TRANSFORMATION OF MAIZE ANTHOCYANIN REGULATORY GENES, *B-PERU* AND *C1*, INTO ORCHID

INTRODUCTION

Orchids, the most beautiful flower, comprise a unique group of plants. They are greatly attracted ornamental plant in the world due to the modified vegetative structures and the wonderful flowers which are varied fragrances, brilliant colors, remarkable range of sizes, manifold shapes, and varied forms. Taxonomically, they present the most highly evolved family among monocotyledon with 600-800 genera and 25,000-35,000 species which occupy top position among all the flowering plants valued for cut flower production and potted plant. Researchers, therefore, have been being in an effort to create a new cultivar through both conventional breeding and genetic engineering techniques. Unfortunately, conventional breeding, which is an artificial crossing between species or hybrids of two natural genera, is limited due to the fact that orchid is long reproduction cycle, slow seed maturation and poor fertility. Namely, genetic engineering, which directly introduces foreign genes into target tissue, is an alternatively powerful technique for crop improvement.

One of the objectives to develop orchid flower is to obtain a novel flower color which is the result of an accumulation of secondary metabolites. Of these, an important flavonoid pigments have been accumulated in flowers, fruits, and foliage of plant ranging from bryophytes to angiosperms. The difference in either structural or regulatory genes involved in the flavonoid biosynthesis pathway causes the variation in the color (Martin *et al.*, 1991; Holton and Cornish, 1995; Mol *et al.,* 1998; Winkel-Shirley, 2001).

This research, therefore, is focused on genetic engineering of two maize transcription factor genes, *B-peru* gene, and *C1* gene involving flavonoid pigments, in *Dendrobium* orchid to accomplish a new perspective of orchid flower color. In this study, not only the new agronomical important flavonoid compounds was introduced into *Dendrobium* orchid, but also the development of the high efficiency routine

system for orchid transformation *via* bombardment was established in transgenic orchid.

The objectives of this study are:

1. To study on temporal expression of structural and regulatory genes involved in flavonoid biosynthesis of *Dendrobium* orchid flower.
2. To analyze the factors affecting *Dendribium* bombardment technology by transient Gus expression.
3. To evaluate the transcriptional control of flavonoid biosynthesis by transforming the maize *B-peru* and *C1* regulatory genes into orchid.

LITERATURE REVIEWS

1. Botany of Orchid

Orchid is a monocotyledonous plant in orchidaceae family, containing more than 800 genera, grouped approximately into 74 sub tribes, 20 tribes, and 5 subfamilies, and widely distributed in all parts of the world except the major desert and arctic circles. There are probably more than 25,000 species of orchids in the world and over 100,000 hybrids. They are found naturally grown on trees, rocks, in meadows or marshes generally divided into three groups; terrestrial orchids (1) grown on the ground with their fleshy roots in the soil and require uniformly supply of water like ordinary plants, epiphyte orchids (2) grown on the tree but they can produce their own food from air and water, and saprophyte orchids (3) are devoid of chlorophyll and grown on decayed organic matter. Orchids are also divided into two major groups according to structure (Sanford, 1974).

1. Monopodial orchid: this group does not make separate new stem each season and does not have a rhizome. The single stem increases in height throughout the lift period. New leaves appear during growth and the flowers appear from the axils of the leaves at the upper portion of the plants. The leaves are arranged in two rows, one opposite the other, or the leaves of one row alternate with those of the other and are leathery in texture and persistent for several years. Aerial roots are produced from the nodes. For propagation, one can cut off the top of the plant to have another plant and new roots will arise from the stem itself.

2. Sympodial orchid: this group has rhizomes which are able to develop a new stem and each new stem produces its own set of roots. The stems are generally thickened and bulbous called pseudobulb, very useful device for storage of water and food which enable the plants to withstand drought. The pseudobulbs are formed by the swelling and consolidation of the base of each new stem between the first pair of true leaves, and one, two or more leaves are produced from the apex of the

pseudobulbs. A portion of the plant including the rhizome is separated to propagate into a new plant.

The leaves of most orchids are basically green and usually entire, fleshy, glabrous, leathery, parallel veined, cylindrical, linear or lanceolate and with an out coat of wax which resists drying. The leaf pores are sunken in the pits which minimize the air movement through the openings. The roots of orchid are very interesting, especially in the root of epiphytic types covered by a whitish spongy and pulpy coat called velamen which protects the inner conductive channel of the root, absorbs water from damp air and helps to cling to any surface it comes in contact. In the sympodial orchids, the roots are produced from rhizome. The green tip on the root is an important indicator of healthy growth. In general, the roots of orchids are cylindrical, often threadlike, branched, and of varying length; some are furnished with numerous root hairs. The tubers are arisen from a bud at the base of the stem and are storage organs. They undergo a period of rest during the cold and dry season and the leaves die and wither.

The structure of orchid flowers is unique among floral plants. The orchid blossoms consist of the fifteen organs which are arranged as usual in five whorls. The first outer whorl consists of three sepals, second inner whorl comprises of three petals, and six stamens in two whorls of three each makes third and fourth whorl. The innermost fifth whorl constitutes three stigmas out of which one stigma is incapable of receiving pollen and modified into wonderful rostellum and two general confluent stigmas. There are three approximately equal size sepals, one dorsal and two laterals. Although the sepals look like petals, they are normally narrower than the petals. When the flower opens, the sepals may become enlarged and colored.

Orchids always have three petals. Two are normal and the third becomes a highly specialized structure called a lip or labellum. The two lateral petals flank the greatly enlarged flamboyant bottom lip which is usually highly modified to attract and, in some cases, trap potential pollinators. The lip may be differently colored or marked, ruffled or pouch shaped, decorated with crests, tails, horns, fans, warts, hairs,

teeth, or other decorations attractive to their selected pollinator. The style and stamens are joined to form a column which is a finger-like structure carrying the reproductive organs. It occupies the centre of the flower and is the primary feature distinguishing the orchidaceae from all other families of plant. At the top of the column is the male anther which contains packets of pollen called pollinia located under the anther cap, operculum. Below the anther is the stigmatic surface, a shallow, usually sticky cavity in which the pollen is placed for fertilization. The pollen of orchid cannot be dispersed by the wind and carried to the stigma to effect fertilization. Pollination can be effected through man or insect by placing one or more pollinia on the stigma of an orchid flower. This leads to the fertilization of the egg in the ovules contained in the ovary. Within few hours after fertilization, the sepals and petals begin to wither; the ovary swells and the ovules gradually develop into seeds. The ovary turns into a seed capsule and finally matures in six months to well over a year. The seeds of orchid are very minute and do not contain the normal endosperm which causes of lacking the capability of germinating in the wild without the aid of a fungus

2. Flavonoid

Flower colors are mainly produced by flavonoids, carotenoids, and betalains. Carotenoids are largely responsible for the production of yellow and orange flowers such as sun flower and tomato (Bartley and Scolnik, 1995). Betalains are yellow to red nitrogenous compounds derived from tyrosine and are distributed only in *Caryophyllales* (Stafford, 1991). Flavonoids have a wide range of colors from pale yellow to red, purple, and blue. Flavonoids, a major class of ubiquitous plant secondary metabolites, play a role in a range of biological processes, such as stress responses, defense against bacterial, fungal and viral diseases, and offer the color acting as a signal to attract pollination of insects and birds. Moreover, for plant genetic transformation, genes involving flavonoid biosynthesis was used as a reporter gene to determine gene expression (Ludwig *et al*., 1990).

The basic structure of flavonoid molecule has similarity in A-ring hydroxylation at the 5^{th} and 7^{th} position and difference in the B and C- ring

substitutions (Figure1). Flavonoids were divided into 12 major groups: chalcone, aurone, flavone, flavonol, flavonone, dihydrochalcone, catechin, flavan-3-4-diol, biflavonoid, isoflavonoid, proanthocyanidin and anthocyanin (Madhuri and Arjula, 1999). Various reactions, such as hydroxylation, methylation, methoxylation, glycosylation, acylation and oxidation/ reduction in the central pyran nucleus and the B ring generally cause of the structural diversity and the color.

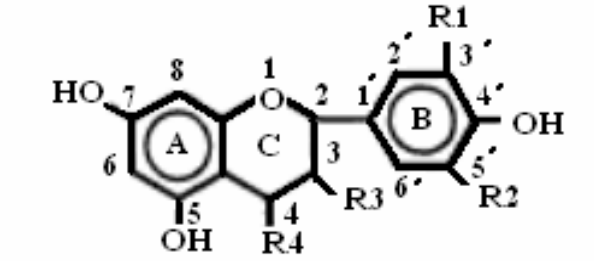


Figure1 Basic structure of flavonoid molecule.
Source: Madhuri and Arjula, 1999

3. Anthocyanin Biosynthesis

Anthocyanins are a colored class of flavoniod and accumulate in vacuoles. There are six major groups: pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin, (Tanaka, 1998). As shown the anthocyanin pathway in figure2, two precursors, malonyl-CoA and *p*-coumaroyl-CoA are catalyzed by chalcone synthase at three acetate units of malonyl-CoA and *p*-coumaroyl-CoA to produce the yellow-colored tetrahydroxychalcone, which is then isomerized by chalcone isomerase to the colorless naringenin. By flavanone 3-hydroxylase, naringenin can convert to dihydrokaempferol, DHK, which can be hydroxylated by flavanoid-3′-hydroxylase to produce dihydroquercetin (DHQ) or by flavanoid-3′, 5′-hydroxylase to produce dihydromyricetin (DHM) and F3′,5′H can also convert DHQ to DHM. The dihydroflavonols, DHK, DHQ, and DHM, are reduced by dihydroflavonol 4-reductase to flavan-3, 4-*cis*-diols (leucoanthocyanidins). Finally, chemical reaction, oxidation, dehydration and glycosylation of the different leucoanthocyanidins can produce brick-red pelargonidin, red cyanidin and blue delphinidin (Holton and Cornish, 1995).

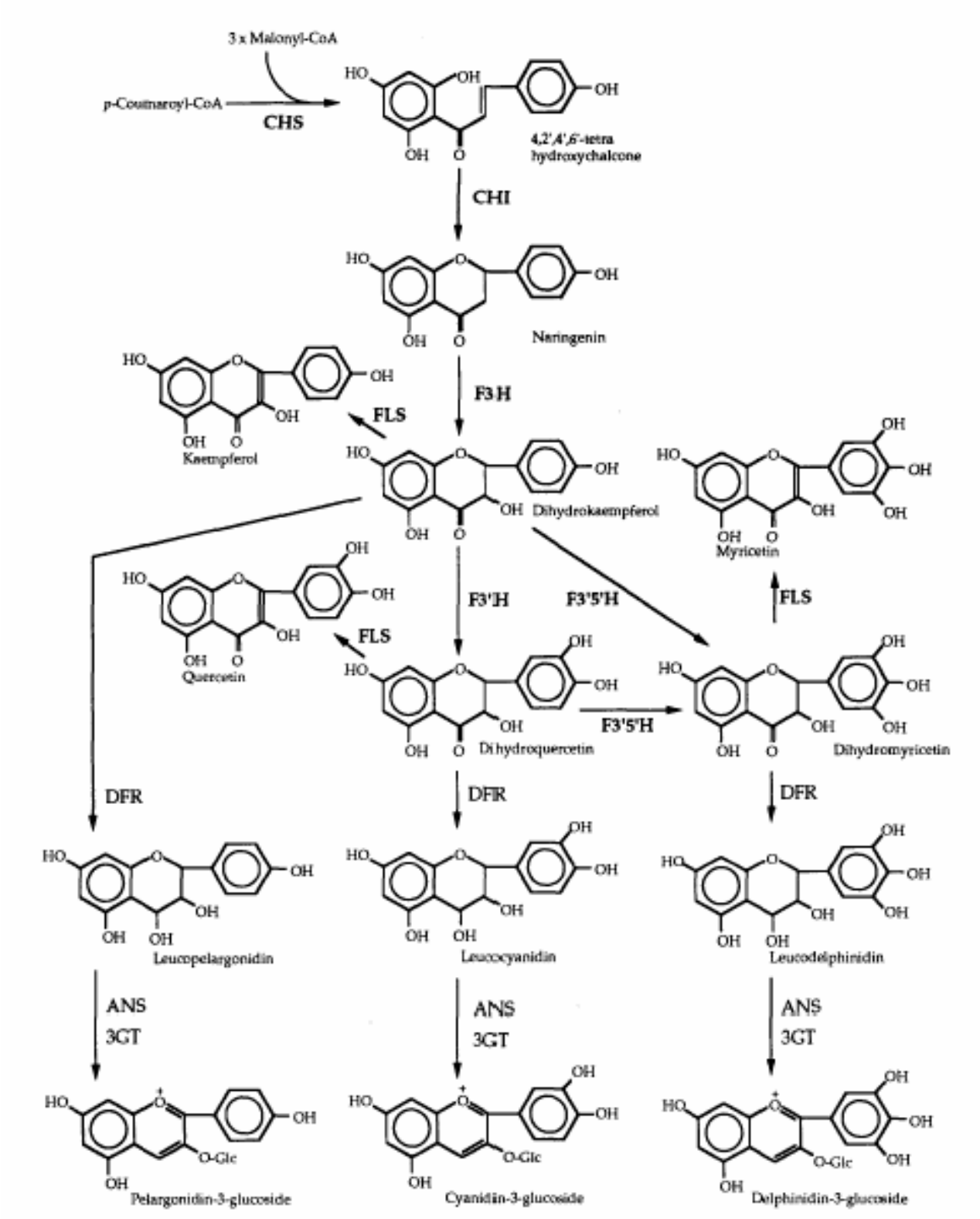


Figure2 Schematic anthocyanin pathways.

Source: Stevenson, 1991

Besides the structures of anthocyanin, pH changes, intermolecular stacking (self-association of anthocyanin and co-pigmentation with polyphenols), and intra-molecular stacking of aromatically modified anthocyanins, metal complex, and cell shapes give almost infinite flower colors. Anthocyanins are red and stable at a low pH but bluer when the pH is basic. The stacking and metal complexities contribute to stabilize anthocyanins and flower color, especially blue color (Brouillard and Dangles, 1994).

4. Molecular Genetic of Anthocyanin Biosynthesis

The genetic and molecular analyze revealed that the pathway is governed by a number of loci dispersed across the plant genome and regulated by distinct regulatory gene families in a temporal and spatial manner. With regard to genetic and biochemical analyze, the genes involved anthocyanin biosynthesis was divided to three distinct classes (Madhuri and Arjula., 1999).

Class I: structural genes encoding enzymes driving in the pathway

Class II: regulatory genes encoding transcription factor proteins that up-regulate the structural genes

Class III: genes that alter the intensity and distribution of pigments as modifying genes by not yet clearly defined mechanism

4.1. Class I: Structural Genes

Eight core structural genes encoding the structural proteins which are specific for anthocyanin biosynthesis are *chalcone synthase* (*chs*) gene, *chalcone isomerase* (*chi*) gene, *flavanone-3-hydroxylase* (*f3h*) gene, *flavonoid-3′-hydroxylase* (*f3′h*) gene, *flavonoid-3′, 5′-hydroxylase* (*f3′,5′h*) gene, *dihydroflavonol 4-reductase* (*dfr*) gene, *anthocyanidin synthase* (*ans*) gene, and *UDP glucose flavonoid 3-O-glucose transferase* (*ufgt*) gene. In all angiosperms, genes of the pathway roughly divided into two groups; the upstream genes encoding enzymes for non-anthocyanin as a precursor are CHS, CHI, F3H, F3′H, and F3′, 5′H and the downstream genes

coding the enzymes for anthocyanin products are DFR, ANS, and UFGT (Martin *et al.*, 1991).

4.2. Class II: Regulatory Genes

Regulatory genes encode transcription factors to modulate the groups of genes through binding at *cis*-acting DNA of the structural genes and to act with the general transcription machinery, chromatin remodeling proteins and/or other transcription factors (Broun, 2004) in a temporal and spatial manner for regulating expression of structural genes. In maize, regulatory genes involving anthocyanin biosynthesis were divided into two group gene families.

1. *Myc* (*myelocytomatosis viral oncogene*) gene family encodes a protein with characteristic acidic N- terminal domain sharing homology with transcription activators and a basic C terminus that is homologous with helix loop helix (bHLH) motif of *myc* family of protooncogenes (Ludwig *et al*., 1989). Maize, *R1* (red) */B1* (booster) gene family was presented. *R1* is genetically complex consisting of many genes, while *B1* allele is a single gene. The members of *R* gene family, *P, Sn*, and *Lc*, are derived from an unidentified single ancient gene that duplicated intra-chromosome to be the *R1* complex on chromosome 10 (Eggleston *et al*., 1995; Walker *et al*., 1995). After the event, *Sn* and *Lc* laid about two units distal to *R1* (Ludwig *et al*., 1989; Tonelli *et al*., 1991). The members of *B1* locus, *B-I* and *B-Peru,* are laid on chromosome 2 and also highly homologous to *Lc* (Goff *et al*., 1990).

The *R/B* gene regulates *chs*, *chi*, *f3h*, *dfr*, *ans*, *flavonoid 3-glucosyl transferase (Bz)* genes in various plant tissues (Dooner, 1983; Bodeau and Walbot, 1992; Deboo *et al*., 1995). The numbers of the *R* locus, the *Sn* gene determines pigmentation in aleurone, anther, seedling and also affects anthocyanin accumulation in the scutellar node, mesocotyl, leaf bases, midrib and ovary integuments, the *Lc* gene controls accumulation in the midrib, ligules auricle, glumes, lemma, palea and pericarp (Coe, 1985), and the *R-Navajo, R-nj,* pigments the crown of the kernel. The

one group, the *B* gene, produces red-purple pigmentation of most photosynthetic tissue (Styles *et al*., 1973). The number of the *B* gene: *B-I* gene confers intensive pigmentation most vegetative tissues such as roots, leaf sheath, blades, auricles, stem, husks, cob and glumes (Coen, 1986; Styles *et al*., 1973) and the *B-Peru* gene gives strong expression in seed, but induces weakly pigmentation in some vegetative parts (Selinger and Chandler, 1999).

2. *Myb* (*myeloblastosis viral oncogene*) gene family encodes a protein consisting of the basic N- terminal with homology to *Myb* protooncogenes and an acidic C-terminus with features of transcription activator (Paz-Ares *et al*., 1986; 1987; 1990). In maize, *C1* (*colorled-1*)/ *Pl* (*plant color*) genes were presented. The *C1* and *Pl gene* regulates the expression at least two structural genes, *chs*, *chi*, *dfr*, and *Bz1* genes (Grotewald and Peterson, 1994). *C1* induces anthocyanin pigmentation in the seed tissues, aleurones and embryos (McCarty *et al*., 1989), while *Pl* induces the pigmentation in most of plant body, seedlings tissues, pericarps, the maternally derived seed integument (Cone and Burr, 1989; Cocciolone and Cone, 1993), and in cultured maize cells (Grotewald *et al.*, 1998).

In snapdragon, three-anthocyanin regulatory genes, *Delila* (*Del*), *Eluta*, and *Rosea* have been isolated. *Del* gene encodes a potential protein with extensive homology to products of the *R* gene family in maize (Goodrich *et al.*, 1992) and the amino acid sequence was extensive homology with *Lc* gene, *B* gene, and *R-S* gene. Two regions were particularly well conserved at residues 16-190 of the N-terminus and at residues 438-497 of the C-terminus showed 61% identity and 60% identity, respectively. The latter region containing a sequence of 53 amino acids similar to the HLH domain conserved in several other eukaryotic regulatory genes strongest similarity the HLH domains of *myc* proteins. *Del* gene regulates the spatial distribution of *Pal* transcription. A mutation of *Del* gene reduces the levels of *Inc* gene transcription in the tubes but no affect in the lobes of flower (Almeida *et al.*, 1989). *Eluta* gene is a semi-dominant gene that restricts pigmentation in the flower, concentrating anthocyanin biosynthesis to the central face, the inner edges of the two back petals and a ring at the base of the tube.

Anthocyanin1 (*an1*)*, an2*, *an4*, and *an11* genes were identified and required for transcription of *dfr*, *rt*, and *glutathione S-transferase* genes but not *chs* and *chi* genes in petunia (Quattrocchio *et al.*, 1993). *An1* gene and *an11* gene are required for transcription of structural anthocyanin biosynthetic genes in all pigmented tissues, while *an2* gene controls the transcription in the flower limb and *An4* controls expression in the anthers.

In Arabidopsis, the functional *transpararent testa1* (*tt1*) gene, *tt2*, and *tt8* genes are required for the expression of the anthocyanin genes during seed formation. *Tt1* gene encodes a WD-repeat-containing protein probably representing an ortholog of the petunia *an11* product (Walker *et al.*, 1999), while *tt8* gene encodes a bHLH domain protein for *dfr* gene expression (Nesi *et al.*, 2000). The *tt2* gene isolated from seed coat is specific to R2R3 MYB domain protein and necessary for the expression of flavonoid late biosynthesis genes (Nesi *et al.*, 2001).

As shown in Figure3, the regulation of the structural genes by transcription factors described for several species indicate transcription factors regulating specific subsets of the structural genes. C1, PL, R, LC, SN and B protein regulate anthocyanin production and P protein which controls phlobaphene production in maize. DELILA regulates specific anthocyanin biosynthetic genes in *A. majus* and AN1, AN2 and AN11 regulates specific anthocyanin biosynthetic genes in *Petunia*.

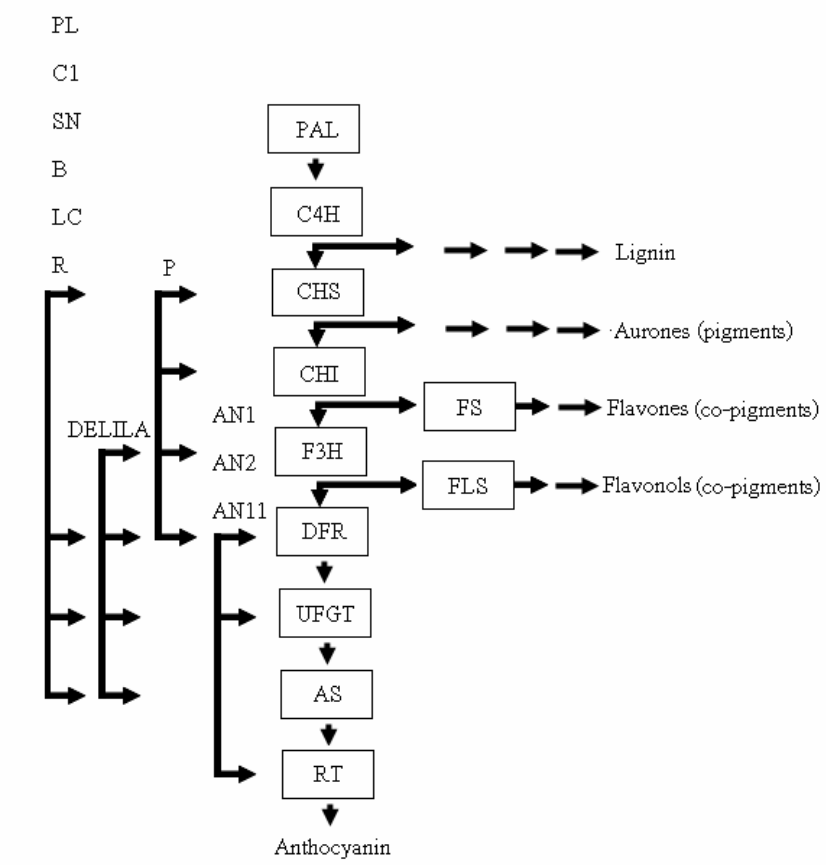


Figure3 The structural genes and their transcription factors involved in phenylpropanoid and anthocyanin biosynthesis.

