THESIS FOR THE DEGREE OF DOCTOR OF AGRICULTURE

Inheritance of Floral pigmentation in *Aerides japonicum* and Chimeral Variegation through Mutagenesis in Korean Native *Cymbidium*

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by

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DEPARTMENT OF HORTICULTURE

GRADUATE SCHOOL

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博 士 學 位 論 文

나도풍란의 화색유전과 돌연변이육종을 이용한 한국자생 심비디움의 키메라 반입현상에 관한 연구

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濟州大學校 大學院

園 藝 學 科

崔 至 鎔

2000 年 6 月

Inheritance of Floral pigmentation in *Aerides japonicum* and Chimeral Variegation through Mutagenesis in Korean Native *Cymbidium*

CHOI, JI YONG

(Supervised by professor So, In Sup)

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF AGRICULTURE

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敎授指導 蘇 寅 燮

崔 至 鎔

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2000 年 6 月

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崔至鎔의 農學 博士學位 論文을 認准함.

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LIST OF ABBREVIATION

AFLP	amplified fragment length polymorphism
DES	diethyl sulphate
DMF	N,N-dimethylformamide
DNA	deoxyribonucleic acid
dNTP	dATP, dTTP, dCTP, and dGTP
EI	ethylene imine
EMS	ethyl methanesulfonate
GST	glutathione S-transferase
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
IPTG	isopropyl-β-D-thiogalactoside
kb	(kilobase) of a DNA or RNA strand: 10^3 base pairs (bp)
kDa	kilodalton: 10^3 dalton
MNU	N-methyl-N-nitroso urethane
MT	metallothioneins
MTL	metallothionein-like metalloproteins
NMU	nitrosomethylurea
NTSYS	Numerical Taxonomy System

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PCR	polymorphic band score
PVP	polyvinylpyrrolidone
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SCAR	sequence characterized amplified regions
SDS	sodium dodecyl sulfate
SSH	suppression subtractive hybridization
UBC	University of British Columbia
UPGMA	unweighted pair group method with average
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Inheritance of Floral Pigmentation in *Aerides japonicum* and Chimeral Variegation through Mutagenesis in Korean Native *Cymbidium*

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(Major in Floriculture and Orchid Breeding)

Advisor : Prof. Dr. So, In Sup

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SUMMARY

The present studies were performed on *Aerides japonicum* in relation to the inheritance of floral pigmentation. In this study, anthocyanins in the flower, the cross hybridization through second generation, the SCAR marker system to identify the floral pigmentation on the early stage of breeding, and the exploration of genes related to flower pigmentation by

suppression subtractive hybridization (SSH) were investigated.

The pigment of sepals and labellum of *Aerides japonicum* was anthocyanins peaked at about 524 nm, while *A. japonicum alba* did not show any peak in the range of wavelength related to anthocyanins.

Aerides japonicum (a *R_*, red colored sepals and lip) when crossed with *R_* red produced only red colored progeny, while *A. japonicum alba* (a *rr*, white sepals and lip) when crossed with *rr* white produced only flowers with white sepals and lips. The cross between F_1 of *A. japonicum* and F_1 of *A. japonicum alba* segregated essentially as a 1:1 ratio in the progeny.

UBC351, UBC375, and UBC396 specific primer pairs were selected as useful markers to distinguish *Aerides japonicum* from *A. japonicum alba* in early stage of breeding. The identification of SCAR marker, UBC351U_{720} primer, showed the segregation of crossing between *Aerides japonicum* and *A. japonicum alba* essentially as a 1:1 ratio.

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The 16 newly generated sequences of *Aerides japonicum* by suppression subtractive hybridization (SSH) are deposited in the Genbank, DDJB, and EMBL. These genes were all related to pigmentation with one exception of methallothionein-like protein. Especially, glutathione S-transferase played a crucial role in stabilisation

and detoxification of anthocyanins by transport to the vacuole. The other genes such as dormancy-associated protein, lipid transfer proteins, germin-like protein gene and 3-hydroxy-3-methylglutaryl-CoA synthase were related to biosynthesis of anthocyanins.

The present works were performed on *Cymbidium goeringii* and *C. kanran* in relation to chimeral variegations. In this study, mutagenesis for inducing various linear chimeras, microscopic observation, and characteristics of genetic relationship to chimeras using RAPD analysis were also investigated.

Survival rates of *Cymbidium* rhizome treated with the mutagen were declined as time elapsed, while the percent of mutant occurrence was increased. 3,000 mg/L EMS for 2 hours was regarded to be the level of LD_{50}. Cuttings into pieces of rhizome were shown as a higher percentage of variegated shoots than the control, and 500 ㎛ thickness was the least size to permit survival and variegation.

When rhizome appeared to be lighter in color (greenish to light green, yellowish, and whitish), greater extent of variegated variants could be expected than respective opposites in the ordinary green. There was a positive correlation between the chlorophyll content of rhizomes and that of leaves. Number of stomates per unit area was less in the

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variegated portion than the normal.

Primarily, mutated rhizomes induced margined (white- and yellow-) and medioalbinated (white- and yellow-) leaves. During the period of subculture, an albino was obtained from white-margined leaves by the replacement of layers, while a normal green leaf was obtained from yellow-margined, white-/yellow-medioalbinated leaves.

RAPD analysis showed mutually identical DNA pattern between induced chimera and normal green plant restored by replacement of layers.

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INTRODUCTION

The Orchidaceae include over 800 genera and over 25,000 known species of monocotyledonous herbaceous perennial plants of nearly worldwide distribution (Liberty Hyde Bailey Hortorium, 1976). Orchidaceae is evolutionally the most advanced plant family and may well be the largest (though "the largest" claims are made to compare to other families). It is also the most hybridized family. Existing hybrids are the result of crosses between (1) distinct forms of one species (intraspecific hybrids); (2) species within a genus (intra or infrageneric hybrids); and (3) different genera (intergeneric hybrids that produce hybrid genera). The latter are of particular interest because hybrids between different genera are uncommon in other genera. Even more remarkable are the facts that crosses can be made between several genera, and the number of intergeneric hybrids is still increasing (Arditti, 1992).

Orchids are found everywhere, and therefore it is not surprising that they have long been appreciated by many cultures (Arditti, 1992). Confucius (551-479 B.C.) mentioned orchids in his writings. He speakes of the fragrance of lan (orchids) in the home, indicating that the Chinese were using orchid flowers to decorate their homes (Sheehan,

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1992).

Few plants surpass the orchids in distribution throughout the world, variability of growth habits, and the magnificent spectrum of colors produced by their flowers and leaves (Arditti and Ernst, 1971; Strauss and Arditti, 1972). To a very large extent, this wealth of colors is due to anthocyanins. Singly or in combinations with other pigments, they contribute to delicate pastels, dazzling yellows, brilliant reds, dull browns, and exciting purples (Arditti and Fisch, 1977).

Variations within species are not unusual among orchids since they are usually cross-pollinated plants (Vajrabhaya, 1977). Color variation such as alba (entirely white) and semi-alba (colored-lip, white sepals and petals) may be found within a single species: For example, *Cattleya warscewiczii* 'Firmin Lambeau' is an alba clone, whereas *Cattleya warscewiczii* 'Frau Melanie Beyrodt' is a semi-alba (Mehlquist, 1958; Stoery and Kamemoto, 1960). Also, in *Aerides japonicum* (lip with purplish spots, sepals with purplish transverse bars at base), is found the variation such as alba (entirely white) in nature rarely. Such rarity value is very important in horticulture. It is important that we should know the inheritance to produce a new variety and mass propagation through cross hybridization. But there has been not much documented about inheritance of pigmentation in *Aerides japonicum* and ways of producing alba yet.

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In this study, therefore, the inheritance of floral pigmentation in *Aerides japonicum* by the cross hybridization through the second generation, anthocyanins in the flower, SCAR marker system to identify the floral pigmentation on the early stage of breeding, and the detection of genes related to flower pigmentation by suppression subtractive hybridization (SSH) were of interest to investigate.

