokinins and IAA in the fractions collected from HPLC was done by Multiple Reaction Monitoring of $[MH]^+$ (dwell time 0.1 s) and the appropriate product ion. All data were processed by the MDS Sciex software. Results were expressed in pmol or nmol of cytokinins or IAA per unit of plantlet or tissue fresh weight (FW).

5.2.4 Cloning of *D.* Chao Praya Smile *CKX* (*DCPSCKX*) gene

RNA from shoot apices of BA-induced plantlets was isolated using the Plant RNeasy extraction kit (Qiagen, Hielden, Germany), followed by cDNA synthesis from 1 µg of total RNA using random hexamers and ThermoScript™ Reverse Transcriptase (Invitrogen). One twentieth volume of each cDNA was used as a template for PCR amplification using the following primers for the target gene: *DCPSCKX*: 5'-TCTCCCCTCACTCATTCACC-3' (forward), 5'-ATCTCACGCTTTGAGGTGCT-3' (reverse). The PCR products were isolated from 1 % agarose gels following electrophoresis and cloned into pGEM-T Easy (Promega, Madison, WI) and sequenced. Nucleotide sequences were analyzed using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI 3730*xl* DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Nucleotide and amino acid sequence alignments were generated using Clustal W (Thompson *et al.*, 1994).

5.2.5 Gene expression analysis by semi-quantitative RT-PCR

Total RNA was isolated using the Plant RNeasy extraction kit (Qiagen, Hielden, Germany). For time course analyses of BA-induction of gene expression, non-induced and BA-induced (11.1 µM) *D.* Chao Praya Smile plantlets were harvested on day-0, 20, 38, 54 and 80 after treatment and total RNA was extracted. For tissue-specific expression analysis, total RNA was isolated from shoot apices (sa), stems and leaf bases (l + lb), stem bases (sb) and leaves (l) from non-induced and BA-induced (11.1 µM) plantlets after 54 days of culture (Fig. 4.2). Total RNA was also isolated from the roots of non-induced plantlets. For treatment-specific expression analysis, total RNA was isolated from the shoot apices of plantlets treated with 11.1 µM BA, 22.2 µM iP, 22.2 µm iPR, 0.5 µm IAA + 11.1µm BA or 2 µm TIBA after 54 days of culture. Total RNA extracted for the various analyses was reverse-transcribed for RT-PCR using the same sets of primers for the cloning of *DCPSCKX*. PCR amplifications began with a 2 min denaturation at 94 ˚C and continued for 40 cycles at 94 ˚C for 40 s, 58 ˚C for 1 min, and 72 ˚C for 1 min, followed by a 7 min extension at 72 ˚C. *Actin* gene was used as control and PCR was carried out with the primers 5'-GCTGCTCGTGACCTGACTGA-3' (forward) and 5'-ACGGAACCTCTCAGCTCCAA-3' (reverse), using the same amplification protocol. Each PCR product was resolved on 1 % agarose gels and stained with SYBR® Safe (Invitrogen). Bands were visualized and analyzed using the ImageJ software (v1.42, Wayne Rasband, NIH). To minimize sample variations, mRNA expression of the target gene was normalized relative to the expression of the house keeping gene *Actin*. All experiments were repeated three times for cDNA prepared for each batch of plantlets or tissues.

5.2.6 Effects of iP, iPR, IAA and TIBA on induction of flowering

Eight-week-old protocorms of *D.* Chao Praya Smile germinated from seeds were cultured in modified KC liquid culture medium supplemented with 2 % (w/v) sucrose, 15 % (v/v) coconut water in 100 ml Erlenmeyer flasks on rotary shakers at 120 rpm. The liquid media were also supplemented with iP (0 – 44.4 μM), iPR (0 – 44.4 μM), IAA (0 – 2.5 μM, and 11.1 μM of BA) or TIBA (2,3,5-triiodobenzoic acid, 0 – 10 μM). The plantlets were sub-cultured in the liquid medium after 20 and 38 days of culture and transferred to the two-layer modified KC medium in Magenta GA7^{TM} containers after 54 days of culture. All cultures were incubated at 25 ± 2 ˚C and a 16 h photoperiod of 40 μmol m^{-2} s^{-1} from daylight fluorescent lamps and observed for flowering.

5.3 Results

5.3.1 Retention times for the cytokinins and IAA separated by HPLC

All unlabeled analytes {Z, iP, DHZ and their respective derivatives [riboside (-R), glucoside (-G) and nucleotide (-MP)], BA and IAA} and their respective deuterium-labeled internal standards were well separated by HPLC in less than 50 min (Fig. 5.1a and 5.1b, respectively). For each type of cytokinin, the nucleotide was eluted first followed by the glucoside, free base and the riboside. When the analytes and their deuterium-labeled internal standards were injected into the HPLC, each internal standard was co-eluted with its analyte (Fig 5.1c). Retention times for the analytes and internal standards were summarized in Table 5.1. Each co-eluted pair of analyte and internal standard was collected separately for quantification in ESI-MS/MS.

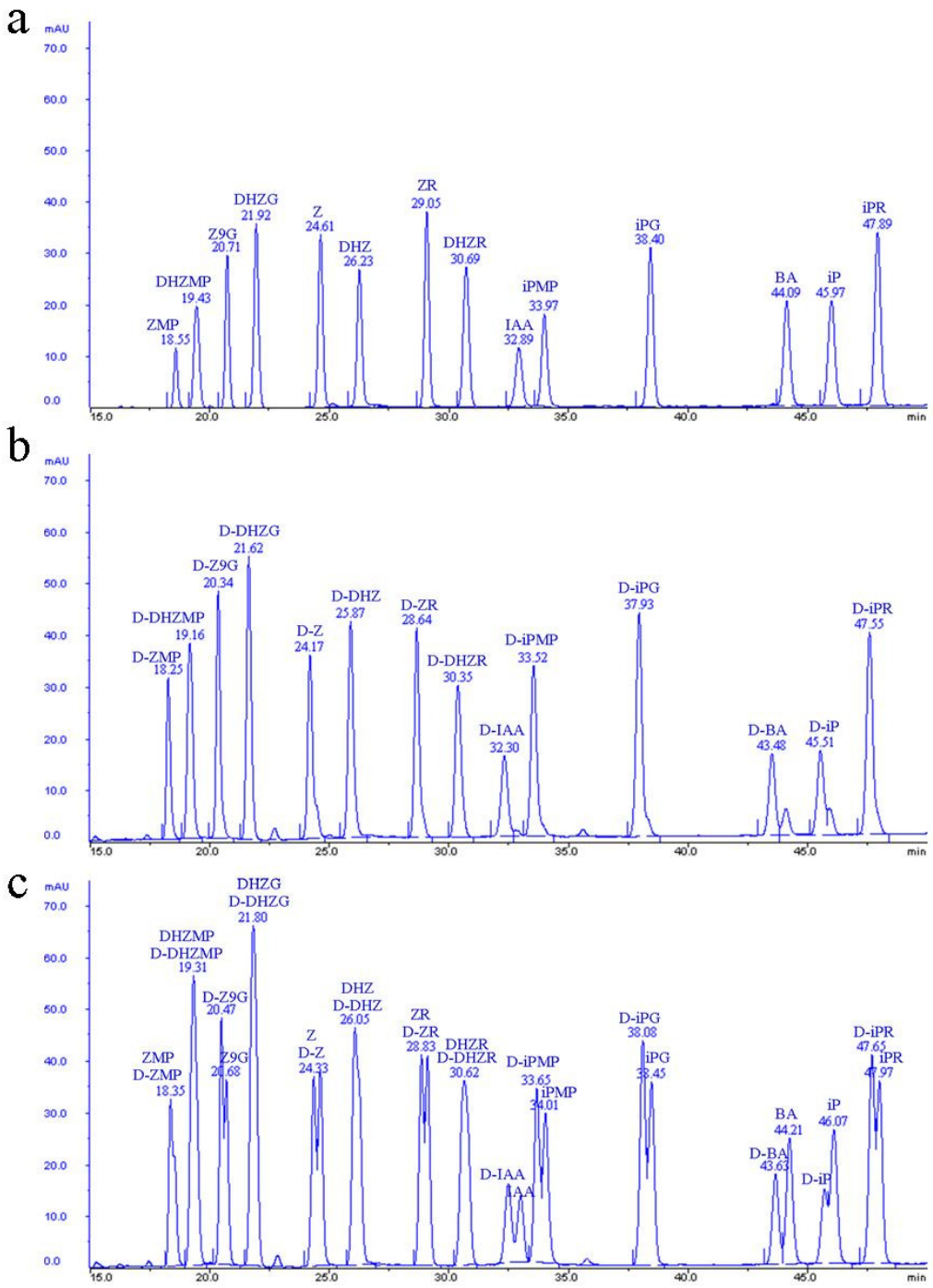

Fig. 5.1 Separation of cytokinins and IAA by HPLC. Zeatin (Z), isopentenyladenine (iP), dihydrozeatin (DHZ), their derivatives [ribosides (-R), glucosides (-9G) and nucleotides (-MP)], 6-benzyladenine (BA) and indol-3-acetic acid (IAA) were separated by using XBridge™ C18 column (Waters). a) HPLC profile of unlabeled cytokinins and IAA standards; b) HPLC profile of deuterium-labeled (D-) cytokinins and IAA internal standards; and c) co-elution of unlabeled and deuterium-labeled standards.

Table 5.1 Precursor-to-product ion transitions used in the quantification of cytokinins and IAA in *D.* Chao Praya Smile by ESI-MS/MS.

Analytes	Retention time (min)	Transition	Internal standards	Retention time (min)	Transition
Z	24.61	220.4 > 136	d_5-Z	24.17	225.5 > 136.9
ZR	29.05	352.4 > 136	d_5-ZR	28.64	357.2 > 136.9
Z9G	20.71	382.3 > 136	d_5-Z9G	20.34	387.3 > 225.1
ZMP	18.55	432.4 > 220	d_5-ZMP	18.25	437.6 > 225
iP	45.97	204.2 > 136	d_6-iP	45.51	210.4 > 136.9
iPR	47.89	336.4 > 136	d_6-iPR	47.55	342.2 > 136.9
iP9G	38.40	366.2 > 136	d_6-iP9G	37.93	372.1 > 136.9
iPMP	33.97	416.5 > 136	d_6-iPMP	33.52	422 > 136.9
DHZ	26.23	222.3 > 148.1	d_3-DHZ	25.87	225.6 > 149
DHZR	30.69	354.4 > 222	d_3-DHZR	30.35	357.2 > 225.1
DHZ9G	21.92	384.4 > 222	d_3-DHZ9G	21.62	387.4 > 225
DHZMP	19.43	434.6 > 222	d_3-DHZMP	19.16	437.3 > 225
BA	44.09	226.1 > 91	d_7-BA	43.48	233.5 > 98.1
IAA	32.89	176.5 > 130	d_5-IAA	32.30	181.6 > 134

5.3.2 Quantification of cytokinins and IAA by ESI-MS/MS

Standard solutions comprising the unlabeled analytes and their respective deuterium-labeled internal standards were used to identify the appropriate precursor-to-product ion transition in ESI-MS/MS. The mass spectra of the diagnostic product ions for Z-type, iP-type, DHZ-type cytokinins, BA and IAA were shown (Fig. 5.2 to 5.5). In the case of Z, D-Z, ZR, D-ZR, Z9G, iP-type cytokinins and their internal standards, product ions that represented protonated adenine (m/z 136) were selected as they formed the major product ions in the fragmentation of precursor ions. Similarly, product ions that represented protonated zeatin free base (m/z 220 or m/z 225 in deuterated internal standards) were selected for D-Z9G, ZMP and D-ZMP while product ions that represented protonated dihydrozeatin free base (m/z 222 or m/z 225 in deuterated internal standards) were selected for DHZR, D-DHZR, DHZ9G, D-DHZ9G, DHZMP and D-DHZMP. In the case of DHZ and D-DHZ, the second major product ions with m/z of 148 and 149, respectively, were selected because they produced the same major product ions of m/z 136. Fragmentation of BA and D-BA produced single product ion peaks at m/z 91 and 98, respectively, which represented the benzyl ion. Major product ions of m/z 130 and 134 were produced in the fragmentation of IAA and D-IAA, respectively, and were therefore selected. The precursor and characteristic product ion for each unlabeled analyte and their deuterium-labeled internal standards that were used for the quantification of cytokinins and IAA were summarized in Table 5.1. Calibration curves generated for the cytokinins and IAA were linear in the concentration range of 0 – 1000 pmol (R^2 values of 0.9276 – 1).

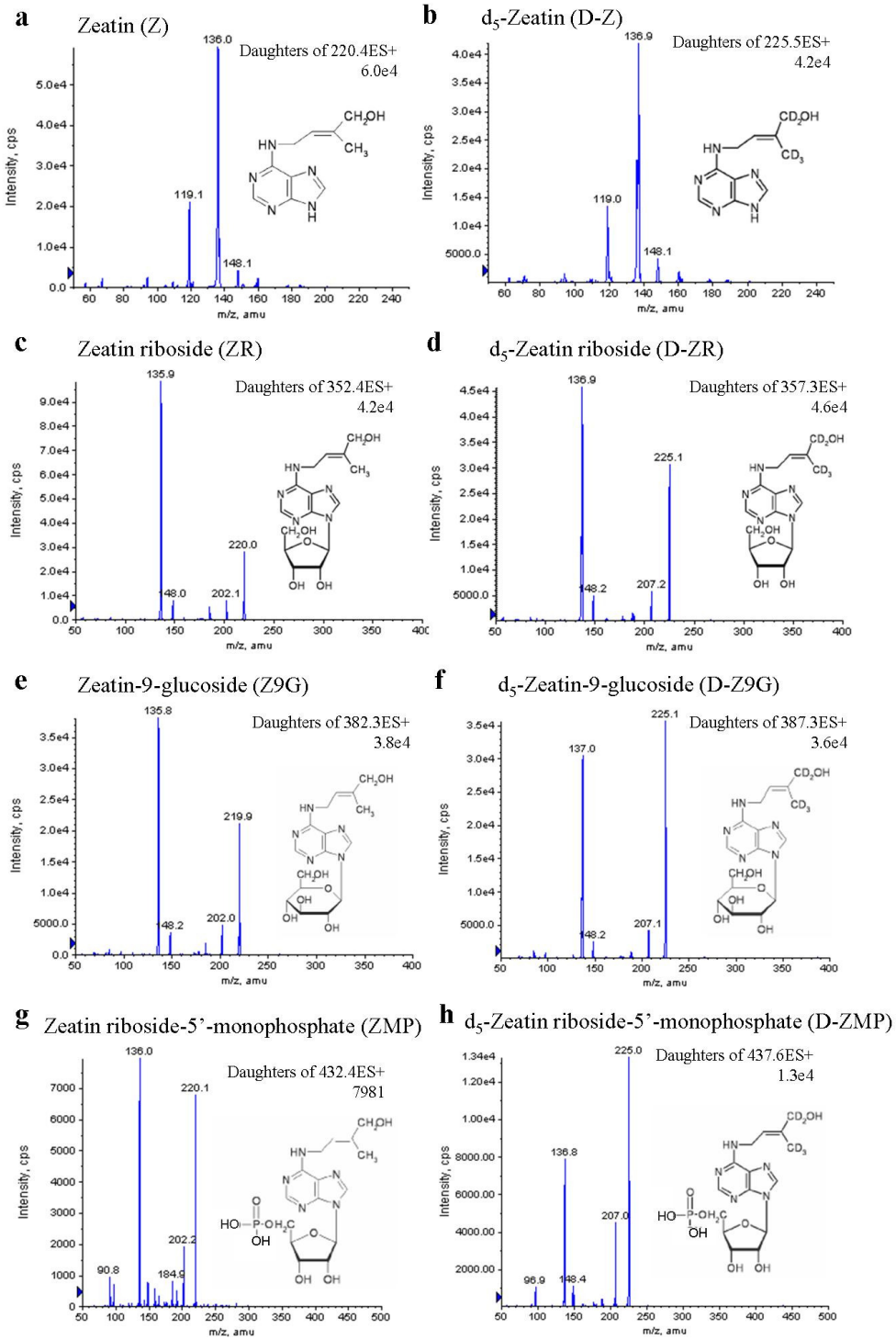

Fig. 5.2 Fragmentation patterns for labeled and unlabeled Z-type cytokinin standards. All compounds were analyzed in the positive-ion mode. a) Precursor (m/z 220.4) and product (m/z 136) ions of Z. b) Precursor (m/z 225.5) and product (m/z 136.9) ions of D-Z internal standard. c) Precursor (m/z 352.4) and product (m/z 136.9) ions of ZR. d) Precursor (m/z 357.3) and product (m/z 136.9) ions of D-ZR internal standard. e) Precursor (m/z 382.3) and product (m/z 136) ions of Z9G. f) Precursor (m/z 387.3) and product (m/z 225.1) ions of D-Z9G internal standard. g) Precursor (m/z 432.4) and product (m/z 220.1) ions of ZMP. h) Precursor (m/z 437.6) and product (m/z 225) ions of D-ZMP internal standard.