Source: Martin, 1996

5. Metabolic Engineering Regulatory Genes Involved in Anthocyanin Biosynthesis

Metabolic engineering is the redirection of the enzymatic reactions for new compounds production in an organism to improve the production of existing compounds or to mediate the degradation of compounds. To date, flavonoid, one of the most important secondary metabolites, has been studied on genetically metabolic engineering focusing on the structural genes and the regulatory genes involved in the pathway. Modifications of secondary metabolic storage products have been generally more successful than manipulations of primary and intermediary metabolism (Stitt, 1995) demonstrated that the study an alternative approach through up-regulation of the endogenous genes by modify the ectopic expression of the genes encoding

transcription factor has much achievement than attempt to increase expression of structural genes in the pathway. These studies, therefore, had been significant innovator in manipulation of metabolic pathway, demonstrating the potential of transcription factor to regulate flavonoid biosynthesis in the plant (Table1).

One of the early report of using this approach to manipulate plant biochemistry was the engineering of maize cells to express the *Lc* gene as a highly potential visible marker for selecting stably transformed line because of no require a complicated biochemical assay or expensive histochemical staining for visualization of the gene expression **(**Luwig *et al*., 1990; Bowen, 1992). The initial report about the transcription activator involved flavonoid biosynthesis became using for plant manipulation when Goff *et al.* (1990) bombarded maize *B-peru* and *B-I* cDNA regulating the expression of *A1* and *Bz1* genes into colorless *r* aleurone and embryo tissues of maize. All tissue tested receiving *B-peru* gene, or *B-I* gene exhibited numerous single cells containing pigment but expression of *C1* gene alone displayed no pigmented cells, indicating both the *B-peru* or *B-I* gene and *C1* gene interaction is required to active the anthocyanin biosynthesis pathway. In 1998, De Majnik *et al.* examined the anthocyanin phenotype produced in white clover, *Trifolium repens*, using transformation of the *B-peru* gene. The tissue expressing *B-peru* displayed a unique pattern of anthocyanin accumulation in the leaf of which pigmentation declined in intensity in the oldest stage. However, Ray *et al*. (2003) demonstrated that *B-peru* and *C1* transgenic populations of alfalfa forage (*Medicago sativa*) under high light intensity or low temperature displayed no visible phenotype changes though these transgene were expressed at detectable levels, but the strongly expression of the *Lc* transgenic lines accumulating red/purple pigments was observed, supporting the emerging picture of *Lc* transgenic specific patterns of expression in other species.

Establishing transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* by introducing the transcription factor genes were performed by introduction of the *C1* gene and the *Lc* genes into tissues of Arabidopsis and tobacco (Lloyd *et al*., 1992). The expression of the *C1* gene and the *Lc* gene showed anthocyanin accumulation in root, petal, and stamen tissues of *A. thaliana* that normally never produce anthocyanin

and was changeable from pink to intense red in the collar and anther filaments of tobacco, but no changing of expression with *C1* gene alone was observed. Moreover, the use of the *Lc* gene and *C1* gene was also applied in petunia (Quattrocchio *et al*., 1993) and petunia Mitchell (Bradley *et al*., 1998). Transgenic petunia were purple due to higher anthocyanin content in floral and leaves, indicating that regulatory pigmentation genes in maize and petunia are functionally similar, despite the wide evolutionary divergence and the extreme differences in pattern and function of maize and petunia. In petunia Mitchell, the stable expression of *Lc* gene resulted in intense of anthocyanin accumulation in the leaves, stems and sepal, along with lower levels of pigmentation in the flower tube and limb, appearing to be specific to the sub-epidermal layer in all tissues.

Maria *et al*. (1994) reported initial attempts to set up a new approach to developmental study in plant, namely genetically engineered cell fate mapping to manipulate single cells of the shoot apical meristem by microinjection of genes encoding visible markers and follow the fate of the marker during development. The expression vector carrying the *Lc* gene was transformed into mesophyl-derived protoplast of maize embryonic and meristematic tissues. The independent transformation events can be monitored and quantified in living cells when the expression of the product of the induced *Lc* gene can substitute for mutant *R* and *B* loci, resulting in pink/red protoplasts. The use of *Lc* as a dominant marker gene to control anthocyanin accumulation was also investigated in cherry tomato (Goldbrough *et al*., 1996). *Lc* gene gave to greatly enhance anthocyanin accumulation in all green vegetative tissues, including roots, sepals, and main vein of petals under high light condition. The expression of *Lc* gene and *C1* gene is sufficient to upregulate the flavonoid pathway in tomato fresh (Bovy *et al.*, 2002). Simultaneously, expressing the maize *Lc* gene and *C1*, fruit flesh of transgenic tomato that normally does not produce any flavonoids resulted in a strong accumulation of flavonols and a more modest increase the flavanones, but remarkably, not in the accumulation of anthocyanin.

Yu *et al*. (2003) showed a strategy for developing lines of soybeans to much higher level of isoflavones accumulation than wild-type seed for the soy foods providing potentially greater health benefits to consumer. A chimeric gene containing a fusion of maize *C1* and the *Lc* allele of *R* gene family was introduced into soybean embryonic suspension cultures. By combination between activating gene transcription factor activation and blocking competing branch pathway, phenylpropanoid pathway genes were activated, which decreased genistein and increased the daidzein level with a small overall increase, resulting in isoflavone synthesis up to four times higher than in wild-type seed. Furthermore, to enhance anthocyanin biosynthesis in foliage of *Caladium bicolor,* Li *et al*. (2005) established the *Lc* transgenic *C. bicolor*. Expression of *Lc* gene alone was sufficient to promote anthocyanin accumulation in all vegetative tissues, including the roots, stems, and leaves. Of these transgenic plants showed perfect symmetrical green/red leaves.

The phenylpropanoid pathway genes in embryogenic suspension cultures of *Glycine max* were also activated by the maize *C1* gene and *Lc* gene (Yu *et al*., 2003). The expression of *C1* gene and *Lc* gene was sufficient to pigment isoflavonoid up four times higher than in wild-type seed. This strategy was great helpful for the production of soybean foods providing health benefits. However, in two popular ornamental species, lisianthus (*Eustoma grandiflorum*) and a regal pelargonium cultivar (*Pelargonium X domesticum* Dubonnet), no visible phenotype alteration in floral and pigmentation was observed after *Lc* gene introduction (Bradley *et al*., 1999). The expression of *Lc* gene alone was unable to transcriptionally up regulate the flavonoid biosynthesis genes of lisianthus and pelargonium, suggesting the regulatory mechanism divergence in some dicots. The combination of introduced bHLH and MYB factors may be required to increase in some plant species.

With in *Sn* gene coding region for transcription factor was utilized to induce stable anthocyanin expression in hairy roots of several dicots (Damiani *et al.*, 1998). The 12 dicot species were transformed with binary vector harboring the plasmid121.Sn containing the maize *Sn* gene to evaluate anthocyanin pigmentation. Most of dicots tested could not show anthocyanin accumulation in the tissue.

Although the transcription factor from maize function in several dicotyledonous species and exerts to control on the same anthocyanin pathway, the complexity of the regulatory mechanisms of anthocyanin synthesis restricts the use of *Sn* due to many factors which are responsible to activation of anthocyanin biosynthesis, such as gene interaction, environment stimuli, and growth regulator, etc. However, the *Sn* gene could up regulate both anthocyanin and condensed tannin biosynthesis in *Lotus corniculatus*, suggesting that there is shared regulatory mechanism between both species (Robbins *et al.*, 2003). The transgene expression showing effects on anthocyanin accumulation were subtle and restricted to the leaf midrib, leaf base, and petiole tissues and the level of condensed tannins in leave increased up to 1% of the dry weight but other major secondary end-products, lignins, and inducible phytoalexins were unaltered in transactivated lines.

In transgenic black mexican sweet maize cell lines, the pathway of flavonoid metabolism independently controlled by ectopic expression of the appropriate regulatory gene to produce anthocyanins were presented (Grotewald *et al.*, 1998). Cell lines engineered to express the maize *C1* gene and *R* gene accumulate two cyanidin derivatives. In contrast, cell lines expressing *P* gene accumulate various 3-deoxy flavonoids. Moreover, the *C1* gene and *R* gene were also utilized to establish transgenic rice accumulating purple pigments. Gandikota *et al.* (2001) produced transgenic *japonica* rice expressing maize anthocyanin genes by co-bombardment of the Tp309 embryonic calli with the *C1*, the *R*, and the *chs* genes. The expression of stable transgenic plants carrying the *chs* gene showed purple/red pigments accumulation in the leaf blade and leaf sheath, presumably the mutation at the *chs* locus. In 2005, Cocciolone transformed maize *P1* transcription factor gene to synthesize maysin, a flavone glycoside from maize silks that confers natural resistance to corn earworm. The *P1* transgenic showed red pigment at pericarp, cob, husk, and tassel tissues. Interestingly, transformed maize plants containing low or no silk maysin with *P1* gene elevated sufficiently for corn earworm biosis. The gene encoding putative bHLH transcription factor involved anthocyanin biosynthesis was also isolated from rice (*Orysa sativa*) (Hu *et al*., 2000). The *Ra1* gene homology along with the *Lc* gene and the *Del* gene, the *Rb2* gene which lay on chromosome 1

undetermined functionally was inducible pigmentation in maize suspension cells like the maize *Lc* as a control.

In dicots, Mooney *et al*., 1995 showed that *Del* gene, which is a transcriptional factor representative of the *myc* family of *A. majus*, altered pigmentation in two *Solanaceous*, tobacco and tomato. Ectopic expression of *Del* gene leaded to increase anthocyanin accumulation in the tissues of tobacco and tomato even though the phenotypic effects of *Del* gene on tomato and tobacco appeared to be quite different. In tomato, the tissues expressing *Del* gene increased pigmentation in vegetative tissues while, in tobacco, effects were only in the flowers, replying that other factors might act to restrict the overall patterns of expression.

Quattrocchio *et al*. (1998; 1999) showed the expression of *Jaf13* gene and *an2* gene, a bHLH of R family of petunia. Strongly blue pigmentation in anther of *an2* transgenic petunia whose flower was normally white corolla limb and yellow anthers was observed. The *Jaf13* gene displayed increased pigmentation in particular of the veins of sepals, the stem, the inflorescence, and the edges of young leaves. In 2000, Spelt *et al*. isolated and characterized the *an11* gene, which has a highly conserved *R2R3 myb* domain. Analysis of *an11* expression in leaf cells of transgenic petunia showed operation at down-stream of AN2 and AN4 expression by activating transcription of the *dfr* promoter. Over-expression of *an11* gene in transgenic tomato and tobacco lines were studied (Mathews *et al*., 2003). Ectopic expression of *an11* gene to up-regulate was responsible for the color phenotype in which vegetative parts, resulting intense purple spotting on the epidermis and pericarp of the fruit. Moreover, to anthocyanin pigment in *N. tabacum* (SRI) with *Gmyb10* gene, the *R2R3*-type *myb* gene from *G. hybrida*, was also identified (Elomaa *et al*., 2003). The transgenic tobacco lines expressing *Gmyb10* gene was increased high levels of anthocyanin accumulation in leaves and stems, flowers, sepals, anthers, and ovary walls.

Table1 Transcription factors functionally associated with the control of flavonoid pathways in plants

TF	TF family	Species	Plant Metabolites	References
B-peru/or B-I	bHLH	Maize	Maize	Goff *et al.,* 1990
			Trifolium repens	De Majnik *et al.,* 1998
Lc	bHLH	Maize	Maize	Luwig *et al.,* 1990; Bowen, 1992; Maria *et al.,* 1994
			Arabidopsis and tobacco	Lloyd *et al.,* 1992
			Petunia	Quattroocchio *et al*., 1993; Bradley *et al.,* 1998
			Tomato	Goldbrough *et al.,* 1996; Bovy *et al.,* 2002
			Lisianthus (*Eustoma grandiflorum*) and Regal pelargonium cultivar (*Pelargonium X domesticum* Dubonnet)	Bradley *et al.,* 1999
			Glycine max	Yu *et al.,* 2003
			Alfalfa (*Medicago sativa*)	Ray *et al.,* 2003
			Soybeans	Yu *et al.,* 2003
			Caladium bicolor	Li *et al.,* 2005

Table1 Transcription factors functionally associated with the control of flavonoid pathways in plants (Continued)

TF	TF family	Species	Metabolites of transgenic plants	References
Sn	bHLH	Maize	Dicots plant	Damiani *et al.,* 1998
			Lotus corniculatus	Robbins *et al.,* 2003
Rb	bHLH	Maize	Maize	Hu *et al.,* 2000
R family	bHLH	Maize	Black mexican sweet maize	Grotewald *et al.,* 1998
			Japonica rice	Gandikota *et al.,* 2001
DEL	bHLH	Snapdragon	Petunia	Goodrich *et al.,* 1992
			Tobacco and tomato	Mooney *et al.,* 1995
AN2/ JAF13	bHLH	Petunia	Petunia	Quattrocchio *et al.,* 1998; 1999
AN1	bHLH	Petunia	Petunia	Spelt *et al*., 2000
P1	MYB	Maize	Maize	Cocciolone *et al*., 2005
GMYB10	MYB	Petunia	Tobacco	Elomaa *et al*., 2003
AN11	MYB	Tomato	Tomato and tobacco	Mathews *et al*., 2003

6. Genetic Engineering in Orchid

Plant genetic engineering methods began in 1980s to create novel species and traditionally desired agronomic traits for agronomic pest control, for improved nutrient food processing or storage qualities, for specially chemical or pharmaceutical production, or for environmental cleanup uses. Two major methods standing out in the evolution of plant genetic engineering are particle gun or biolistic technology and *Agrobacterium tumifaciens* Ti vector-based system.

Microprojectile bombardment employs high-velocity metal particles to deliver biologically active DNA into plant cells which can be regenerated to whole plant. With the advent of microprojectile or particle gun based, transformations of numerous monocotyledonous species have been reported. The general basic for particle gun technology is the coating of DNA onto particles of metals such as gold, tungsten, or platinum, which then are accelerated sufficiently to penetrate into host cells, protoplast, leaves, stems, hypocotyls, or embryos. Once inside the cell, a portion of DNA diffused from the coated particle into a recipient nucleus, where it comes integrate into a host plant chromosome. The practical result and implication of direct transformation of elite or commercial lines, rather than laboratory lines, is that overall breeding times are shorted.

Because of many factors affecting to orchid transformation, Nan and Kuehnle (1995) studied several parameters for microprojectile bombardment. The results demonstrated that 1.6µm Bio-Rad gold particles were more effective than 1.0µm ASI gold particle, the super-promoter produced transient transformants at least 1.5 times as the single promoter, and the tissues genotype affected transient Gus expression. Moreover, a key role to the transformation success was to adopt the liquid culture system to stimulate actively continued proliferation of the meristematic tissue pre-and post-bombardment and to develop a selection regime that minimizes the toxic influence of dying cell on neighboring transformed cells (Yang *et al.*, 1999)

Antibiotics concentration and date for selection posted bombardment is one of the factors effecting transformation efficiency because of wound caused by bombardment and fail to form calli or protocorm while in competition with unbombarded cells on common medium free of selective agents. Men *et al*. (2003) showed success of genetic transformation of orchid, *D. phalaenopsis* and *D. nobile* by early selection. The chopped protocorms were bombarded with pCAMBIA1301 plasmid encoding an intron-containing *β-glucuronidase* (*gus*) and *hygromycin phosphotransferase* (*hpt*) genes into protocorms and transferred to 1/2 strength MS medium supplemented with 30mg/l hygromycin for 4-6weeks immediately after bombardment. In addition, Knapp *et al*. (2000) altered selective agent for the recovery of three orchid genera, *Brassia, Cattleya,* and *Doritaenopsis* using the *bar* gene. The results showed that protocorms proliferated on selection medium containing 1mg/l bialaphos and transferred to the selection medium containing 3mg/l of the herbicide twice a 2-months intervals was potential to select transgenic cells.

However, using antibiotics and /or herbicides as selective agents is possibly toxic and allergic to human. To avoid the use of antibiotic or herbicide selection agents, Chia *et al*., 1994 developed the marker acting as reporter and selectable gene using the *firefly luciferase* (*Luc*) gene which rapidly screens and isolates. Protocorm-like-bodies (Plbs) were bombarded with tungsten particles coated with plasmids carrying CaMV35S-*luc* gene. Three weeks after bombardment, 1mM luciferin was added to the bombarded tissues. The tissues were identified by virtue of their bioluminescence as monitored by low-light video microscopy in combination with a real-time photon imaging technique. Transformed tissues were selected by excision and allowed to proliferate, and then subjected to a second round of screening. After three rounds of growth and screening, *Dendrobium* tissues expressing luciferase were generated to transgenic orchid plants. In 2003, *ferredoxin-like protein* (*pflp*) gene as selectable marker and *Erwinia carotovora* as the selective agent have been utilized to establish transgenic *Oncidium* orchid (You *et al*., 2003). An expression vector containing a *pflp* cDNA under the control of CAMV35S promoter was transformed into protocorms by particle gun bombardment. *A. tumefaciens* and *E. carotovora*

were used to screen transformants, thereby obtaining transgenic plants without the use of an antibiotic selection agent.

With regard to improve orchid via genetic engineering via microprojectile bombardment, Kuehnle and Sugii (1992) produced transgenic virus resistance-orchid by bombardment with the plasmid pGA482GG/cpPRV4 containing neomycin phosphotransferase (NPTII) and papaya ringspot nirus (PRV) coat protein (CP) genes driven by Nos promoter. Unfortunately, the antibiotic could not be used for long-term selection because such levels suppressed to regenerate the transformed tissues, all kanamycin–resisting tissues contained the Nos-NPTII gene found only one transgenic line carrying the vector-linked PRV CP-gene. However, recently, Liao *et al*. (2004) step forward in orchid improvement by focusing on the resistance of *Phalaenopsis* to CymMV by expressing a virus-derived transgene. A DNA cassette containing a CymMV *coat protein* (*CP*) cDNA and a Nos terminator driven by maize ubiqutine promoter was transformed into the protocorms These plants exhibited enhanced resistance to virus infection, as confirmed by RT-PCR and ELISA. Five among the 13 tested lines in the field showed CymMV protection in more than 50% of their progeny.

The other one of the major method to introduce transgene into plant genome is to use *A. tumifaciens*, a soil borne plant pathogen which is the agent of crown gall disease of dicotyledonous plants. In nature, *A. tumifaciens* enters via wound, generally at or near the soil line or crown. Wounds are caused by implements or insects and by pruning. Once the pathogen has entered its plant host, a segment of DNA called T-DNA, derived from an endogenous plasmid called Ti, randomly insert into a chromosome of the host plant, where it provided genetic instruction to produce auxin, a hormone which results in softening of cell walls, plant cell multiplication and enlargement, and subsequent production of a primary tumor or gall. The attraction of plant biotechnologist to *A. tumifaciens* is its ability to insert DNA into host plant genetic material. Therefore, to utilize its integration characteristic, the genes were removed from the Ti-vector to create a disarmed vector for use in plant

transformation. So far, there are many papers presenting successful genetic transformation in monocotyledon, including orchid.

Yu *et al*. (2001) established a system for the simple recovery of orchid transformants through the use of *Agrobacterium*-mediated transformation. The basis of an optimization of the co-cultivation period was developed by inoculating thin-section explants from plbs of *D.* Madame Thong-In with *A. tumifaciences* strains LBA4404 harboring a binary vector carried the orchid *DOH1 antisense* gene. The transformation was successfully performed through two consecutive stages of co-cultivation, with the first stage occurring on antibiotic-free medium for 3days and subsequent stage on medium containing 50mg/l cabenicillin for 3-4weeks. Expression of the *DOH1 antisense* gene caused abnormal multiple shoot development, indicating a role for DOH1 in the basic plant architecture in orchid.

Belarmino and Mii (2000) applied to use of *A. tumifaciens* transformation in a *Phalaenopsis* (*Doritaenopsis* Coral fantasy X *Phalaenosis* (Baby hat X Ann jessica). Transgenic *Phalaenopsis* was regenerated after co-cultivation of cell clumps with *A. tumifaciens* strains LBA4404 (pTOK233) and EHA101 (pIG121Hm) that harbored *β-glucuronidase* gene and *hygromycin resistance* gene. The efficiency of transformation was markedly increased by 10h co-cultivation in 200μM acetosyringone. Hygromycin-resistant cell clusters were selected after 4-6weeks of culture on new Dogashima medium (NDM) supplemented with 50mg/l hygromycin and 300mg/l cefotaxime. Likely, Chai *et al*. 2002 successfully used agrobacterium transformation system for *Phalaenopsis*. *A. tumifacience* strains LBA4404 harboring a binary vector pTOK223 carrying *gus* and *hpt* genes were transformed into protocorms. The first selection was carried out on regeneration medium containing 3mg/l hygromycin for 2 months for plbs formation under the hygromycin selection. Newly formed plbs from Gus-positive plantlets were selected on the medium containing 1.5mg/l hygromycin repeatedly monthly for four cycles.

In 2003, genetic transformation of *Oncidium* orchid via *A. tumifacience* was performed (Liau *et al*., 2003). An expression vector containing *hptII* gene and *gusA*

gene driven by CaMV35S promoter was introduced into plbs through two stages of co-cultivation, the first stage occurring on antibiotic-free medium 3days and subsequent stage on medium containing 100mg/l timentin for 1 month. The transformed plbs were proliferated on 5mg/l hygromycin selection medium. Gus assay, southern, northern, and western blot hybridization were confirmed the integration, transcription and translation, respectively, in transgenic orchid.