Variegation can be defined as the presence of discrete markings of different colors on an organ or an organism (Marcotrigiano, 1997). In plants, variegation is most frequently manifested as stripes, blotches, and streaks, or by differences in color between leaf margins (so-called magined) and the leaf mid-region (so-called medioalbinated). Variegation types can be categorized as either cell lineage type or noncell lineage type (Kirk and Tilney-Bassett, 1978). Cell lineage variegation occurs in genetic mosaics (individuals with cells of different genotypes) and a plant chimeras is a specific type of genetic mosaic that cannot be seed propagated. In plants with noncell lineage variegation, all cells have the same genotype but the genes responsible for the synthesis or destruction of pigments are expressed in only some of the cells. Noncell lineage variegation patterns can be transmitted sexually from on generation to the next. Nonchimeral noncell lineage variegation is the most common cause of variegation. Variegation and chimerism are phenomena with

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both economic and scientific importances for horticulture and for the study of plant development. Especially in Korea, Japan and China, the vareigated cymbidiums such as *Cymbidium goeringii* and *C. kanran* are invaluable because they are rare in nature. In *Cymbidium*, variegation types can be classified as linear type (such as stripes) and spot type. Therefore, it is most important to know which is the cause of variegated leaves, noncell lineage variegation or chimeral variegation. Because the difference makes a decision about the way of breeding. That is, noncell lineage variegated cymbidiums can be easily bred by cross hybridization and propagated by seeds, while chimeral variegated cymbidiums can be induced by the mutagenesis and propagated asexually. But, there is no sufficient and correct reports about variegation of *Cymbidium goeringii* and *C. kanran* yet.

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In this study, therefore, the mutagenesis for inducing various linear chimeras of *Cymbidium goeringii* and *C. kanran* by the alkylating agent, ethyl methanesulfonate (EMS), the morphological characteristics by microscopic observation, phenomena of replacements during subculture, and characteristics of genetic relationship of chimeras using randomly amplified polymorphic DNA (RAPD) analysis were investigated.

LITERATURE REVIEWS

Few plants surpass the orchids in distribution throughout the world, variability of growth habits, and the magnificent spectrum of colors produced by their flowers and leaves (Arditti and Ernst, 1971; Strauss and Arditti, 1972). To a very large extent, this wealth of colors is due to anthocyanins. Singly or in combinations with other pigments, they contribute to delicate pastels, dazzling yellows, brilliant reds, dull browns, and exciting purples (Arditti and Fisch, 1977).

Anthocyanins (Greek: *anthos*, a flower; *kyanos*, blue) are flavonoids (Latin: *flavus*, yellow) derived from 2-phenylbenzopyran (Bate-Smith, 1949). Flavonoids are phenolic compounds that include a wide rage of colored substances. The most widespread group of pigmented flavonoids are the anthocyanin, which range in color from yellow to red and magenta (Arditii and Fisch, 1977; Taiz and Zeiger, 1991).

Anthocyanins are glycosides that have sugars at position 3 and sometimes elsewhere. Without their sugars, anthocyanins are known as anthocyanidins (the non-water-soluble colored part of the molecule, also called the agylecone) (Taiz and Zeiger, 1991; Salisbury and Ross, 1992).

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Anthocyanidins are usually named after the particular plant from which they were first obtained. The most common anthocyanidin is cyanidin, which was first isolated from the blue cornflower, *Centaurea cyanus*. Another, pelagonidin, was named after a bright red geranium of the genus *Pelargonium*. A third, delphinidin, obtained its name from the genus *Delphinium* (blue larkspur). These anthocyanidins differ only in the number of hydroxyl groups attached to the B ring of the basic flavonoid structure. Other important anthocyanidins include the reddish peonidin (present in peonies), the purple petunidin (in petunias), and the mauve-colored (purplish) pigment malvidin, first found in a member of the Malvaceae, the mallow family (Salisbury and Ross, 1992).

Generally, introduction of hydroxyl groups at the 3 position or in the B ring bathochromically shifts the visible spectrum, yielding bluer hues. In contrast, OH substitution at the 6 or 8 position does not displace the absorption maximum in an consistent way. When existing hydroxyl groups are methylated, the result is invariably a hypsochromic shift of the visible spectrum (bluer hues), but the effect is small (Arditti and Fisch, 1977).

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Most anthocyanins are reddish in acidic solution but become purple and blue as the pH is raised. In larkspur flowers the pH of epidermal cells containing delphinidin increases from 5.5 to 6.5 during aging, and the color changes from reddish purple to purplish blue (Asen

et al., 1975).

The general question of flower-color inheritance in *Sophronitis, Cattleya, Lealia, and Brassavola* hybrids has received wide attention. Color inheritance in *Spathoglottis* has also been studied extensively (Storey, 1950, 1958; Lenz and Wimber, 1959). These studies have led to the assumption that flower color in this orchid is controlled by three independent gene pairs. *Cattleya* has a different type of coloration that purple sap or rosy-purple color type, semi-alba type, and yellow coloration. The rosy-purple color in the various species of *Cattleya* is due to the simultaneous presence of two complimentary color factors, which called C and R. If one or both of these factors is absent, the result is a true albino with no trace of purple sap. This purple color can only produced when the two factors C and R are both present (Hurst, 1913). The purple sap or rosy-purple color are most probably anthocyanins. The C and R hypothesis is now generally accepted as being valid by all orchid breeders and geneticists (Storey and Kamemoto, 1960). Some *Cattleya* forms produce flowers with white sepals and petals, but colored lips which called "semi-alba" is used to describe these combinations (Mehlquist, 1958). In this case, flower color is produced by the three factors C, R, and a additional gene p (Mehlquist, 1958; Storey and Kamemoto, 1960). Yellow coloration in *Cattleya* (which is probably not due to anthocyanins) is controlled by

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γ1, γ2, y1, and y2, but is recessive to purple (Northen, 1962).

Recently, a more reliable and specific PCR-based marker known as sequence characterized amplified regions (SCAR), was developed. SCAR primers are longer than RAPD primers and a highly stringent annealing temperature can be employed that prevents mismatching in the priming site during DNA amplification (Paran and Michelmore, 1993). Unlike RAPD, SCAR pimers can amplify a single locus that appears as a single easily scored band in agarose gel. Kesseli et al. (1994) had made a study of the application of targeted and randomly generated markers (RFLP, RAPD, SCAR, AFLP and SSR) to crop breeding programs and the uses of these markers for forest genetic research. Lawson et al. (1994) researched into marker-assisted selection for two rust resistance genes in sunflower. Kelly and Miklas (1998) studied the role of RAPD markers in breeding for disease resistance in common bean.

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Subtractive cDNA hybridization has been a powerful approach to identify and isolate cDNA of differentially expressed genes (Duguin and Dinauer, 1990; Hara et al., 1991; Hendrick et al., 1984). Numerous cDNA subtractive methods have been reported. In general, they involve hybridization of cDNA from on population (tester) to excess of mRNA (cDNA) from other population (driver) and then separation of the unhybridized fraction (target) grom hybridized common sequences. The

latter step is usually accomplished by hydroxylapatite chromatography (Hendrick et al., 1984), avidin-biotin binding (Duguin and Dinauer, 1990; Sargent and Dawid, 1983), or oligo$(dT)_{30}$-latex beads (Hara et al., 1991). Despite the successful identification of numerous important genes such as the T-cell receptors (Hendrick et al., 1984) by these methods, they are usually inefficient for obtaining low abundance transcripts. These subtraction techniques often require greater then 20 μg of poly(A)+ RNA, involve multiple or repeated subtraction steps, and are labor intensive. Recently, Diatchenko et al. (1996) presented a new PCR-based cDNA subtraction method, termed suppression subtractive hybridization (SSH), and demonstrated its effectiveness. They suggested that the SSH technique is applicable to many molecular genetic and positional cloning studies for the identification of disease, developmental, tissue-specific, or other differentially expressed genes.