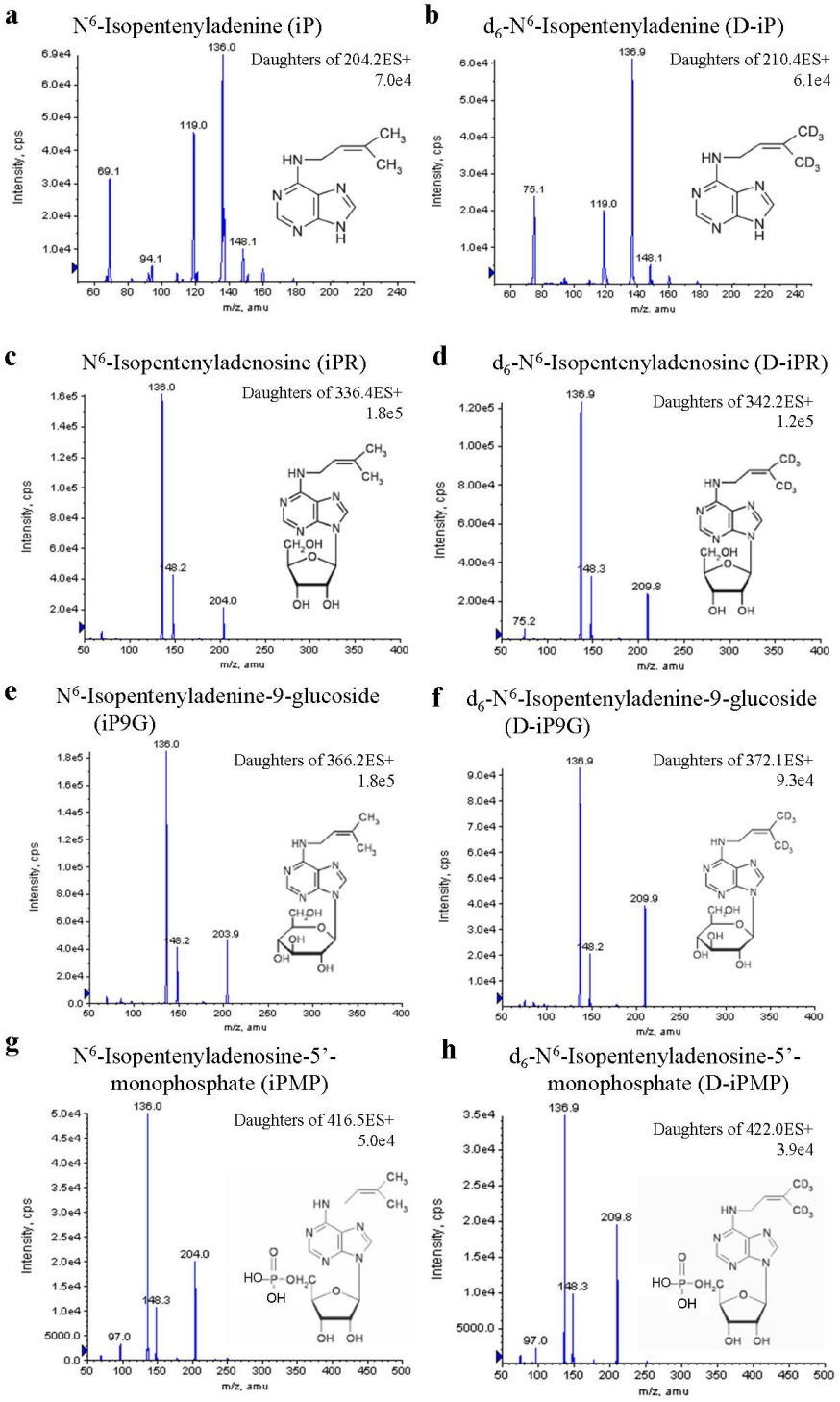

Fig. 5.3 Fragmentation patterns for labeled and unlabeled iP-type cytokinin standards. All compounds were analyzed in the positive-ion mode. a) Precursor (m/z 204.2) and product (m/z 136) ions of iP. b) Precursor (m/z 210.4) and product (m/z 136.9) ions of D-iP internal standard. c) Precursor (m/z 336.4) and product (m/z 136) ions of iPR. d) Precursor (m/z 342.2) and product (m/z 136.9) ions of D-iPR internal standard. e) Precursor (m/z 366.2) and product (m/z 136) ions of iP9G. f) Precursor (m/z 372.1) and product (m/z 136.9) ions of D-iP9G internal standard. g) Precursor (m/z 416.5) and product (m/z 136) ions of iPMP. h) Precursor (m/z 422) and product (m/z 136.9) ions of D-iPMP internal standard.

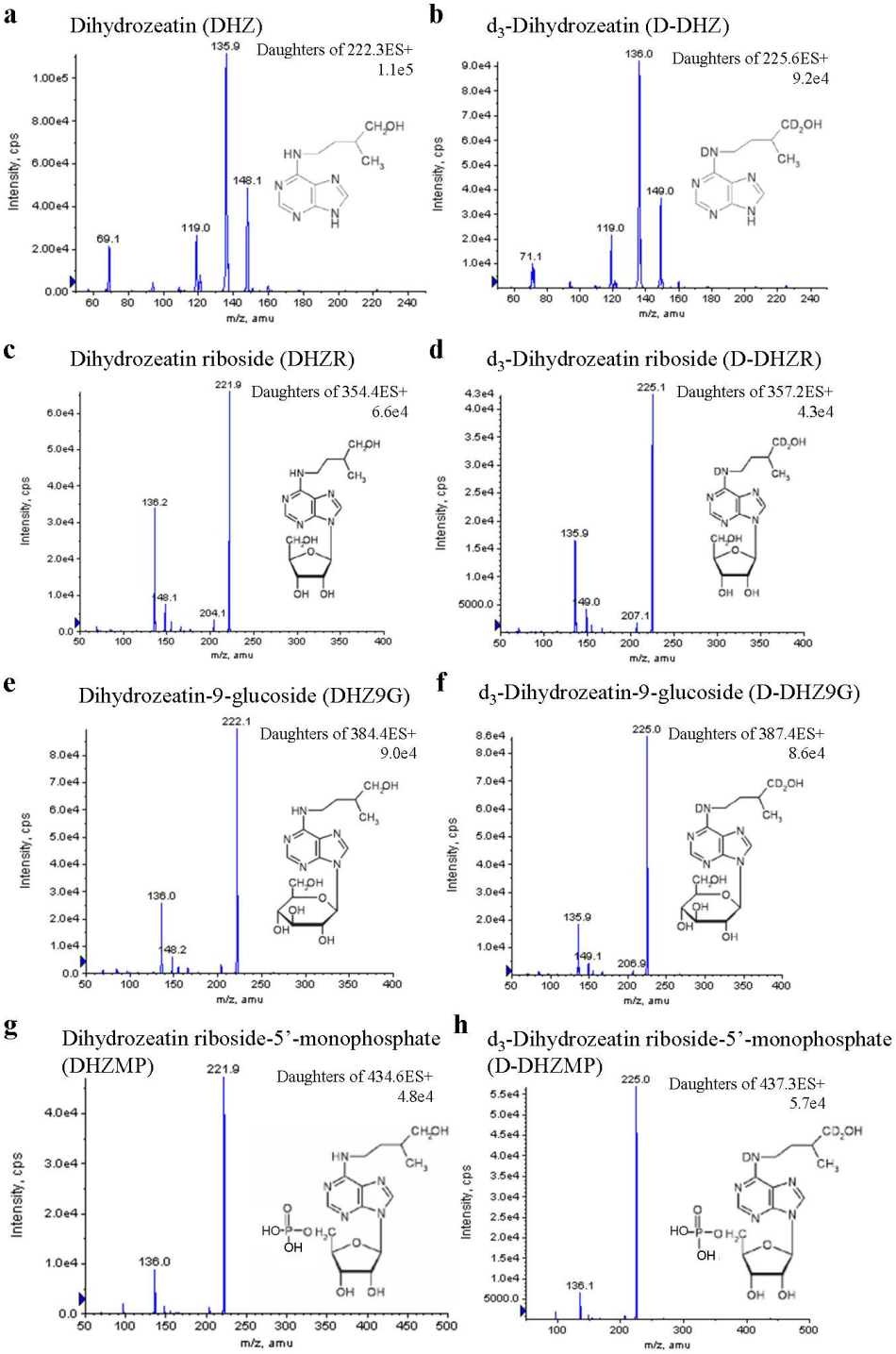

Fig. 5.4 Fragmentation patterns for labeled and unlabeled DHZ-type cytokinin standards. All compounds were analyzed in the positive-ion mode. a) Precursor (m/z 222.3) and product (m/z 148.1) ions of DHZ. b) Precursor (m/z 225.6) and product (m/z 149) ions of D-DHZ internal standard. c) Precursor (m/z 354.4) and product (m/z 221.9) ions of DHZR. d) Precursor (m/z 357.2) and product (m/z 225.1) ions of D-DHZR internal standard. e) Precursor (m/z 384.4) and product (m/z 222) ions of DHZ9G. f) Precursor (m/z 387.4) and product (m/z 225) ions of D-DHZG internal standard. g) Precursor (m/z 434.6) and product (m/z 221.9) ions of DHZMP. h) Precursor (m/z 437.3) and product (m/z 225) ions of D-DHZMP internal standard.

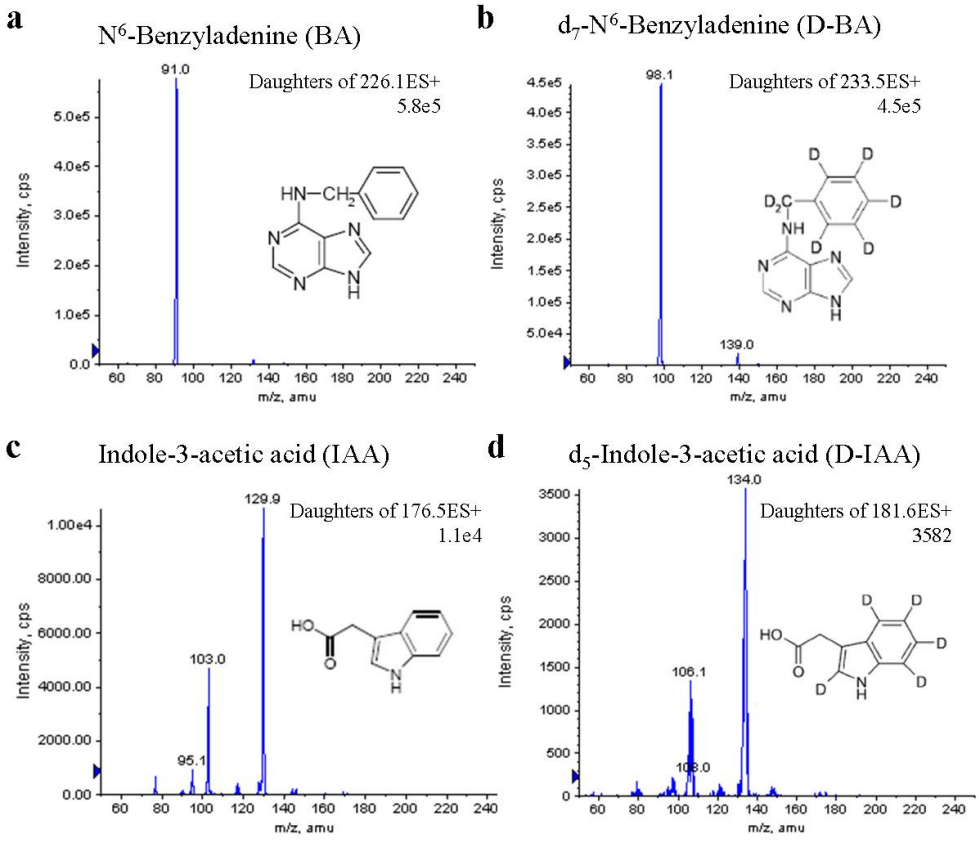

Fig. 5.5 Fragmentation patterns for labeled and unlabeled BA and IAA standards. All compounds were analyzed in the positive-ion mode. a) Precursor (m/z 226.1) and product (m/z 91) ions of benzyladenine. b) Precursor (m/z 233.5) and product (m/z 98.1) ions of D-BA internal standard. c) Precursor (m/z 176.5) and product (m/z 129.9) ions of IAA. d) Precursor (m/z 181.6) and product (m/z 134) of D-IAA internal standard.

5.3.3 Changes in cytokinins and IAA at different growth stages

The levels of total cytokinins (excluding BA) in *D.* Chao Praya Smile plantlets increased dramatically when they were grown in liquid media as compared to protocorms (Fig. 5.6). The concentrations of cytokinins remained in the range of 6 – 8 nmol g^{-1} FW during their growth in liquid media for 54 days. BA-induced plantlets contained similar concentrations of cytokinins as non-induced plantlets (Fig. 5.6) during floral transition (54 days after culture, Fig. 4.3 and 4.5). The levels of total cytokinins in both BA-induced and non-induced plantlets started to decrease when they were grown in the two-layer media. Floral buds were produced in BA-induced plantlets after 80 days of culture (Fig. 4.3). At this stage, the BA-induced plantlets contained approximately 70 % more cytokinins than the non-induced plantlets that remained vegetative.

The Z-type cytokinin represented the predominant cytokinin that accounted for more than 70 % of the total cytokinins throughout the growth of BA-induced plantlets from vegetative to flowering stage (Fig. 5.7). At different growth stages, iP- and DHZ-type cytokinins fluctuated between 12 – 21 % and 5 – 15 %, respectively, of the total cytokinins. It was noted that the proportion of Z-, iP- and DHZ-type cytokinins in BA-induced plantlets during floral transition (54 days after culture) was not significantly different from the non-induced vegetative plantlets.

Z and Z9G were the most prominent Z-type cytokinins in BA-induced plantlets in which their concentrations were in the range of 1 to 4 nmol g^{-1} FW throughout the vegetative growth to floral transition (Fig. 5.8). Besides, BA-induced plantlets contained approximately 500 pmol g^{-1} FW of ZR and less than 100 pmol g^{-1} FW of ZMP during these growth stages. At the stage of floral bud development, BA-induced plantlets contained 50, 80 and 100 % more of Z, Z9G and ZR than the non-

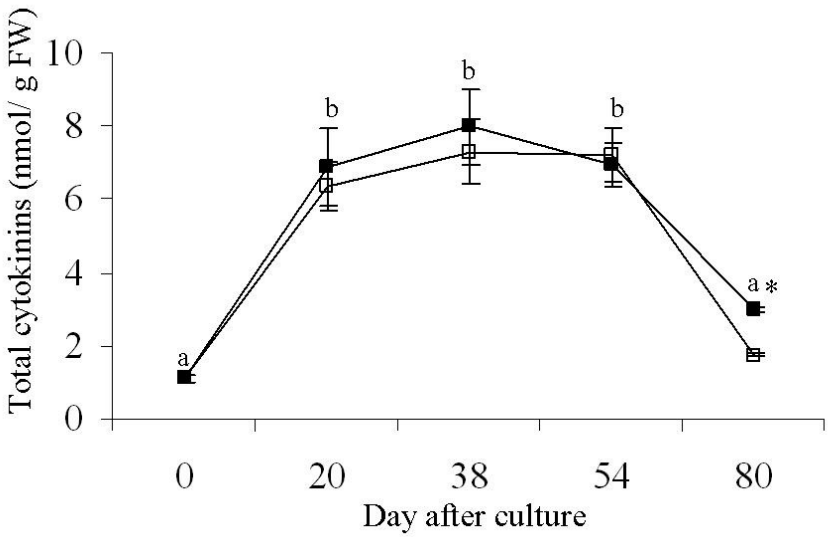

Fig. 5.6 Levels of total cytokinins (excluding BA) in non-induced (open symbols) and BA-induced (closed symbols) *D.* Chao Praya Smile at different days after culture. Same letters above the closed symbols indicate no significant difference among the levels of total cytokinins in the BA-induced plantlets (One-Way ANOVA Tukey's test at 95 % confidence level). Asterisk following the letter indicates significant difference in the levels of total cytokinins between the BA-induced and non-induced plantlets at the specific day after culture (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

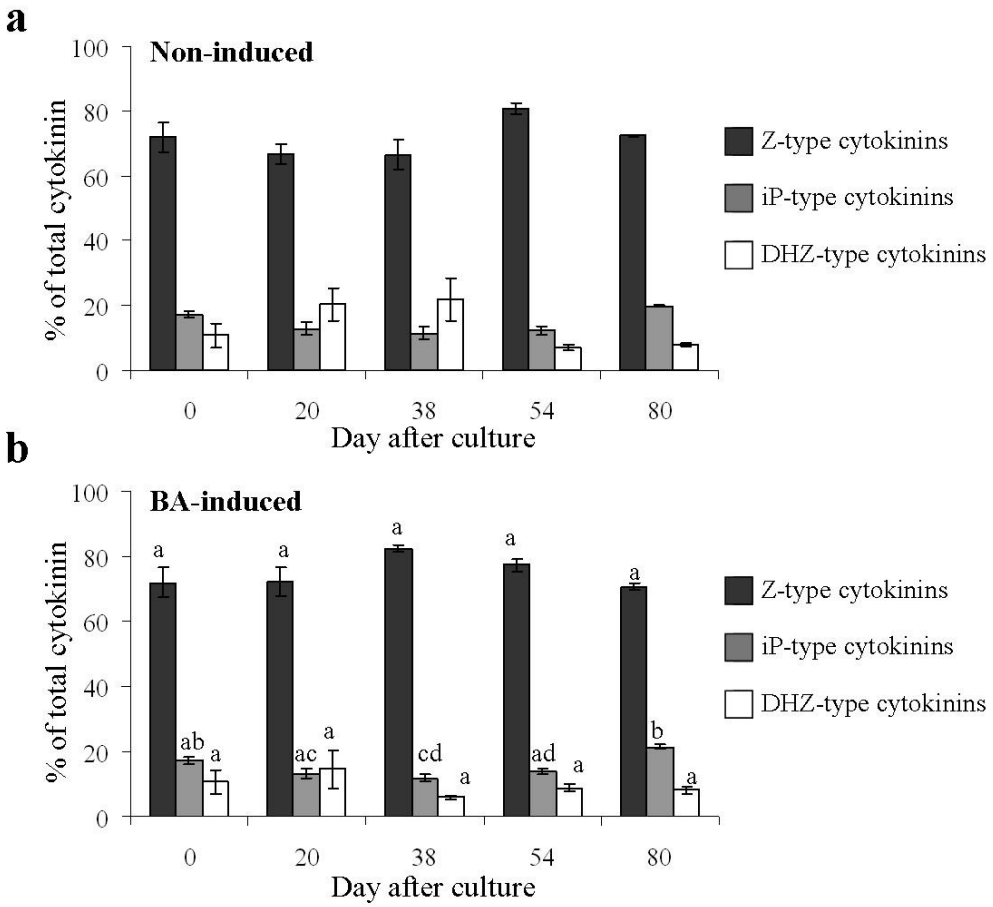

Fig. 5.7 Percentage composition of Z-, iP- and DHZ-type cytokinins in non-induced (a) and BA-induced (b) *D.* Chao Praya Smile at different days after culture. Same letters above the bars indicate no significant difference among the percentage compositions of Z-, iP- or DHZ-type cytokinins in the BA-induced plantlets (One-Way ANOVA Tukey's test at 95 % confidence level). Vertical bars denote SE. n = 3.