7. Constitutive Promoters to Control Gene expression

7.1. Act-1 Promoter

Actin gene found as family gene in all higher plant is an essential component of the eukaryotic cell cytoskeleton (Pollard and Cooper, 1986), such as cell shape determination, cell division, organelle movement, and extension growth. Actin promoters are likely to be active in all tissues due to fundamental component of the plant cell cytosketleton (Seagull, 1989). The *Rac1* 5$^{/}$-noncoding exon sequence was separated by a 447-bp intron (intron1) from the first coding exon and conserved between plant and animal cytoplasmic *actin* gene is essential for gene expression, rated by a 447-bp intron (intron1) from the first coding exon. In rice, there are at least eight actin-like sequences per haploid genome; four of these have been isolated and shown to differ from each other in the tissue-and stage-specific abundance of their respective transcripts (Reece *et al*., 1990). In situ characterization of Act1-*gus* gene expression revealed that this promoter has a virtually constitutive pattern of activity in both the vegetative and reproductive tissues of transgenic rice plants. It is active in most sporophytic cell types, such as gametophytic pollen tissue, floral, stigma, stamen tissue, leaf trichome, bulliform, bundle sheath cells, and root epidermal cells (Zhang *et al*., 1991). Rice *Act-1* gene involved in the determination of Act1 transcript abundance in whole organ (McElroy *et al*., 1990).

7.2. Ubi-1 Promoter

Ubiquitin gene, which is one of the most conserved proteins, is highly abundant in the cytoplasm as a free monomer (Christensen *et al*., 1992). This protein has been implicated in many vital cellular processes, such as protein turnover, chromatin structure, cell cycle control, DNA repair, and response to heat shock and other stress. *Polyubiquitin* genes have been isolated and sequenced from a wide range of organisms comprising eight different phyla (Callis and Vierstra, 1989). The maize ubiquitin gene was screened from a maize genomic library with maize cDNA clone and S1 mapping of the Ubi-1 gene was used to define the transcription start site (Christensen *et al*., 1992). Several sequence elements common to promoters of many other eukaryotic genes are found in the Ubi-1 5′ flanking sequence.

7.3. CaMV35S Promoter

A double-stranded DNA cauliflower mosaic virus that infects members of the *Cruciferae* is approximately 8 kb (Hohn *et al*., 1982). Because expression of the 35S promoter does not require any viral-encoded protein, host nuclear factors must be responsible for its activity (Guilley *et al*., 1982). The 35S promoter region was isolated as a *Bgl*II fragment extending from –941 to +208 with respect to the transcription start site mapped for the 35S RNA found in CaMV infected turnip leaves and has been used extensively in dicot transformation. This promoter can be divided into two domains, A and B, each with distinct cell specificity. Expression from domain A (-90 to +8) is strongest in the radical pole of the endosperm and in root tissue of seeding and mature plants. Expression from domain B (-343 to –90) is strongest in the cells adjacent the cotyledon of the endosperm, in the cotyledons of the embryo and seedlings and in the leaves and stem of mature plants. The combination of domain A with domain B results expression in most tissues at all stages of development.

To determine ability of promoter for orchid transformation, Anzai *et al*. (1996) studied different constitutive promoters for control transgene expression,

showing that all promoters of CaMV*35S* gene, maize *ubiquitin* gene and rice *actin* gene could be expressed in *Phalaenopsis* using transient assay of *gus* gene. The plbs derived from the leaf segment culture were bombarded by gold particles coated with pMSP38 and pWI-Gus DNA containing the *bar* gene and the *gus* gene, respectively, driven by CaMV35S promoter and selected on the modified VW medium supplemented 1mg/l bialaphos for 2 months and 5mg/l bialaphos for 1-2 months to accomplish putative transgenic *Phalaenopsis*.

MATERIALS AND METHODS

Materials

1. Plant Materials

1.1. Plant Tissues for RNA Dot Blot Hybridization

The flower tissues of *Dendrobium* orchids in different developmental stages are used in the study of temporal expression of the structural and regulatory gene as following: the green closed flowers of 1.0cm in length (Stage1), the green closed flowers of 2.5-3cm in length (Stage2), the flower colored at the edges of the petals of 3-4cm in length (Stage3), the blooming flowers (Stage4), and the fully blooming flower (Stage5).

1.2. Plant Tissues for Bombardment

Flower tissues and protocorms of *Dendrobium* Jaquelyn Thomas obtained from Rapee Sagarik Orchid Garden, Kasetsart University, Thailand, were utilized to study the physical parameters for bombardment in orchid.

2. Medium

The media used for tissue culture and selection as shown in Appendix Table1

3. Bacterial Strain

Escherichia coli strains XL1-Blue was utilized as a host cell for gene cloning.

4. Plasmids

4.1. The Plasmids Used to Vector for Bombardment Transformation

Five plasmids carrying different constitutive promoters fused to a reporter and/or a selectable marker gene and Nos terminator were used as a vector for each bombardment transformation (Table2).

Table2 Various plasmids utilized to be the vectors for orchid transformation (Appendix Figure1).

Plasmid	Promoter	Reporter gene	Selective gene	Provided by
1. pACT1-D	Act-1	*Uid A* gene	-	Prof. Ray Wu
2. pAHC27	Ubi-1	*Uid A* gene	-	Prof. H. Uchimiya
3. p2K7	CaMV35S	*Uid A* gene	-	Prof. Jame Dale
4. pMNK1005	Ubi-1	*S65Tgfp* gene	*hph* gene	CSIRO, Australia
5. pTG0063	Act-1		-	CAMBIA, Australia

4.2. The Plasmids Containing the Maize Transcription Factor Genes Involved in Anthocyanin Biosynthesis

Two plasmid clones containing the maize flavonoid transcription factor genes, *B-peru* and *C1*, inserted in p35SBpcDNA and p35SC1, respectively, driven by CaMV35S promoter and Nos terminator were kindly given by Prof. Vicki L. Chandler (Department of Plant Science, University of Arizona, Tucson, USA) (Appendix Figure2).

5. Primers

5.1. Primers for Amplification of Specific Genes by PCR (Table3)

Table3 Oligonucleotide primers utilized for PCR detection

Gene	Primer	Sequence	Size
1. *hph*	1.1 HPT(S)135	5′-GCC TCC AGA AGA AGA TGT TG-3′	0.5-kb
	1.2 HPT(A)136	5′-ATG TCC TGC GGG TAA ATA GC-3′	
2. *gfp*	2.1 GFP1(S)59	5′-GCG GAT CCA TGG TGA GCA AG-3′	0.7-kb
	2.2 GFP2(A)60	5′-GGG CGG CCG GTT TAC TTG TA-3′	
3. *B-peru*	3.1 R3(S)123	5′-CGC GCG CTC CTG GCC AAG A-3′	1.5-kb
	3.2 NOS(A)188	5′-GAG GAT TCA ATC TTA AGA AAC TT-3′	
4. *C1*	4.1 C1(S)161	5′-AAC AAA GGG GCA TGG AC-3′	1.1-kb
	4.2 NOS(A)188	5′-GAG GAT TCA ATC TTA AGA AAC TT-3′	

5.2. Primers for Amplification Specific Genes by RT-PCR (Table 4)

Table4 Oligonucleotide primers utilized for RT-PCR analysis.

Gene	Primer	Sequence	Gene Code	Position	Size
1. *B-peru*	1.1 R3(S)123	5′-CGC GCG CTC CTG GCC AAG A-3′	X57276.1 *Zea* mays *B-Peru*	616	0.8-kb
	1.2 R2(A)117	5′-ATG AC(A/T/C/G) (G/T)G(A/T/C/G) GT(A/T/C/G) TT(G/A) GA(G/A) GCA CCT-3′	X57276.1 *Zea* mays *B-Peru*	1478	
2. *C1*	2.1 C1-3(S)215	5′-AAC AAA GGG GCA TGG AC-3′	AF485899.1 *Dendrobium* sp (MYB8)	89	0.4-kb
	2.2 C1-4(A)217	5′-TTG ATG TCG TTG TCC GT-3′	AY083610 *Oryza* sativa *myb*.	398	
3. *chs*	3.1 CHS3(S)177	5′-CGT TTC ATG ATG TAC CAA CAG-3′	Z67988.1 *Callistephus* chinensis CHS	507	0.3-kb
	3.2 CHS4(A)178	5′-CAA TGT TCT TCG AGA TCA GCC C-3′	AB201530.1*Lilium* speciosumLs CHS	844	
4. *chi*	4.1 CHI7(S)213	5′-GCC GTT AAG TGG AAG GGT AA-3′	AB054802.1 *Lotus* corniculatus CHI	193	0.4-kb
	4.2 CHI8(A)218	5′-AG(C/T) ACT GCT TCA GAT AAT (C/A)G-3′	M68326.1 *Antirrhinum* majus	573	

Table4 Oligonucleotide primers utilized for RT-PCR analysis (Continued).

Gene	Primer	Sequence	Gene Code	Position	Size
5. *f3h*	5.1 F3H9(S)131	5′-GGC GGC AAG AAG GGC GGC GGC TT-3′	AB070931.1| *Polygonum* hydropiper PhF3H-1 mRNA	232	0.5-kb
	5.2 F3H10(S)132	5′-ATG GTG CCG GGG TCG GTG TG-3′	X58138.1| *Hordeum* vulgare mRNA for F3H	717	
6. *f3′,5′h*	6.1 F3′,5′H3(S)152	5′-CAA TCG TCC ACC TAA TGC AGG-3′	Z22544.1 *Petunia* hybrida F3',5'H	323	1.2-kb
	6.2 F3′,5′H3(A)1535	′-GAG TAA CCA TAG CTT CAA GAG-3′	Z22544.1 *Petunia* hybrida F3',5'H	1518	

Methods

1. Study on Temporal Expression of Structural and Regulatory Genes Involved in Flavonoid Biosynthesis in *Dendrobium* Orchid Flower

1.1. Isolation of Total RNA

Total RNA was extracted from orchid flowers by grinding the tissue into fine powder in liquid nitrogen. Add the pre-warmed (65^{O}C) RNA extraction buffer (1.4% SDS, 2% PVP, 0.5M NaCl, 0.1M sodium acetate, 0.05M EDTA, pH. 8.0, and 0.1% β–mercaptoethanol) to previously ground samples at flower tissue: buffer (1:12), and then vortex to mix and incubate at 65 ^{O}C for 30min. Centrifuge at 6,500 rotations per minute (rpm) for 10min at room temperature to separate the starch and other cell debris and transfer the supernatant to a new tube. Add 0.25volume of 4M potassium acetate, pH 4.8, mix by inversion and incubate on ice for 30min. Then centrifuge at 10,000rpm for 20min at 4^{O}C, carefully pipette the supernatant to a new tube. Add 6M lithium chloride to a final concentration of 3M and incubate overnight at 4 ^{O}C. Centrifuge at 15,000rpm for 30min at 4 ^{O}C to RNA pellet and resuspend the pellet with DEPC-treated H_2O. Add phenol: chloroform (1:1) to separate protein and precipitate the total RNA with ethanol.

1.2. RNA Blotting

Pre-wet the blotting membrane by placing the membrane (Genescreen membranes, NEN^{TM} life science production) in 6XSSC solution (0.9M Nacl, 0.09M trisodium citrate) and assemble the Bio-Dot apparatus (Bio-Rad) according to the instruction (Figure4). Immediately dissolve 10µg total RNA with 500µl ice-cold 10mM NaOH and 1mM EDTA and pull the RNA samples through by a gentle vacuum. Rinse with 500µl ice-cold 10mM NaOH and 1mM EDTA to wash through any sample on the side of the wells, until the sample wells are dry. Then disassemble the Bio-Dot apparatus, remove the blotted membrane and rinse with 2X SSC and 0.1% SDS. Bake the membrane under vacuum for 2 h at 80^{O}C.

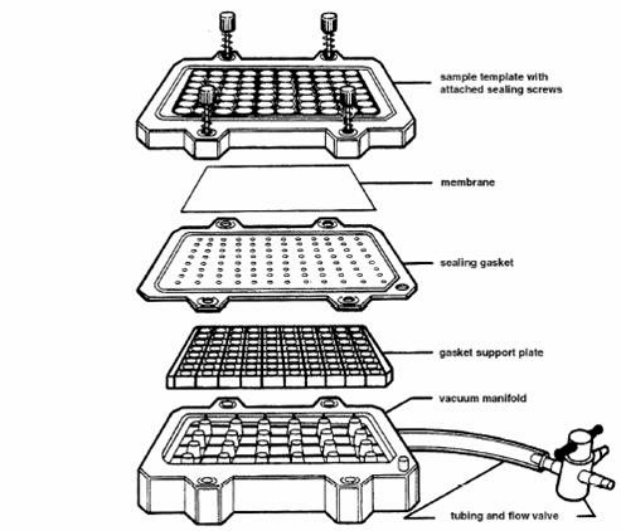


Figure4 Dot blot apparatus.
Source: Bio-Rad, USA

1.3. Labeling Probe Using Reading To Go™ DNA Labelling Beads (α-^{32}P-dCTP) (Amersham Pharmacia,USA)

Before label specific probes, generate the fragment of probes by cutting plasmid containing the structural genes, *chs, chi, dfr*, and *f3h*, and the regulatory genes, *B-peru* and *C1*. Denature the DNA fragments by heating for 2-3min at 95-100 ^{O}C, place on ice for 2min and immediately spin down. Add the following reagents to the tube containing the reaction mix bead: 45µl denatured DNA (25-50ng), 5µl (α-^{32}P) dCTP (3000Ci/mmol), and adjust the total volume of the reaction to 50µl with distilled H_2O. Mix by gently pipetting up and down and incubate at 37 ^{O}C for 5-30min.

1.4. Hybridization and Autoradiography

Gently shake the nylon membrane in prehybridization solution (0.25M $NaHPO_4$, pH 7.2, 1 mM EDTA, 0.5%, blocking reagent, 5% SDS) at 55 ^{O}C for 1h. Denature the radiolabeled probe by boiling for 5min, add 30µl denatured probe to prehybridization solution, and continually incubate overnight. First wash the membrane for 5 min at room temperature in 2X SSC and second wash in 0.5X SSC

and 0.1% SDS at 55 ^{O}C by shaking for 15min. Establish an autoradiograph by exposing the filter overnight to X-ray film (Kodak, USA) at -80 ^{O}C, and then fix and develop the film with fixative and developer.

2. Analyze Optimum Parameters for *Dendrobium* Bombardment Using Transient GUS Expression

2.1. Parameters for Bombardment

2.1.1. Tissue Culture

Pre-cultured three month-old protocorms in liquid ½ MS (Murashige and Skoog, 1962) and ½ VW (Vacent and Went, 1949) (Appendix table2) supplemented with 1.5% sucrose and 7.5% coconut water, shaking at 100rpm to produce secondary protocorms. 1d prior to biolistic experiment, the secondary plbs were placed in solid MS medium supplemented with 1.5% sucrose and 0.8% manitol for osmotic adjustment.

2.1.2. Bombardment Transformation

The pActin1-D plasmid was coated onto microparticles using $CaCl_2$ and spermidine as described by Christou (1991). To study the efficiency of the parameters for transient gene expression, the bombardment conditions were compared as following: gold particles (1.0μm in diameter from Bio-Rad) and tungsten particle (1.0-1.3μm in diameter from Bio-Rad), two levels of helium pressure, 900 and 1,100 pound squire inch (psi), two different sizes of target tissue, approximately 1-2mm and 3-5mm in diameter, and two types of particle guns, Biolistic PDH-1000/He (Bio-Rad, USA) and modified helium particle inflow gun (PIG) (CAMBIA, Australia) with vacuum chamber at a pressure of 28inches of mercury.

2.1.3. Transient GUS Histochemical Assay

3d after bombardment, the flower tissues were examined for transient GUS activity by histochemical assay according to Jefferson *et al*. (1987). Immerse the bombarded tissues in 1mM X-gluc containing 100mM sodium phosphate buffer, pH 8.0, 0.5mM potassium ferricyanide, and 0.5mM potassium ferrocyanide. Incubate overnight at 37^{o}C. Remove the chlorophyll by immerse in 70% ethanol to examine and count the blue spots occurred on the tissues.

2.2. Promoters for Orchid Flower Expression

2.2.1. Culture Condition

Before bombardment, the fresh sepals of *Dendrobium* Jaquelyn Thomas were surface-sterilized by immersing for 15min in 10% Clorox and rinsed three times with sterile distilled water. The sterilized sepals were placed onto wetted 3MM paper placed on a Petri dish.

2.2.2. Coating Plasmid DNA onto Particles

Each of plasmid, pActin1-D, pAHC27, or p2K7, was coated onto microparticles using $CaCl_2$ and spermidine as described by Christou (1991). Before coating, dispersed 4mg gold particles (Bio-Rad, USA) in 100ml cool absolute ethanol with sonicater for 15min, and centrifuge at 12,000rpm for 2min. Resuspend the particles with 500µl water and discard water by centrifuge. Coat the particles by adding 40µg plasmid, 2µl 10X loading DNA, 2µl 1M spermidine, and 2µl 25% poly ethylene glycon, gently mix the solution for 10 min at room temperature. Then centrifuge at 12,000rpm for 1min and discard the supernatant. Finally, wash the coated particles with 1ml absolute ethanol and discard and resuspend the coated particles with 100µl absolute ethanol and use 10µl of solution per once bombardment.

2.2.3. Microprojectile Bombardment of Orchid Flower

The coated plasmids were accelerated to the sterilized sepal of *D.* Jaquelyn Thomas by PHD 1000/Helium biolistic device using following parameters: helium gas at pressure of 600psi, target distance of 12cm, and vacuum chamber at 25inchHg. Transient GUS Histochemical Assay was determined the Gus activity 1d after bombardment.

3. Maize Regulatory Genes Transformation

3.1. Regulatory Gene Construction into Plant Vector

3.1.1. Fragment Preparation

The fragments of 1,794bp of *B-peru* gene and 1,167bp of *C1* gene obtained from cutting the p35SBPcDNA with *Pst* I/ *Sal* I and p35SC1 with *Bam* HI/ *Kpn* I, respectively, were recombined with TG0063 plasmid, (pAct-1:Nos) in the same restriction enzyme sites (Figure5 and 6).

-1 promoter and Nos terminator.

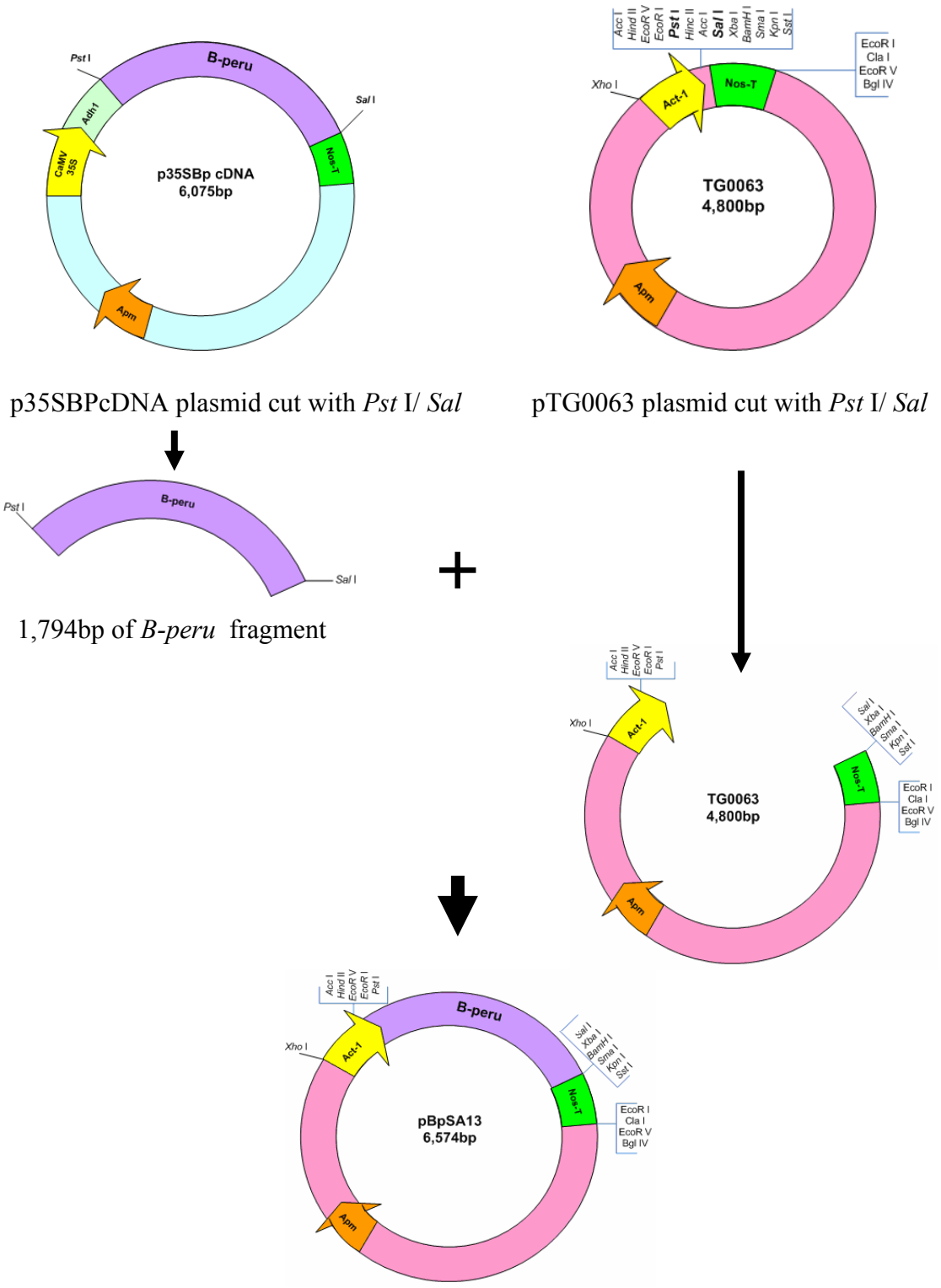
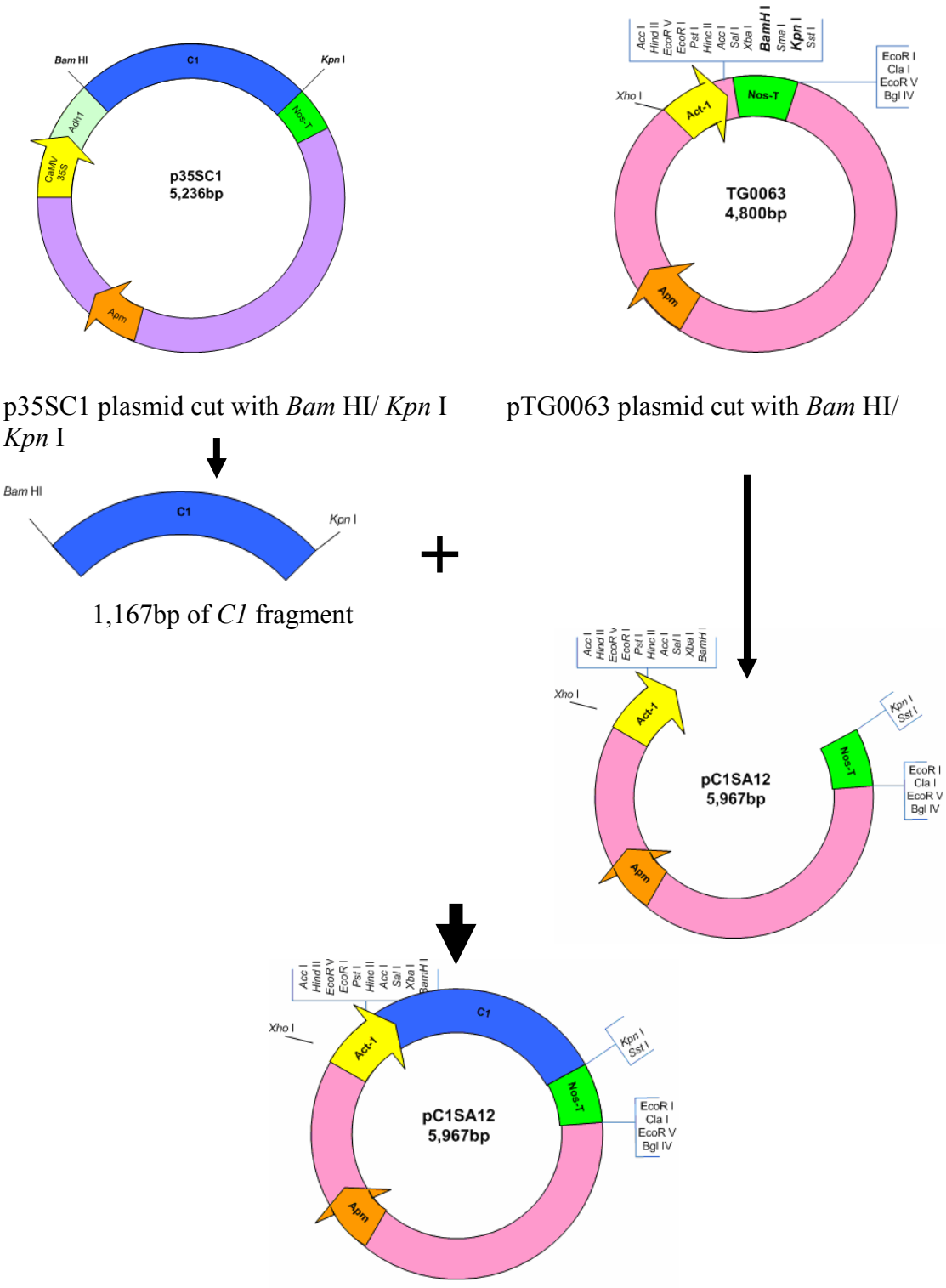