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Variegation can be defined as the presence of discrete markings of different colors on an organ or an organism (Marcotrigiano, 1997). In plants, variegation is most frequently manifested as stripes, blotches, and streaks, or by differences in color between leaf margins (so-called magined) and the leaf mid-region (so-called medioalbinated). Variegation types can be categorized as either cell lineage type or noncell lineage type (Kirk and Tilney-Bassett, 1978). Cell lineage variegation occurs in genetic mosaics (individuals with cells of different genotypes) and a

plant chimeras is a specific type of genetic mosaic that cannot be seed propagated. In plants with noncell lineage variegation, all cells have the same genotype but the genes responsible for the synthesis or destruction of pigments are expressed in only some of the cells. Noncell lineage variegation patterns can be transmitted sexually from on generation to the next. Nonchimeral noncell lineage variegation is the most common cause of variegation.

Histologists have been reluctant to utilize chimeras in ontogenetic studies because of their concern that "they may behave differently from the normal form" (Clowes, 1956). Nougarde (1967) stated that "These chimeras are really only particular cases that do not provide proof of the existence of independent layers in normal and nonchimeric apices." But variegation and chimerism are phenomena with both economic and scientific importance for horticulture and for the study of plant development. Stewart et al. (1974) reported the competition and accommodation between apical layers and their derivatives in the ontogeny of chimeral shoots of *Perlrgonium* x *hortorum*. They suggested that there are independent apical layers and cell lineages derived from them in nonchimeral shoots, and that their contribution in normal ontogeny is like that of the layers in Dpl W_1 chimeras. Stewart and Dermen (1979) had made a study of the developmental anatomy of apically stable periclinal chloroplast chimeras

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in a number of monocotyledonous genera. Marcotrigiano and Morgan (1988) studied the origin of adventitious shoots from leaf cuttings and cultured leaf tissue of chlorophyll-deficient cell lines of *Sansevieria trifasciata*.

The induction of mutations in higher plants is widely practiced. The physical mutagens used include X-ray, gamma rays, and fast and thermal neutrons, while the chemical mutagens include ethyl methane sulphonate (EMS), diethyl sulphate (DES), ethylene imine (EI), propane sultone, N-methyl-N-nitroso urethane (MNU) and some of its related compounds, and also sodium azide (Gottschalk and Wolff, 1983). The preferred technique for sexually propagated crops is to irradiate dry seeds prior to germination as these are easier to handle than moistened seeds, or shoots, and chemicals are more inclined to produce unwanted side effects, especially loss of vigour and reduced fertility. Nevertheless, once the right conditions for a plant are worked out, chemical mutagens are extremely useful and widely used (Tilney-Bassett, R.A.E, 1986).

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The mutagen treatments discussed so far are aimed at altering nuclear genes; on other occasions the plastid genes are the main targets. Many of the variegated-leaf chimeras have arisen as spontaneous plastid mutants followed by sorting-out into periclinal chimeras. Experimentally, plastid mutation, leading to chimera formation, has been chemically induced by EMS, particularly in tobacco (Dulieu, 1965, 1967; Deshayes,

1973), and more recently in a number of crop species - peas, carrots, soybeans, lentils, and radishes (Miller et al., 1980, 1984). Another effective compound is nitrosomethylurea (NMU), which has been successfully applied in snapdragon, sunflower and the zonal perlargonium (Kirk and Tilney-Bassett, 1978), and more recently in *Lycopersicon esculentum*, *L. hirsutum* and *L. pennelli* (Hosticka and Hanson, 1984) and *Saintpaulia ionantha* (Pohlheim and Beger, 1974).

This work was performed on *Aerides japonicum* in relation to the inheritance of floral pigmentation and the mutagenesis for inducing various linear chimeras of *Cymbidium goeringii* and *C. kanran* by the alkylating agent, ethyl methanesulfonate (EMS).

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CHAPTER 1. Inheritance of Floral Pigmentation in *Aerides japonicum*

ABSTRACT

The present studies were performed on *Aerides japonicum* in relation to the inheritance of floral pigmentation. In this study, anthocyanins in the flower, the cross hybridization through second generation, the SCAR marker system to identify the floral pigmentation on the early stage of breeding, and the exploration of genes related to flower pigmentation by suppression subtractive hybridization (SSH) were investigated.

The pigment of sepals and labellum of *Aerides japonicum* was anthocyanins peaked at about 524 nm, while *A. japonicum alba* did not show any peak in the range of wavelength related to anthocyanins.

Aerides japonicum (a *R_*, red colored sepals and lip) when crossed with *R_* red produced only red colored progeny, while *A. japonicum alba* (a *rr*, white sepals and lip) when crossed with *rr* white produced only flowers with white sepals and lips. The cross between F_1

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of *A. japonicum* and F_1 of *A. japonicum alba* segregated essentially as a 1:1 ratio in the progeny.

UBC351, UBC375, and UBC396 specific primer pairs were selected as useful markers to distinguish *Aerides japonicum* from *A. japonicum alba* in early stage of breeding. The identification of SCAR marker, $UBC351U_{720}$ primer, showed the segregation of crossing between *Aerides japonicum* and *A. japonicum alba* essentially as a 1:1 ratio.

The 16 newly generated sequences of *Aerides japonicum* by suppression subtractive hybridization (SSH) are deposited in the Genbank, DDJB, and EMBL. These genes were all related to pigmentation with one exception of methallothionein-like protein. Especially, glutathione S-transferase played a crucial role in stabilisation and detoxification of anthocyanins by transport to the vacuole. The other genes such as dormancy-associated protein, lipid transfer proteins, germin-like protein gene and 3-hydroxy-3-methylglutaryl-CoA synthase were related to biosynthesis of anthocyanins.

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INTRODUCTION

Aerides japonicum is a favorite orchid because it's flower has attractive fragrance and color. This flower blooms in early summer and distributes in Cheju Island, Korea.

Variations within species are not unusual among orchids since they are usually cross-pollinated plants (Vajrabhaya, 1977). Color variation such as alba (entirely white) and semi-alba (colored-lip, white sepals and petals) may be found within a single species. In *Aerides japonicum* (lip with purplish spots, sepals with purplish transverse bars at base), is found the variation such as alba (entirely white) in nature rarely. Such rarity value is very important in horticulture. It is important that we should know the inheritance to produce a new variety and mass propagation through cross hybridization. But there has been not much documented about inheritance of pigmentation in *Aerides japonicum* and ways of producing alba yet.

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Recently, a more reliable and specific PCR-based marker known as sequence characterized amplified regions (SCAR) was developed. SCAR pimers can amplify a single locus that appears as a single easily scored band in agarose gel. Also, subtractive cDNA hybridization has been a powerful approach to identify and isolate cDNA of differentially

expressed genes. These molecular biological techniques will support to elucidate the inheritance of pigmentation and genes related to pigmentation of *Aerdes japonicum.*

This work was performed on *Aerides japonicum* in relation to the inheritance of floral pigmentation. The present studies included anthocyanins in the flower, the cross hybridization through second generation, the SCAR marker system to identify the floral pigmentation on the early stage of breeding, and the exploration of genes related to flower pigmentation by suppression subtractive hybridization (SSH).

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MATERIALS AND METHODS

(1) Flower color in *Aerides japonicum*

Anthocyanins in the labellum and sepals of flowers in *Aerides japonicum* (Fig. 1.1, left) and *A. japonicum alba* (Fig. 1.1, right) were extracted with 10 mL of 1% HCl in methanol for 24 hours at 4℃ (Goh et al., 1985; Suh et al, 1996). The extract was then diluted, if necessary, and the absorbance was read in the range of the wavelength from 450 nm to 650 nm with a spectrophotometer (Berckman DU-64, USA). *Aerides japonicum alba* is one of variations of *A. japonicum*

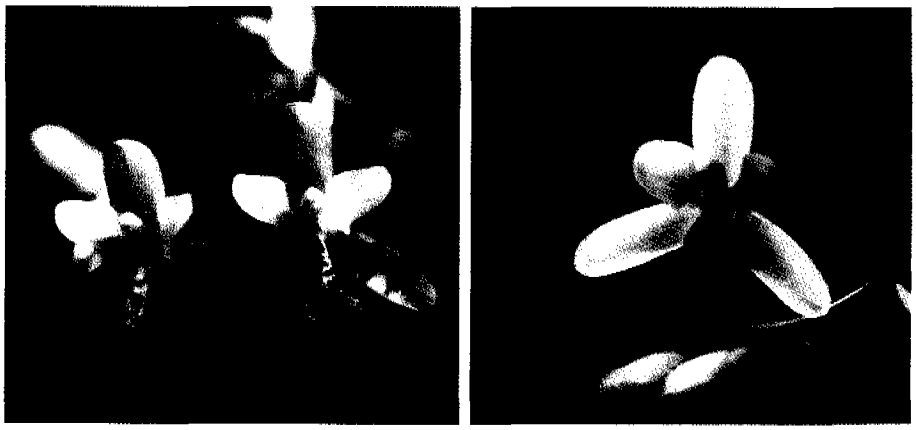

Fig. 1.1. Flowers of *Aerides japonicum* (left) and *A. japoncum alba* (right).