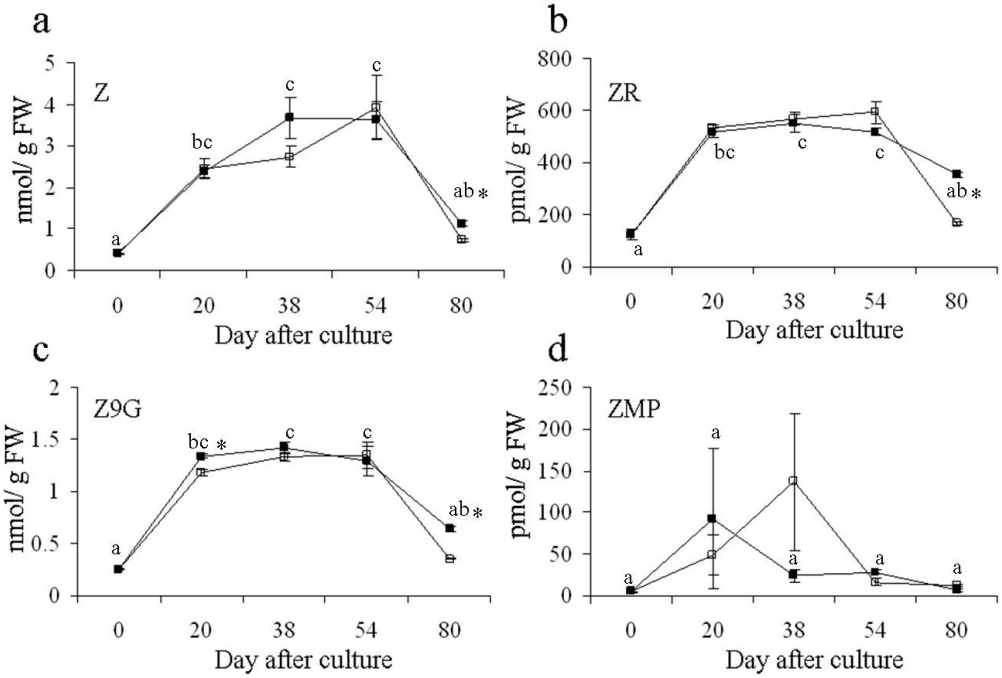

Fig. 5.8 Concentrations of zeatin (Z) (a), zeatin riboside (ZR) (b), zeatin-9-glucoside (Z9G) (c) and zeatin riboside-5'-monophosphate (ZMP) (d) in non-induced (open symbols) and BA-induced (closed symbols) *D.* Chao Praya Smile at different days after culture. Same letters above the closed symbols indicate no significant difference among the concentrations of cytokinins in the BA-induced plantlets (One-Way ANOVA Tukey's test at 95 % confidence level). Asterisks following the letters indicate significant difference in the concentrations of cytokinins between the BA-induced and non-induced plantlets at the specific day after culture (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

induced plantlets (Fig. 5.8a, b and d).

BA-induced plantlets contained 150 to 300 pmol g^{-1} FW of iP-type cytokinins throughout the growth from vegetative phase to flowering (Fig. 5.9). At floral transition, BA-induced plantlets contained approximately 68 % more of iPR than the vegetative non-induced plantlets (Fig 5.9b). It was also observed that BA-induced plantlets contained significantly more iP-type cytokinins (in the range of 70 to 100 %) than the non-induced plantlets, when floral buds were being formed. On the other hand, the concentrations of DHZ-type cytokinins in BA-induced *D.* Chao Praya Smile remained quite constant at different growth stages (Fig. 5.10). It was noted that BA-induced plantlets contained 135 % more of DHZMP during floral buds development (Fig. 5.10d).

The concentration of BA in BA-induced plantlets was continuously increasing during the vegetative growth and reached its peak during floral transition at 12.7 ± 3 nmol g^{-1} FW (Fig. 5.11a). Its concentration was reduced in the two-layer culture medium when flower buds were produced. In contrast, BA was not detected in non-induced plantlets. Conversely, the concentration of IAA in BA-induced plantlets was reduced when the treatment started and remained it less than 1 nmol g^{-1} FW at different growth stages till flowering (Fig. 5.11b).

The ratios of total cytokinins (excluding BA) to IAA were calculated in non-induced and BA-induced plantlets at various growth stages (Fig. 5.12). The results showed that the CKs/IAA ratio was 7.2 folds higher in BA-induced plantlets than the non-induced plantlets at floral transition. It was also noted that at the stage of floral bud development, BA-induced plantlets showed a 2.8 folds higher CKs/IAA ratio than the vegetative non-induced plantlets.

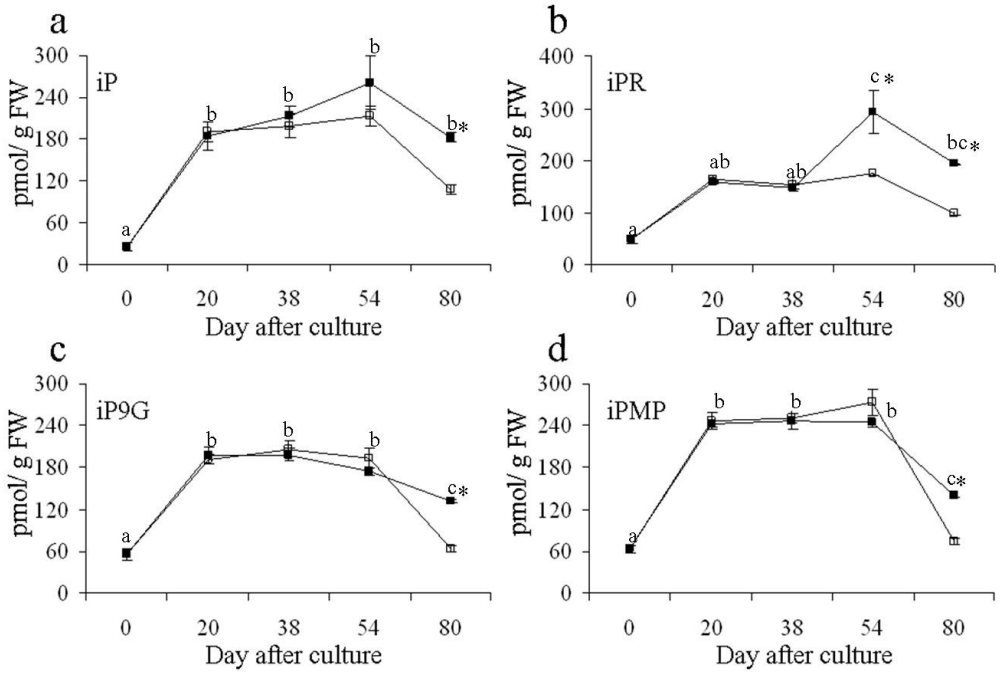

Fig. 5.9 Concentrations of isopentenyladenine (iP) (a), isopentenyladenosine (iPR) (b), isopentenyladenine-9-glucoside (iP9G) (c) and isopentenyladenosine-5'-monophosphate (iPMP) (d) in non-induced (open symbols) and BA-induced (closed symbols) *D.* Chao Praya Smile at different days after culture. Same letters above the closed symbols indicate no significant difference among the concentrations of cytokinins in the BA-induced plantlets (One-Way ANOVA Tukey's test at 95 % confidence level). Asterisks following the letters indicate significant difference in the concentrations of cytokinins between the BA-induced and non-induced plantlets at the specific day after culture (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

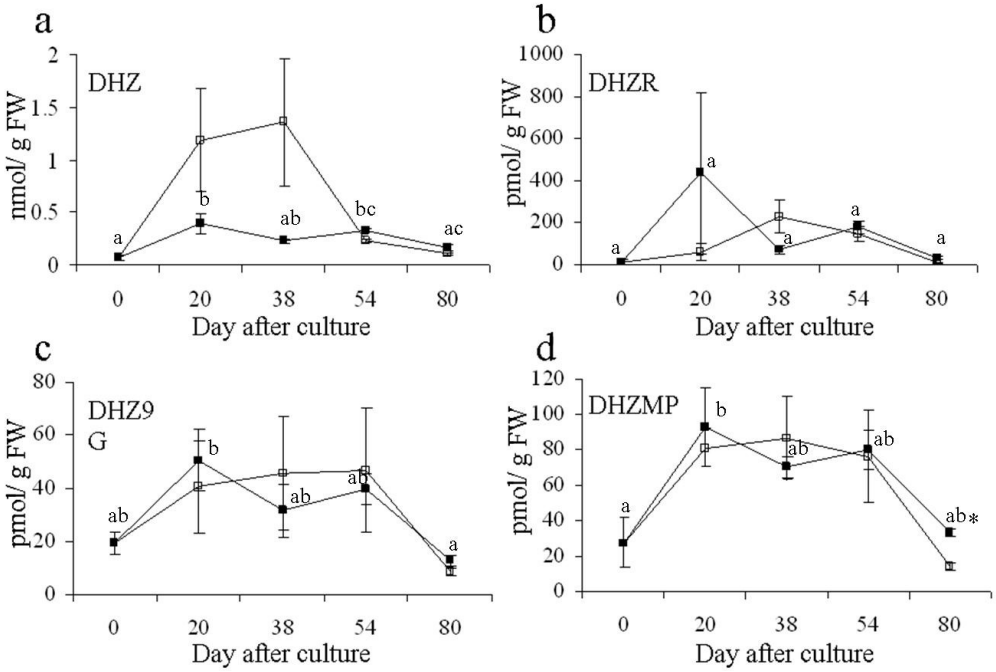

Fig. 5.10 Concentrations of dihydrozeatin (DHZ) (a), dihydrozeatin riboside (DHZR) (b), dihydrozeatin-9-glucoside (DHZ9G) (c) and dihydrozeatin riboside-5'-monophosphate (DHZMP) (d) in non-induced (open symbols) and BA-induced (closed symbols) *D.* Chao Praya Smile at different days after culture. Same letters above the closed symbols indicate no significant difference among the concentrations of cytokinins in the BA-induced plantlets (One-Way ANOVA Tukey's test at 95 % confidence level). Asterisks following the letters indicate significant difference in the concentrations of cytokinins between the BA-induced and non-induced plantlets at the specific day after culture (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

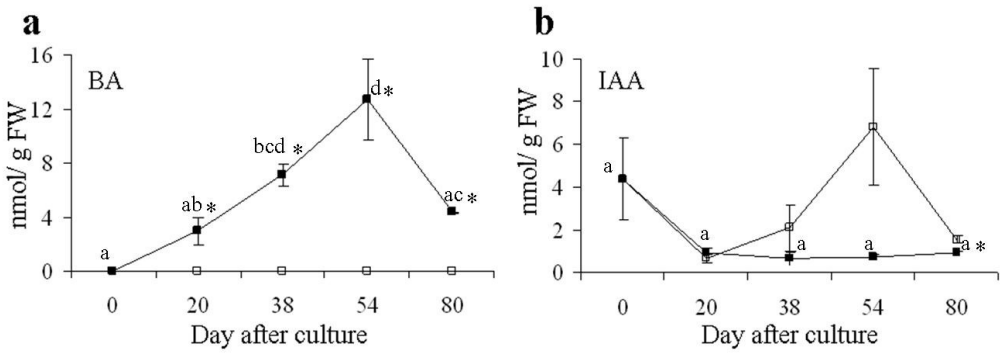

Fig. 5.11 Concentrations of benzyladenine (BA) (a) and indole-3-acetic acid (IAA) (b) in non-induced (open symbols) and BA-induced (closed symbols) *D.* Chao Praya Smile at different days after culture. Same letters above the closed symbols indicate no significant difference among the concentrations of BA or IAA in the BA-induced plantlets (One-Way ANOVA Tukey's test at 95 % confidence level). Asterisks following the letters indicate significant difference in the concentrations of BA or IAA between the BA-induced and non-induced plantlets at the specific day after culture (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

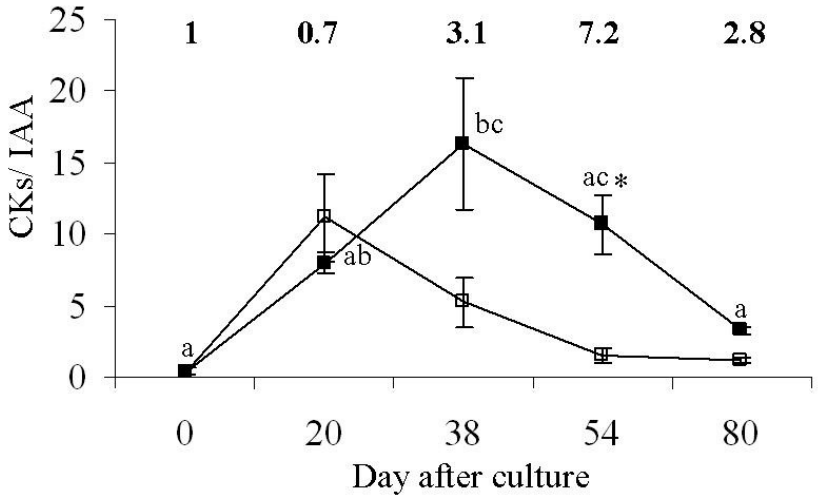

Fig. 5.12 Ratios of total cytokinins (excluding BA) to IAA (CKs/IAA) in non-induced (open symbols) and BA-induced (closed symbols) *D.* Chao Praya Smile at different days after culture. Same letters above the closed symbols indicate no significant difference among the CKs/IAA in the BA-induced plantlets (One-Way ANOVA Tukey's test at 95 % confidence level). Asterisk following the letters indicates significant difference in CKs/IAA between the BA-induced and non-induced plantlets at the specific day after culture (2-Sample t-test at 95 % confidence level). Numbers above the symbols indicate the results of CKs/IAA (BA-induced) divided by CKs/IAA (non-induced) at the specific day after culture. Vertical bars denote SE. n = 3.

5.3.4 Changes in cytokinins and IAA in various plant tissues

Cytokinins and IAA in the shoot apex, stem and leaf base, stem base and leaf were analyzed in each of the BA-induced plantlets after 54 days of culture, which was at the stage of floral transition. The positions of these tissues were indicated in Fig. 4.2. Relative distances of these tissues, with respect to the shoot apex, were also as illustrated in Fig. 5.13. The shoot apices of both non-induced and BA-induced plantlets were enriched with Z-, iP- and DHZ-type cytokinins. The concentrations of Z-, iP- and DHZ-type cytokinins in the shoot apices were approximately 4, 2 and 4 times more, respectively, than those in other plant tissues (Fig. 5.14). In addition, there were about 9 % more of iP-type cytokinins in the shoot apices of the BA-induced plantlets than the non-induced plantlets. Beneath the shoot apices, the stems and leaf bases of BA-induced plantlets were found to contain more Z- and iP-type cytokinins (26 and 52 %, respectively) than non-induced plantlets (Fig. 5.14a and b). Similarly, the stem bases of BA-induced plantlets, which were located farthest away from the shoot apices along the stem axis, also contained more Z- and iP-type cytokinins (99 and 90 %, respectively). Therefore, in comparison to non-induced plantlets, a gradient of significantly higher levels of iP-type cytokinins were found along the stem axes of BA-induced plantlets; however, cytokinin concentration decreased basipetally. In contrast, DHZ-type cytokinin level in BA-induced plantlets was not significantly different from that of the non-induced plantlets in all the tissues analyzed (Fig. 5.14c). Leaves of BA-induced and non-induced plantlets contained similar concentrations of the three types of cytokinins (Fig. 5.14a, b and c).

Among the cytokinins analyzed, other than BA, which was used to induce flowering, iPR level was found to increase most significantly in the shoot apex of BA-induced plantlet at floral transition; thus was approximately 20 % more than that in

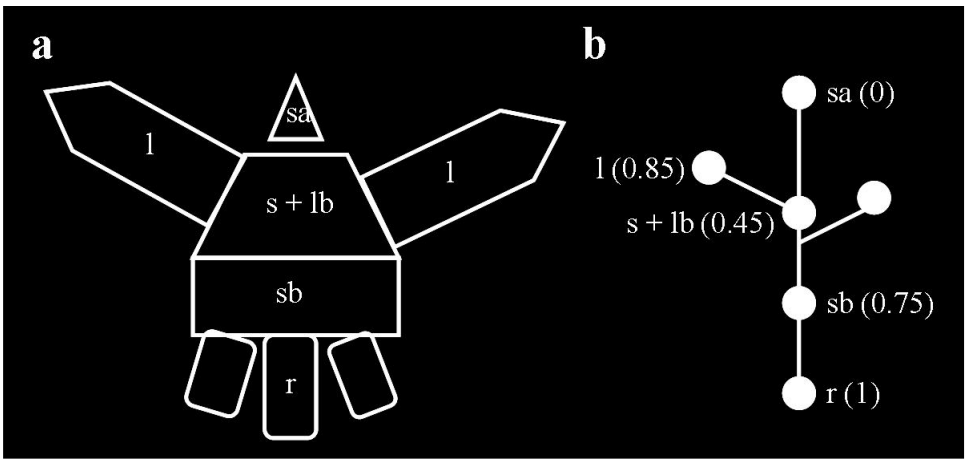

Fig. 5.13 Relative distances of various tissues in *D.* Chao Praya Smile plantlet. (a) Schematic representation of a non-induced *D.* Chao Praya Smile after 54 days of culture. Various tissues were indicated in the drawing at which cytokinins and IAA were analyzed. sa, s + lb, sb, r and l indicate shoot apex, stem + leaf base, stem base, root and leaf, respectively. (b) Relative distances of various tissues in the plantlet with respect to the shoot apex. The shoot apex and root were arbitrarily assigned as 0 and 1, respectively, in distances. Numbers in the parentheses indicate the relative distances of each tissue measured from the center of the tissue to the shoot apex.