Figure5 The pBpSA13 gene construction: the pBpSA13 plasmid consisted of 1.79-kb of maize *B-peru* gene of p35SBPcDNA driven by Act-1 promoter and Nos terminator of pTG0063 expression vector.



p35SC1 plasmid cut with *Bam* HI/ *Kpn* I

pTG0063 plasmid cut with *Bam* HI/ *Kpn* I

1,167bp of *C1* fragment

Figure6 The pC1SA12 gene construction: the pC1SA12 plasmid consisted of 1.16-kb of maize *C1* gene of p35SC1 driven by Act-1 promoter and Nos terminator of pTG0063 expression vector.

3.1.2. Purification of Gene Fragments

Separate the cut products by electrophoresis in 0.8% agarose gel and excise target band from the gel. The DNA fragments were extracted and purified by Gene Clean Kit (Bio101, Vista, California). Add 500µl NaI and incubate at 55 ^{O}C for 5min and then add glass milk suspension. Mix with pipette and incubate for 5min and centrifuge at 12,000rpm for 1min at room temperature. Wash pellet with 500µl new wash, centrifuge at 12,000rpm for 1min at room temperature for three times and dry glass milk/ DNA complex in vacuum for 8min. Resuspend the DNA in 20µl of TE buffer (10mM Tris-HCl, pH.8.0 and 1mM EDTA) for 5min at 55 ^{O}C, briefly mix, centrifuge at 12,000rpm for 2min at room temperature and then pipette the supernatant into a new tube carefully.

3.1.3. Ligation of the Regulatory Genes into Plant Vector TG0063

Insert the cleaned fragment into TG0063 vector (CAMBIA, Australia) with the following reaction: 5µl 2X ligation buffer, 0.5µl cut vector TG0063, 3µl of the fragment DNA and 1µl T4 DNA ligase (3unit/ µl), then mix the reactions and incubate overnight at 16 ^{O}C.

3.1.4. Transformation into *E. coli*

3.1.4.1. Preparation of Competent Cells

Prepare competent *E. coli* cells according to the method described by Sambrook *et al.* (1989). Culture a single colony of XL1-Blue strain in 2ml Liquid Bloth (LB) medium Luria-Bertani Broth (10g/l Bacto tryptone, 5g/l Bacto yeast extract, and 5g/l NaCl, pH. 7.5) in a glass tube overnight and then transfer 100µl overnight culture into 250ml Erlenmeyer flask with 50ml LB medium, incubate at 37^{o}C with shaking until the growth of culture reached exponential phase (3-4h). Transfer the culture medium into sterile centrifuge tubes and cool down to 4^{o}C by storing the tubes on ice for 10min. Centrifuge at 4,000rpm 4^{o}C for 10min and take off

the supernatant. Resuspend the cells in 3.5ml ice-cold TFB (100ml/l 1M MES, 8.91g/l MnCl2.4H2O, 7.46g/l KCl, and 0.8g/l Hexaminecobalt trichloride). Centrifuged at 5,000rpm 4^{o}C, for 5min and resuspend in 3.5ml ice-cold TFB, store the tube on ice for 5min. Add 130µl dimethyle formamide (DMFO) and 7µl 2-β mercapto-ethanol and store the tube on ice for 5 min. Aliquot the competent *E. coli* cells to 100µl for each transformation.

3.1.4.2. Transformation of *E. coli* using Heat Shock

Transformation was performed according to the method described by Sambrook *et al.* (1989). Place 100µl competent cells aliquot on ice, mix with 10ng plasmid DNA and incubate on ice for 30min. Heat shocked the mixed solution at 42^{o}C for 1min and immediately chilled on ice for 2min. Transfer into 1ml SOC medium (20g/l Bacto tryptone, 5g/l Bacto yeast extract, 0.5g/l NaCl, 10 ml/l of 250mM KCl, 5ml/l of 2M $MgCl_2$, and 20ml/l of 1M glucose) and incubated at 37^{o}C for 1h and then spread on LB agar plates containing 50µg/ml ampicillin, and incubated at 37^{o}C overnight.

3.1.5. Plasmid Purification by Alkaline Lysis

The rapid alkaline lysis method is performed according to the method described by Sambrook *et al.* (1998). Grow the bacteria in 3ml LB broth supplemented with 50µg/ml ampicillin at 37^{o}C with shaking until the cell growth reached log phase, centrifuged at 12,000rpm for 5min at 4^{o}C. Resuspend the bacterial cells in 100µl cold GTE solution (50mM glucose, 25mM Tris-Hcl, pH. 8, and 10mM EDTA), vortex and keep on ice. Add 200µl freshly prepared 0.2N NaOH and 1% SDS solution, immediately mix by inverting the tube three times. Store the tube on ice and add 150µl cold 3M potassium acetate, pH 4.8. Mix gently by inverting the tube for 10sec and then store on ice for 15min. Centrifuge at 12,000rpm for 5min at 4^{o}C. Transfer the supernatant to a new tube. Precipitate the plasmid with 2volume of absolute ethanol and 0.3M sodium acetate and place for 2min at room temperature.

Centrifuge at 12,000rpm for 5min, then washed once with 70% ethanol. Dry the pellet and resuspend in 50µl sterile deionized water.

3.1.6. Digestion with Restiction Endonuclease

Plasmid digestion with various restriction enzymes is performed using the buffer provided with the enzymes under the conditions recommended by the manufacturers to confirm the recombinant plasmid. When digestion of DNA with two different enzymes was required, in the case where reaction conditions (buffer and temperature) are compatible, both enzymes are added simultaneously.

3.2. Maize Regulatory Genes Transformation

3.2.1. Bombardment of Orchid Flower

3.2.1.1. Culture Condition

The fresh sepals of *D.* Jaquelyn Thomas were surface-sterilized as described in 2.2.1.

3.2.1.2. Bombardment

To evaluate the maize transcription factor gene expression in orchid flower, pBpSA13 and pC1SA12 plasmids were coated onto gold particles using $CaCl_2$ and spermidine as described by Christou (1991) and bombarded into the petal of *D.* Jaquelyn Thomas by PHD 1000/Helium biolistic device using following parameters: helium gas at pressure of 600psi, target distance of 12cm, and vacuum chamber at 25inchHg. The anthocyanin pigmentation in floral tissues was observed 7d after transformation under a stereomicroscope.

3.2.2. Bombardment of Orchid Protocorms

3.2.2.1. Culture Condition

Immerse a green capsule of *D.* Jaquelyn Thomas in 70% ethanol for surface-sterilization, and rinse three times with sterile distilled water. Take out the seed from sterilized capsule and produce the primary protocorms by culturing in solid VW medium supplemented with 2% sucrose, 10% homogenized ripe banana, 10% potato water extract, 15% coconut water, 0.2% charcoal, and 0.7% agar, and continually proliferate primary protocorms to produce secondary protocorms in 30ml liquid MS medium containing 1.5% sucrose and 7.5% coconut water with shaking at 100rpm, sub-culturing twice a week. 7d prior to transformation, these tissues were sub-cultured to produce newly active protocorms, which would be used as a target tissue for bombardment.

3.2.2.2. Bombardment Condition

4h before bombardment, place the protocorms in a circle with a diameter of 25mm on solid MSM (Appendix2) for osmotic pretreatment. Three plasmids coated on gold microparticles described by Christou *et al.* (1991) were co-bombarded into the protocorm with He gas pressure of 1,100psi, the distance from macrocarrier to target tissue set at 90mm, and vacuum chamber at 25inch Hg using a PDH 1000/Helium biolistic device. After bombardment, bombarded tissues were maintained in the dark growth chamber for 3d before culturing in solid MS medium under a 16h photoperiod with cool white light at 25 ^{o}C.

3.3.2.3. Selection of Transformants

3d after bombardment, transformed tissues were initially selected in solid MSHyg5 medium for 45d. Proliferating protocorms were chopped and transferred to solid MSHyg25 medium. The green tissues were maintained in 25µg/ml selective media for 45d nd subsequently selected in solid MSHyg30 for

another 30d. Control explants were also selected in the same way. Later, the hygromycin-resistant protocorms were transferred to solid MS medium without selective agent for regeneration of plantlets.

3.2.2.4. GFP Detection

The bombarded protocorms having been in selective media for 7, 45, 90, or 120d were observed for their GFP expression with a fluorescence microscope equipped with a BZA filter, and the images were captured in real time with a Nikon coolpix5400 camera attached to the fluorescence microscope using Nikon software.

3.2.2.5. DNA Analysis

3.2.2.5.1 DNA extraction

Genomic DNA from fresh leaves of individual lines of hygromycin-resistant plantlets and unbombarded plants were extracted using the CTAB method (Murray and Thomson, 1980). Add pre-warmed (60^{O}C) DNA extraction buffer (2% hexadecyltrimethyl ammonium bromide, 1.4M NaCl, 20mM Na_2EDTA, and 100mM Tris-HCl, pH. 8.0) to ground samples at leave tissues: buffer (1:12) and vortex to mix and incubate at 60 ^{O}C for 30min, and then add 500µl chloroform: isoamyl alcohol (24:1), vortex for 30sec. Starch and other cell debris are pelleted by centrifugation at 12,000rpm for 8min at 4^{O}C, and transfer the supernatant to a new tube. Add 0.67volume of 4M potassium acetate, pH 4.8, mix by inversion and incubate on ice for 10min. Precipitate by centrifuge at 12,000rpm for 8min at 4^{O}C. Wash with 70% ethanol and add 30µl sterilized water to resuspend the DNA.

3.2.2.5.2. *Hph* and *Gfp* Detection

3.2.2.5.2.1. PCR Amplification

Preliminary screened the integration of the target gene, the DNA from leave of individual transgenic lines and unbombarded plants were used for amplification of internal *hph* and *gfp* genes with the pairs of specific primers. The thermal cycling program was performed as following: 35 cycles of 1 min at 95^{O}C, 1 min at 60^{O}C and 2 min at 72^{O}C. The expected PCR products of the *hph* and *gfp* genes are 590 and 800, respectively.

3.2.2.5.2.2. PCR and Genomic Southern Blot Hybridization

To confirm the integration of the *hph and gfp* genes in chromosomes of the transgenic orchid, 1µg PCR products of *hph* and *gfp* genes and 10µg gDNA digested with *Sac*I were separated in 1% agarose gel and blotted onto Hybond N^{+} nylon membrane (Amersham Bioscience, UK) using capillary blotting with 10X SSC. The *hph* and *gfp* probes were labeled using Gene Images Alkphos Direct labeling and detection system (Amersham Bioscience, UK). Add the following reagents to 100ng denatured DNA tube as following: 10µl reaction buffer, 2µl labeling reagent, 10µl cross linker working solution. Adjust the total volume of the reaction to 32µl with sterilized distill water and incubate overnight at 37 ^{o}C. The Blots were hybridized with labeled DNA probes at 60^{O}C in hybridization buffer (0.25M $NaHPO_4$, pH 7.2, 1mM EDTA, 0.5% Blocking Reagent, 5% SDS). After hybridization, the blots were washed twice for 10min in the first wash buffer (2M Urea, 0.1% SDS, 50mM $NaH_2PO_4.7H_2O$, pH 7.0, 150mM of NaCl, 1mM $MgCl_2$, 0.2% Blocking reagent) at 60 ^{O}C and twice for 5min with the second wash buffer (1M Tris Base, 2M NaCl) at room temperature. CDP Star Detection was used for signal detection.

3.2.2.5.3. *B-peru* and *C1* Detection

3.2.2.5.3.1. PCR Amplification

The DNA from leave of individual transgenic lines and unbombarded plants were used for amplification of internal *B-peru*, and *C1* genes with the pair of specific primers to initially detected samples of the integration of the target genes. The thermal cycling program was performed as following: 35 cycles of 1min at 95^{O}C, 1min at 60^{O}C and 2min at 72^{O}C.

3.2.2.5.3.2. Southern Blot Hybridization

10µg gDNA of the putative transgenic lines and the untransformed plant were digested with *Eco* RI and separated on 1% agarose gel. The DNA were denatured by soaking with 0.25% HCl for 15min and denaturation solution (0.4M NaOH and 0.4M NaCl) for 30min. The denatured DNA is then transferred to a Hybond-N^{+} nylon membrane with alkaline transfer method (Sambrook *et al.*, 1989), rince the membrane with neutralization solution 4(0.5M Tris-Hcl, pH. 7.2 and 1.0M NaCl), and dry at room-temperature for30 min. The *B-peru* and *C1* fragments were labeled with Reading To Go^{TM} DNA Labelling Beads (α-^{32}P) dCTP) (Amersham Bioscience, UK). Denatured DNA fragments were pipetted to a tube containing the reaction mix bead and add 2µl (α-^{32}P) dCTP (3000 Ci/mmol). Mix by gently pipetting, and incubate at 37 ^{O}C for 1h. Hybridize the blots with the labeled probe in hybridization solution (0.25M $NaHPO_4$, pH 7.2, 1mM EDTA, 0.5% Blocking Reagent, 5% SDS) at 55 ^{O}C overnight and wash the membrane for 5min at room temperature in 2X SSC, then second wash in 0.5X SSC and 0.1% SDS at 55 ^{O}C for 1 min. Expose the signal overnight to X-ray film at -80 ^{O}C.

3.2.2.6. RNA analysis

3.2.2.6.1. RNA Extraction

The RNA from leaf tissues of putative transgenic and unbombarded lines were extracted using Hot Phenol (Wilkins and Smart, 1996). Add 250µl pre-warmed (80^{O}C) RNA extraction buffer (0.1M LiCl, 1% SDS, 100mM Tris-HCl, pH 8.0, and 20mM EDTA) and 250µl pre-warmed phenol to previously ground samples at leave tissues: buffer (1:12), vortex to homogenize for 30sec and incubate at 80^{O}C for 30sec. Add 250µl Chloroform: Isoamyl alcohol (24:1), vortex for 30sec. Centrifuge at 12,000rpm for 10min at 4 ^{O}C and transfer the supernatant carefully to a new tube. Add 1volume 6M LiCl and mix by inversion; incubate on ice for 4h. Centrifuge at 12,000rpm for 10min at 4 ^{O}C. Resuspend the pallet in 250µl DEPC-treated water. Add 0.1volume 3M NaOAc and 2volume absolute ethanol, incubate at 4 ^{O}C overnight. Centrifuge 12,000rpm for 15min at 4^{O}C and discard the supernatant. Wash the pallet with 70% ethanol and centrifuge at 12,000rpm for 5min at 4 ^{O}C. Dry the pallet and resuspend in 30µl DEPC-treated water.

3.2.2.6.2. Northern Blot Hybridization

Add 10X MOP solution (0.2M MOPS, pH. 7.0, 20mM Sodium Acetate, and 10mM EDTA, pH. 8.0) and 18ml formaldehyde in warmed 1.2% agarose gel and set the gel for 30 min at room temperature. Prepare the RNA by mixing the following in a sterile microcentrifuge tube: 5µg the total RNA in 10µl DEPC-treated water, 3.5µl 10X MOP solution, 17.5µl 50% formamide, 6.2 µl 37% formaldehyde, and 1µl of 200µg/ml EtBr solution and then adjust the total volume to 35 µl. Heat the solution at 65 ^{O}C for 15 min, immediately chill on ice, and add 3.5µl loading buffer (6X loading buffer, 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). Load the RNA sample solution in each well and run the gel submerged in 1X MOPs solution. Transfer denatured RNA to Hybond-N+ by capillary blotting using 10X SSC and hybridize

with the fragment probes (Gene Images Random Prime Labeling Module, Amersham Bioscience, UK) overnight at 55 ^{O}C in hybridization solution (5X SSC, 0.1% SDS, 5% Dextran sulphate, and 1/20 liquid block dilution). Wash the blots for 5 min at room temperature in 2X SSC and for 15 min at 55 ^{O}C in 0.5X SSC and 0.1% SDS. Place blots in 1/10 dilution of liquid blocking agent in bufferA (100mM Tris-HCl,300mM NaCl, pH 9.5) and then in diluted conjugate for 1h at room temperature each step. Wash in 0.3% Tween20 in bufferA at room temperature with agitation for 10 min three times. Detect the signal using CDP star detection kit.

3.2.2.6.3. RT-PCR

Pipette the total RNA into a microcentrifuge tube, and adjust the total volumn to 30µl with DEPC-treated water. Then place this tube into the heating block at 65 ^{O}C for 10min, rapidly cools on ice for 2min. Pipette 30µl denatured total RNA into a tube containing beads (Ready-To-Go, Amersham Bioscience, UK), add 3µl oligodT17 and specific antisense primer, and place at room temperature for 1min. Gently mix and briefly spin. Incubate the reaction at 37 ^{O}C for 60min to synthesize first-strand cDNA. The cDNA fragment of the regulatory genes and structural genes involved in anthocyanin biosynthesis is generated. The Mastercycler gradient is used to amplify the products through 94 ^{O}C for 1min, 55 ^{O}C for 1min, 72 ^{O}C for 2min for 30 cycles with the following conditions; 36µl sterile water, 3µl of cDNA template, 5µl 10X PCR buffer, 1µl 10mM dNTP, 1µl DNA polymerase, and 2µl a pair of specific primer.

RESULTS AND DISCUSSION

1. Study on Temporal Expression of Structural and Regulatory Genes Involved in Anthocyanin Biosynthesis of Orchid Flower

With regard to RNA isolation, the greatly different yield of total RNA extracted from 2g of sepal and petal parts in each flower developmental stage were observed. The results showed that total RNA extracted from the *Ascocendra* flower gave higher concentration than *Dendrobium* flower (Figure7A). Within the *Dendrobium* varies, the flowers of those having thick sepal and the petal are rich in polysaccharide such as in *D.* Pinky Sem 'Sabin' giving less yield of total RNA than the variety with lower polysaccharide like *D.* Jaquelyn Thomas (Figure7B, C). Moreover, the quantity of total RNA from different stages of flower development was also different. The flower developing from bud in the first stage to nearly blooming flower in the third stage gave higher yield than the blooming flower in the forth and fifth stages of development in which the flower is getting mature.

Because of low cell density and rich of polyphenol compounds and polysaccharide nature in mature or thick flowers tissues of orchid, the yield of total RNA is typically low. Due to polyphenol compounds which normally accumulated in the plant tissues, such as phenolic, terpene, tannin, were able to bind the RNA, changing its chemical structure; furthermore, chemical structure of polysaccharide and nucleic acid are similar, their agglutination occur simultaneously in high salt concentration of 6M LiCl solution in RNA isolation step (Wikins and Smart, 1996). To solve the problem of polysaccharide interrupt, 2% polyvinyl pyrolidone (PVP) supplemented in RNA extraction buffer is to complex polysaccharide in the lyses step and 4M potassium acetate (KoAc) is to agglutinate polysaccharide prior to the RNA extraction, making increasing of quantity of total RNA in the orchid flower, particularly in the blooming and think flower in the late stage of development.