Fig. 1.5. A photograph showing the flower pigmentation and segregation according to the different hybridization.
(Top) *Aerides japonicum alba* × *A. japonicum alba*
(Middle) *Aerides japonicum* × *A. japonicum*
(Bottom) *Aerides japonicum alba* × *A. japonicum*

which has not reddish pigment on sepals and labellum. It is not granted as a scientific name yet, so it is used as "*Aerides japonicum alba*" in this study.

(2) Cross hybridization to produce alba variation

Cross hybridization was carried out for the matching as follows:

(1) *Aerides japonicum* × *A. japonicum*

(2) *Aerides japonicum alba* × *A. japonicum alba*

(3) Progeny between *Aerides japonicum* themselves × progeny between *A. japonicum alba* themselves.

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After the six months of pollination, seeds were germinated in Hyponex medium (Kano, 1968; Nagashima, 1993). Germinated seedlings were subcultured in vitro. Basal culture medium for subculture was consisted of 2.0 g/L Hyponex (N:P:K=6.5:6.0:19:0) supplemented with 50.0 g/L banana extract, 25.0 g/L potato extract, 4.0 g/L peptone, 2.0 g/L activated charcoal, and 30.0 g/L sucrose. The medium was adjusted to pH 5.7 prior to autoclaving and gelled with 6.5 g/L agar. Cultures were maintained in a growth chamber at 25±2℃ under cool-white fluorescent light (1.5 klux) from 0800 to 2400 $_{HR}$ and in darkness from

2400 to 0800 $_{HR}$. Young seedlings were hardened in greenhouse, and induced flowering.

(3) SCAR maker to identify the floral pigmentation

Plant materials and DNA isolation

The genotypes used for making the marker system to detect the floral pigmentation in the early stage of breeding were *Aerides japonicum*, *A. japonicum alba*, and F_1 seedlings of hybridization between them.

Genomic DNA was isolated from *Aerides japonicum* using PVP, SDS, and chloroform extraction (Kim et al., 1997). The DNA samples had $OD_{260}/ OD_{280} \fallingdotseq 1.8$ and $OD_{260}/OD_{230} \fallingdotseq 1.8 \sim 2.0$.

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RAPD reactions and construction of SCARs marker

Reaction mixtures in 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.1% Triton X-100, 37.5 μg/ml BSA and 1.5mM $MgCl_2$ contained 0.018 units/μL Taq DNA polymerase (ABGENE, England); 100 μM dNTP mix (dATP, dTTP, dGTP, and dCTP); 0.2 μM primer; and about 5 to 10 ng total DNA in a final volume of 20 μL, respectively. Reaction mixture was overlayed with mineral oil (Sigma Co., USA), mixed and

centrifuged for 2 min at $2\times10^3\times g$. RAPD primers were obtained from the University of British Columbia (UBC) (Table 1.1).

Amplification of genomic DNA was performed in a PCR system PTC-100 thermal controller (MJ Research, INC., USA) programmed for 5 min at 95℃ (initial denaturation) and 45 cycles of 15 sec at 94℃ (denaturation), 60 sec at 37℃ (annealing), 90 sec at 72℃ (extension), followed by 10 min at 72℃ (final extension). Reaction products were resolved by electrophoresis in 1.4% agarose gels and visualized by ethidium bromide staining. Discrete and visible DNA segments on the gels were recorded by the particular size based on a sized 1 Kb DNA marker (GIBCO BRL/Life Technologies, Gaithersburg, MD, USA). The RAPD PCR analysis for each condition was at least twice.

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Taq DNA polymerase-generated RAPD bands excised from agarose gels and purified using a Geneclean III kit (Bio 101, Co.) were cloned into pGEM-T Easy vector (Promega Co.) and introduced into competent *E. coli* JM109 according to the supplier's protocol. Recombinants were identified as white or blue colonies on LB-ampicillin medium supplemented with X-gal and IPTG medium. Plasmid DNA was isolated from several independent transformants by alkaline lysis using the rapid procedure of Zhou et al. (1990). Restriction enzymes were used according to the manufactur's instructions, Boehringer Mannheim. The cloned RAPD markers were subjected to automatic sequencing using T7

Table 1.1. The list of 30 random primers and their sequences used in RAPD for construction of sequence-specific primers of *Aerides japonicum*.

No.[z)]	Sequences	GC content (%)
303	5'-GCGGGAGACC-3'	80
304	5'-AGTCCTCGCC-3'	70
305	5'-GCTGGTACCC-3'	70
309	5'-ACATCCTGCG-3'	60
310	5'-GAGCCAGAAG-3'	60
311	5'-GGTAACCGTA-3'	50
313	5'-ACGGCAGTGG-3'	70
315	5'-GGTCTCCTAG-3'	60
318	5'-CGGAGAGCGA-3'	70
322	5'-GCCGCTACTA-3'	60
327	5'-ATACGGCGTC-3'	60
333	5'-GAATGCGACG-3'	60
334	5'-TGGACCACCC-3'	50
335	5'-TGGACCACCC-3'	70
336	5'-GCCACGGCGA-3'	70
337	5'-TCCCGAACCG-3'	70
347	5'-TTGGCGAACG-3'	60
348	5'-CACGGCTGCG-3'	80
349	5'-GGAGCCCCCT-3'	80
351	5'-CTCCCGGTGG-3'	80
352	5'-CACAACGGGT-3'	60
353	5'-TGGGCTCGCT-3'	70
354	5'-CTAGAGGCCG-3'	70
358	5'-GGTCAGACCT-3'	80
362	5'-CCGCCTTACA-3'	60
364	5'-GGCTCTCGCG-3'	80
372	5'-CCCACTGACG-3'	70
375	5'-CCGGACACGA-3'	70
389	5'-CGCCCGCAGT-3'	80
396	5'-CGCCCGCAGT-3'	60

[z] Accession number of UBC (the University of British Columbia) primer set.

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and SP6 sequencing primers. Sequence- specific primers were designed by adding 10 bases to 5' and 3' end of the original 10-mers to amplify sequence characterized amplified regions (SCARs) as described by Paran and Michelmore (1993).

PCR for SCAR marker

Amplification of genomic DNA using putative SCARs markers was performed in a PTC-100 thermal cycler (MJ Research Inc.) programed for 5 min at 95℃ and 30 cycles of 30 sec at 94℃, 2 min at 50~65℃, and 2 min at 72℃. PCR was carried out in 25 μl of reaction solution mixture [10 mM Tris · HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM each of dNTP; and 1.25 units of Taq DNA polymerase (Takara Shuzo, Kyoto)] with the templates of the plant DNAs (0.1 μg) and the manufactured primers (0.25 μM each).