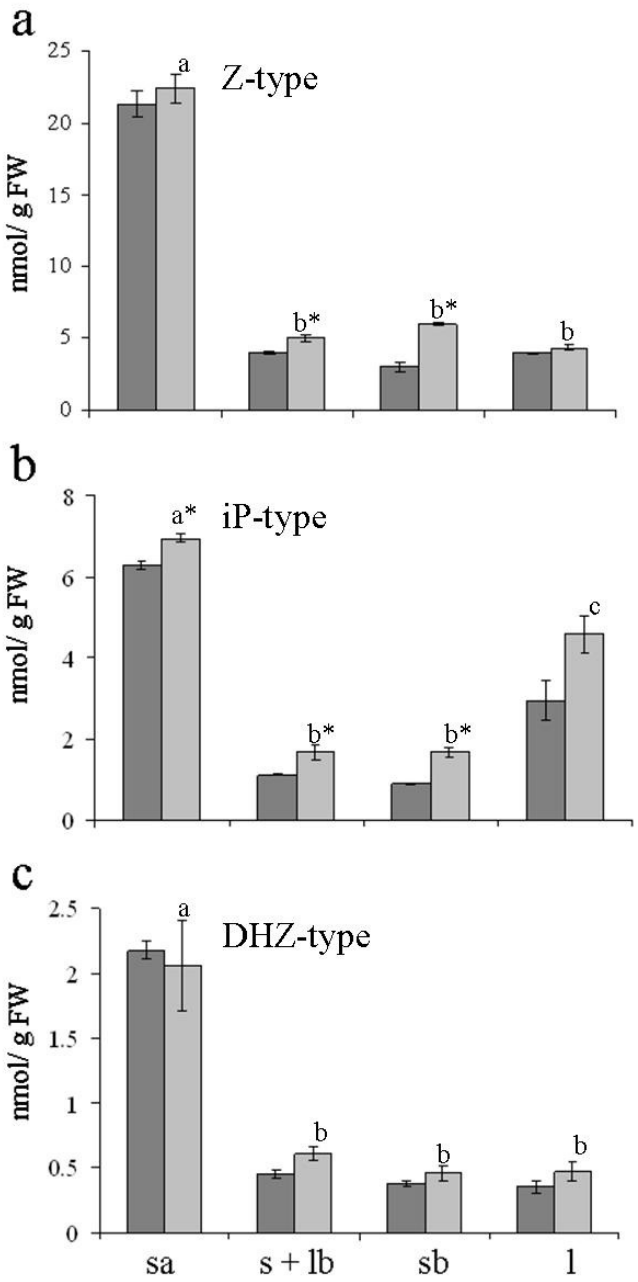

Fig. 5.14 Distribution of Z- (a), iP- (b) and DHZ-type (c) cytokinins in various tissues of non-induced (dark grey bars) and BA-induced (light grey bars) *D.* Chao Praya Smile plantlets during floral transition. sa, s+lb, sb and l refer to shoot apex, stem + leaf base, stem base and leaf, respectively. Same letters above the light grey bars indicate no significant difference among the concentrations of cytokinins in the BA-induced plantlets (One-Way ANOVA Tukey's test at 95 % confidence level). Asterisks following the letters indicate significant difference in the concentrations of cytokinins between the BA-induced and non-induced plantlets in the specific type of tissue (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

the non-induced plantlet (Fig. 5.15). Moreover, there were 82 % more of iPR in the leaves of BA-induced plantlet during the change from a vegetative to reproductive phase (Fig. 5.16). Besides iPR, there were also 11 % more of Z and iP9G in the shoot apex of BA-induced plantlet. In comparison to the non-induced plantlets, the stems and leaf bases of BA-induced plantlets showed 24 to 37 % more of Z, ZR, Z9G, iP, iP9G and iPMP (Fig. 5.17). Similarly, there were 61 to 114 % more of these cytokinins in the stem base of BA-induced plantlet (Fig. 5.18). In contrast, IAA level reduced by approximately 50 and 64 % in the shoot apices and stems and leaf bases, respectively (Fig. 5.15 and 5.17). BA was detected in all tissues of BA-induced plantlet. There was a gradient of BA along the stem axis of BA-induced plantlet in which the stem base contained approximately 5.5 times more BA than the shoot apex (Fig 5.15, 5.17 and 5.18). In contrast, BA was undetected in non-induced plantlets. Apart from this, cytokinins and IAA were also analyzed in the roots of non-induced plantlet. Roots were not produced in the BA-induced plantlet during floral transition. Roots of non-induced plantlet contained IAA and Z at concentrations above 5.5 nmol g^{-1} FW (Fig. 5.19). They were followed by Z9G and ZR which were present in the range of 1.2 to 2.5 nmol g^{-1} FW. The iP-type cytokinins and DHZ were also detected and their levels ranged between 0.47 to 0.71 nmol g^{-1} FW.

The ratios of the levels of total cytokinins (excluding BA) to IAA at various distances relative to the shoot apex in non-induced and BA-induced plantlets were shown in Fig. 5.20. It was noticed that during floral transition, CKs/IAA ratio was two-fold higher in the shoot apex of BA-induced plantlet as compared to that in the non-induced plantlet. Farther away from the shoot apex, the CKs/IAA ratios were similar in BA-induced and non-induced plantlets (Fig. 5.20).

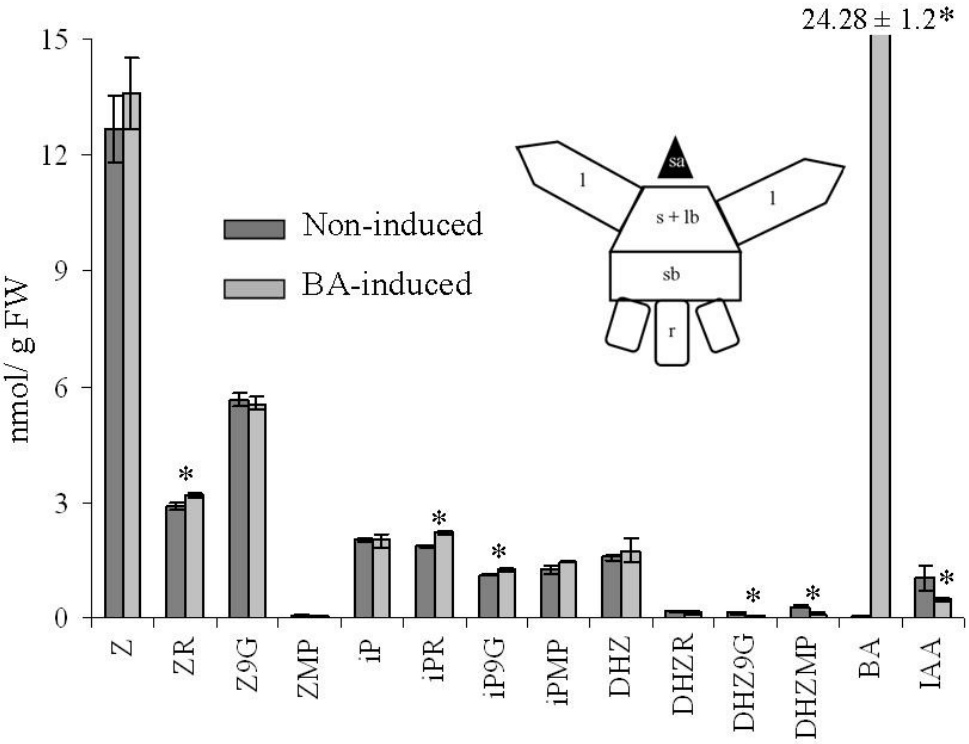

Fig. 5.15 Concentrations of cytokinins and IAA in the shoot apices of non-induced and BA-induced *D.* Chao Praya Smile plantlets during floral transition. Asterisks indicate significant difference in the concentrations of cytokinins or IAA between BA-induced and non-induced plantlets (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

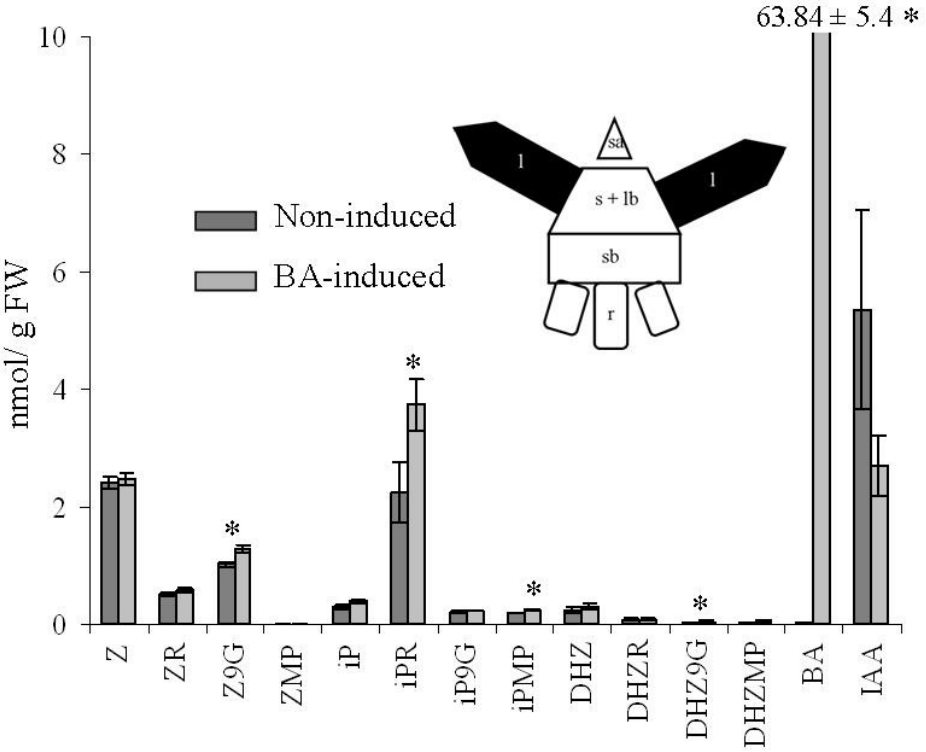

Fig. 5.16 Concentrations of cytokinins and IAA in the leaves of non-induced and BA-induced *D.* Chao Praya Smile plantlets during floral transition. Asterisks indicate significant difference in the concentrations of cytokinins or IAA between BA-induced and non-induced plantlets (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

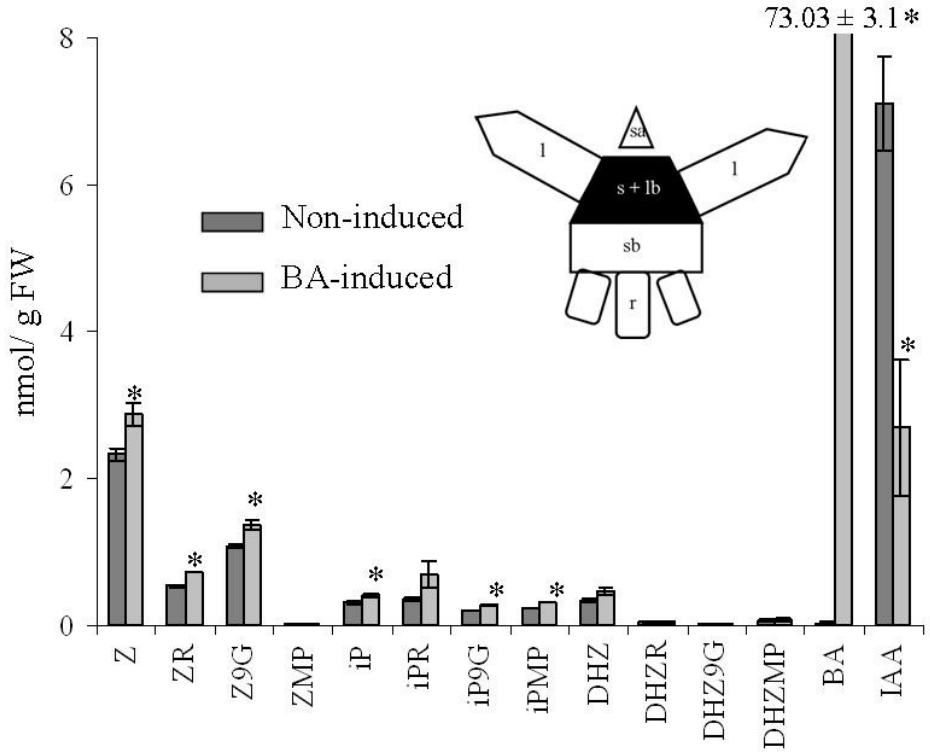

Fig. 5.17 Concentrations of cytokinins and IAA in the stems and leaf bases of non-induced and BA-induced *D.* Chao Praya Smile plantlets during floral transition. Asterisks indicate significant difference in the concentrations of cytokinins or IAA between BA-induced and non-induced plantlets (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

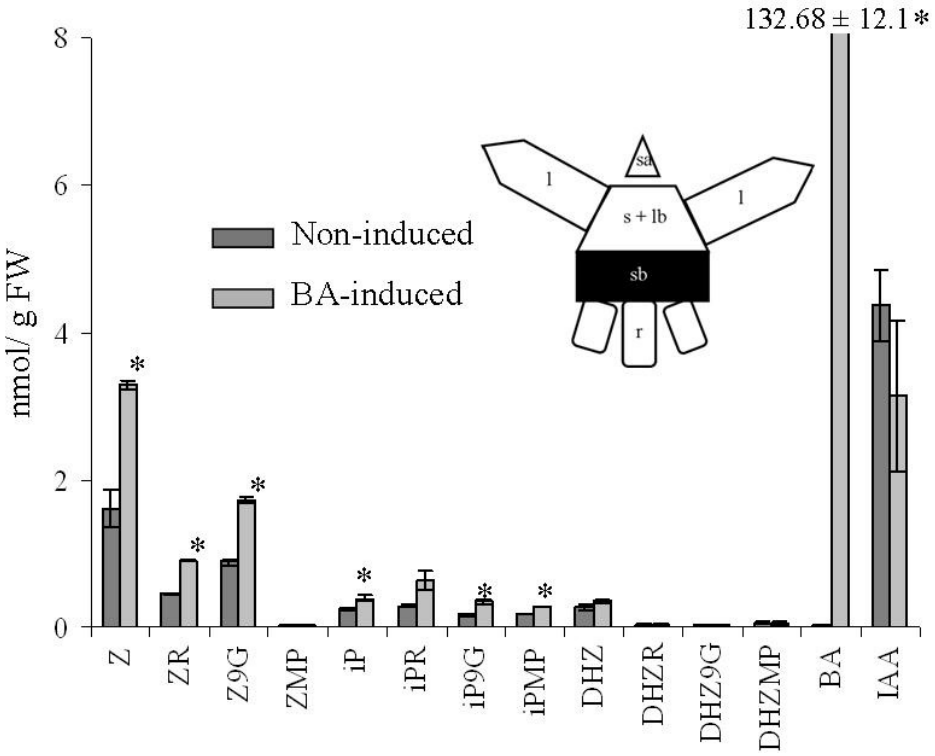

Fig. 5.18 Concentrations of cytokinins and IAA in the stem bases of non-induced and BA-induced *D.* Chao Praya Smile plantlets during floral transition. Asterisks indicate significant difference in the concentrations of cytokinins or IAA between BA-induced and non-induced plantlets (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

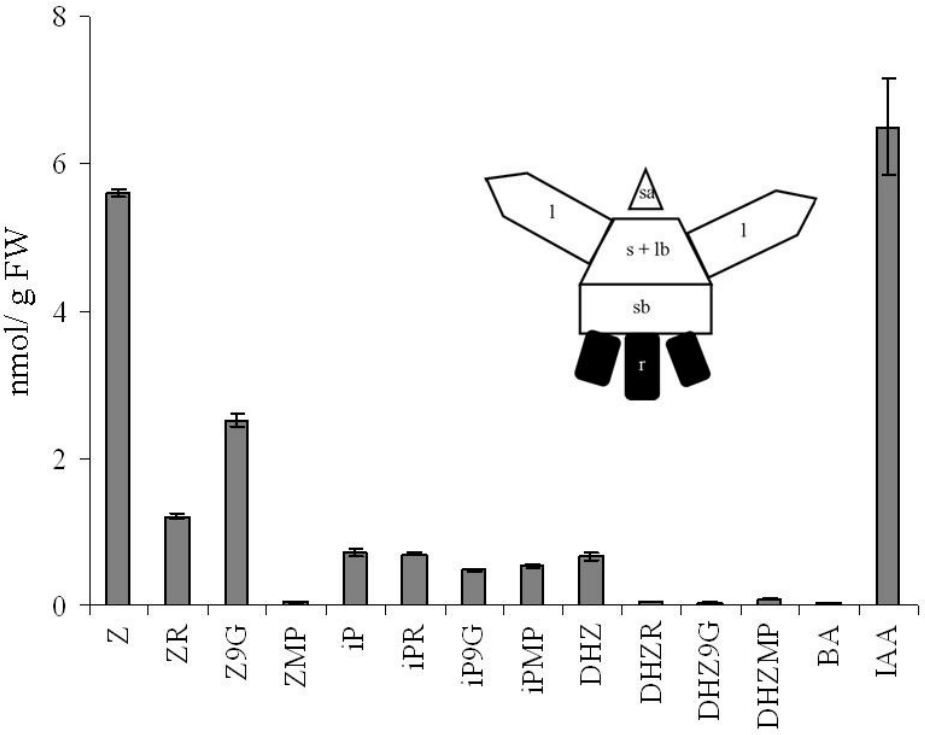

Fig. 5.19 Concentrations of cytokinins and IAA in the roots of non-induced *D.* Chao Praya Smile plantlets after being grown in liquid medium for 54 days.

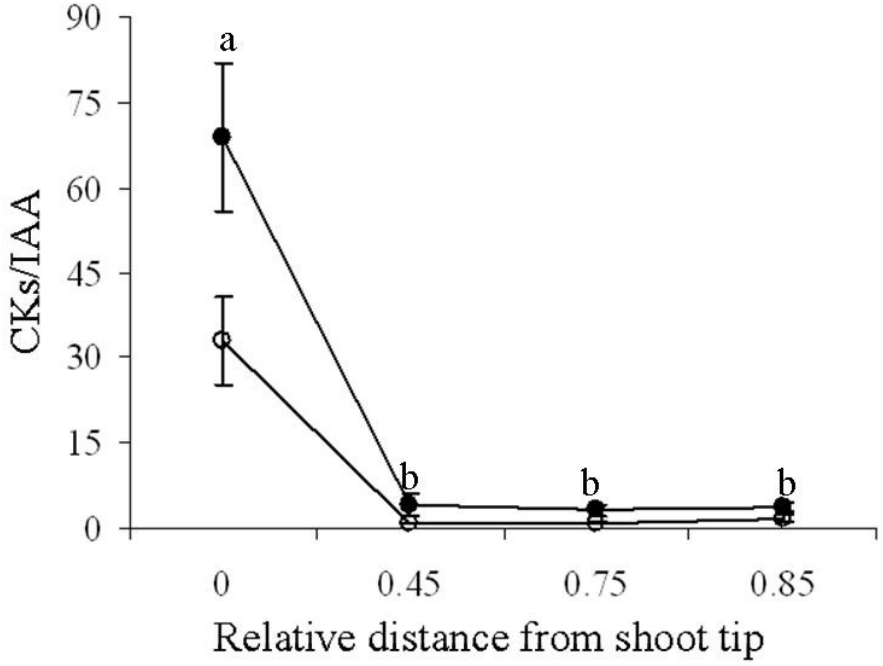

Fig. 5.20 Ratios of the levels of total cytokinins (excluding BA) to IAA (CKs/IAA) in BA-induced (closed circles) and non-induced (open circles) *D.* Chao Praya Smile plantlets at various distances from the shoot apices after 54 days of culture. During this time, the BA-induced plantlets were undergoing floral transition. Same letters above the closed circles indicate no significant difference among the CKs/IAA in BA-induced plantlets (One-Way ANOVA Tukey's test at 95 % confidence level). Vertical bars denote SE. n = 3.