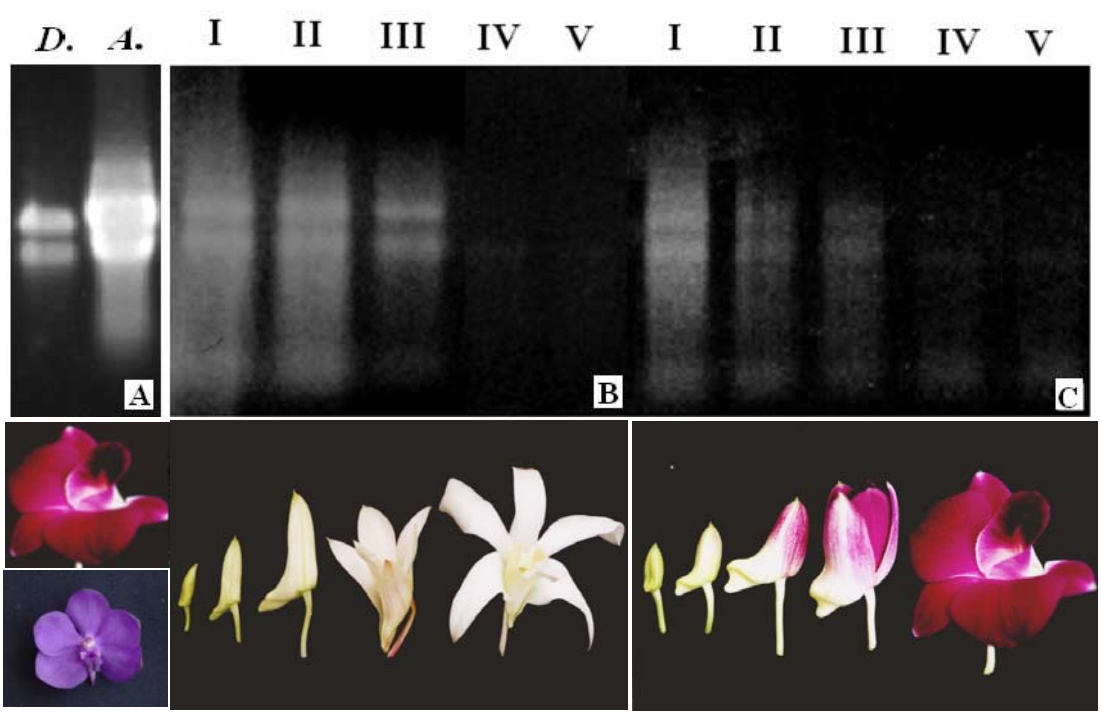


Figure7 The total RNA extracted from the orchid flower: (A) 10µg of total RNA extracted from flower at all stages of development: *D*: *Dendrobium* and *A.*: *Ascocendra*, total RNA during developmental stages from flower bud to completely blooming flower: (B) *D.* Jaquelyn Thomas, (C) *D.* hybrid 'Honny': I: flower bud in length of 1.0cm, II: closed flowers in length of 2.5-3cm, III: the flower colored at the edges of the petals in length of the 3-4cm, IV: the blooming flowers, and V: the completely blooming flower.

Three heterologous in genes encoding biosynthesis enzyme and regulatory genes encoding MYC and MYB transcription factors related to anthocyanin biosynthesis were utilized to study their expression patterns during flower development. RNA from five stages of flower from flower bud to fully blooming flower was analyzed by dot blot hybridization. 10µg of total RNA from each stage of floral development of white flowers extracted from *D.* hybrid 'Yuki White', *D.* Jaquelyn Thomas, *D.* hybrid 'Sanan White', *D.* hybrid 'Honny', and *D.* Shavin White 'White 5N' and of red- purple, blue-purple and orange flowers obtained from *D.* Pinky Sem 'Sabin', *D.* Kiyoshi Izumi, *Vascostylis* Pine Rivers, and *A.* Su-Fun Beauty, respectively, (Figure8) were blotted and hybridized with the probes of two

representative transcription factor genes involved anthocyanin pathway of maize, *B-peru* and *C1* gene encoding *myc* and *myb* transcription factors, respectively, and three major of structural genes, including *chs* and *chi* genes obtained from *A. majus* and *f3h* gene of *Vascostylis* Pine Rivers.

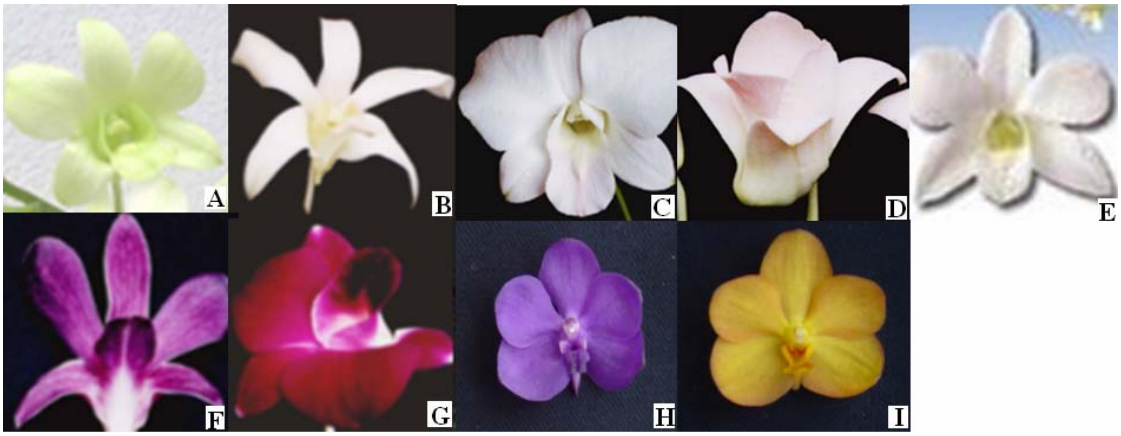


Figure8 The samples of orchid flower used in the study of temporal expression of the regulatory and structural genes involved in anthocyanin biosynthesis: (A) *D.* hybrid 'Yuki White', (B) *D.* Jaquelyn Thomas, (C) *D.* hybrid 'Sanan White', (D) *D.* hybrid 'Honny', (E) *D.* Shavin White 'White 5N', (F) *D.* Kiyoshi Izumi, (G) *D.* Pinky Sem 'Sabin', (H) *Vascostylis* Pine Rivers, and (I) *A.* Su-Fun Beauty.

1.1. Expression Pattern of Structural Gene involved in Anthocyanin Biosynthesis of Orchid Flowers

The expression levels of the *chs*, *chi*, and *f3h* genes encoding anthocyanin biosynthesis enzymes in orchid flower during flower color development were investigated. RNA dot blot hybridization using hetelozygous probe indicated that the *chs* transcripts were found in all flowers tested with the different expression pattern. The high expression was found not only in initial but also in late stage of floral development. Figure9A showed that the expression at the fourth and fifth stages of flower development was observed. The *chs* transcripts were poorly detectable in the initial developmental stage of *D.* hybrid 'Yuki White' flower, and in

the late stage of development of the white flower of *D.* Jaquelyn Thomas of which blooming flower slightly accumulated anthocyanin pigments at the edge. Although, *chs* transcripts of *D.* Kiyoshi Izumi and *Vascostylis* Pine Rivers flower in which accumulated blue-purple anthocyanin pigments were found abundant in all developmental stages, the higher level of the *chs* expression was detected in white flower of *D.* hybrid 'Sanan White'. Moreover, strong expression of *chs* gene during floral development was observed in white flower of *D.* hybrid 'Honny' and *D.* Shavin White 'White 5N', and in orange flower of *A.* Su-Fan beauty. The highest expression was shown in initial developmental stage of red-purple flower of *D.* Pinky Sem 'Sabin', particularly in the third developmental stage of the flower becoming coloration. Dooner, 1983 revealed that, in maize, *chs* expression occur independently of R gene family, which normally controls the biosynthesis gene transcription. In Antirrhinum and Petunia spp. Flower, the early structural genes are activated by another set of regulatory genes (Mogano *et al.*, 1996; Quattrocchio *et al*., 1998). Base on the results, the *chs* transcripts corresponding to heterologous probes were detected in all variety, indicating that *chs* transcription for anthocyanin pigmentation might be differently regulated from other anthocyanin biosynthesis genes.

On the contrary, in the initially floral developmental stage, the transcription of *chi* gene was rarely detectable in the white flower of *D.* hybrid 'Yuki White', *D.* Jaquelyn Thomas, *D.* hybrid 'Sanan White', and *D.* hybrid 'Honny' and low level of *chi* expression was obtained of the white flower of *Dendrobium* Shavin White 'White 5N', and of the blue-purple flower of *D.* Kiyoshi Izumi, and *Vascostylis* Pine Rivers (Figure8B), while high level of *chi* expression was found in the flowers of *D.* Pinky Sem 'Sabin' and *A.* Su-Fun beauty accumulating the red-purple and orange pigments. No any signal was obtained in all varieties tested in the forth and fifth stages of flower development. Corollas of *Callistephus chinesis* (China aster) and *Dianthus caryophyllus* (Carnation) are devoid of CHI activity, resulting in the production of yellow flowers due to the accumulation of chalcone pigments (Huhn *et al*., 1978; Forkmann, 1980). Similarly, as the results shown *chi* gene was expressed in the colored flower at initial stage of flower development, the *chi* gene may be one of the important structural genes determining the anthocyanin pigmentation in orchid flower.

Although, no any anthocyanin pigment were observed in all developmental stages of *D.* hybrid 'Yuki White' flower, the similar pattern of the expression of *f3h* and *chi* genes was obtained. Figure8C showed the weakly detectable level of *f3h* gene expression in the initial and in the latest floral developmental stages of the white flower of *D.* hybrid 'Honny' and in the latest stage of the white flower of *D.* Jaquelyn Thomas. The high expression of *f3h* gene was found in the white flower of *D.* hybrid 'Sanan White' and in the blue-purple flower of *D.* Kiyoshi Izumi and *Vascostylis* Pine Rivers at the initial stages of flower development. The white flower of *D.* Shavin White 'White 5N', the red-purple flower of *D.* Pinky Sem 'Sabin', and the orange flower of *A.* Su-Fun Beauty conferred strong *f3h* expression (Figure8C). Undoubted, the white flower of *D.* hybrid 'Sanan White' and *D.* Shavin White 'White 5N' showed high level of *f3h* gene expression. Due to the fact that they are hybrid in which some structural genes, including *f3h* gene still express, while other structural genes may be mutated, consequently, anthocyanin pigmentation could not be completely biosynthesized.

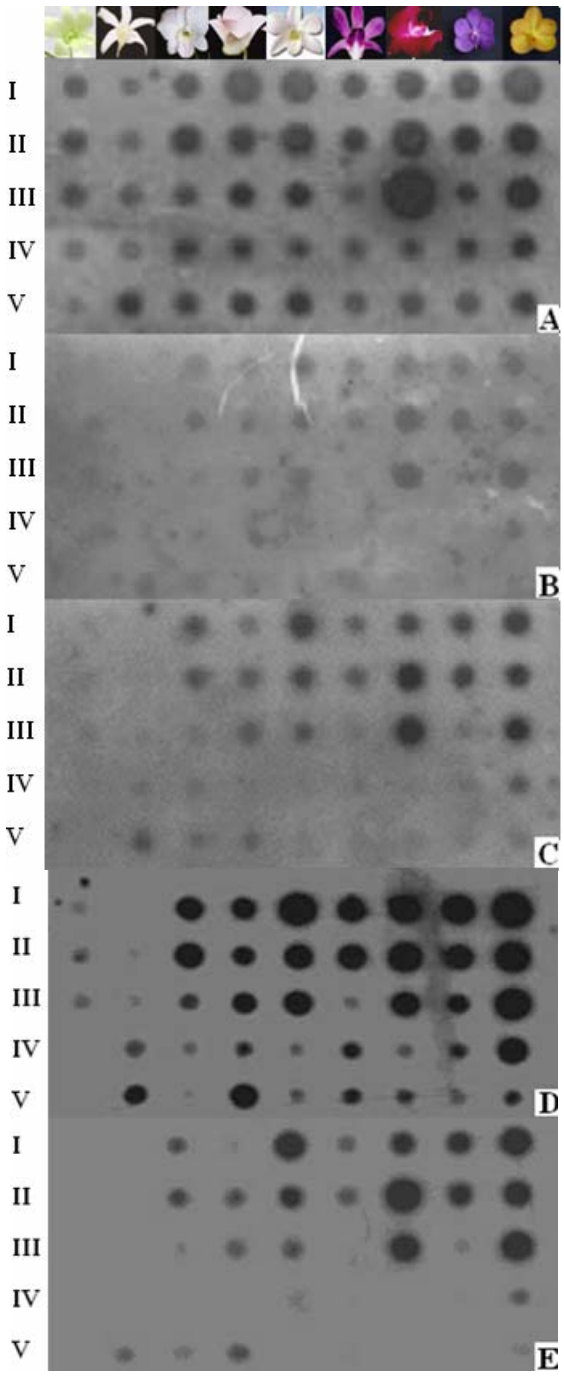


Figure9 RNA dot blot analysis showing the expression of the structural and regulatory genes involved in anthocyanin biosynthesis at various stages of the orchid flower development. 10μg of total RNA of orchid varieties were blotted and hybridized with the fragment probe: (A) *chs* gene, (B) *chi* gene, (C) *f3h* gene, (D) *Myc* gene, and (E) *Myb* gene. The numbers of orchid were shown at the top. The five different stages of flower development were indicated at the left.

1.2. Expression Pattern of Transcription Factor Genes Involved in Anthocyanin Biosynthesis of Orchid Flowers

The expression pattern of regulatory genes was elucidated using the heterologous cDNA clone from maize as a probe. The results demonstrated that almost all flowers tested had strong *Myc* gene expression in the flower bud and decreased through the developmental stages. Low levels of *Myc* expression were observed at the forth and the fifth stages of flower development, except white flower of *D.* Jaquelyn Thomas and of *D.* hybrid 'Honny' which showed low expression in the late developmental stage having anthocyanin accumulation in the flower tissue. Interestingly, only one of the white flowers of *D.* hybrid 'Yuki White' was not able to detect the signal of the *myc* transcription compared with the signal of other varieties (Figure8D). All flower samples accumulated anthocyanin pigments, such as orange flower of *A.* Su-Fun Beauty, red-purple flower of *D.* Pinky Sem 'Sabin', and blue-purple of *D.* Kiyoshi Izumi and *Vascostylis* Pine Rivers and partially accumulated anthocyanin in the white flowers, including *D.* hybrid 'Sanan White', *D.* hybrid 'Honny', and *D.* Shavin White 'White 5N' showed high level of *Myc* expression (Figure8D) and the highest *Myc* expression was observed in the orange flower of *A.* Su-Fun Beauty. The *Myc* transcripts were strongly detectable in the blooming flower accumulating anthocyanin, demonstrating that *Myc* gene stringently up-regulated the structural genes expression in orchid flowers for coloration. Base on the results that the high level of *Myc* transcripts were shown in white flowers which there were no or partially anthocynain accumulation, the mutant might be occurred in *cis*-element of the structural gene encoding the enzyme in the pathway; consequently, there are no anthocyanin accumulation in the white flower.

The expression pattern of *Myb* transcription factor gene was rather different from the expression pattern of *Myc* transcription factor. Figure8E demonstrated that the equal levels of its expression was found in the blue-purple flower of *D.* Kiyoshi Izumi and *Vascostylis* Pine Rivers, and in the orange flower of *A.* Su-Fun Beauty at the early stage of floral development, but the *Myc* transcripts in the red-purple flower of *D.* Pinky Sem 'Sabin' were increased through the flower

development from flower bud to the third stage in which flower become blooming. Low or undetectable levels of *Myb* expression in the colorful flowers were obtained at the late stages. However, the similarity of the expression pattern of *Myc* and *Myb* expression were weak in signals were found in the blooming flower of *D.* Jaquelyn Thomas and of *D.* hybrid 'Honny' partially accumulating the anthocyanin pigments. It is implied that the *Myc* transcription factor is also significant to regulate the anthocyanin biosynthesis gene in the orchid flower.

Base on the results, only *D.* hybrid 'Yuki White' is truly white flower in which there was no any anthocyanin accumulation, low or undetectable level of expression of structural and regulatory genes, but *chs* gene was not obtained. While the other white flower including *D.* Jaquelyn Thomas, *D.* hybrid 'Sanan White', *D.* hybrid 'Honny', and *D.* Shavin White 'White 5N' which partially accumulated anthocyanin pigments showed various expression of the genes. Due to the fact that, the flower of *D.* Jaquelyn Thomas and *D.* hybrid 'Sanan White' flowers normally accumulated anthocyanin pigments in the blooming flower and *D.* hybrid 'Sanan White' and *D.* Shavin White 'White 5N' are hybrid cultivars, their expression was obtained in the white flowers of these varieties. The high expression levels of *chs* and *f3h* structural genes demonstrated that both genes still have expressed in white flower, but *chi* structural gene might be mutated, consequently, there are no or partial anthocyanin accumulations in their white flowers. The results indicated that the *chi* and *f3h* genes, but not *chs*, conferred the high expression at the early flower developmental stages of orchid. Mutation of at least one of structural gene locus of orchid would block anthocyanin biosynthesis, giving rise to uncolored or partially colored flowers. In addition, with regard to the flower accumulating anthocyanin, including *D.* Kiyoshi Izumi, *D.* Pinky Sem 'Sabin', *Vascostylis* Pine Rivers, and *A.* Su-Fun Beauty, the level of gene expression was rather different. The orange flower of *A.* Su-Fun Beauty and red-purple flower of *D.* Pinky Sem 'Sabin' had higher expression of the genes involved in anthocyanin biosynthesis than blue-purple flower of *D.* Kiyoshi Izumi and *Vascostylis* Pine Rivers. It is implied that the levels of gene

expression in each step of the pathway might be significantly critical for different types of pigment accumulation. However, the detail is not clear.

Transcription factors belonging to the bHLH gene family and HTH gene family are important regulatory components in anthocyanin biosynthesis pathway (Atchey and Fitch, 1997). Base on the results, in spit of white flower, the *chs* gene transcripts could be detected, demonstrating that neither the MYC nor MYB transcription factor involved in the pathway might up-regulate the *chs* transcripts. It is possible that there are other group of gene family might control the expression of *chs* gene. Jackson *et al*. (1992) revealed that the product of *Del* gene encoding MYC gene transcription factor protein involved in anthcaynin biosynthesis in flower of *A. majus* also acts as a repressor of the *chs* gene expression in the flower lobe mesophyll. The mutant of *Eluta* and *Rosea* genes belonging to *Myc* transcription factor decreased the expression of *f3h* (Bartlett, 1989). Similarly, the results displayed that the abundant *chi* and *f3h* transcripts found in the flowers accumulating the anthocyanin pigments showed strongly relative to *B-peru* transcription factor, MYC transcription factor might modulate the expression of at least *chi* and *f3h* transcripts.

2. Analyze Optimum Parameters for *Dendrobium* Bombardment Using Transient GUS Expression

2.1. Effective Parameters for Bombardment

To determine the optimal condition for particle bombardment, the transient GUS activity of transformed *Dendrobium* orchids was examined, comparing types of particle, gold and tungsten, levels of helium gas pressure, 900 and 1,100Psi, and sizes of target tissue, small size (1-2mm in diameter) and large size (3-5mm in diameter). Transient GUS expression was observed in the secondary protocorms after bombardment with pActin-1D plasmid carrying the *gus* gene driven by Act-1 promoter and Nos terminator. The initial observation found that the range from macrocarrier to target tissue had a significant effect on the death of bombarded tissue. The target distance of 60mm greatly damaged the bombarded small tissue, such that

99.66% of bombarded small protocorms were dead, while the distance of 90 mm caused only 14.12% to die (data not shown). Furthermore, the blue spots on protocorms with the flight distance of 90mm (Figure10B) were more spreader-out than 60mm (Figure10A). The target distance of 90mm, therefore, was a suitable parameter to be used for further orchid transformation.

No significant transient GUS expression of protocorms bombarded with different microparticles was found. The number of GUS spots per protocorm after bombardment with gold particles, 1.0(m in diameter from Bio-Rad, and tungsten particles, 1.0-1.3(m in diameter from Bio-Rad, were 28.16 and 23.44, respectively (Table5). The round surfaced spherical gold particles produced well spread blue spots (Figure10C), while the irregular surfaced of sharp tungsten particles gave a large blue patch (Figure10D) because of the aggregation during DNA coating. Furthermore, since the sharpness and toxicity of tungsten particles also damaged tissues (Sanford et al. 1993; Hunold et al. 1994) the number of surviving tissues after bombardment with gold was more than with tungsten (Table5). Russell et al. (1992) showed no difference between tungsten and gold particles on the transformation rate of tobacco, but the rate of stable transformation with gold was four times higher than that of tungsten; consequently, gold particles are remarkably more efficient for genetic transformation.

No effect of helium gas pressure on GUS activity was observed. The difference in the number of blue spots from helium gas pressure of 900 and 1,200Psi was not significant (Table5). Nan and Kuehnle (1995); Yang et al. (1999) and also found no difference in transient GUS expression after bombardment at various pressures of helium gas in Dendrobium orchid transformant. Nevertheless, with a better survival transformation rate for bombardment at 1.100Psi, this pressure was recommended for orchid bombardment.

Two types of particle gun; Biolistic PDS-1000/He and modified helium particle inflow gun, PIG (CAMBIA, Australia) were also compared for their efficiency in genetic transformation. The result showed that the blue spots on the

protocorm bombarded with the Biolistic PDS-1000/He (Figure10E) were more intense than those using the PIG gun (Figure10F). The Biolistic PDS-1000/He was not only superior in controlling bombardment parameters, but also helpful in decreasing contamination (Nan and Kuehnle, 1995) and safer than the modified helium particle inflow gun (Klein et al., 1987). Thus, the Biolistic PDS-1000/He was considered to be an efficient gun for establishing transformants.

Type of orchid tissues was another critical point effecting to gene transformation success. The active dividing orchid protocorms were widely used for genetic transformation as previously reported in Dendrobium (Kuehnle and Sugii, 1992; Chia et al., 1994; Yu et al., 1999; Yu et al., 2001; Men et al., 2003). Nan and Kuehnle (1995) showed that the protocorm-like body of Dendrobium orchid was the target tissue that yielded the highest GUS expression. The sizes of the protocorms, however, also affected their survival percentage and transient GUS expression. Table5 showed that the survivals of small and large protocorm were 61.45% and 100%, respectively. It was demonstrated that bombardment could damage small protocorms, but did not affect the large protocorms. Although, because of the fact that transgene integration is favored in cells which are in the M-and G2-phases to cells in the S-and G1-phases (Iida et al., 1991), the small protocorms with most of their cells in the dividing state gave a higher number of blue spots and more efficient transformation than the large protocorms with most of their cells in stationary state (Table5). The results suggested that the target tissue must be actively dividing cells, which can be obtained by sub-culturing them in liquid medium to stimulate cell division prior bombardment to accomplish orchid transformation.

Table5 *Gus* gene expression and survival percentage of orchid protocorms 3d after bombardment with different parameters.