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(4) Suppression subtractive hybridization (SSH) to explore the genes of flowers

Total RNA extraction

Total RNA extraction

Total RNA isolation of flowers of *Aerides japonicum* and *A.*

japonicum alba were processed following four parts: effective disruption of cells or tissues, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity, removal of contaminating DNA and proteins (Chamczynski and Sacchi, 1987). Elution tube containing the purified RNA was stored at -70°C.

mRNA isolation

In a sterile, RNase-free tube, 0.1-1.0 mg of total RNA was brought to a final volume of 500 μL in RNase-free water and incubated 65℃ heating block for 10 min. Three mictoliter of the Biotinylated-Oligo(dT) Probe (Promega Co.) and 13 μL of 20X SSC was added to the RNA, and then mixed gently and incubated at room temperature until completely cooled. The entire contents of the annealing reaction was added to the tube containing the washed SA-PMPs and incubated at room temperature for 10 minutes. The SA-PMPs using the magnetic stand carefully removed the supernatant without disturbing the SA-PMP pellet and washed the particles four times with 0.1X SSC (0.3 ml per wash) by gently flicking the bottom of the tube until all of the particles were resuspended. After the final wash, the aqueous phase were removed as much of as possible without disturbing the SA-PMP particles. To elute the mRNA, the final SA-PMP pellet was resuspended in 0.1 ml of the RNase-Free Water and gently resuspended the particles

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by flicking the tube. The SA-PMPs were magnetically captured and transferred the eluted mRNA aqueous phase to a sterile and RNase-free tube.

Determination of total RNA and mRNA concentration and purity

The yields of total RNA and mRNA were determined spectrophotometrically at 260 nm, where 1 absorbance unit (A_{260}) equals 40 μg of single-stranded RNA/ml. The purity was also estimated by spectrophotometry from the relative absorbances at 230, 260 and 280nm (i.e., A_{260}/A_{280} and A_{260}/A_{230}).

PCR-select cDNA Subtraction

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cDNA synthesis

Double-stranded cDNA was synthesized using Superscrip reverse transcriptase (Gibco BRL).

Representational difference analysis of cDNAs (cDNA RDA)

The DNA in which target sequences were to be found was called "Red-tester", and the reference DNA was called "Alba-driver". First, both DNA samples were digested with a restriction enzyme that generates blunt ends. The tester DNA was then divided into two

portions, each of which was ligated to unique adaptors that facilitate suppression PCR.

Double stranded cDNA was digested with the four-cutter *Rsa* I (GT ↓AC) to obtain maximum representation, ligated to adaptors and amplified by PCR to generate amplicons. Adaptors were consist of Adaptor 1 and Adaptor 2R, and which ligated *Rsa* I digested cDNA using T4 DNA ligase (Fig. 1.2).

Subtractive hybridization

Two microliters of driver ds cDNA (600 ng) was added to each of two tubes containing 2 μL of adaptor 1- and adaptor 2-ligated tester cDNA (20 ng). The samples were mixed, ethanol precipitated, and then resuspended in 1.5 μL of hybridization buffer (50 mM Hepes, pH 8.3 / 0.5 M NaCl / 0.02 mM EDTA, pH 8.0 / 10% (wt/vol) PEG 8000). The solution was overlaid with moneral oil, the DNAs were denatured (1.5 mim, 98℃), and then allowed to anneal for 8h at 68℃. After this first hybridization, the two samples were combined and a fresh portion of heat-denatured driver (≒150 ng) in 1.5 μL of hybridization buffer was added. The sample was allowed to hybridize for an additional overnight at 68℃. The final hybridization was then diluted in 200 μL of dilution buffer (20 mM Hepes, pH 8.3 / 50 mM NaCl / 0.2 mM EDTA), heated at 72℃ for 7 min and stored at -20℃.

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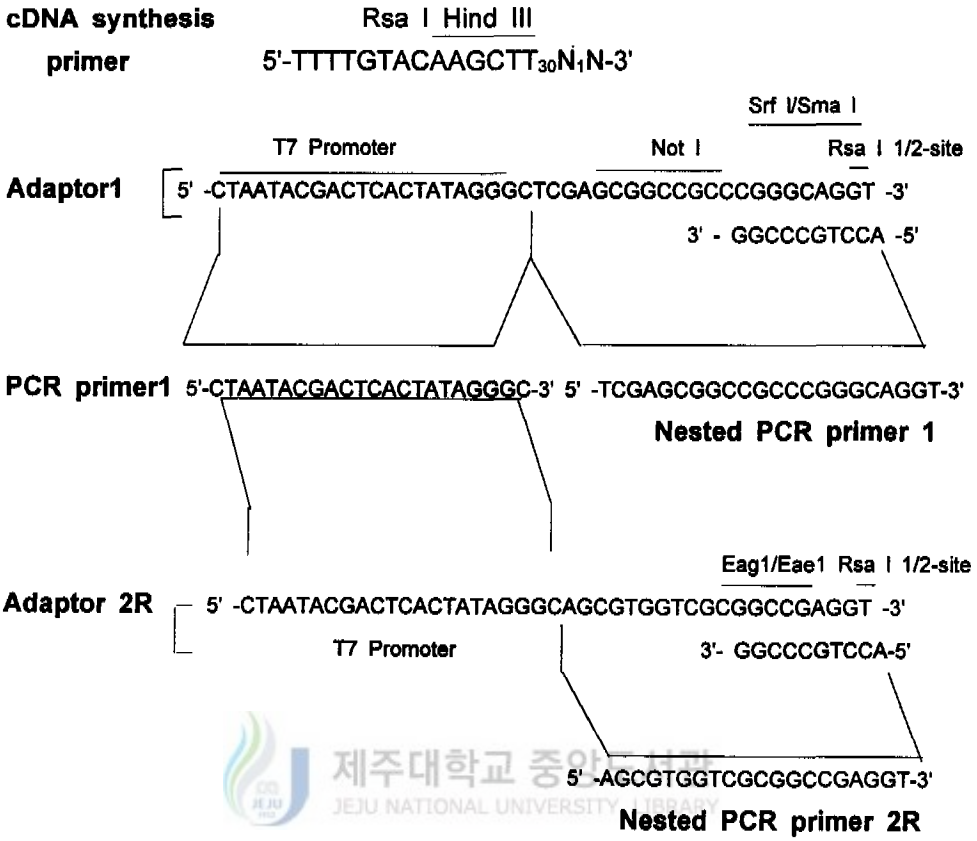

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Fig. 1.2. Sequences of the suppression subtractive hybridization cDNA synthesis primer, adaptors, and PCR primers. when the adaptors are ligated to *Rsa* I-digested cDNA, the *Rsa* I site is restored.

PCR Amplification

For each subtraction, I performed two PCR amplification. The primary PCR was conducted in 25 μL. It contains 1 μL of diluted, subtracted cDNA, 1 μL of PCR primer 1 (10 μM), and 22 μL of PCR master mixture prepared using the Advantage cDNA PCR Core Kit (Clonetech Co.). PCR was performed with the following parameters: 7 5℃ for 7 min; and 30 cycles at (94℃ for 10 sec; 66℃ for 30 sec; 7 2℃ 1.5 min). The amplified products were diluted 10-fold in deionized water. Some of the product (1 μL) was then used as a template in secondary PCR for 10 cycles under the same conditions used for the primary PCR, except PCR primer 1 was replaced with nested PCR primer PN1 and PN2. The PCR products were analyzed by 1.4% agarose gel electrophoresis.

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Cloning into a TA vector

After evaluation of the subtraction efficiency the subtracted library cDNA was cloned directly into pGEM-T Easy vector using a TA cloning kit (Promega). Approximately 100 ng PCR-amplified cDNA were ligated without further purification into 50 ng vector and the ligation mixture was introduced into bacterial strain JM109 (Promega). For library screening the titre was determined and bacteria were plated

onto 10×10 cm agar plates containing 100 μg/ml ampicillin, 100 μM IPTG and 50 μg/ml X-Gal. Plates were incubated at 37℃ until small colonies were visible then incubated further at 4℃ until blue/white staining could be clearly distinguished.

Analysis of the Subtracted cDNA clone

DNA sequencing was performed by automated means at the Bionet Co. (The University of Hanyang, Korea). Nucleic acid homology searches were performed using the BLAST (Altschul et al., 1997) program through internet servers at the National Centers for Biotechnology Information (National Institutes of Health, Bethesda). Alignment of genes was performed using DNAStar program for IBM PC computer.