5.3.5 Cloning and expression of *DCPSCKX* in *D.* Chao Praya Smile

RT-PCR was carried out using shoot apex total RNA as template and primers that were designed based on the conserved regions in *Dendrobium* "Sonia" and *Dendrobium* huoshanense. A PCR product band of approximately the correct size (465 bp) was resolved on agarose gel electrophoresis and subsequently purified. Nucleotide sequence analysis revealed that the isolated fragment represented the partial sequence of *DCPSCKX*, showing 99 and 97 % sequence similarities the corresponding regions of *CKX*s from *D.* "Sonia" and *D.* huoshanense, respectively (Fig. 5.21). The fragment also showed 98 and 88 % sequence similarities with the corresponding regions of CKX proteins from *D.* "Sonia" and *D.* huoshanense, respectively (Fig. 5.22). The cloned fragment also included part of the characteristic FAD-binding domain of *CKX* genes. The isolated fragment was therefore used as a probe to analyze *DCPSCKX* expression in *D.* Chao Praya Smile.

Expression of *DCPSCKX* was found to increase dramatically in BA-induced plantlets and remained significantly higher than the non-induced plantlets throughout different stages of growth (Fig. 5.23). Thus, it was apparent that *DCPSCKX* expression was induced by BA treatment. The expression of *DCPSCKX* in the plantlets increased by more than 33 times after they were grown in BA-containing liquid medium for 20 days as compared to untreated protocorms. During floral transition, the expression of *DCPSCKX* was 17.4 higher in BA-induced plantlets as compared to the non-induced plantlets which remained vegetative at time. The expression of *DCPSCKX* was quite homogenous in the shoot apex, stem and leaf base, stem base and leaf of BA-induced plantlets during floral transition (Fig. 5.24)

Fig. 5.21 Nucleotide alignment of partial *DCPSCKX* with *CKX*s from *D.* "Sonia" (*DSCKX1*; AJ294542) and *D.* huoshanense (*DhCKX*; EF213077). Sequences were aligned using the Clustal W program (Thompson *et al*., 1994). The positions of nucleotides are given on the right. The stars below each line of alignment indicate conserved sites.

Fig. 5.22 Amino acid alignment of partial DCPSCKX with CKXs from *D.* "Sonia" (DSCKX1; CAC17752) and *D.* huoshanense (DhCKX; ABM98099). Sequences were aligned using the Clustal W program (Thompson *et al*., 1994). The line represents the consensus FAD-binding region. FAD-binding motifs (GHS) are underlined. The positions of amino acids are given on the right. The stars below each line of alignment indicate conserved sites.

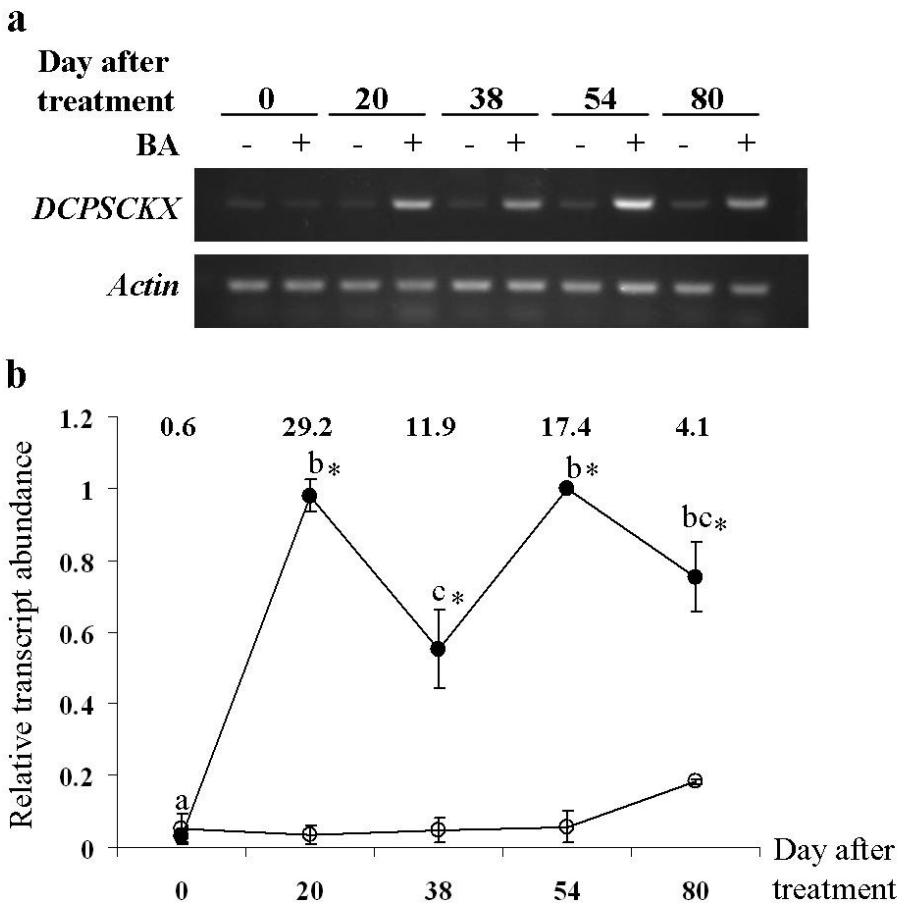

Fig. 5.23 Expression of *DCPSCKX* at various growth stages in *D.* Chao Praya Smile. (a) Semi-quantitative RT-PCR analysis of *DCPSCKX*. Flowering was induced using 11.1 µM of BA and non-induced plantlets were used as controls. Total RNA was extracted and reverse transcribed for the analysis. Expression of *Actin* gene was used as control. (b) Relative levels of *DCPSCKX* transcripts for BA-induced (closed circles) and non-induced (open circles) plantlets at various stages. Transcript levels of BA-induced plantlets after 54 days of culture were arbitrarily set as 1. Transcript levels for *DCPSCKX* products were determined by intensity-based quantification of each product using the ImageJ software. Numbers above the symbols indicate the results of relative transcript abundances (BA-induced) divided by relative transcript abundances (non-induced) at the specific day after culture. Same letters above the closed circles indicate no significant difference among the relative transcript abundances in BA-induced plantlets (One-Way ANOVA Tukey's test at 95 % confidence level). Asterisks following the letters indicate significant difference in relative transcript abundances between the non-induced and BA-induced plantlets at the specific day after culture (2-Sample t-test at 95 % confidence level). Vertical bars denote SE. n = 3.

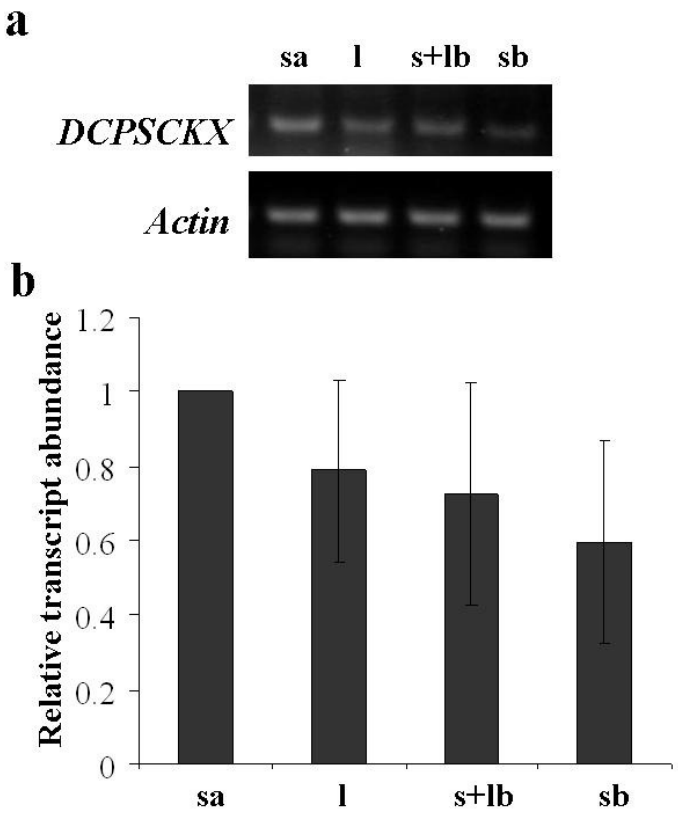

Fig. 5.24 Expression of *DCPSCKX* in different tissues of BA-induced *D.* Chao Praya Smile plantlets at floral transition. (a) Semi-quantitative RT-PCR analysis of DC*PSCKX* in different tissues. sa, l, s+lb and sb refer to shoot apex, leaf, stem and leaf base and stem base, respectively. Total RNA was extracted from the respective tissues and reverse transcribed for the analysis. Expression of *Actin* gene was used as control. (b) Relative levels of *DCPSCKX* transcripts in different tissues of BA-induced plantlets. Transcript level of *DCPSCKX* in shoot apex was arbitrarily set as 1. Transcript levels for *DCPSCKX* products were determined by intensity-based quantification of each product using ImageJ software. Vertical bars denote SE. n = 3.

5.3.6 Effects of iP, iPR, IAA and TIBA on induction of flowering

Only a very low percentage of the plantlets (2 ± 1.7 %) were induced to flower by 22.2 µM of iP (Table 5.2). In contrast, iPR could induce flowering, as efficient as BA, with the highest percentage of flowering (36 ± 6.7 %) achieved at 22.2 µM. On the other hand, IAA at as low as 0.25 µM, completely nullified the flowering-inductive effect of BA. Interestingly, addition of the auxin transport inhibitor, TIBA, to the culture medium was found to induce flowering in *D.* Chao Praya Smile at 1 – 10 µM (Table 5.2). The highest percentage of flowering (30 ± 8.7 %) was obtained with the addition of 2 µM TIBA. Nonetheless, it was observed that approximately 5 % of the plantlets flowered in media containing no plant growth regulator (Table 5.2). Flowering in plant growth regulator-free media was not consistent because only one or two plantlets flowered in one or two replicates of the triplicates. In addition, only inflorescence stalks were produced in these plantlets and no floral buds were formed.

Growth of *D.* Chao Praya Smile plantlets was not affected by the various plant growth regulators tested (Fig. 5.25 and Table 5.3). Plantlets grown in media containing iP, iPR, IAA or TIBA showed similar morphology as well as FW, dry weight, number of leaf and leaf size as those grown in medium containing BA.

5.3.7 Expression of *DCPSknox* and *DCPSCKX* in shoot apices of plantlets treated with BA, iP, iPR, IAA and TIBA

The expression of *DCPSknox* was highest in the shoot apices of *D.* Chao Praya Smile plantlets treated with BA (Fig 5.26). This gene was also expressed in the shoot apices of iP, iPR and TIBA treated plantlets, which showed flowering at their respective treatment concentrations, at levels lower but close to that in BA-induced

Table 5.2 Effects of cytokinins (iP and iPR), auxin (IAA) and auxin transport inhibitor (TIBA) on flowering induction in *D.* Chao Praya Smile.

% of flowering					
	Concentration (μM)				
	0	4.4	11.1	22.2	44.4
iP	0	0	0	2 ± 1.7	0
iPR[#]	3 ± 1.7a	17 ± 6.0ab	32 ± 4.4b	36 ± 6.7b	30 ± 0b
	Concentration (μM)				
	0	0.25	0.5	1	2.5
IAA (with 11.1 μM BA)	33 ± 4.4	0	0	0	0
	Concentration (μM)				
	0	1	2	5	10
TIBA[#]	5 ± 2.9a	25 ± 25.0ab	30 ± 8.7b	13 ± 1.7ab	8 ± 1.7a

Plantlets were grown in liquid medium with varying concentrations of iP, iPR, IAA (and 11.1 μM BA) or TIBA for 54 days followed by the two-layer medium. Scoring of inflorescence production was made at 10 weeks in the two-layer medium.

[#]Same letters following the numbers indicate no significant difference among the percentages of flowering (One-Way ANOVA Tukey's test at 95 % confidence level). n = 3.

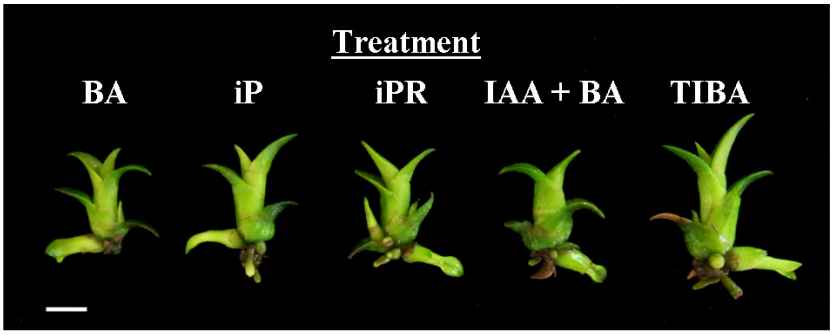

Fig. 5.25 Morphology of *D.* Chao Praya Smile plantlets after growing in liquid medium supplemented with BA (11.1 µM), iP (22.2 µM), iPR (22.2 µM), IAA (0.5 µM) + BA (11.1 µM) or TIBA (2 µM) for 54 days. Bar = 8 mm.

Table 5.3 Characteristics of *D.* Chao Praya Smile plantlets after growing in liquid media supplemented with BA (11.1 µM), iP (22.2 µM), iPR (22.2 µM), IAA (0.5 µM) + BA (11.1 µM) or TIBA (2 µM) for 54 days.

Characteristics	BA	iP	iPR	IAA + BA	TIBA
Fresh weight[#] (g)	0.50 ± 0.02a	0.52 ± 0.02a	0.56 ± 0.04ab	0.61 ± 0.04ab	0.66 ± 0.04b
Dry weight[#] (g)	0.044 ± 0.003a	0.048 ± 0.003a	0.049 ± 0.004a	0.056 ± 0.005a	0.058 ± 0.004a
Water content[#] (%)	91.2 ± 0.3a	91.0 ± 0.5a	91.2 ± 0.3a	90.8 ± 0.4a	91.2 ± 0.2a
Plant height[#] (mm)	14.6 ± 0.3a	15.3 ± 0.3a	15.1 ± 0.3a	15.1 ± 0.4a	17.6 ± 0.6b
Leaf (fully expanded)					
Number	4 ± 0	4 ± 0	5 ± 0	4 ± 0	4 ± 0
Length[#] (mm)	8.5 ± 0.6a	8.7 ± 0.5a	9.1 ± 0.4ab	8.8 ± 0.4a	10.7 ± 0.5b
Width[#] (mm)	4.9 ± 0.4a	4.6 ± 0.2a	4.7 ± 0.2a	5.3 ± 0.5a	4.9 ± 0.2a
Roots					
Number[#]	4 ± 0a	5 ± 0a	5 ± 1a	5 ± 1a	5 ± 0a
Length[#] (mm)	3.3 ± 0.3ab	3.5 ± 0.3ab	4.1 ± 0.4a	3.7 ± 0.4ab	2.6 ± 0.2b
Number of lateral shoot	2 ± 0	2 ± 0	3 ± 0	2 ± 0	2 ± 0

[#]Same letters following the numbers of each characteristic indicate no significant difference among the characteristic (One-Way ANOVA Tukey's test at 95 % confidence level). n = 10.

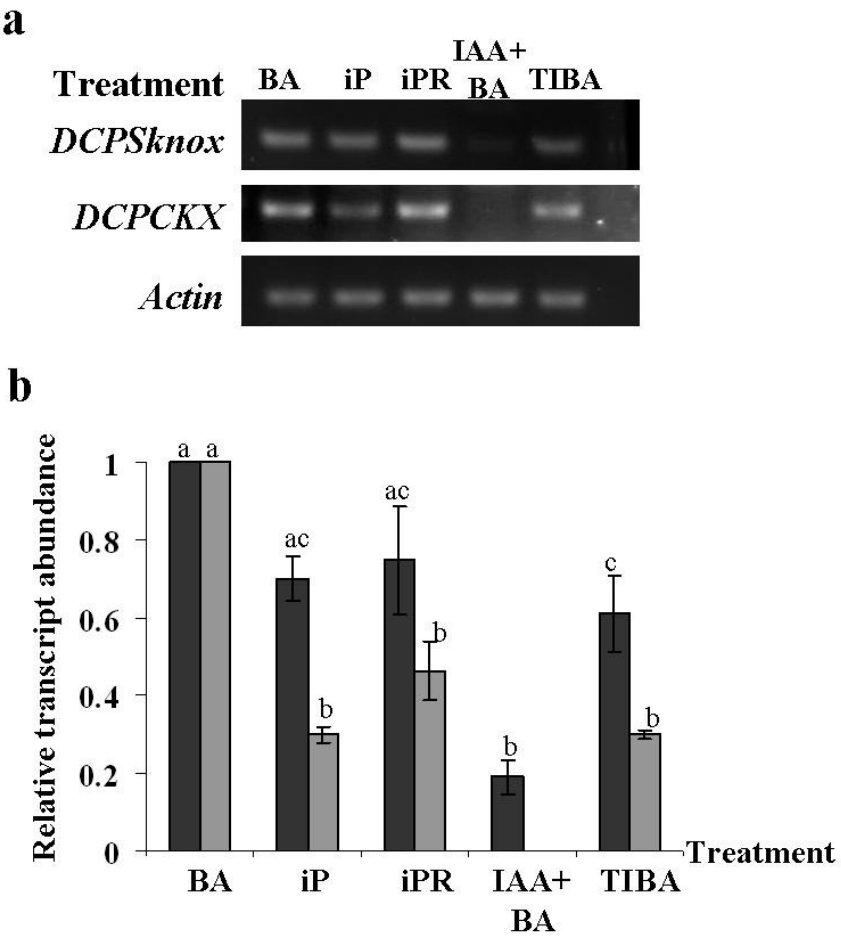

Fig. 5.26 Expression of *DCPSknox* and *DCPSCKX* in shoot apices of *D.* Chao Praya Smile plantlets treated with various plant growth regulators after 54 days of culture. The plantlets were treated with BA (11.1 µM), iP (22.2 µM), iPR (22.2 µM), IAA (0.5 µM) + BA (11.1 µM) or TIBA (2 µM). (a) Semi-quantitative RT-PCR analysis of *DCPSknox* and *DCPSCKX* in the shoot apices of plantlets subjected to various treatments. Total RNA was extracted from shoot apices of the plantlets and reverse transcribed for the analysis. Expression of *Actin* gene was used as control. (b) Relative levels of *DCPSknox* (dark grey bars) and *DCPSCKX* (light grey bars) transcripts in shoot apices of plantlets subjected to various treatments. Transcript levels of *DCPSknox* and *DCPSCKX* in shoot apices of BA-treated plantlets were arbitrarily set as 1. Transcript levels for *DCPSknox* and *DCPSCKX* products were determined by intensity-based quantification of each product using the ImageJ software. Same letters above the bars indicate no significant difference among the relative transcript abundances (One-Way ANOVA Tukey's test at 95 % confidence level). Vertical bars denote SE. n = 3.

plantlets. In contrast, the expression of *DCPSknox* in the shoot apices of plantlets treated with IAA and BA simultaneously, in which no flowering was induced, was significantly lower than that in flowering plantlets. Therefore, the expression of *DCPSknox* was in the decreasing order of BA > iPR > iP > TIBA >> IAA + BA treatments.