Parameters	Average number of GUS spots per protocorm	Survival percentage (%)
Type of particles		
Gold	28.16a	65.77
Tungsten	23.44a	57.14
He gas pressure (psi)		
900	25.26b	48.89
1,100	26.34b	73.99
Size of target protocorm		
Small	26.95[D]c	61.45
Large	24.65c	100.00

D: comparison with equal surface area of protocorm

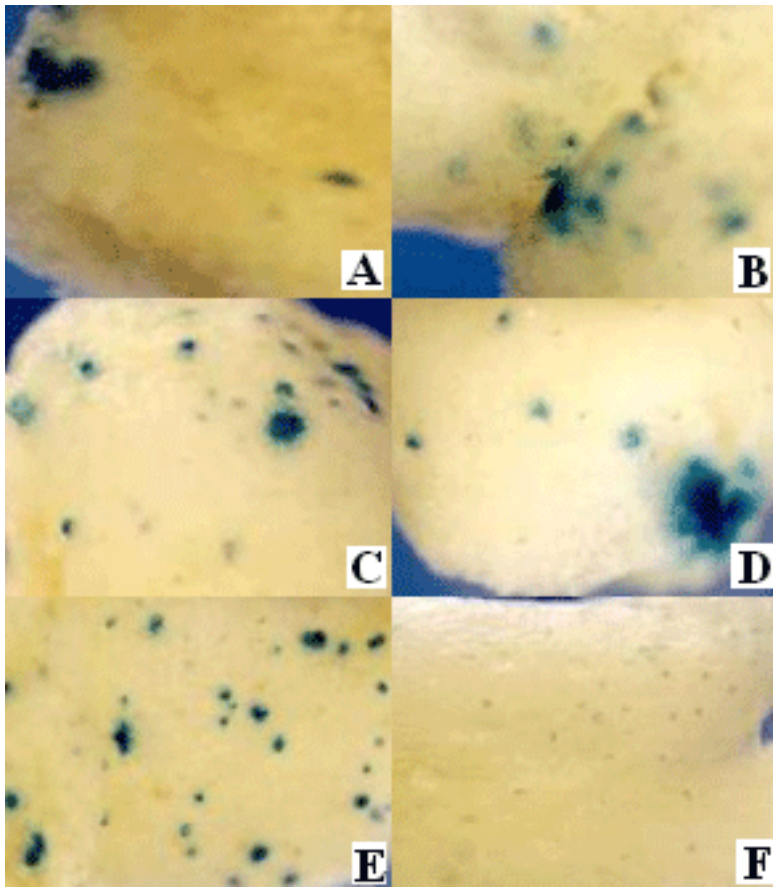


Figure10 Comparison of affecting parameters for bombardment in orchid protocorm: (A) target distance of 60mm, (B) target distance of 90mm, (C) gold particle, (D) tungsten particle, (E) Biolistic PDH1000/He, and (F) modified particle inflow gun.

2.2. Promoters for the Expression of Gene in Orchid Flower

Each of the plasmids consisting of a constitutive promoter, Act-1, Ubi-1, and CaMV35S, was delivered into fresh sepal of *D.* Jaquelyn Thomas to evaluate for floral expression. Transient GUS expression in bombarded sepal tissues was determined by histochemical assay in 1d after bombardment. The results displayed the difference of the ability of each promoter for driving *gus* transgene expression. Although, the Act-1 and CaMV35S promoter were active in the *Dendrobium* sepal, *gus* gene expression under the control of Act-1 promoter in the examined sepal was more activity than under the control of CaMV35S. The high level of expression with Act-1 promoter appeared throughout the sepal base (Figure11A). Likely, the CaMV35S promoter worked particularly well within the vascular tissue (Figure11B, C). Interestingly, no any blue spots were observed in orchid sepal under the control of Ubi-1 promoter, indicating that the maize Ubi-1 promoter might not be active in the sepal and the vascular cells of orchid flower (Figure11D).

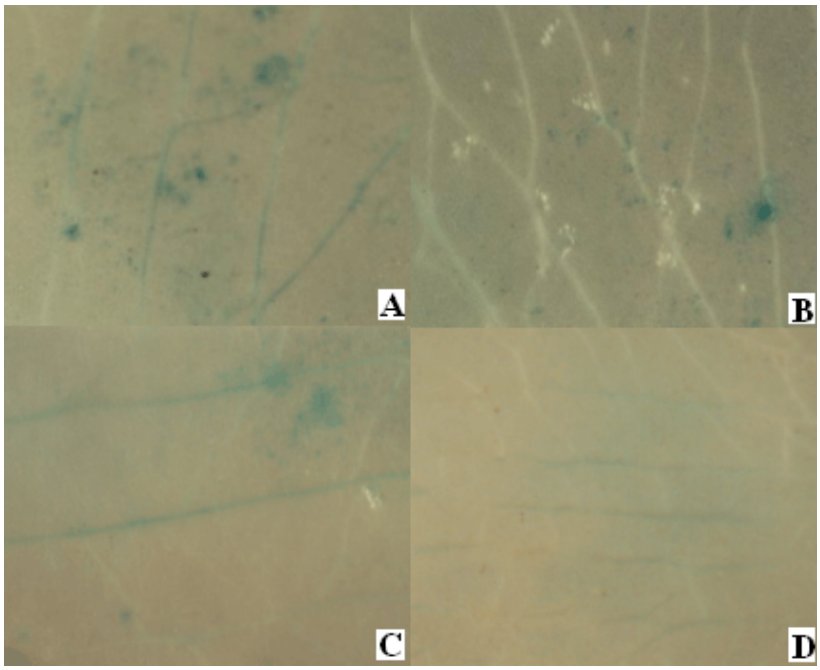


Figure11 Transient GUS activity in sepal of *Dendrobium* orchid with different promoters: (A) Act-1 promoter, (B, C) CaMV35S promoter, and (D) Ubi-1promoter.

In order to determine an effective promoter to specifically control gene expression, the sepals of *Dendrobium* orchid were used to evaluate the efficiency of three kinds of a constitutive promoter. Rice Act-1, maize Ubi-1 and CaMV35S promoters were constructed in pAct1-D, pAHC27 and p2K7 plant vectors, respectively, for *gus* gene transient expression assay in those orchid tissues through microprojectile bombardment. Although, previous reports revealed the high potential of the CaMV35S promoter for control expression of *gus* gene in callus of *Phalaenopsis* (Anzai *et al*., 1996), *Dendrobium* (Tee *et al*., 2003), and protocorms of *Dendrobium* hybrid (Yu *et al*., 1999), in the sepals of *Dendrobium* orchid, rice Act-1, but not Ubi-1 promoter showed stronger level of Gus expression than CaMV35S promoter. As actin gene is a component of the plant cell cytoskeleton, it is activate in all cell types (Seagull, 1989; Zhang *et al*., 1991). In transgenic maize, the Act-1 promoter gave the high level expression in shoot and floral meristematic tissues (Zhong *et al*., 1996) and in the most cell types of rice (Zhang *et al*., 1991); whereas, CaMV35S promoter is known as an efficient promoter in dicotyledon. All cells of transgenic rice were not detected (Tereda and Shimamoto, 1990; Cornejo, 1993). Wilkinson *et al*. (1997) demonstrated that CaMV35S promoter could active in leaves but not in pollen of all *Arabidopsis* lines. In anther and pollen tissues, CaMV25S expression was strong in transgenic tobacco but limited in transgenic petunia. Moreover, CaMV35S promoter was expressible in most cells and tissue types of transgenic cotton (Sunilkumar *et al*., 2002) and strawberry (Toki *et al*., 1992), implying that the level of CaMV35S expression might depend on the tissue types.

The promoter can be active due to a trans-acting factor presenting in the cell recombines with a *cis*-element contained in promoter (Benfey and Chua 1989). As result showed no any Gus expression under the control of Ubi-1 promoter in the flower, recognizing between *cis*-elements of Ubi-1 promoter and trans-acting factor presenting in the flower might be limited. Ubi-1 promoter was highly potential to express in active dividing and growing cell (Carnejo *et al*., 1993; Stephen, 2004). A potato ubiquitin, Ubi-3, showed the highest expression in meristematic tissue and declined during leaf expansion in transgenic potato and rose (Gurbarino and Belknap, 1994). In transgenic rice, Ubi-1 promoter showed highest expression in the root tips

and lowest in the mature root. Ubi-1 promoter may play a key role in cell division of eukaryote (Takimoto 1994; Plesse, 2001**)**, suggesting that Ubi-1 is low ability in mature orchid flower containing differentiated cells.

3. Maize Regulatory Genes Transformation

To engineer flower color-modified orchid, two maize regulatory genes, *B-peru* gene encoding helix-loop-helix proteins of the *Myc* transcription factors and *C1* genes encoding R2R3 proteins of the *Myb* transcription factor from maize which were broadly used to modify the expression of the genes encoding the biosynthetic enzymes in transgenic plants were inserted into a cassette containing Act-1 promoter. As the rice Act-1 promoter is a potential promoter to drive the *B-peru* and *C1* expression for anthocyanin pigmentation in *Dendrobium* orchid flower, the sense strand fragments of 1.79-kb of *B-peru* gene and 1.16-kb of *C1* gene obtained from digesting the p35SBPcDNA and p35SC1 with *Pst* I/ *Sal* I and *Bam* HI/ *Kpn* I, respectively, were constructed into pTG0063 plant expression vector. After construction, the pBpSA13 plasmid (Figure5) and pC1SA12 plasmid (Figure6) consisting of *B-peru* and *C1*, respectively, under the control of Act-1 promoter and Nos terminator were obtained. The reconstruction plasmids were then utilized for floral orchid improvement via bombardment transformation.

3.1. Transient Expression in Fresh Orchid Flower

To test whether the possibleexpression of either the *B-peru* gene, *C1* gene, or recombination between *B-peru* and *C1* induce the expression of structural genes to accumulate anthocyanin pigments in orchid flower, the intact sepal of *D.* Jaquelyn Thomas was bombarded with the gold particles coated with pBpSA13 (*B-peru*), pC1SA12 (*C1*), and pBpSA13 (*B-peru*) and pC1SA12 (*C1*) plasmids. After bombardment for one week, the white bombarded sepal tissues were observed for the anthocyanin accumulation spot under a stereomicroscope. Figure12B showed that the anthocyanins were accumulated in the tissue co-bombarded with both pBpSA13 (*B-peru*) and pC1SA12 (*C1*). No any anthocyanin pigments spots were observed in the

sepal tissue bombarded with either pBpSA13 (*B-peru*) or pC1SA12 (*C1*) alone (Data not shown). The results demonstrated the combination of B-peru and C1 transcription factor proteins were able to drive the anthocyanin structural gene transcriptions in orchid flowers. It revealed that ectopic expression of *B-peru* in combination with the C1 MYB protein was able to induce the transcription for pigmentation.

Figure12 The anthocyanin pigment spots in the sepal cells: (A) negative-control, flower bombared with blank particles and (B) the flower co-bombarded with pBpSA13 and pC1SA12.

Such an interaction is proposed to promote a conformational change in either C1 or B-peru protein leading to enhance DNA recognition and binding ability (Goff *et al*., 1990). R/B proteins act in concert with C1 protein, which contains a myb domain to activate transcription of at least four structural genes in the anthocyanin pathway (Paz-Ares *et al*., 1987; Goff *et al*., 1990; Ludwig and Wessler, 1998; Roth *et al*., 1991; Bodeau and Walbot, 1992). These data conferred a useful piece of information on biosynthesis of flower pigmentation in orchid, and facilitate our understanding of modification of flower color by genetic engineering.

3.2. Selection and GFP Expression in Transgenic Orchids

As the maize transcription factor genes possibly induce the anthocyanin accumulation in orchid flower, three plasmids, pBpSA13 (*B-peru*), pC1SA12 (*C1*), and pMNK1005 (*hph* and *gfp*), coated on gold microparticles were delivered into the secondary activating protocorms of *D.* Jaquelyn Thomas. After bombardment, the treated tissues were maintained in the dark growth chamber for 3 d before culturing in

solid MS medium supplemented with different levels of hygromycin reagent for selection. The various stages of GFP expression of bombarded protocorms were observed during the time period of hygromycin selection. Our previous study showed that solid MSHyg30 media was able to completely inhibit the growth of all *Dendrobium* protocorms within two months; consequently, it was used for the selection of transgenic orchids in this experiment.

For 45d of the first selection, the bombarded protocorms were weakly selected from solid MSHyg5 media (Figure13E). The protocorms after bombardment showed small green fluorescent spots of transformed cells and red-orange backgrounds of untransformed cells (Figure13A). Within 30d of the first selection, 15.33% of all bombarded protocorms turned brown and died before the remaining green tissue further proliferated rapidly (Figure13F). The spots of green fluorescent were bigger, whereas almost all of the tissues would consist of unbombarded cells (Figgure13B). It was implied that the medium containing 5 mg/l hygromycin could maintain and allow proliferation of the transformed tissues. All surviving protocorms were subsequently transferred into solid MSHyg25 medium for 45d. Although approximately half of the protocorms deteriorated and died (Figure13G), the green fluorescent spots were expanded and became larger (Figure13C). At this selection stage, the percentage of living protocorms was 50.96%. Yang *et al.* (1999) suggested that to obtain transformed cells, DNA has to be integrated into the plant genome at an early stage which immediately stimulates cell division during antibiotic selection. Hence, stepwise selection in hygromycin at 5 and 25mg/l was beneficial in eliminating the untransformed cells and simultaneously promoting cell division in transformed tissues.

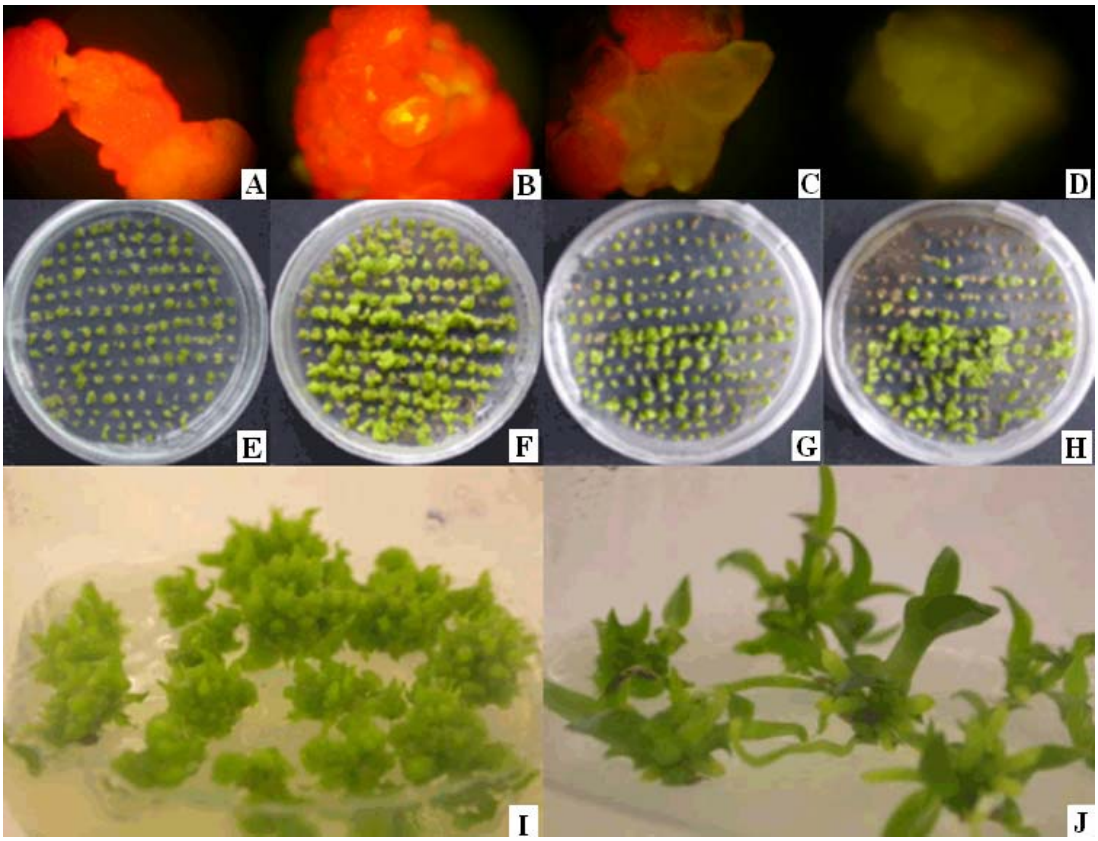


Figure13 Characters of GFP-expressing protocorm during hygromycin selection: (A, E) 3 days, (B, F) 45 days, (C, G) 90 days, (D, H) 120 days, (I) hygromycin-resistance plbs, and (J) putative transgenic plantlets.

Surviving tissues in 25mg/l hygromycin were transferred into solid MSHyg30 medium for 30d. Figure13H showed that transformed tissues were still green and subsequently proliferated, while untransformed tissues were completely dead after the selection. Hygromycin-resistant protocorms resulting from the selection strategy contained *gfp*-transformed cells that could express GFP and did not obtain non GFP-expressing cells (Figure13D). The transformation efficiency of 19.87% was rather high when compared with the former reports (Nan and Kuehnle, 1995, Anzai *et al.*, 1996, Yang *et al.*, 1999, Yu *et al.*, 1999, Knapp, 2000, Men *et al.,* 2003, You *et al.*, 2003). After the selection process, the hygromycin-resistant tissues which initially formed shoots were transferred into solid MS medium without antibiotic (Figure13I) to produce putative transgenic plantlets (Figure13J). However, the level of hygromycin as a selective agent and timing for selection in *Dendrobium*

orchids also greatly depend on species. Yu *et al.* (1999) reported that hygromycin at 50 mg/l for 60d completely suppressed the growth of untransformed *D.* hybrid 'MiHua' protocorms and transgenic *D.* Phalaenopsis and *D.* Nobile plantlets were obtained by selecting from 30mg/l hygromycin for 105d (Men *et al.,* 2003), while in our experiment, the concentration of hygromycin needed to eliminate untransformed cells from transformed cells of *D.* Jaquelyn Thomas protocorm was 30mg/l for 60d.

The concentration and timing for the selection process are also important factors that determine the efficiency of transformation (Tee *et al.*, 2003). Normally, orchid protocorms for transformation are slowly proliferating and might be chimera because of de novo embryogenesis, which directly regenerate into plantlets without passing through the callus, especially in *Dendrobium* (Kuehnle and Sugii, 1992). Besides, Yang *et al.* (1999) demonstrated that selection in solid medium with a high concentration of selective agent immediately after bombardment could not obtain transgenic orchids, while delayed selection would be of very low efficiency (Yu *et al.*, 1999; Men *et al.*, 2003) and would often obtain chimeras (Christou and Ford, 1995). Hence, in this experiment, selection was divided into three steps.

Using too high a level of antibiotic to discriminate untransformed orchid tissues might result in chimeras due to escape (Chia *et al.*, 1994), or death of transformed tissue. The problem of chimeras has also been reported in transgenic soybean (Christou *et al.*, 1989) and papaya (Fitch *et al.*, 1990). Moreover, long-term selection with low level of antibiotic would inhibit the regeneration of transgenic orchid (Kuehnle and Sugii, 1992) and not completely suppress the growth of untransformed tissues (Griesbash, 1994, Nan and Kuehnle, 1995). The inhibitory activity of antibiotics on regeneration of transgenic plantlets has also been reported in *Nicotiana* (Kiernan *et al.*, 1989) and *Oryza* (Dekeyser *et al.*, 1989). To solve these problems, a stepwise selection was adapted using solid MS medium containing hygromycin at 5mg/l and 25mg/l each for 45d prior selection in 30mg/l for 30d to avoid the occurrence of chimeras or the inhibition of plantlet formation. The results demonstrated the accomplishment of this selection strategy to recover non-chimera orchid transformants with high efficiency.

3.3. PCR and Southern Blot Analysis

3.3.1. *Hph* and *gfp* Genes

PCR analysis showed the presence of exogenous *hph* (Figure14A) and *gfp* (Figure14C) in the positive control (lane 2) and all eigh33t putatively transgenic orchids, 6, 12, 34, 35, 36, 37, 39, 40, (lane 4-11) with two different sets of *hph* and *gfp* primers. There was no amplification of DNA from negative control (lane3). Southern blot analysis of the PCR products presenting *hph* (Figure14B) and *gfp* (Figure14D) showed 0.5-kb and 0.7-kp bands which hybridized with *hph* and *gfp* fragment probes, respectively. All eight independent putative transgenic lines contained both 0.5-kb *hph* and 0.7-kb *gfp* fragments (lanes 4-11). Neither the *hph* nor the *gfp* hybridization signal was found in the untransformed control line (lane 3).

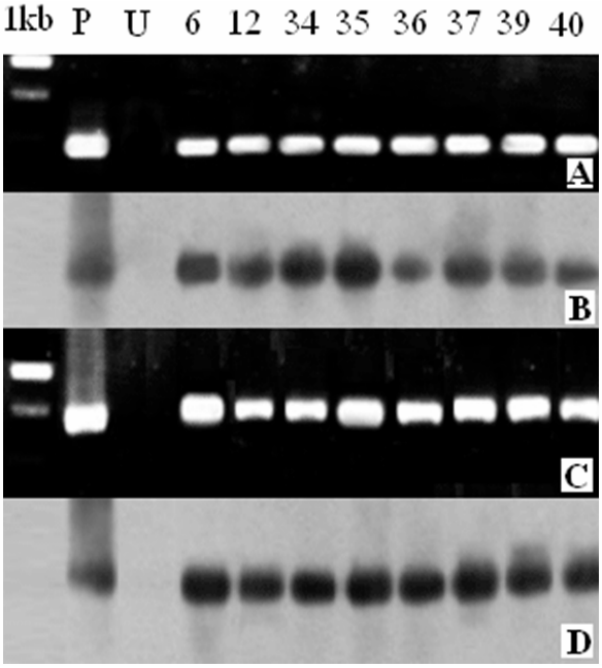


Figure14 PCR and PCR southern blot hybridization of *hph* and *gfp* genes: (A) the 0.5-kb of *hph* fragment of PCR analysis, (B) of PCR Southern blot hybridization, (C) the 0.7-kb of *gfp* fragment of PCR analysis, and (D) of PCR Southern blot hybridization. 1kb-marker (lane1), positive (P) control (lane 2), unbombarded (U) line (lane 3) and independent putative transgenic lines (lane 4-11).

To confirm *gfp* integration in the genome of the putative transgenic plantlets, 10µg of genomic DNA from leaves of unbombarded orchid and eight transgenic lines were digested with *Sac*I and hybridized with a 0.7-kb of *gfp* fragment probe. As this plasmid carries a unique *Sac*I site, digestion of the genomic DNA of putative transformants with *Sac*I would generate a different fragment for each integrated copy. Southern blot hybridization, therefore, provided copy numbers of the *gfp* in the genome of the putative transgenic plantlets. Unique *gfp* specific banding patterns, indicating independent transformation events, occurred in all putatively transgenic lines (Figure15). The hybridization revealed one of the transgenic lines, 39, carried one copy (lane 9), while 36 (lane 7) lines and 37 (lane 8) carried two copies, 12 (lane 4), three copies, 6 (lane 3) and 35 (lane 6), four copies, and 34 (lane 5) and 40 (lane 10), five copies, indicating multiple integrations in the genome of the transgenic orchids. A previous result for bombardment transformation in *Dendrobium* orchid also showed multiple copies of gene (Yu *et al.*, 2001). A 0.7-kb band of pMNK1005 plasmid digested with *Nco*I was detected (lane1), whereas no signal of genomic DNA from leaf of unbombarded orchid was obtained (lane2). The results demonstrated that this strategy could deliver transgene into the genome producing stable transgenic lines.