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RESULTS AND DISCUSSION

(1) Flower color in *Aerides japonicum*

The colors of orchid flowers vary from pure white (as in *Phalaenopsis amabilis*) to nearly black (portions of the labellum of *Coelogyne pandurata*). Between these extremes, orchid flowers can be

blue (*Vanda coerulea*), green (some *Cymbidium* species and hybrids), yellow (*Anguloa clowesii*), orange (*Cattleya aurantiaca*), red (*Renanthera coccinea*), purple (which is to many a typical orchid color, as in some *Cattleya* hybrids) and numerous combinations and blends. In nature, flower pigmentation plays an important role in attracting pollinators. Horticulturally, the color of orchid flowers is an important reason for their popularity (Arditti, 1992).

Aerides japonicum alba produces flowers without red stripes and spots on sepals and lip (Fig 1.1, right). The spectrum showed that *A. japonicum alba* did not have any anthocyanin (Fig. 1.3, dotted-line). *Aerides japonicum* produces flowers with red stripes on a cream sepal and red spots in a white lip (Fig 1.1, left). The spectrum showed that almost all of color is due to anthocyanin (peaked at about 524 nm) (Fig. 1.3, solid-line). Other studies (Arditti, 1992; Arditti and Dunn, 1969) indicated that this anthocyanin was probably a cyanidin-based anthocyanin.

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(2) Cross hybridization to produce alba variation

Variations within species are not unusual among orchids since they are usually cross-pollinated plants. Color variation such as alba

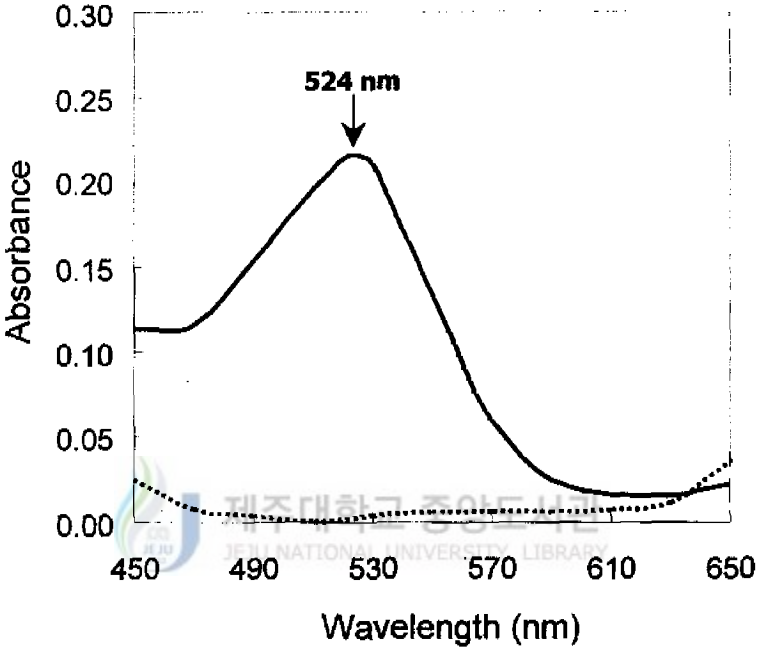

Fig. 1.3. Spectrum from sepals and labellum of *Aerides japonicum* (solid line) and *A. japonicum* alba (dotted line).

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(entirely white) and semi-alba (colored-lip, white sepals and petals) may be found within a single species (Vajrabhaya, 1977).

Aerides japonicum (a *R_*, red colored sepals and lip) when crossed with *R_* red produced only red colored progeny, presumably of the *R_* genogype. *A. japonicum alba* (a *rr*, white sepals and lip) when crossed with *rr* white produced only flowers with white sepals and lips, presumably of the *rr* genotype. The term "alba" was used to describe these type. The cross between F_1 of *A. japonicum* and F_1 of *A. japonicum alba* produced 286 colored and 237 alba hybrids, essentially a 1:1 ratio (Fig. 1.4, 1.5). Therefore, the pigmentation of sepals and labellum in *Aerides japonicum* is controlled by a single dominant gene (*R*) and flower color in this species due to cyanidin-based anthocyanins. The presence of anthocyanins in *Cymbidium* (especially *C. goeringii*) that is similar to color of *Aerides japonicum* is also apparently due to a single dominant gene (*C*) (Lenz and Wimber, 1959; Strauss and Arditti, 1972). Evidence for such a gene has been obtained from a cross between *Cymbidium* Blue Smoke (colored and probably *Cc*) and *Cymbidium* Vale of Cashmir (colored and probably Cc), which produced progeny in the ratio of 3 (1 *CC* and 2 *Cc*, all three colored) : 1 (*cc*, white) (Lenz and Wimber, 1959). And the pigment, especially anthocyanins may be dominant in inheritance of orchids and inherited across generic lines. Anthocyanins present in *Broughtonia sanguinea*

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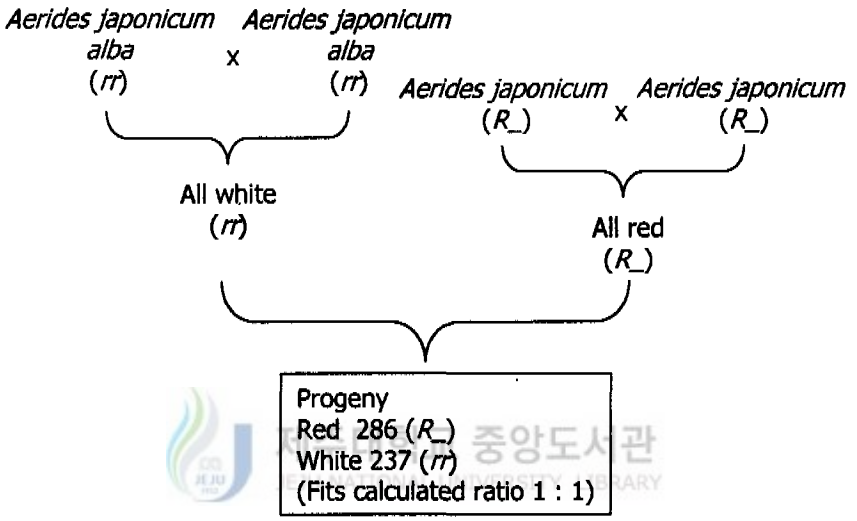

Fig. 1.4. Pedigree of the progeny of *Aerides japonicum* with assigned hypothetical genotypes.

were also detected in its hybrids, but *Cattleytonia* xRosy Jewell contained additional pigments (Arditti, 1969). *Vanda coerulea* contains a blue-mauve anthocyanin R_f 0.12 which may be similar to the one from *Vanda* xProfusion (Sanford et al., 1964). This is interesting since *Vanda* xRothschildiana, the seed parent of *V.* xProfusion, is as hybrid of *V. coerulea*. A new bronze coloration was produced by crossing *Phalaenopsis amboinensis* flowers containing gold carotenoids with *Doritaenopsis* Grebe flowers containing magenta anthocyanins (Griesbach, 1984).

Therefore, it is suggested that the pigment of *Aerides japonicum* flowers is a cyanidin-based anthocyanins and may be inherited through two hybrids dominantly. To obtain *A. japonicum alba* massively, it needs to cross between *A. japonicum alba* themselves and propagate by seeds in vitro.

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(3) SCAR maker for detecting the floral pigmentation

The sequences of the 20-mer SCAR primers obtained by the RAPD primer extension are given in Table 1.2. SCAR primers were synthesized and used in amplification reaction.

PCR amplification with the selected UBC351, UBC375, and

Table 1.2. Sequence-specific primer pairs derived from cloned RAPD bands.

No.[z]	Sequences	Annealing temp(℃)
UBC 351U_{720}	5'-CTC CCG GTG GGG TTG GGA TA-3'	56
UBC 375L_{2030}	5'-CCG GAC ACG AGT ATG TGG GA-3'	58
UBC 396U_{1832}	5'-CGC CCG CAG TTG CAC GGA AA-3'	65

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[z] Underlined sequences are derived from the original RAPD primers. The number and letters preceding the U(upper) and L(lower) and subscript (size of marker in bp) refer to the primer used to generate the marker. UBC = The University of British Columbia.