Similar to *DCPSknox*, expression of *DCPSCKX* was the highest in the shoot apices of BA-induced plantlets (Fig 5.25). Shoot apices of plantlets treated with iPR, TIBA and iP showed similar expression of *DCPSCKX*, in the range of 30 – 46 % of that in BA-induced plantlets. The expression of *DCPSCKX* was undetected in the shoot apices of plantlets treated with IAA and BA simultaneously. Thus, the expression of *DCPSknox* was in the decreasing order of BA > iPR > TIBA, iP treatments.

5.4 Discussion

Most naturally occurring cytokinins are adenine derivatives and can be classified by the configuration of their N^6-side chain as either isoprenoid or aromatic. The development of sensitive analytical methods for the determination of cytokinin levels in plant tissues was crucial to elucidate the roles of cytokinins. Various methods have been developed for the analysis of cytokinins in plant tissues and they include HPLC, gas chromatography and capillary electrophoresis (Tarkowski *et al.*, 2009) for instance. In the present study, a sensitive and efficient HPLC-ESI-MS/MS method for the separation and quantification of various cytokinins and IAA extracted from *D.* Chao Praya Smile was developed (Fig. 5.1 to 5.5 and Table 5.1). The development of this method was important because it represented a tool to investigate the changes in levels of cytokinins and IAA in the *D.* Chao Praya Smile plantlets upon

flowering induction using BA. HPLC was suggested as a particularly suitable technique for cytokinin analysis in view of the facts that cytokinins exhibit gradients in polarity and are readily detected by UV absorbance (Chen *et al.*, 1987). Among all the methods developed, HPLC-ESI-MS/MS represented the most common method in cytokinin analysis (Tarkowski *et al.*, 2009). A HPLC-ESI-MS/MS with Multiple Reaction Monitoring was developed as a fast and sensitive method for the determination of 16 compounds including IAA and its metabolites (Prinsen *et al.*, 1997). It was anticipated that HPLC-MS/MS would continue to become important tool in cytokinin analysis in plant science because, apart from sensitivity, this method could provide structural information based on the fragmentation patterns and cytokinins could be analyzed without the need of derivatization (Tarkowski *et al.*, 2009).

Analysis of cytokinins and IAA in flowering-induced plants was crucial towards the understanding of flowering mechanism in view of the fact that their contents could change in flowering-induced plants and could therefore be directly related to flowering. In the analysis of cytokinins at various growth stages during flowering induction in *D.* Chao Praya Smile, iPR level increased by more than 1.5 folds during floral transition (Fig. 5.9b). Furthermore, there were 20 % more of iPR at the shoot apex of the plantlet during this transitional phase (Fig. 5.15). The iPR in the shoot apex of *D.* Chao Praya Smile during floral transition could be transported from the leaves because there was even a higher concentration of iPR in the leaf tissues during floral transition (Fig. 5.16). The present findings were in accordance with other reports on different plant species which have indicated the role of cytokinins, especially the iP-type cytokinins, in floral transition. In *Arabidopsis*, cytokinin analysis in leaves and leaf exudate in long-day (LD)-induced plants has indicated the

role of cytokinins as a component of floral stimulus of leaf origin (Corbesier *et al.*, 2003). The concentrations of iPR and iPMP markedly increased in the leaf exudate during floral transition upon LD induction (Corbesier *et al.*, 2003). Similarly, the concentrations of iP-type cytokinins increased in the leaf tissues of *Arabidopsis* following flowering induction using triacontanol, cerium and lanthanum (He and Loh, 2002). In another LD plant *S. alba*, iP and iPR levels were found to increase in the leaf exudate following inductive LD treatment (Lejuene *et al.*, 1988). In addition, there was a 1.5 – 2 folds increase in the levels of iP-type cytokinins in the phloem sap feeding the shoot apex (Lejeune *et al.*, 1994). In orchid hybrids such as *Dendrobium* Second Love (de Melo Ferreira *et al.*, 2006) and *Dendrobium* Madame Thong-In (Sim *et al.*, 2008), plantlets induced to flowering *in vitro* using thidiazuron and BA, respectively, were also found to contain significant higher levels of iP and iPR during floral transition as compared vegetative plantlets. During flower bud development in *D.* Chao Praya Smile, the levels of Z-type and iP-type cytokinins were found to increase. However, it was found in *Humulus lupulus* that during flower development, the levels of Z-type and iP-type cytokinins reduced as compared to vegetative development (Villacorta *et al*., 2008). It was noted in the examples discussed above, various modes of flowering induction have been employed which included appropriate daylength, chemicals and plant growth regulators. Therefore, all the findings appeared to imply that iP and iPR were essential and commonly involved in the initiation of flowering in various plant species regardless of the modes of induction. However, it is difficult to conclude whether several folds increase in the cytokinins can induce different physiological phenomena.

In the monopodial orchid *Aranda* deborah, a flowering gradient has been reported to exist along the stem axis and it diminished basipetally (Goh, 1975). The

nature of the axillary shoots produced upon decapitation, whether vegetative or reproductive, was correlated to their position along the stem axis (Goh, 1975). Buds nearer to the apex developed into inflorescences while those further away from the apex developed into vegetative shoots (Goh, 1975). The flowering gradient has also been observed in other monopodial orchids such as *Holttumara* Maggie Manson and *Aranthera* James Storie (Hew and Yong, 1997). Goh (1974) suggested that the flowering gradient could be regulated by the levels of growth substances along the stem axis, especially auxin, based on the observation that auxin application stimulated vegetative shoot production in decapitated orchids. The hormonal nature of the flowering gradient was further substantiated by the findings showing that the aerial root tips closer to the apex of flowering *Aranda* Noorah Alsagoff contained higher level of cytokinins than those in non-flowering plant (Zhang *et al.*, 1995). In this study, a gradient of iP-type cytokinins was found to exist along the stem axis, with concentrations decreasing towards the basal part of BA-induced *D.* Chao Praya Smile plantlet at floral transition (Fig. 5.14). Therefore, it was likely that a flowering gradient, which was iP-type cytokinins in nature, existed in this *Dendrobium* hybrid.

High cytokinin/IAA ratios have been reported to be essential in the flowering of certain plant species such as bromeliads and longan (Mercier and Endres, 1999; Hegele *et al.*, 2008). Similarly, floral transition in *S. alba* was characterized by a decrease in the IAA/cytokinins ratio in the apical bud (Sotta *et al.*, 1992). In *Vanda* Miss Joaquim, the endogenous auxin was lower when grown under high irradiance that was in favor of flowering, and vice versa (Hew and Yong, 1997). It was suggested that the ratio of cytokinin/auxin, rather than the absolute amount of auxin, was the important determinant of flowering in this orchid. Auxin was suggested to control apical dominance by repressing local biosynthesis of cytokinins in the apical

bud (Tanaka *et al.*, 2006). Therefore, cytokinin application probably promoted flowering by releasing the buds from apical dominance controlled by auxin (Hew and Yong, 1997). This could also explain the flowering-promoting effect of a high cytokinins/IAA ratio in plant. In flowering-induced *D.* Chao Praya Smile plantlets, the cytokinins/IAA ratio at floral transition was more than 7 times higher than that in the vegetative plantlet (Fig. 5.12). Moreover, the ratio of cytokinins/IAA in the shoot apices of flowering-induced plantlets was approximately two folds of those in the non-induced plantlets (Fig 5.20). Higher cytokinins/IAA ratio in the BA-induced plantlets could be attributed to inhibited root growth in these plantlets. Roots were produced extensively in non-induced plantlets and were found to be a significant source of IAA (Fig. 5.19). Therefore, the results obtained probably indicated that, similar to the case of *Vanda* Miss Joaquim, it was the cytokinin/IAA ratio and not the absolute cytokinin concentration that determined *in vitro* flowering in *D.* Chao Praya Smile. Nonetheless, the cytokinins/IAA ratio in the flowering-induced *D.* Second Love at floral transition was not higher than that in the vegetative phase (de Melo Ferreira *et al.*, 2006) probably due to limited cytokinins analyzed. In view of the fact that BA accumulated in the shoot apices of the induced plantlets, it was possible that exogenously applied BA could act directly on SAM and induce flowering; and cytokinin (including BA) might have a real meaning as hormone function. The involvement of endogenous cytokinins in floral induction could be further clarified by investigating the expression of cytokinin biosynthesis genes and the changes of endogenous cytokinins during floral transition without applying exogenous BA.

CKX, which has substrates specificity for iP and iPR, plays a significant role in cytokinin metabolism in plants by catalyzing the irreversible degradation of cytokinins (Mok and Mok, 2001). The results obtained showed that *DCPSCKX* was

markedly upregulated in *D.* Chao Praya Smile plantlets treated with BA, and remained significantly higher than that of the non-induced plantlets at various growth stages (Fig. 5.23). Similarly, *D.* "Sonia" and *D.* huoshanense *CKX* genes were found to be rapidly upregulated in the presence of BA (Yang *et al.*, 2003; Wang *et al.*, 2008b). The *CKX* gene isolated from maize was also shown to be rapidly induced by exogenous application of BA at concentration as low as 0.01 µM (Brugière *et al.*, 2003). A higher expression of *DCPSCKX*, which is associated with the degradation of cytokinins, in the BA-induced plantlets should have resulted in reduced cytokinin contents in the plantlets. However, the total cytokinin contents in these plantlets were not reduced and their concentrations were similar to those of the non-induced plantlets at different growth stages (Fig. 5.6). One of the possible explanations for this observation was the stimulation of cytokinin biosynthesis in BA-induced plantlets. It has been proposed that exogenously applied BA could trigger a positive feedback loop that involved cytokinin biosynthesis to increase the cytokinin content in plant (Kamínek *et al.*, 1997). The cytokinins synthesized would in turn serve as substrates that stimulated the expression of *CKX* gene. The enhancement of CKX activity by exogenous application of cytokinins could be under transcriptional or translational regulation as it was sensitive to inhibitors of RNA and protein synthesis (Chatfield and Armstrong, 1988).

The homogenous expression of *DCPSCKX* in different tissues in *D.* Chao Praya Smile plantlets at floral transition (Fig. 5.24) probably indicated that *DCPSCKX* response to BA treatment was not tissue specific and cytokinin degradation could take place in all tissues. It could further imply that the cytokinin biosynthesis stimulated by BA application, if it did exist in *D.* Chao Praya Smile, was taking place in the entire

plantlet during floral transition. Other *CKX* homologues in *D.* Chao Praya Smile could be investigated to understand the roles of these genes in the plantlets.

The significant role of iPR in the flowering of *D.* Chao Praya Smile was further substantiated by its capability to induce flowering at efficiency on par with BA (Table 5.2). It was deduced that iPR probably acted downstream upon BA treatment and participated directly in flowering initiation in the SAM, in view of its accumulation at the shoot apex during floral transition. The iPR was not derived from its free base because treatment with iP hardly induced flowering in *D.* Chao Praya Smile (Table 5.2). On the other hand, the inhibitory role of IAA on flowering was verified by treating BA-induced plantlets with this plant growth regulator (Table 5.2). IAA has also been shown to inhibit the photoperiodic induction of flowering in *Pharbitis nil* (Kęsy *et al.*, 2008). It was possible that IAA inhibited flowering in *D.* Chao Praya Smile by disrupting the cytokinin/auxin ratio in the plantlets as it was found that TIBA, an auxin transport inhibitor, was able to induce flowering in this orchid hybrid (Table 5.2). TIBA application was shown to reduce the amount of IAA below the site of application when it was applied at the internode nearest to the shoot tip (Ross, 1998) and increased the endogenous cytokinin concentrations (Ito, 2001). Therefore, it was likely that TIBA induced flowering in *D.* Chao Praya Smile (Table 5.2) by reducing the IAA content and thereby maintaining a higher cytokinin/IAA ratio which was in favor to flowering. The flowering-promoting effect of TIBA has also been shown in *Tulipa gesneriana* (Geng *et al.*, 2005). However, TIBA was shown to prevent *in vitro* flowering of *Cymbidium niveo-marginatum* Mak (Kostenyuk *et al.*, 1999) probably indicating the specificity in flowering mechanisms in different orchid species. The effects of exogenously applied Z, ZR, DHZ and DHZR on flowering induction could also be investigated which may provide

suggestive information on the action of these naturally occurring cytokinins; especially Z which present at high level in *D.* Chao Praya Smile.

DCPSknox and *DCPSCKX* were probably involved in the BA induction of flowering in *D.* Chao Praya Smile as it was shown that IAA treatment of BA-induced plantlets, which nullified the flowering effect of BA (Table 5.2), drastically reduced the expression of these two genes (Fig. 5.26). In addition to the role of controlling shoot development, *DCPSknox* might contribute to flowering by increasing the cytokinin contents in the plantlets. KNOX proteins have been reported to induce the expression of cytokinin biosynthesis gene in *Arabidopsis* (Yanai *et al.*, 2005) and rice (Sakamoto *et al.*, 2006) and hence increased the cytokinin content in the plants. In the case of iP and iPR, there was a huge difference in the percentage of flowering despite the similar levels of expression of *DCPSknox* and *DCPSCKX* (Fig. 5.26). This probably indicated the specific role of iPR in flowering induction. *DCPSCKX* was induced because iP and iPR are substrates of CKX (Mok and Mok, 2001). In *D.* "Sonia", *CKX* gene was strongly induced by BA and moderately by iP and iPR (Yang *et al.*, 2003), as was observed in *D.* Chao Praya Smile (Fig. 5.26). TIBA is not a substrate for CKX. However, *DCPSCKX* was expressed in TIBA treated plantlets (Fig. 5.26) probably because TIBA increased the cytokinin contents in the plantlet. TIBA has been shown to increase the cytokinin levels in the shoot of Japanese pear (Ito, 2001). More work would be needed to further elucidate the role of *DCPSknox* and *DCPSCKX* in flowering induction.

Bernier *et al.* (1993) have proposed a model of regulatory loop involving sucrose and cytokinins in the control of floral transition in *S. alba*, a member of the Brassicaceae family. In the model, upon perception of LD by leaves, starch was mobilized in the leaves and stem while sucrose was transported to both the apical

meristem and roots via the phloem. This was followed by the transport of ZR and iPR in the xylem from roots to leaves. The final step involved the transport of iP, which could be produced locally or converted from iPR, in the phloem from leaves to the apical meristem. The apical meristem, enriched with sucrose and iP, then proceeded to floral transition (Bernier *et al.*, 1993). The model has been further verified by Havelange *et al.* (2000) using experiments that interrupted phloem and xylem transport by bark girdling and growing plants in 100 % relative humidity.

The model proposed for *S. alba* could not be adopted for *D.* Chao Praya Smile. Firstly, Tropical *Dendrobium* hybrids like *D.* Chao Praya Smile was most likely day neutral plants, which were indifferent to daylength (Hew and Yong, 1997). Therefore, LD perception that stimulated the translocation of sucrose, which was the first step in the proposed model that appeared to trigger the cascade of events leading to flowering initiation, might not take place in *D.* Chao Praya Smile. In addition, the results obtained showed that sucrose treatment was insufficient to induce flowering in *D.* Chao Praya Smile (section 3.3.2) and that BA remained as the most important factor in flowering induction (Table 3.1). Secondly, root to leaf transport of ZR and iPR as proposed in *S. alba* was unlikely to take place because BA treatment greatly inhibited root production in *D.* Chao Praya Smile (Fig 4.4, Table 4.1). Thirdly, local synthesis of iP or conversion of iPR to iP was not seen in the leaves of BA-induced *D.* Chao Praya Smile because its iP concentration was not higher than that in the non-induced plantlet (Fig. 5.16). Instead, iPR content was higher in the leaves of BA-induced plantlet (Fig. 5.16), as it was observed in the shoot apex (Fig. 5.15). Therefore, it was plausible that iPR, and not iP, participated in flowering initiation in *D.* Chao Praya Smile. Moreover, iPR could induce flowering in *D.* Chao Praya Smile at efficiency similar to BA (Table 5.2). In conclusion, the flowering mechanism might

not have been conserved between *Brassicaceae* and *Orchidaceae*; the differences could arise from the different modes of flowering induction. Nonetheless, it was apparent that iP-type cytokinins were significant in the flowering of these two plant species.

5.5 Concluding remarks

A sensitive and efficient HPLC-ESI-MS/MS method to analyze the changes in concentrations of cytokinins and IAA in flowering-induced *D.* Chao Praya Smile was developed. It was shown that the level of total cytokinins was not affected upon BA treatment and that the concentration of iPR significantly increased in the plantlets during floral transition. Besides, it was also found that the cytokinins/IAA ratio in the BA-induced plantlets was more than 7 times higher during floral transition than the non-induced vegetative plantlets, primarily the result of reduced IAA content in BA-induced plantlets. Cytokinins and IAA were also analyzed in the various tissues of *D.* Chao Praya Smile undergoing floral transition. Similarly, it was found that the shoot apices of BA-induced plantlets contained 20 % more of iPR than the non-induced plantlets. It was likely the iPR was transported from the leaves which contained the highest concentration of iPR. Besides, a gradient of iP-type cytokinins was observed along the stem axes of *D.* Chao Praya Smile plantlets during floral transition, and this was speculated as floral gradient. The shoot apex of *D.* Chao Praya Smile was also found to exhibit a higher cytokinin/IAA ratio during floral transition as a result of reduced IAA content. Therefore, it was possible that the endogenous cytokinin/IAA ratio, and not the absolute amount cytokinins, determined flowering in *D.* Chao Praya Smile. *DCPSCKX* was cloned from *D.* Chao Praya Smile and its expression was

drastically upregulated upon BA treatment. Finally, the inductive and inhibitory roles of iPR and IAA, respectively, were verified in *D.* Chao Praya Smile.