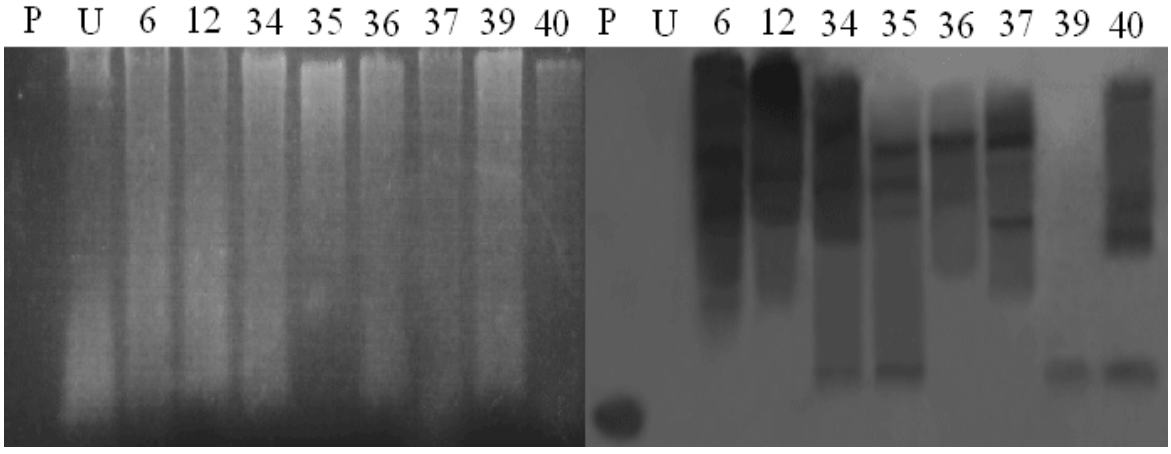


Figure15 Genomic southern blot hybridization of *gfp* gene: 0.1µg of plasmid DNA (P) 10µg of genomic DNA digested with *Sac*I from leaves of untransformed plant (U) and eight transgenic plantlets (lane 3-10) hybridized with *gfp* fragment probe.

3.3.2. *B-peru* and *C1* genes

After transformation and regeneration, the integration of the transgenes in the chromosome of the population of new genotype exhibiting resistance to hygromycin and expression of GFP were detected by PCR and southern blot analysis. The positive control (P), genomic DNA extracted from leave of unbombarded line (U), and all fourteen putatively transgenic lines resisting to hygromycin and expressing GFP (lines1-14), were amplified to present exogenous *B-peru*, and *C1* genes with two pairs of specific gene primers to both transgenes and Nos terminator as shown in Figure16. The PCR analysis indicated that all of the number of these primary transgenic events, hygromycin-resistance plantlet expressing GFP had no *B-peru* and *C1* genes (Figure17). Only 3/14 of putative transgenic lines expressing GFP and HPH contained the 1.7-kb of *B-peru* gene and 1/9 of putative transgenic lines contained both of the 1.7-kb of *B-peru* and 1.1-kb of *C1* genes. No amplification of DNA from negative control was observed (U).

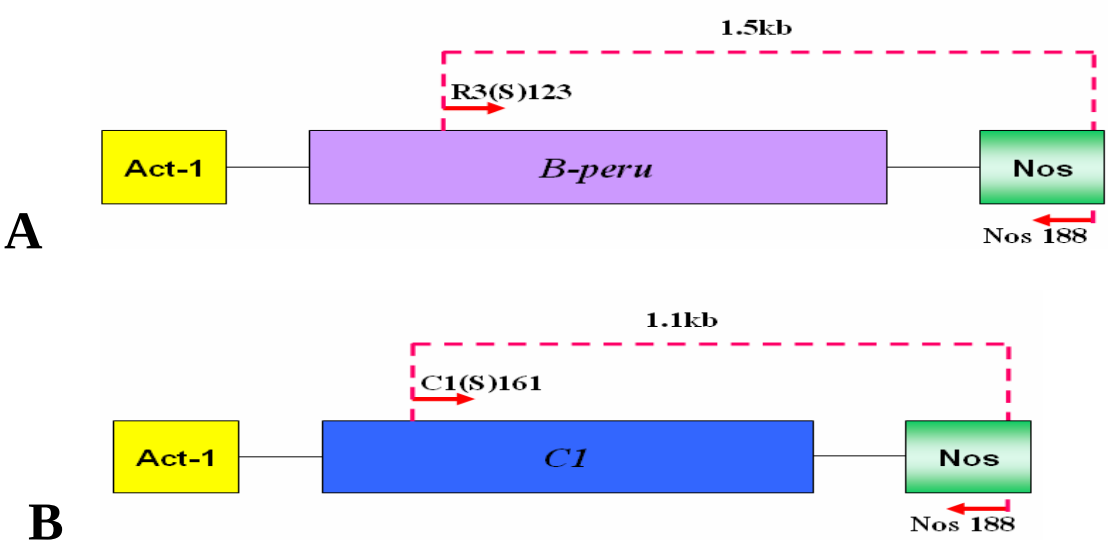


Figure16 The position of the specific primer for transcription factor gene and Nos terminator: (A) pBpSa13 and (B) pC1SA12.

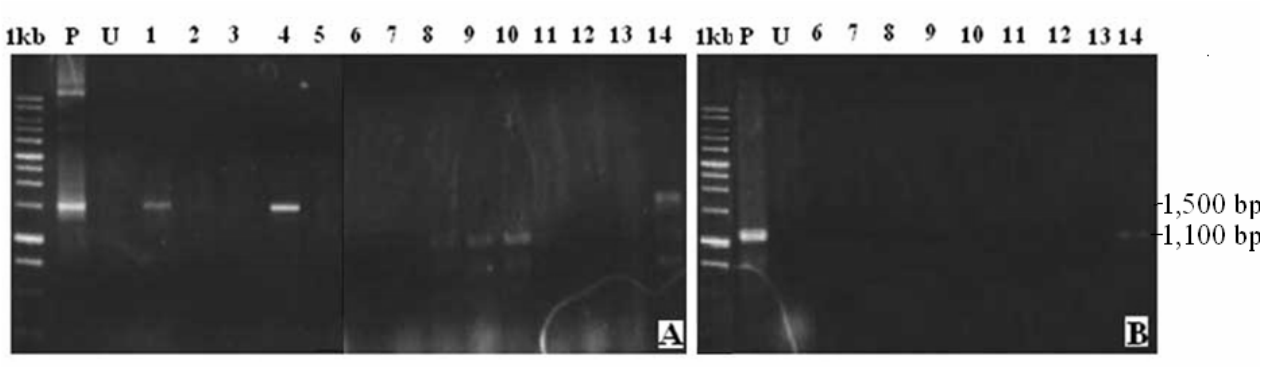


Figure17 PCR analysis of hygromycin-resistance plantlet expressing GFP using two pairs of specific primers to the transcription factor gene and Nos terminator: (A) the 1.5-kb of internal fragment of *B-peru*/Nos primers and (B) the 1.1-kb of internal fragment of *C1*/Nos primers. 1-kb marker (lane1), positive control (P), unbombarded line (U) and independent putative transgenic lines (1-14).

Southern Blot Hybridization was used to confirm the *B-peru* and *C1* transgenes integration in the chromosome of the putative transgenic orchids. 10μg of genomic DNA extracted from leaves of all positive detection of transgenic lines and the unbombarded line was digested with *Eco*RI and hybridized with the 1.2-kb of *B-peru* and 1.1-kb of *C1* fragment probes. Since pBpSA13 and pC1SA12 plasmids containing two *Eco*RI sites at the end of the transgenes, digestion of the genomic DNA of putative transformants with *Eco*RI would generate a fragment. The hybridization, therefore, revealed one band of 1.7-kb of *B-peru* probe (Figure18A) and 1.1-kb of *C1* probe (Figure18B). The results showed that, although the putative transgenic lines showed positive-PCR analysis, all of the independent lines were not contained 1.7-kb of *B-peru* gene or 1.1-kb of *C1* gene fragments. The results revealed that all of 14 independent lines, 14.1-14.6, contained both of the 1.7-kb of *B-peru* gene and 1.1-kb of *C1* gene. The 1.7-kb of *B-peru* gene, but no 1.1-kb of *C1* gene was found in the 1.1 and 1.2 independent lines and no any signals were observed in 4.1 and 4.2 lines. Neither the *B-peru* nor the *C1* hybridization signal was found in the untransformed control line (U).

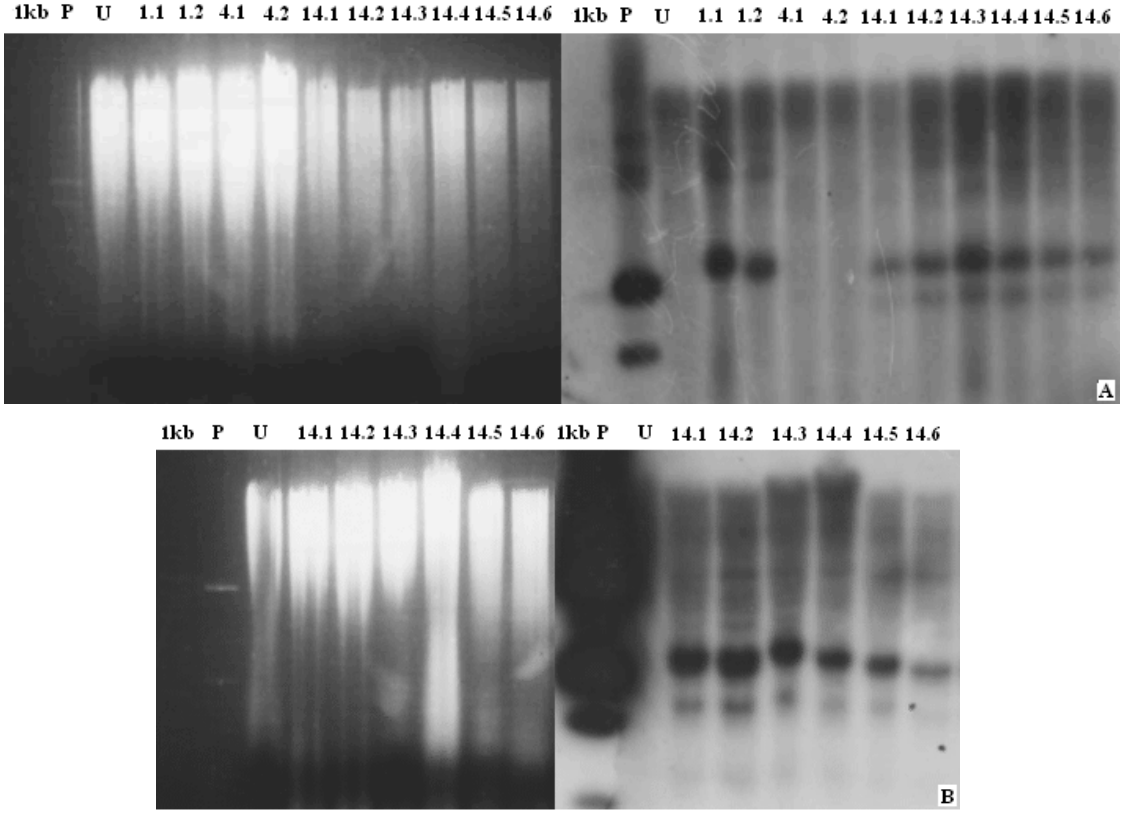


Figure18 Genomic southern blot hybridization of putative transgenic independent lines: 0.1μg of plasmid DNA (P), 10μg of genomic DNA digested with *Eco*RI from leaves of untransformed plant (U) and transgenic plantlets(1, 4, 14) hybridized with *B-peru* fragment probe (A) and with *C1* fragment probe (B).

3.4. Anthocyanin Accumulation in Putative Transgenic Orchid

Although two transcription factor genes were clearly detected on the southern blot hybridization, only a few clones of the transgenic population bombarded with *B-peru* and *C1* genes displayed phenotypic changing. Most of *B-peru* or *C1* transgenic plantlets remained green and did not show phenotypic color changing (Figure19B). One of the putative transgenic lines with both *B-peru* and *C1* transcription factors showed accumulation of purple pigment which was visible on the surface of epidermal layer of leaf sheaths (Figure19D-F). In contrast to non-transformed parents showed poorly expression of the MYB and MYC transcription factors remained green (Figure19A). However, the red-purple invisible coloration

became within 14d. Moreover, the abnormal plantlets were found in the transgenic population during regeneration, showing many shoots, dark-green leaves, and short internode which were unable to separate each plantlet (Figure19C). From the results demonstrated that *B-peru* and *C1* transcription factor genes were potential to accumulate the composition of anthocyanin by inducing the structural gene transcription.

Interestingly, in spite of bombardment with maize *b-peru* and *C1* transcription factor, the difference expression pattern was occurred. Quattrocchio *et al*. (1999) revealed that two ways could be explained the differences of expression pattern is that the regulators of flower pigmentation in Antirrhinum and Petunia spp. are not related to C1 and R from maize or C1- and R-related genes do control flower pigmentation but regulatory differences are due to divergent evolution of the target structural genes.

Figure19 Phenotype resulting from the transcription factor gene expression in putative transgenic orchid: (A) unbombarded plantlet, (B) normal growth of the putative transgenic plantlet, (C) abnormal growth of the putative transgenic plantlet, and (D-F) the transgenic orchid showing the anthocyanin accumulation in epidermal layer of leaf sheath.

3.5. The maize *B-peru and C1* Gene Expression Analysis in Transgenic Orchids

3.5.1. Northern Blot Analysis

The transgene expressions of *B-peru and C1* transgene were determined in the putatively transgenic orchid plantlet tissues by Northern Blot hybridization. 10μg of RNA samples extracted from the leaves of independently transgenic lines and unbombarded lines were used to analyze the transcription factor *B-peru* gene (MYC family) expression event. Figure20 showed that the untransformed tissues (negative control), the *B-peru/C1* putative transgenic line (line1), the *B-peru* putative transgenic line (line3), and abnormal development transgenic plantlet (line4) which had no anthocyanin accumulation, had poorly expression of the *B-peru* transgene. In contrast, the *B-peru/C1* transgenic plantlet (line2) which displayed a red-purple phenotype had strong transcripts of 1.7kb of *B-peru* transgene, demonstrating that the *B-peru* gene had a major effect on the level of steady-state transcripts of the gene encoding the anthocyanin biosynthesis enzymes in transgenic orchid. Namely, the MYC family is a gene required to induce the anthocyanin accumulation in the transgenic *Dendrobium* plantlet, while the abnormal phenotype might be caused by the *Myc* gene integration.

So far, *B-peru* transcription factor belonging to *Myc* gene family involved flavonoid biosynthesis and induced anthocyanin accumulation in transgenic plants. For example, the expression of *B-peru* gene conferred accumulating anthocyanin pigments in the maize cells (Goff *et al.*, 1990). All tissue tested receiving *B-peru* gene exhibited numerous single cells containing purple pigment. De Majnik *et al.* (1998) demonstrated that *B-peru* gene expression was the cause of the anthocyanin pigmentation in white clover, *Trifolium repens*.

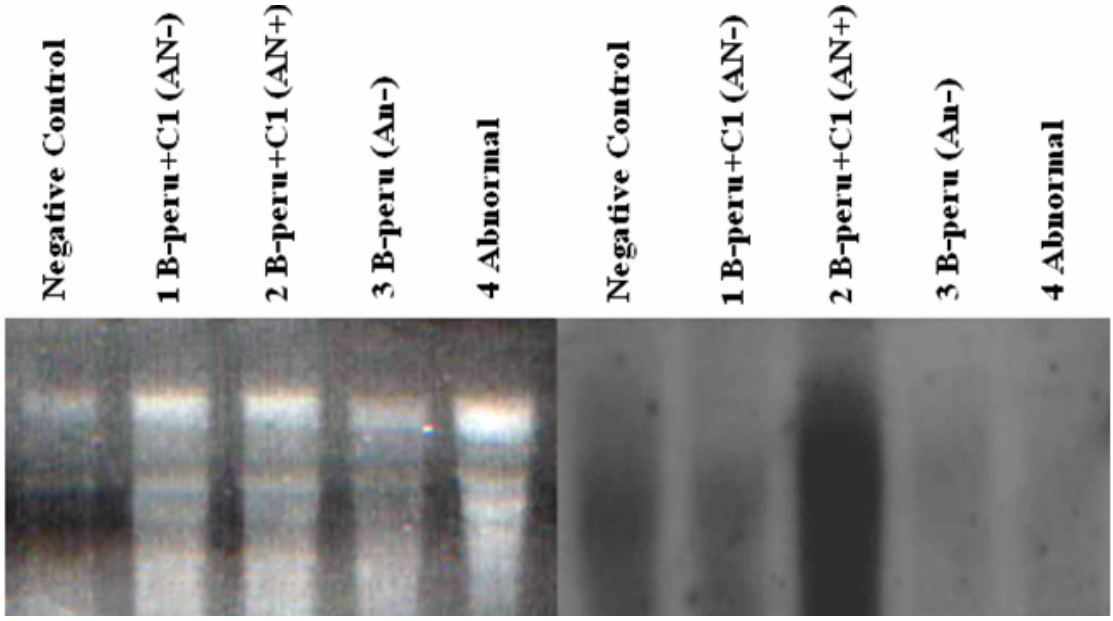


Figure20 Northern blot analysis of *Myc* transcription factor, maize *B-peru*, gene involved in anthocyanin biosynthesis: 10µg of total RNA of unbombarded control (U) and putative transgenic orchids (lines1-4) were blotted and hybridized with maize *B-peru* probe.

3.5.2 RT-PCR Analysis

The level of maize *B-peru* and *C1* transgene expression were compared with the level of endogenous anthocyanin structural gene expression in all independent transgenic lines. The function of those two candidate regulatory genes, *B-peru* (*Myc* family) and *C1* (*Myb* family), to control the anthocyanin structural gene transcriptions in the transgenic orchids were examined by RT-PCR. Total RNA extracted from the leaves of unbombarded line, independent transgenic lines (line1-3) and an abnormal growth transgenic line were used to study their expressions.

3.5.2.1. *B-peru* (*Myc* family) Gene Expression

As shown in Figure21B, abundant levels of *B-peru* transcripts were found in the transgenic line bombarded with *B-peru* gene alone (line3) and in the abnormal growth transgenic line. The *B-peru* expression in *B-*

peru/C1 transgenic normal line (line1) and the *B-peru/C1* line shortly accumulating anthocyanin (line2) were poor. Without doubt, only in independent transgenic line2, the high level expression of *B-peru* gene was observed when it showed anthocyanin accumulation at stems and leaves (Figure19D-E). When the RT-PCR was performed while this line had no anthocyanin accumulation, consequently its expression rapidly decreased. On the contrary, a high level of *B-peru* expression was observed in the abnormal developmental line and in the line bombarded with *B-peru* gene alone. Both lines did not have any anthocyanin pigments.

3.5.2.2. *C1* (*Myb* family) Gene Expression

With regard to C1 expression, transcription was observed in all lines tested. Figure 21C showed that in the unbombarded line and the transgenic orchid which was not bombarded C1 transgene expression was also detectable. It was implied that Myb transcription factor gene normally expresses in leaves of Dendrobium orchid. The *C1* gene belonging to MYB transcription factor possibly act as an enhancer to recombine with the MYB transcription factor to induce the expression of structural genes in the pathway.

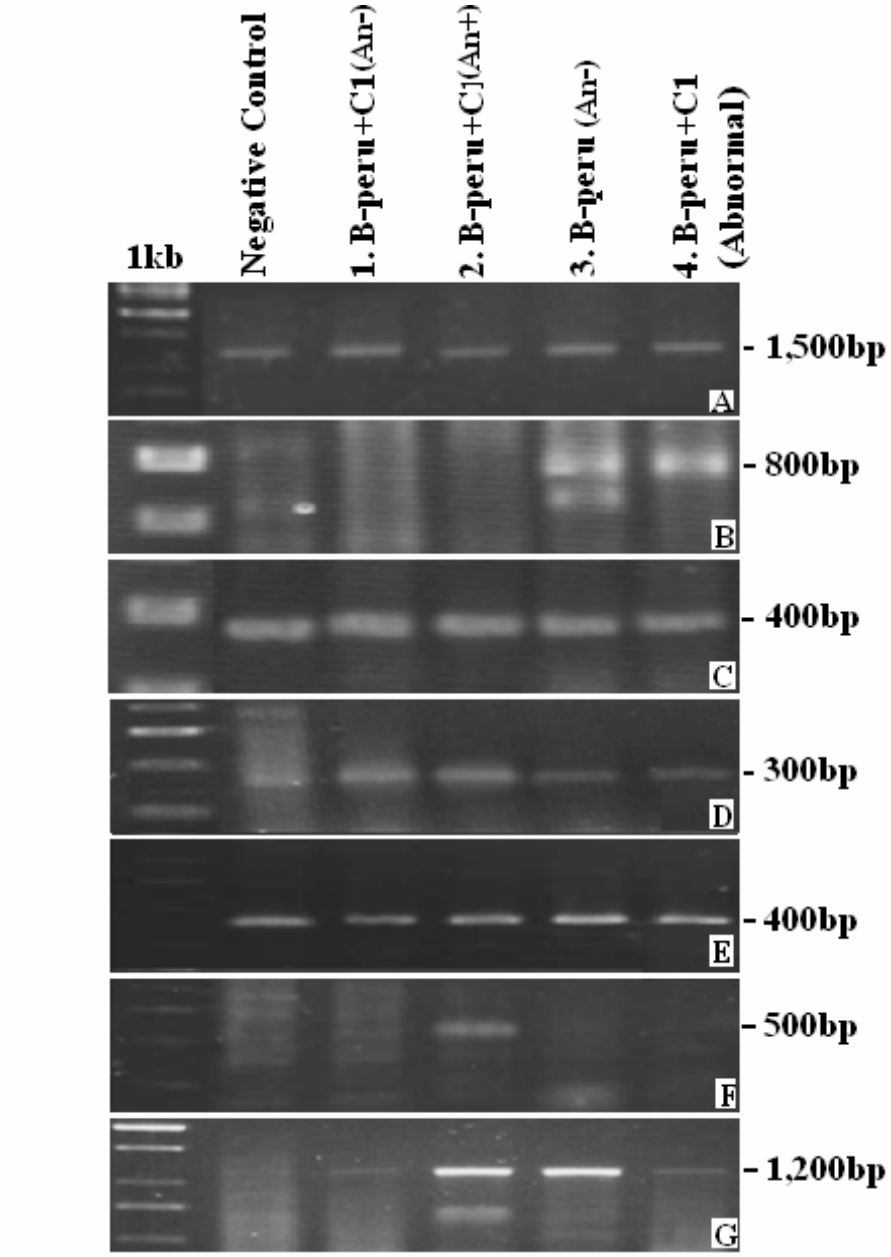


Figure21 Applification by RT-PCR of cDNA with specific primers to detect gene expression of four endogenous anthocyanin genes and two maize transcription factor trans-genes in putative transgenic orchids (line1-4). 5μg of total RNA isolated from leaf of samples of untranformed and transgenic orchids were amplified. The arrow was indicated the PCR product: (A) 1.5-kb of *actin* gene served as a positive control, (B) 0.8-kb of *B-peru* transcription factor gene, (C) 0.4-kb of *C1* transcription factor gene, and four structural genes; (D) 0.3-kb of *chs* gene, (E) 0.4-kb of *chi* gene, (F) 0.5kb of *f3h* gene, and (G) 1.2-kb of *f3′,5′h* gene.