UBC396 specific primers was performed using the DNA extracted from *Aerides japonicum* and *A. japonicum alba* (Fig. 1.6). The 720 bp fragment of UBC351 primer and the 2030 bp fragment of UBC375 primer were recovered in *A. japonicum*, while there was not recovered any band in that size in *A. japonicum alba*. The 1832 bp fragment of UBC396 pirmer was recovered in *A. japonicum alba*, while there was not recovered any band in *A. japonicum*. As compared with RAPD and SCAR, there is no difference between the profile in the same size (Fig. 1.6). In higher plant, photosynthetic electron transport and CO_2 fixation occur within chloroplasts. Chloroplast DNA has the conserved sequences with the species, and inherited maternally from one generation to next (Kim and Jansen, 1998; Friesen et al., 1999).

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These results suggest that UBC351, UBC375, and UBC396 specific primer pairs can be useful marker to distinguish *Aerides japonicum* from alba.

The identification SCAR maker for progenies of cross between *Aerides japonicum* and alba obtained with $UBC351U_{720}$ primer (Fig. 1.7). The 720 bp fragments was recovered in 9 of 14 randomly selected progenies (Fig. 1.7) and totally 31 of 50 randomly selected progenies, essentially a 1:1 ratio.

The primer pairs of $UBC351U_{720}$ were made from the decamer, UBC 351 primer, and produced the sequence-specific primers according

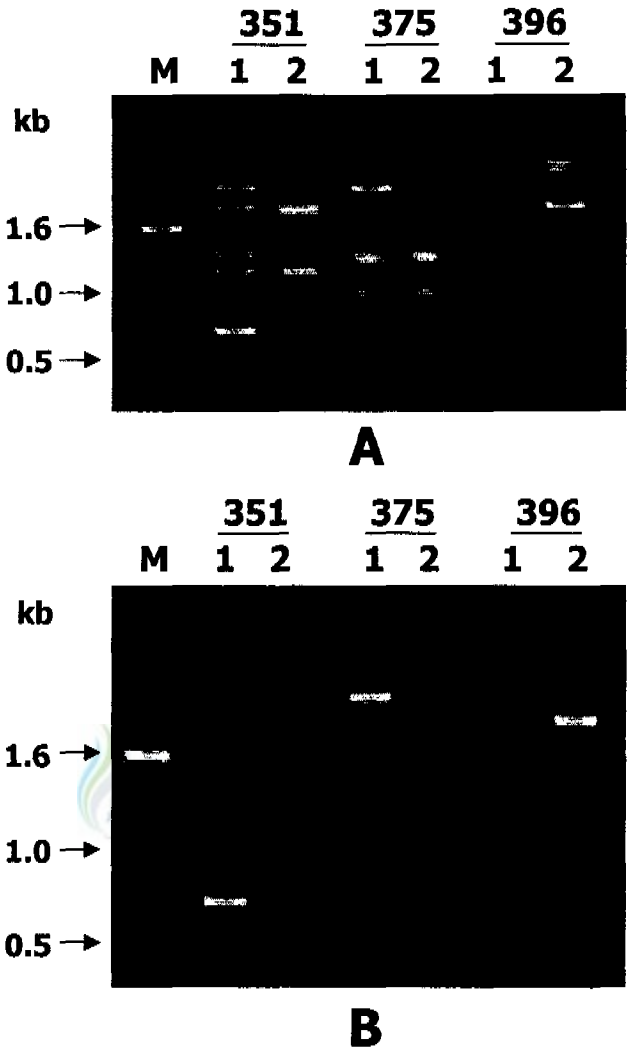

Fig. 1.6. DNA profiles obtained from *Aerides japonicum* (lane 1) and alba (lane 2) with the primers UBC 351, UBC 375, and UBC 396.

M : 1Kb DNA ladder (GIBCO-BRL, Gaithersdurg, Md.).

A : RAPD profiles

B : Selected bands as a marker

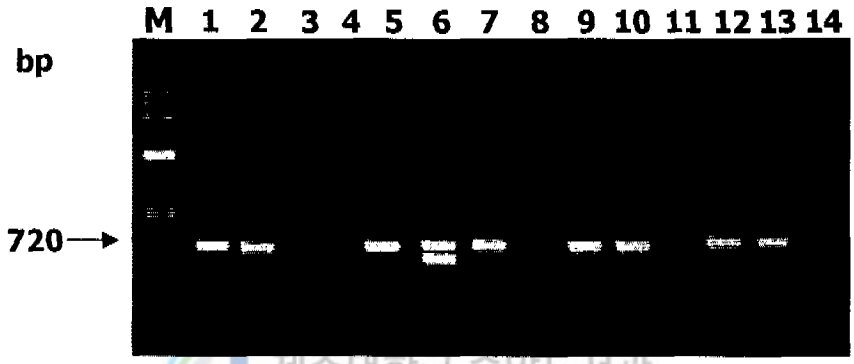

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Fig. 1.7. Amplification products obtained with sequence-specific primers UBC351U720. Lanes are randomly selected progeny DNA which are crossed between *Aerides japonicum* and *A. japonicum alba*.

to their size. In lane 6, the additional band appears in the lower size about 700 bp. Paran and Michelmore (1993) have reported that alleles were amplified from both parents in the basic population so that a dominant RAPD locus was converted into a codominant SCAR locus in several cases. So, this result could be indicated that the amplified bands with UBC351U_{720} specific primer may be located in the different alleles.

Thus the information obtained with SCAR sequence-specific primer pairs is available to identify between colored progeny and alba progeny in the early stage of breeding that will be shorten the breeding period. This result suggests that the inheritance of *Aerides japonicum* flowers pigment may be controlled by a single dominant gene.

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(4) Suppression subtractive hybridization (SSH) to explore the genes of flowers

Total RNA isolation of *Aerides japonicum* was substantially free of DNA and contaminating protein. The yield of total RNAs were determined spectrophotometrically at 260 nm, where 1 absorbance unit (A_{260}) equals 40 μg of single-stranded RNA/mL. The purity was also estimated by spectrophotometry from the relative absorbances at 230, 260, and 280 nm (i.e., A_{260}/A_{280} and A_{260}/A_{230}). The RNAs were shown

an A_{260}/A_{280} ratio of 1.8-2.2. The ratio of 28S to 18S rRNAs were approximately 2:1 by ethidium bromide staining, indicate that no gross degradation of RNA has occured. mRNA was determined spectrophotometrically in the same way as total RNA (Fig. 1.8).

The DNA in which target sequences to be found were called "Red-tester", and "Alba-tester", and the reference DNA was called "driver". First, both DNA samples were digested with a restriction enzyme that generates blunt ends. The tester DNA is then divided into two portions, each of which is ligated to unique adaptors that facilitate suppression PCR.

To determine if a sample was completely digested, electrophoresis was performed 2.5 μL of uncut, ds cDNA and 2.5 μL of Rsa I-digested cDNA on a 1% agarose/EtBr gel in 1X TAE buffer. Double stranded cDNA derived from poly A^+ RNA appears as a smear from 0.5-10 Kb. After Rsa I digestion, the average cDNA size was smaller (0.1-3Kb) and smear completely (Fig. 1.8).

The single round of subtractive hybridization in the PCR-Select technique included two hybridization steps. In the first hybridization step, an excess of driver DNA was added to each population of adaptor-ligated tester. The samples were heat-denatured and allowed to anneal. After several hours, due to the second-order kinetics of hybridization (Milner et al., 1995), ss molecules corresponding to high-

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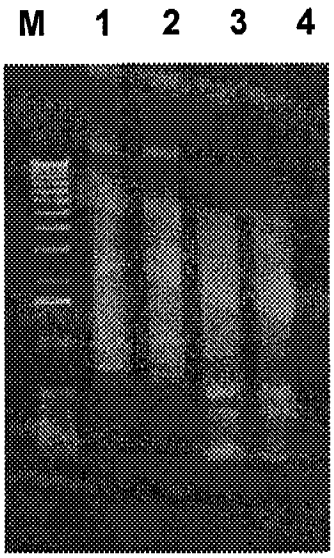

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Fig. 1.8. Result of *Aerides japonicum* ds cDNAs before and after *Rsa* I digestion.