Chapter 6

CONCLUSION

In this study, flowering in *Dendrobium* Chao Praya Smile was successfully induced within 6 months from seed germination in a two-layer medium using 6-benzyladenine (BA). With careful selection of morphologically normal seedlings prior to transfer to the two-layer medium, more than 70 % of the plantlets could be induced to flowering *in vitro*. The functionality of these *in vitro*-developed flowers was shown through sporad analysis and pollen grain germination. The *in vitro*-developed flowers could be self-pollinated to produce seedpods and viable seeds in culture. This indicated the capability of *D.* Chao Praya Smile to undergo a complete life cycle from seed germination to the production of next-generation seeds entirely in culture within a period of 11 months. It also implied that orchid breeding could be fast-tracked using *in vitro* flowering and seed production in culture in view of the facts that conventional orchid breeding in field-grown plants could take up to several years.

Morphological examination and histological analysis revealed that *D.* Chao Praya Smile proceeded to floral transition as early as 54 days after BA treatment in liquid medium. Subsequent transfer to the two-layer medium allowed the development of floral buds. It was also shown that the expression of *DCPSknox*, which played a role in the maintenance of indeterminacy of shoot apical meristem (SAM), was gradually reduced during floral transition, probably indicating the commitment of BA-induced plantlets to flowering. On the other hand, it was found that both complete and incomplete flowers were produced *in vitro*. Segregation of colors was observed in the complete flowers developed *in vitro* with varying intensity of pink coloration in the lips, petals and sepals.

These flowers could be classified into 4 types based on the color intensity in the floral organs. To investigate the cause of color segregation, *DCPSCHS* gene, which was involved in anthocyanin production, was cloned and its expression was investigated. The results obtained indicated that color segregation *in vitro*-developed flowers was probably naturally occurring as BA-induced and non-induced plantlets showed similar expression levels of *DCPSCHS*. It could also signify that the *in vitro* flowering system developed was suitable for early evaluation of flower characteristics.

A HPLC-ESI-MS/MS method was developed to analyze the changes in levels of cytokinins and indole-3-acetic acid (IAA) in *D.* Chao Praya Smile in relation to BA-induction of flowering. The content of iPR was found to increase in the plantlets during floral transition. The shoot apex of the plantlet was found to contain more iPR during this phase change which could be transported from the leaves. The significant role of iPR in flowering was further substantiated by its capability to induce flowering in *D.* Chao Praya Smile just like BA. The IAA content in BA-induced plantlets was lower than that in the non-induced plantlets primarily due to inhibited root growth as the roots were found to contain predominantly of IAA. The roots of non-induced plant were found to contain predominantly IAA. However, the roots might not be the main source of IAA in view of the fact that comparable concentrations of IAA were also detected in other tissues of the plantlets. In addition, the shoot apices, leaves and stems of BA-induced plantlets were also found to contain IAA during floral transition even when roots were not produced in these plantlets. It was reported that the shoot apex was the main site of IAA biosynthesis (Normanly *et al.,* 2004). Therefore, it was likely that IAA was synthesized in the shoot apices and transported to other tissues in *D.* Chao Praya Smile plantlets. It was also

possible that IAA was synthesized in the roots of *D.* Chao Praya Smile although evidence was not presented in this study. It could be proposed that a higher cytokinins/IAA ratio, instead of the absolute amount of cytokinins, could be essential to flowering as it was found in the plantlet during floral transition. The inhibitory effect of IAA was verified as it could nullify the flowering-inductive effect of BA at concentration as low as 0.25 μM.

Cytokinin analysis and expression study of *DCPSCKX* at different growth stages provided some clues on the possible mechanisms underlying the BA-induction of flowering in *D.* Chao Praya Smile (Fig. 6.1). It could be proposed that BA induced flowering in the plantlets by stimulating a positive feedback loop that relied on cytokinin biosynthesis (Kamínek *et al.*, 1997). This could explain well for the presence of substantial amount of cytokinins in the plantlets despite the consistent and dramatic increase of *DCPSCKX* expression, which catalyzed the degradation of cytokinins. With enhanced cytokinin biosynthesis, iPR could be produced through the dephosphorylation of iP nucleotides, which represented the initial products of cytokinin biosynthesis (Kakimoto, 2003b). The iPR accumulated in the shoot apex, which could be synthesized locally or transported from the leave, then participate in flowering induction. Equally important was that BA treatment inhibited root growth in the plantlets, which in turn reduced the amount of root-produced IAA and generated a higher cytokinin/IAA ratio in the shoot apex that promoted flowering. However, the direct action of BA on flowering induction should not be neglected in view of the fact that BA was accumulated in all tissues of the plantlets, including the shoot apices. The role of exogenously applied BA could only be examined under the condition of no cytokinin biosynthesis. Nonetheless, the actual role of iPR, which accumulated in the shoot apex, in flowering induction

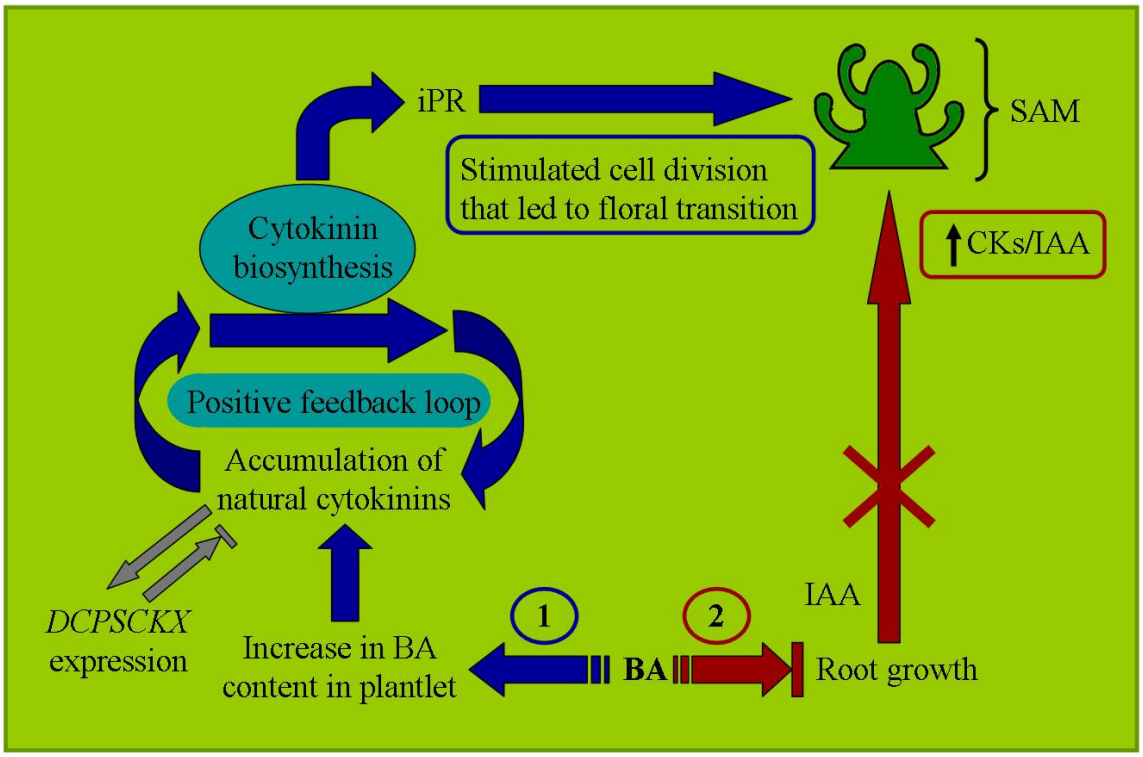

Fig. 6.1 Proposed mechanisms underlying the BA-induction of *in vitro* flowering in *D.* Chao Praya Smile. BA treatment increased the BA content in the plantlets which in turn caused the accumulation of natural cytokinins through a positive feedback loop that involved cytokinin biosynthesis (route 1, blue arrows). The natural cytokinins accumulated served as substrates and stimulated the expression of *DCPSCKX*. iPR, which could be produced through dephosphorylation of iP nucleotides generated in cytokinin biosynthesis, stimulated cell division in the SAM and subsequently led to flowering. On the other hand, BA treatment inhibited root growth in the plantlets (route 2, red arrows). In this case, root-produced IAA was reduced and a higher cytokinins/IAA ratio that promoted flowering was created in the SAM.

remained to be elucidated. It was possible that iPR initiated flowering by stimulating cell division in the SAM. Enhanced cell division was shown to be essential before floral transition could take place (Jacqmard *et al*., 2003).

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APPENDIX

Hee KH, Loh CS, Yeoh HH (2007) Early *in vitro* flowering and seed production in culture in *Dendrobium* Chao Praya Smile (Orchidaceae). Plant Cell Reports 26: 2055-2062.

Plant Cell Rep (2007) 26:2055–2062
DOI 10.1007/s00299-007-0421-9

CELL BIOLOGY AND MORPHOGENESIS

Early in vitro flowering and seed production in culture in *Dendrobium* Chao Praya Smile (Orchidaceae)

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Received: 15 May 2007 / Revised: 19 July 2007 / Accepted: 22 July 2007 / Published online: 7 August 2007

Abstract Plantlets of *Dendrobium* Chao Praya Smile maintained in vitro were induced to flower, which produced viable seeds within about 11 months. A two-layer (Gelrite-solidified layer topped with a layer of liquid medium of the same volume and composition) culture system containing benzyladenine (BA) at 11.1 μM induced the highest percent of flowering (45%) in plantlets within 6 months from germination. The percentage of inflorescence induction was increased to 72% by pre-selecting morphologically normal seedlings prior to two-layer culture. Plantlets in culture produced both complete (developmentally normal but smaller than flowers of field grown plants) and incomplete flowers. Pollen and female reproductive organs of in vitro-developed complete flowers were morphologically and anatomically similar to flowers of field grown plants. In addition, 65% of the pollen grains derived from in vitro-developed flower were tetrad suggesting that regular meiosis occurred during microsporogenesis. The percentage of germination of pollen grains derived from in vitro-developed flowers and flowers of field grown plants, incubated on modified Knops' medium for 8 days, were 18.2 and 52.8%, respectively. Despite a lower percentage of germination of the pollen grains derived from in vitro-developed flowers, flowers induced in culture could be self-pollinated and developed seedpods with viable seeds. Nearly 90% of these seeds developed into protocorms on germination in vitro. These seedlings were grown in culture and induced to flower in vitro again using the same procedure.

Communicated by P.P. Kumar.

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Keywords *Dendrobium* Chao Praya Smile · In vitro flowering · Seed production in culture · Sporad analysis · Pollen germination

Introduction

The increase in popularity of orchids in Asia, Europe and the United States has led to continued increase in worldwide orchid production (Winkelmann et al. 2006). Also, with increasing demand for orchid cut-flowers and potted plants, the need to generate new commercial cultivars is constantly expanding. Conventional orchid breeding is time consuming, irrespective of the demand for new clones, because orchid propagation requires a long period of in vitro culture. Orchid breeding involves pollination, seedpod maturation, protocorm development, in vitro growth of seedlings and subsequent ex vitro establishment of seedlings. The entire breeding cycle could be between 3 and 5 years depending on the genotypes involved (Kamemoto et al. 1999). For instance, it has been shown that breeding *Dendrobium* hybrids could take up to 5 years (Fadelah 2006). This is primarily due to the long juvenility of these orchids which can span up to 30 months. Juvenility refers to the early phase of plant growth during which flowering does not occur normally under natural conditions (Hew and Yong 1997).

To keep in pace with the increasing demand, methods for rapid in vitro propagation of orchids have been developed (Martin and Madassery 2006; Kuo et al. 2005; Nayak et al. 2002; Park et al. 2002). To overcome the long juvenile phase of orchid cultures, protocols to induce early in vitro flowering have been developed in several *Dendrobium* orchids (Sim et al. 2007; Ferreira et al. 2006; Wang et al. 1997). These early in vitro flowering protocols could shorten the time required for flowering, which could be

used to get an early indication of floral characteristics. But more importantly, in vitro flowering could be used to fast-track breeding, provided viable seed production can be realized with such a system. Production of viable orchid seeds in culture following crossing has not been reported to date. The objectives of our study were: (1) to induce in vitro flowering in *Dendrobium* Chao Praya Smile; (2) to produce seedpods in culture with viable seeds by self-pollinating the in vitro-developed flowers; and (3) to examine pollen and ovule development in flowers developing in vitro and in field. In this paper, we report early seed production in culture in *Dendrobium* Chao Praya Smile and discuss its application in orchid breeding.

Materials and methods

Plant materials, culture media and culture conditions

Flowers of *Dendrobium* Chao Praya Smile (*Dendrobium* Pinky × *Dendrobium* Kiyomi Beauty) were self-pollinated. The seedpods were harvested 120 days after pollination. The seeds obtained from the seedpods were germinated aseptically in 90 mm petri dishes with 25 ml of modified Knudson C medium (KC, Knudson 1946) supplemented with 2% (w/v) sucrose, 15% (v/v) coconut water and 0.3% (w/v) Gelrite. All media were adjusted to pH 5.3 before autoclaving at 121°C for 20 min.

Eight-week-old protocorms were transferred to 50 ml of modified KC liquid culture medium containing (mg l^{-1}): $MgSO_4 \cdot 7H_2O$ (250), KH_2PO_4 (500), $(NH_4)_2SO_4$ (250), $Ca(NO_3)_2 \cdot 4H_2O$ (500), $MnSO_4 \cdot H_2O$ (5.68) and EDTA-Fe (28) supplemented with 2% (w/v) sucrose and 15% (v/v) coconut water in 100 ml Erlenmeyer flasks on rotary shakers at 120 rpm for proliferation. The liquid media were also supplemented with benzyladenine (BA) at 0–22.2 μM. After three rounds of sub-culturing in the liquid medium at 3-week intervals, the seedlings were transferred to two-layer (Sim et al. 2007) modified KC medium (containing the same composition as the modified KC liquid culture medium) in Magenta GA7^{TM} containers. The two-layer culture media consisted of 50 ml of Gelrite-solidified medium topped with a layer of liquid medium of the same volume and composition. All cultures were incubated at 25 ± 2°C and a 16 h photoperiod of 40 μmol m^{-2} s^{-1} from daylight fluorescent lamps.

Sporad analysis of pollinia

Pollinia were transferred from in vitro-developed flowers and flowers of field-grown plants onto a slide using a pair of fine forceps after removing the operculum. The pollinia were mounted in a drop of water and teased apart with a scalpel. One drop of acetocarmine (1%, w/v) was added to the pollen and observed under the microscope.

Pollen germination in vitro

Three in vitro-developed flowers that were open for 3 or 4 days were chosen. Four halves of pollinia from each flower were transferred, respectively, in a laminar flow hood onto 5 ml of solidified modified-Knops' medium in 35 mm petri dishes. The modified-Knops' medium consisted of (in mg l^{-1}) H_3BO_3 (100), $Ca(NO_3)_2 \cdot H_2O$ (300), $MgSO_4 \cdot 7H_2O$ (200), KNO_3 (100), sucrose (5%, w/v) and Gelrite (0.3%, w/v). Observation for pollen grain germination was carried out after 2, 4, 8 and 12 days of incubation at 28°C. For observation under microscope, the germinated pollen grains were transferred from the solidified modified-Knops' medium onto a glass slide with a drop of water. The pollen grains were teased apart with the aid of a needle and a blade. One drop of acetocarmine (1%, w/v) was then added to the pollen grains. For each pollinium, 250–300 pollen grains were observed for germination.

In vitro pollination and seed production in culture

Plantlets that bore freshly-opened complete flowers were transferred to fresh two-layer KH medium. These in vitro-developed flowers were self-pollinated in a laminar flow hood using a pair of forceps. Upon pollination, the plantlets were observed for seedpod formation. At 120 days after pollination, the seedpods were harvested and cut open. Seeds from these in vitro-developed seedpods were germinated on modified KC medium. Seedlings grown from these seeds were further induced to flowering. All statistical analyses were carried out using One-way ANOVA Tukey's test at 95% confidence level.

Results

Inflorescence induction in vitro

Dendrobium Chao Praya Smile was induced to flower within 6 months from germination using BA in two-layer culture (Fig. 1a). The highest percent of flowering (45%) was induced in plantlets at 11.1 μM BA (Table 1). Plantlets grown in BA-free medium did not produce inflorescence. Each flowering plantlet produced one inflorescence stalk with an average of three to four flower buds. As for the duration of induction, inflorescences were

produced earliest at 4 weeks upon transfer to two-layer culture at 4.4 and 11.1 μM of BA (Table 2), with the highest inflorescence induction rate at 11.1 μM of BA after 8 weeks. It was also observed that both complete and incomplete flowers were produced in the plantlets. Complete flowers had all floral organs (Fig. 1b). In incomplete flowers, some of the floral organs were absent (Fig. 1c, d) or they were morphologically distorted (Fig. 1e). About 50% of the flowering plantlets produced only complete flowers while another 44% of the flowering plantlets produced both complete and incomplete flowers on the same inflorescence (Table 1).

Some seedlings died in the liquid culture containing BA. Seedling mortality increased with increasing BA concentration. Thus about 34% of the seedlings (n = 100) were dead at 22.2 μM of BA after 9 weeks of culture in liquid medium, compared to 5% mortality in BA-free liquid medium. In order to secure more seedlings, seedlings were treated with BA only in the two-layer culture. BA treatment at 11.1 μM in two-layer culture was sufficient to induce inflorescence production in 42% of the plantlets (Table 3). In addition, nearly all inflorescences induced in this late-BA-treatment bore flower buds, although the number was lesser than that in consecutive BA treatments in both liquid and two-layer cultures. To further improve inflorescence induction, morphologically normal seedlings in the liquid culture were selected prior to transfer to two-layer culture. This screening process increased the inflorescence induction from 45 to 72% at 11.1 μM BA (Table 4). The pre-selection method was useful as none of the morphologically abnormal seedlings produced inflorescence.