3.5.2.3. Structural Gene Expression

To assay the alteration of the level of flavonoid gene transcription in transgenic orchid, RT-PCR analysis was used to elucidate four structural gene expressions. The four key structural genes in this study, including *chalcone synthase* (*chs*), *chalcone isomerase* (*chi*), *flavonoid 3 hydroxylase* (*f3h)* and *flavonoid 3′,5′ hydroxylase* (*f3′,5′h)*. The results showed that abundance of *chs* and *chi* transcripts was detected in all of *Dendrobium* orchid tested both of unbombarded and bombarded plantlets (line1-4). The *chs* and *chi* transcripts were not increased in putative transgenic orchid compared to unbombarded orchid (Figure21D, E). As shown in the Figure20, the levels of *chs* and *chi* transcripts were not related to the level of maize *B-peru* transgene expression. Therefore the gene expression level of *chs* and *chi* gene might be normally expressed in *Dendrobium* orchid. *B-peru* transcription factor gene might not up-regulate the transcription of *chs* and *chi* gene for anthocyanin accumulation in tissues of the transgenic populations.

On the contrary, Figure21F demonstrated that the *f3h* gene expression was higher in the transgenic line accumulating red-purple anthocyanin pigments (line2) compared to the lines which had no visible anthocynain accumulation (line1, 3, and 4). Similarly, no *f3′,5′h* transcription was found in unbombarded lines but detectable in transgenic lines which had no anthocyanin accumulation (Figure21G), while abundance of *f3′,5′h* transcripts was obtained in the transgenic line showing red-purple pigments phenotype in the epidermal of leaf sheath (line2).

The results demonstrated that the transgenic line containing purple on the leaves clearly accumulated more *f3h* and *f 3′, 5′h* transcripts compared with leaves of the lines without accumulated anthocynanin pigments. Clearly, the red-purple pigment will be accumulated when the *f3h* was active and this event could be induced by *Myc* (*B-peru*) together with *Myb* (*C1*) regulator. Martin *et al.* (1996) revealed that the *R* and *C1* genes in *Z. mays* are required for expression of all anthocyanin genes, while the regulators of *P. hybrida* and *A. majus* control only a

subset of structural genes. In rose and chrysanthemum, the purple delphinidin derivatives do not synthesize because of weak expression of *f3′,5′h* (Elomaa and Holton, 1994). In the flower tube of snapdragon, the anthocyanin biosynthesis genes were also divided into two groups. One is *chs* and *chi* in the early stage prior to pigmentation and the others are *f3h*, *dfr*, *ans* and *ufgt* involving in coloration (Martin, 1991). As the first two enzymes of the pathway, CHS and CHI, are precursors for flavone as well as anthocyanin biosynthesis, abundance of their expression was found, while the switching of gene expression from F3H to F3′,5′H is considered critical for sepal pigmentation by changing the biosynthesis pathway from flavone to anthocyanin. It is implied that *f3h* and *f3,5′h* genes were significant for the moderation of anthocyanin pigmentation, producing the purple flower pigmentation in orchid. In addition, the results showed the abundance of *f3h* and *f3′,5′h* transcription was rather relative to the expression of MYB gene, revealing that the regulatory gene *B-peru* affects the steady-state of transcripts of *f3h* gene encoding enzyme to up-regulate the transcripts of *f3h* and *f3′, 5′h* encoding the enzymes in the anthocyanin pathway of orchid.

CONCLUSION

To date, anthocyanin pigmentation in the ornamental flower is a key feature not only for attracting pollinator, but also human due to a cut flower and pot plants are becoming increasingly popular in the international market. Understanding in the molecular mechanism of pigment formation in the orchid flower is important. In this experiment, RNA dot blot hybridization was used to determine the transcription level of the flavonoid biosynthesis gene in orchid flowers during development. The results demonstrated that MYC transcription factor might modulate the expression of at least *chi* and *f3h* transcripts in orchid flower, while *chs* transcripts might be regulated by the other groups. In order to accomplish transgenic orchid, three constitutive promoters affected to transgene expression in flower of *D.* Jaquelyn Thomas were used. The rice Act-1 and CaMV35S promoters, but not maize Ubi-1 promoter would be highly potential to considerably improve flower quality. Moreover, the optimal conditions for orchid transformation are to use of the small size protocorm bombarded with the helium gas pressure of 1,100Psi delivering the gold particles by Biolistic He1000/PDH.

Consequently, to improve flower of *Dendrobium* orchid via genetic engineering, maize *B-peru* cDNA encoding the *Myc* transcription factor and maize *C1* cDNA encoding the *Myb* transcription factor were constructed into TG0063 plant vector consisting of a rice Act-1 promoter and Nos-terminator and then co-bombarded with pMNK1005 plasmid containing *gfp* and *hph* genes into actively dividing protocorms of *D.* Jacquelyn Thomas. The highly efficient transformation was found (over 19%) with non-chimera tissue by sequential selection in hygromycin at 5mg/l and 25mg/l before a stringent selection regime at 30mg/l for complete eliminatation of unutransformed cells. In addition, the introduction of the *B-peru* and *C1* cDNA under the control of Act-1 promoter and Nos terminator into the protocorms showed the new phenotype. *B-peru/C1* transgenic stems and leaves had a distinct purple color compared with the green stems of the non-transformed plantlet. PCR and Southern Blot analysis were used to confirm transgene integration in chromosome of transgenic orchid.

Northern Blot hybridization and RT-PCR analysis were utilized to determine the expression of the gene involved in anthocyanin biosynthesis. Abundance of *B-peru* transcripts in transgenic line accumulated the red-purple pigments in stem and leave was found. RT-PCR analysis demonstrated that neither *chs* nor *chi* gene expression in transgenic orchid significantly alter anthocyanin accumulation. On the contrary, *f3h* and *F3′, 5′h* transcripts were rarely detectable in transgenic lines remained green but stronger in the genotype accumulated the red-purple pigments, demonstrating that F3H and F3′,5′H may be the major- limiting step to modulate anthocyanin pigmentation and the maize *B-peru* possibly up-regulates the *f3h* and *f3′,5′h* transcription in transgenic orchid.

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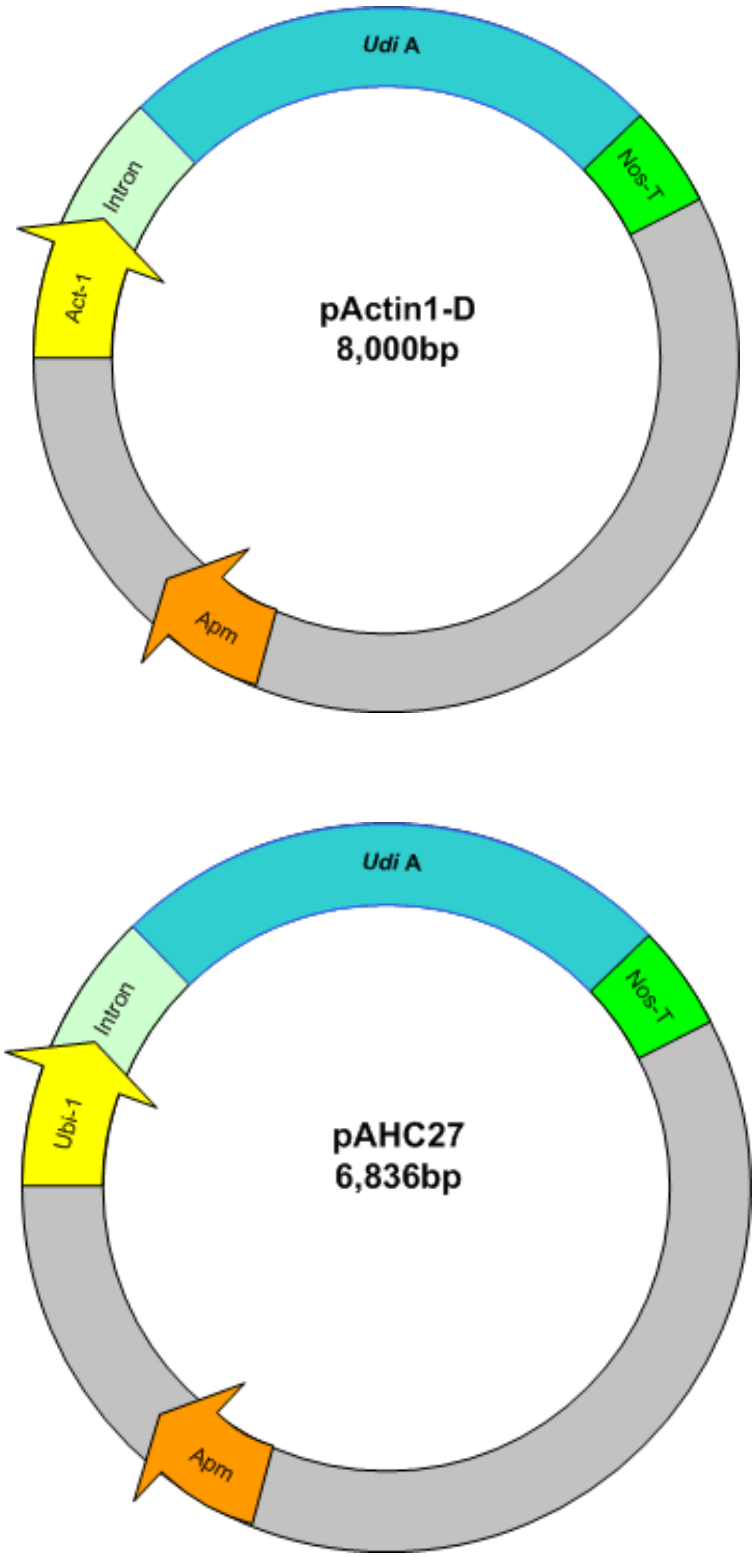
APPENDIX

Appendix Table1 The medium used for tissue culture and selection.

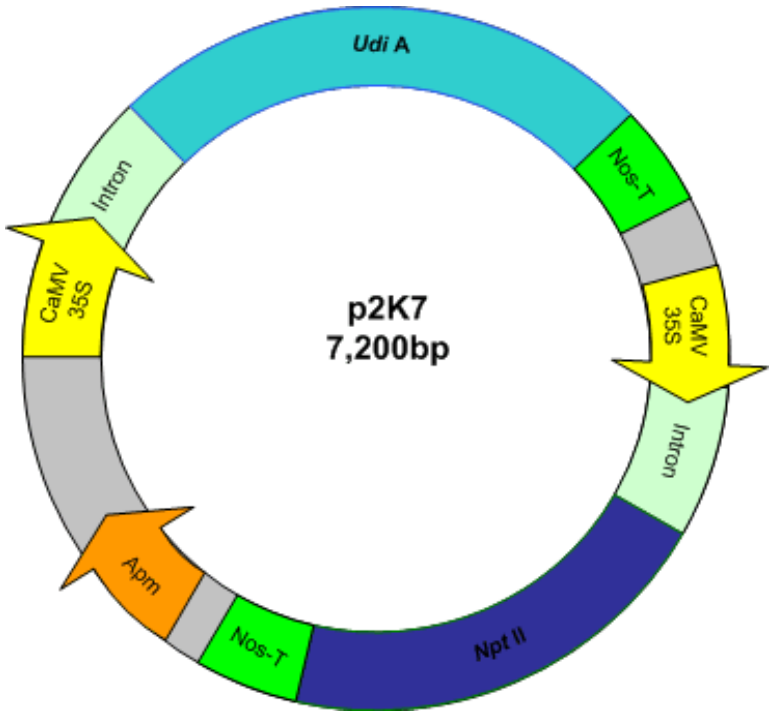
Medium	Components
VW	VW medium supplemented with 2% sucrose, 15% coconut water, 0.6% charcoal, and 0.8% agar
MS	MS medium supplemented with 3% sucrose and 0.8% agar
MS:VW (1:1)	MS and VW medium supplemented with 1.5% sucrose and 7.5% coconut water
MSM	MS medium supplemented with 3% sucrose, 0.8% manitol, and 0.7% agar
MSHyg5	MS medium supplemented with 5mg/l hygromycin, 3% sucrose, and 0.8% agar
MSHyg25	MS medium supplemented with 25mg/l hygromycin, 3% sucrose, and 0.8% agar
MSHyg30	MS medium supplemented with 30mg/l hygromycin, 3% sucrose, and 0.8% agar

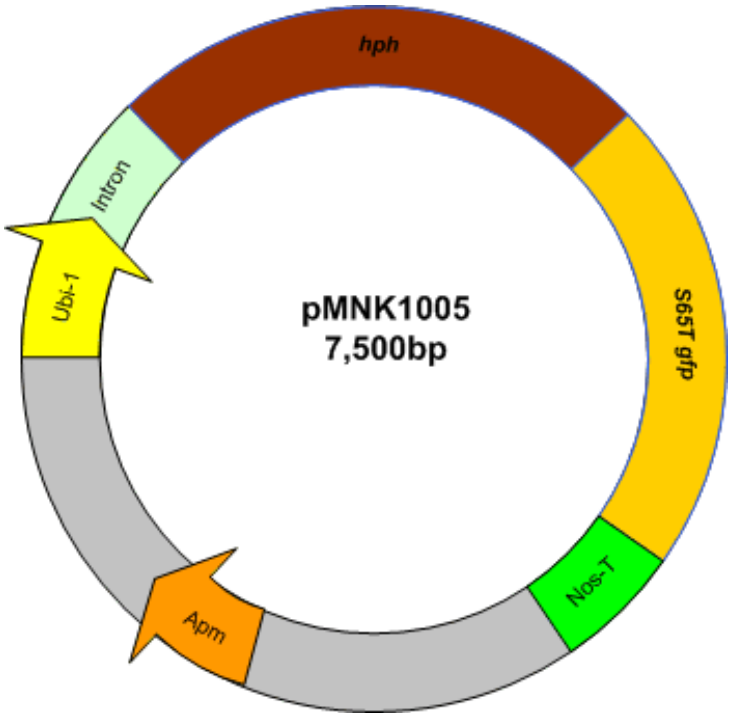
Appendix Table2 Chemical of MS medium (Murashige and Skoog, 1962) and VW medium (Vacin and Went, 1949).

Chemicals	MS (mg/l)	VW (mg/l)
Macroelements		
NH_4NO_3	1,650.00	-
KNO_3	1,900.00	525.00
$CaCl_2.2H_2O$	440.00	-
$MgSO_4.7H_2O$	370.00	250.00
KH_2PO_4	170.00	250.00
$Ca_3(PO_4)_2$	-	200.00
$(NH_4)_2SO_4$	-	500.00
Microelements		
Na_2EDTA	37.30	37.30
$FeSO_4.7H_2O$	27.80	27.80
H_3BO_3	6.20	-
$MnSO_4.4H_2O$	22.30	5.70
$ZnSO_4.2H_2O$	8.60	-
KI	0.85	-
$NaMoO_4.2H_2O$	0.25	-
$CuSO_4.5H_2O$	0.025	-
$CoCl_2.6H_2O$	0.025	-
Organic compounds		
Myo-inositol	100.00	100.00
Glycine	2.00	2.00
Nicotinic Acid (B12)	0.50	0.50
Pyridoxine HCl (B6)	0.50	0.50
Thiamine HCl (B1)	0.50	0.10

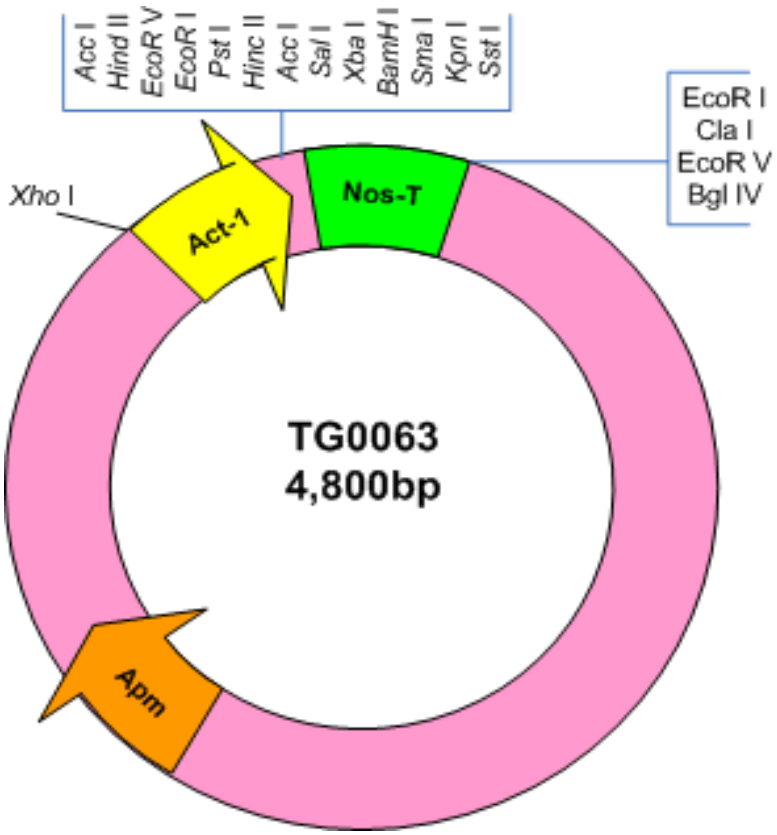


Appendix Figure1 Schematic of the pActin1-D and pAHC27vectors used for genetic transformation.

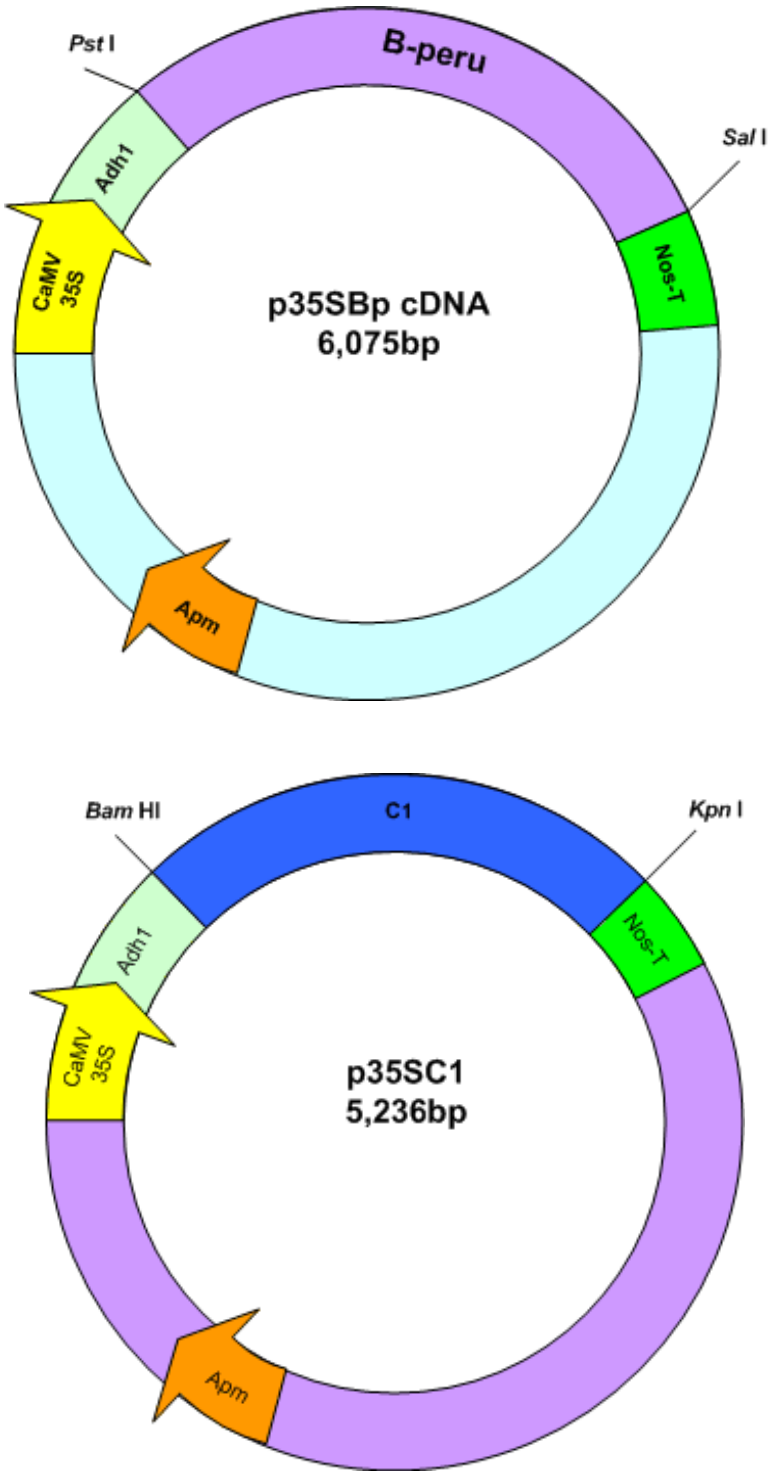




Appendix Figure1 Schematic of the p2K7 and pMNK1005 vectors used for genetic transformation (Continued).



Appendix Figure1 Schematic of the TG0063 vector used for genetic transformation (Continued).



Appendix Figure2 Schematic of the plasmids containing the *B-peru* and *C1* inserted in p35SBpcDNA and p35SC1.