In vitro plantlets produced lesser and smaller flowers than field grown plants. An average of four flower buds were produced in each in vitro plantlet with flower diameter of 2–2.5 cm whereas field grown plants could produce an average of 12 flowers of 4 cm in diameter (Fig. 2a, b). The lengths of stomata on lower epidermis of leaves of the in vitro plantlets and field grown plants were 30.9 ± 2.0 and 38.5 ± 0.4 μm, respectively (Fig. 2c, d). Conversely, in vitro plantlets had higher stomatal density than field-grown plants, 38 ± 0 and 23 ± 1 per mm^2, respectively.

Pollen and female reproductive organs

Three in vitro-developed complete flowers were examined for their pollen grains and female reproductive organs in comparison to flowers of field grown plants. Pollinia derived from the in vitro-developed flowers were green and consisted of four halves. They were waxy, 1.8 mm in length and half the thickness of the pollinia derived from flowers of field grown plants (Fig. 2e, f). Stigma of the in vitro-developed flower was clear and sticky. Column and ovary of the in vitro-developed flower were clearly visible when the flower was dissected along the axis of symmetry (Fig. 2h). These female reproductive organs appeared to be anatomically similar to that in flowers of field grown plants (Fig. 2g), albeit smaller. The ovary of the in vitro-developed flower was found to be approximately 1 cm in length, compared to 1.5 cm in flowers of field grown plants.

Sporad analysis and pollen germination

Observation on the pollen derived from in vitro-developed flower showed 65% normal tetrad and 35% triad (Table 5). Similar observation was obtained in the pollen derived from flowers of field grown plants at which 79% of the

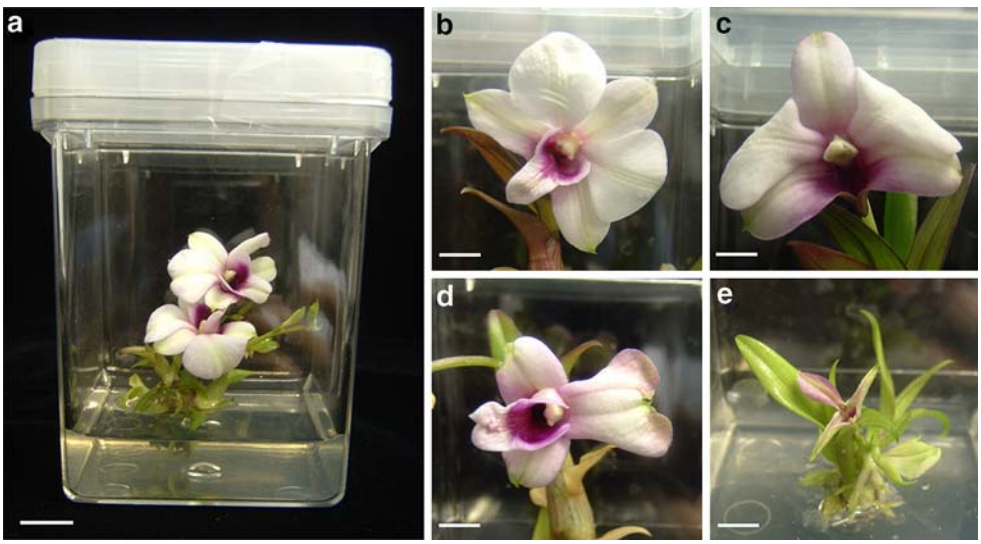

Fig. 1 In vitro flowering and production of complete and incomplete flowers. **a** Flowering in *Dendrobium* Chao Praya Smile in GA7^{TM} container. *Bar* 1 cm. **b** A complete flower. *Bar* 5 mm. **c**–**e** Incomplete flowers lacking floral organs or with totally distorted organs. *Bar* 5 mm

Table 1 Inflorescence induction and flower development in *Dendrobium* Chao Praya Smile

BA (μM)	No. of plantlets	% Plantlet with		% Flowering plantlet producing		
		Inflorescence stalk[a]	Flower bud[b]	Both complete and incomplete flowers[c]	Complete flowers only[c]	Incomplete flowers only[c]
0	22	0	0	0	0	0
4.4	36	14 (5)	8 (3 ± 1) a	67 (2)	33 (1)	0
11.1	20	45 (9)	45 (3 ± 1) a	33 (3)	56 (5)	11 (1)
22.2	15	27 (4)	27 (5 ± 1) a	50 (2)	50 (2)	0

The seedlings were grown in liquid culture for 9 weeks followed by two-layer culture, both of which containing the same concentrations of BA. Scoring of inflorescence production and flower bud formation were made at 10 weeks in two-layer culture when maximum number of flower bud had been formed. Assessment of flower development was made for individual flower bud at bloom

[a] Numbers in the parentheses indicate the number of plantlets with inflorescence stalk

[b] Numbers in parentheses indicate average number of flower bud per inflorescence ± SE. Same letters following the parentheses indicate no significant difference among the numbers of flower bud

[c] Numbers in the parentheses indicate the number of flowering plantlets

Table 2 Effects of BA on early inflorescence induction in *Dendrobium* Chao Praya Smile

BA (μM)	No. of plantlets	Plantlet with inflorescence (%)	
		4 weeks[a]	8 weeks[a]
0	32	0	0
4.4	30	7 (2)	17 (5)
11.1	26	8 (2)	39 (10)
22.2	18	0	22 (4)

The seedlings were grown in liquid culture for 9 weeks followed by two-layer culture, both of which containing the same concentrations of BA. Scoring of inflorescence production was made at 4 and 8 weeks in the two-layer cultures

[a] Numbers in the parentheses indicate the number of plantlets with inflorescence stalk

Table 3 Effects of BA on flowering induction in *Dendrobium* Chao Praya Smile

BA in two-layer culture (μM)	No. of plantlets	% Plantlet with	
		Inflorescence stalk[a]	Flower buds[b]
0	30	0	0
4.4	29	34 (10)	34 (3 ± 0) a
11.1	24	42 (10)	38 (3 ± 1) a
22.2	22	36 (8)	36 (4 ± 1) a

Seedlings were grown in BA-free liquid medium for 9 weeks followed by treatment with BA of various concentrations in two-layer culture. Scoring of inflorescence production and flower bud formation were made at 10 weeks in two-layer culture when maximum number of flower bud had been formed

[a] Numbers in the parentheses indicate the number of plantlets with inflorescence stalk

[b] Numbers in parentheses indicate average number of flower bud per inflorescence ± SE. Same letters following the parentheses indicate no significant difference among the numbers of flower bud

sporads were tetrads. Both the tetrad pollens derived from in vitro-developed flower and flower of field grown plant were in the range of 30–40 μm (Fig. 3a, b). Monad and dyad, which resulted from irregular meiosis, were not observed in both cases. The pollens derived from in vitro-developed flowers and flowers of field grown plants germinated on modified-Knops' medium after 2 days of incubation. After 8 days of incubation, 18.2 and 52.8% of pollens derived from in vitro-developed flowers and flowers of field grown plant, respectively, germinated (Table 5, Fig. 3c).

Seed production

Three out of four in vitro pollinations were successful and led to seedpod development (Fig. 4a). At the time of maturation, the seedpods were 1.5–1.8 cm in length, compared to 2.7–3.0 cm of the seedpods developed in field (Fig. 4b, c). The seedpods developed in vitro were harvested 120 days after pollination when they turned slightly yellowish. These seedpods contained yellowish and dust-like seeds. The seeds were 428 ± 10 μm in length, shorter than those obtained from seedpods developed in the field (684 ± 13 μm; Fig. 4d, e). The seeds produced in the in vitro-developed seedpods were fertile with more than 90% developing into protocorms on modified KC agar medium after 8 weeks. One in vitro-developed seedpod produced 500–1,000 seedlings. These seedlings produced inflorescences upon induction using BA.

Table 4 Effects of seedlings selection on rate of inflorescence induction in *Dendrobium* Chao Praya Smile

BA (μM)	Inflorescence production (%)	
	Morphologically normal plantlets[a]	Morphologically abnormal plantlets[a]
0	0	0
4.4	53 (19)	0
11.1	72 (26)	6 (2)
22.2	22 (8)	0

Selections of 36 morphologically normal and abnormal seedlings, respectively, for each treatment were carried out prior to transfer to two-layer culture

[a] Numbers in the parentheses indicate the number of plantlets with inflorescence

Discussion

Reproduction is an important stage of plant development. In orchids, sexual reproduction can be effected through flowering resulting in the production of seedpod and seeds (Hew and Yong 1997). In this study, *Dendrobium* Chao Praya Smile was shown to flower and produce seeds in culture. Optimal BA concentration was required to induce maximum inflorescence production in the plantlets. Two-layer culture system was adopted in *Dendrobium* Chao Praya Smile because this culture system was reported to promote normal development of flower buds in orchid (Sim et al. 2007). In our experiments, we have observed that plantlets of *Dendrobium* Chao Praya Smile were unable to produce inflorescence when they were cultured on Gelrite-solidified medium.

Plantlets of *Dendrobium* Chao Praya Smile produced complete and incomplete flower concurrently in in vitro culture. As the aim of our study was to produce seeds in culture, production of complete flowers that resemble the flowers of field grown plants was desired. BA was required for normal development of floral buds in roses (Vu et al. 2006), which possibly regulated floral development through genes controlling shoot apical meristem activity (Lindsay et al. 2006). On the other hand, a lesser number of flowers were produced in in vitro plantlets compared to field grown plants. This could be due to the smaller size of in vitro plantlets as reproductive output could be affected by plant size (Sletvold 2002). Despite the production of a lesser number of flowers in in vitro plantlets, breeding success would not be hindered because numerous seeds can be produced in one seedpod and would be sufficient for breeding. In our study, seedlings with abnormal leaf arrangement or non-expanding leaves were not selected for inflorescence induction. These abnormal seedlings could not produce any inflorescence upon BA treatment. Morphological abnormalities in the *Dendrobium* seedlings could be the result of cytokinin activity because cytokinins have been reported to affect the morphogenesis of early seedlings (Nikolić et al. 2006). Selection of morphologically normal seedlings for BA treatment would therefore ensure a higher percentage of inflorescence induction.

Morphologies of pollen and female organs could be correlated to breeding and hybridization success (Fratini et al. 2006). Morphological and anatomical examination of pollens and female organs of in vitro-developed flowers revealed that they were similar to flowers of field grown plants and were therefore probably functional. In the female organs, column connects stigma to ovary and allows

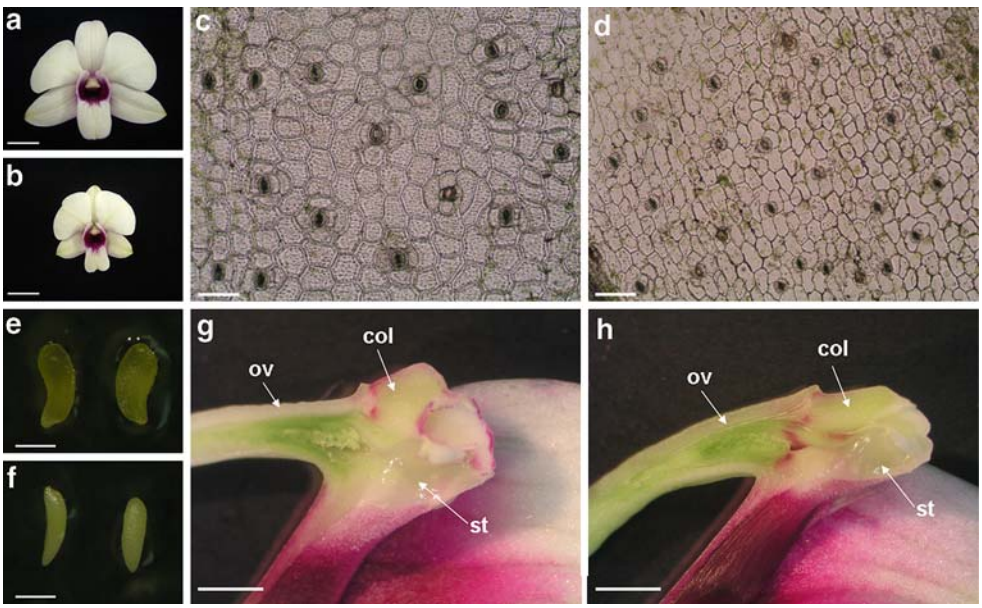

Fig. 2 Comparison of reproductive organs and leaf epidermal peels of *Dendrobium* Chao Praya Smile grown in field and in culture. **a**, **b** Flower of field grown plant and in vitro-developed flower, respectively. *Bar* 1 cm. **c**, **d** Leaf epidermal peels of *Dendrobium* Chao Praya Smile grown in field and in culture, respectively. *Bar* 100 μm. **e**, **f** Pollinia derived from flower of field grown plant and in vitro-developed flower, respectively. *Bar* 1 mm. **g**, **h** Female reproductive organs in flower of field grown plant and in vitro-developed flower, respectively. *col*, *ov* and *st* refer to column, ovary and stigma, respectively. *Bar* 5 mm

Table 5 Sporad formation and in vitro germination of pollen grains derived from flowers of field grown plants and in vitro-developed flowers

Pollen grains derived from	Sporad formation			Pollen germination (%)		
	Total sporad observed	Total sporad observed (%)		Incubation (days)		
		Triad	Tetrad	2	4	8
Flowers of field grown plant	214	21	79	15.5	29.8	52.8
In vitro-developed flowers	280	35	65	3	5.5	18.2

the growth of pollen tubes towards the ovule during fertilization. Thus, production of normal flowers with functional reproductive organs is imperative for successful breeding attempts using in vitro flowering technology.

Pollen quality of the in vitro-developed flowers was assessed by sporad analysis and in vitro pollen germination because it also determines breeding success. Meiotic behavior and sporads formation have been studied in orchids in relation to their fertility (Lee 1994, 1987, 1988; McConnell and Kamemoto 1993). In orchid microsporogenesis, regular meiosis results in four microspores grouped together, called a tetrad. When meiosis is irregular, polyploid spores in the form of monads, dyads or triads will be formed. Pollination of polyploid gametes could result in the formation of sexually sub-fertile or infertile progenies (Teoh 1984). Therefore, high percentage of tetrad formation in the pollen derived from in vitro-developed flower indicated regular meiosis and pollen fertility of in vitro plantlets. In vitro pollen germination is regarded as a reliable test of fertility with the assumption that pollen capable of germination would be fertile pollen (Montaner et al. 2003). However, the rate of pollen germination in vitro largely depends on optimization of the medium (Heslop-Harrison et al. 1984) and this factor has to be taken into consideration while counting germination as an indication of pollen quality. In our study, modified-Knops' medium promoted germination of the pollens derived from in vitro-developed flowers and flowers of field grown plants but germination on this medium was slow and maximum germination was observed after 8 days of

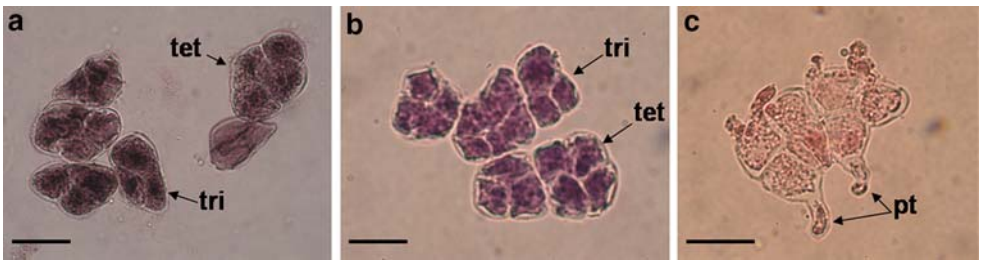

Fig. 3 Sporads and in vitro pollen germination. **a**, **b** Sporads derived from flower of field grown plant and in vitro-developed flower, respectively. *tet* and *tri* refer to tetrad and triad, respectively. *Bar* 30 μm. **c** Germination of pollens derived from in vitro-developed flower on modified-Knops' medium. *pt* refers to pollen tube. *Bar* 30 μm

Fig. 4 Seedpod development and seed production in culture. **a** Formation of seedpod in a plantlet upon self-pollination of an in vitro-developed flower. *Bar* 1 cm. **b**, **c** Seedpods developed in field and in culture, respectively. *Bar* 1 cm. **d**, **e** Seeds produced by field grown plant and plantlet in culture, respectively. *Bar* 100 μm

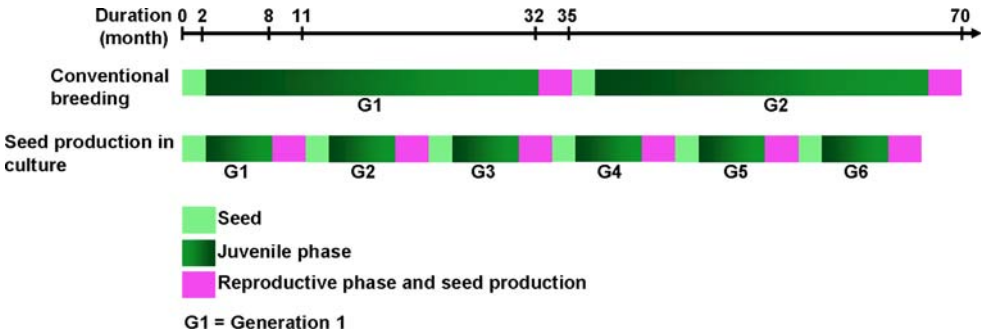

Fig. 5 Comparison of durations between conventional orchid breeding and method of seed production in culture

incubation. However, the percentage of germination of the pollens derived from in vitro-developed flowers was lower than that derived from flowers of field grown plants on this medium.

In this study, we have shown that *Dendrobium* Chao Praya Smile could be induced to flower early and produce seeds in culture. In vitro fruit development and fertile seed production have been reported in *Lycopersicon esculentum* (Rao et al. 2005) and *Pisum sativum* L. (Franklin et al. 2000). Despite the low percentage of germination of pollen derived from in vitro-developed flowers, pollination of in vitro-developed flowers and subsequent seedpod formation have produced a large number of seeds sufficient for breeding purposes. In our protocol, the process from seed germination to production of the next generation seeds in culture has been shortened from over 35 months to only about 11 months. The method of seed production in culture would have produced six generations of progenies with the time required for two generations in conventional orchid breeding (Fig. 5). Therefore, seed production in culture would have tremendous application in orchid breeding in view of the fact that viable seed production is crucial in producing homozygous plants and new hybrids. Therefore, the use of our technology of seed production in culture would shorten the breeding period and in turn significantly decrease the cost of producing new orchid hybrids.

Acknowledgments K. H. Hee was supported by research scholarship from the National University of Singapore (NUS). C. S. Loh and H. H. Yeoh thank NUS for the research grant.

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