M : 1kb ladder (GIBCO BRL)

Lane 1 and 2 : *Aerides japonicum* and *A. japonicum alba* ds cDNA before *Rsa* I digestion, respectively.

Lane 3 and 4 : *Aerides japonicum* and *A. japonicum alba* ds cDNA after *Rsa* I digestion, respectively.

and low-abundance sequences become equalized. In addition, the ss fraction was enriched for tester-specific sequences, as "common" nontarget DNAs anneal with the driver.

During the second hybridization step, the two primary hybridization samples were mixed together. Since the samples were not heat-denatured, only the remaining equalized and subtracted ss tester DNA molecules were able to reassociate to form a new type of hybrid. These new hybrid molecules were ds tester DNA molecules with different adaptor sequences at their ends. Fresh denatured driver DNA was added to further enrich the differentially expressed sequences.

The entire population of hybridized DNA was then subjected to two rounds of PCR to amplify the desired differentially expressed sequences. In the primary PCR, only the molecules with different adaptors at each end can be exponentially amplified (templates with the same adaptor at both ends will form pan-like structures which were suppressed in PCR). In the secondary PCR, nested primers were used to reduce any background while further enriching the differentially expressed sequences (Fig. 1.9).

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After secondary PCR, the enriched, subtracted DNAs were inserted into a T/A-cloning vector and obtained approximately 300 to 400 white colonies. Differential expression of sequences were appeared various size from 200 to 1500 bp (Fig. 1.10, 1.11).

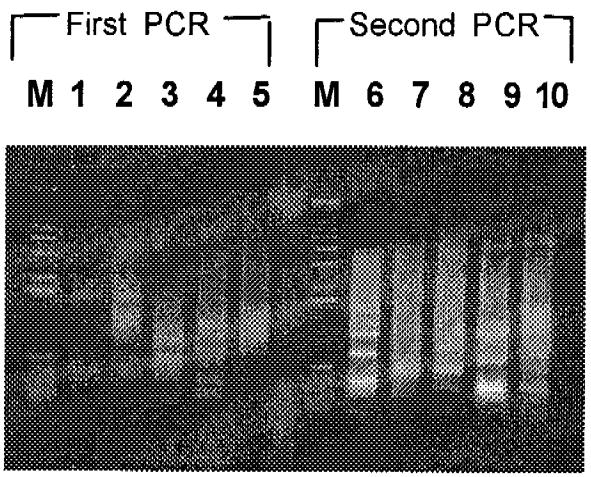

Fig. 1.9. Amplification products generated first (lane 1 to 5) and second (lane 6 to 10) PCR of unsubtract (lane 2, 3, 7, and 8) and subtract hybridization (lane 4, 5, 9, and 10).

M : 1kb ladder (GIBCO BRL)

Lane 1 and 6 : PCR control of substracted skeletal mucle tester

Lane 2 and 7 : unsubtracted common type cDNA digested with *Rsa* I

Lane 3 and 8 : unsubtracted alba type cDNA digested with *Rsa* I

Lane 4 and 9 : subtract hybridization of *Aerides japonicum*

Lane 5 and 10 : subtract hybridization of *Aerides japonicum alba*

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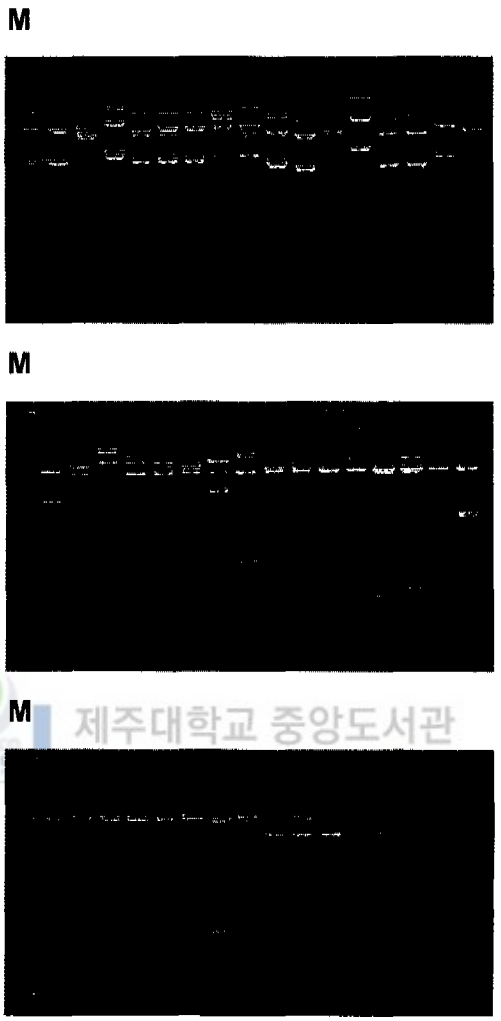

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Fig. 1.10. Restriction enzymes digestion with *EcoR* I of cDNA clones obtained by suppression subtractive hybridization of *Aerides japonicum*.

M : 1kb ladder (GIBCO BRL)

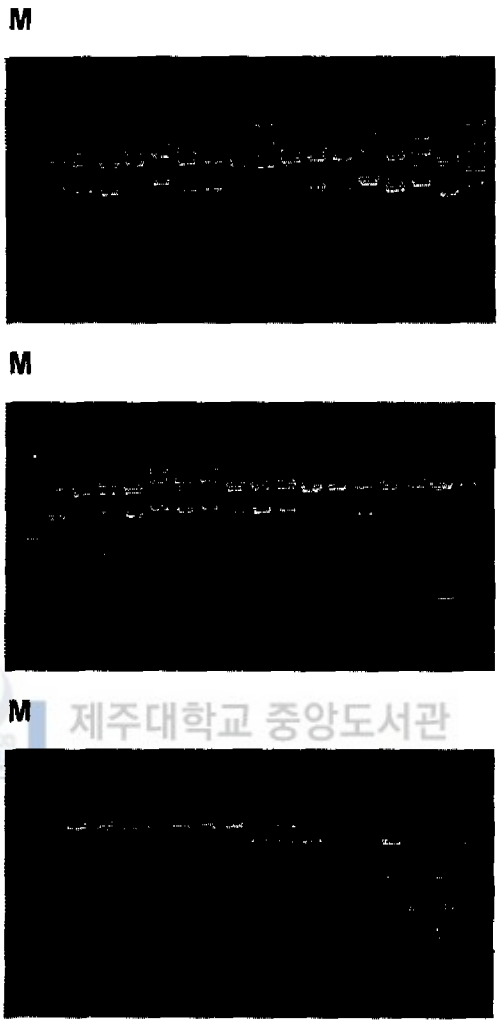

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Fig. 1.11. Restriction enzymes digestion with *EcoR* I of cDNA clones obtained by suppression subtractive hybridization of *Aerides japonicum alba*.

M : 1kb ladder (GIBCO BRL)

The 16 newly generated sequences of *Aerides japonicum* are deposited in the Genbank, DDJB, and EMBL under accession numbers listed in Table 1.3. These genes are all related to pigmentation with one exception of methallothionein-like protein (Fig. 1.12). The occurrence of metallothioneins (MT) or of metallothionein-like metalloproteins (MTL) has been reported for many species of vertebrates and invertebrates (Roesijadi, 1992). These metalloproteins were first discovered and characterised in mammals (Margoshes and Vallee, 1957); they exhibit a low molecular weight (6 to 7 kDa), a high cysteine content with a unique distribution of its residues, an absence of aromatic amino acids, heat-stability and a selective capacity to bind metal ions such as copper, zinc, cadmium and mercury by the formation of tetrahedral metal-thiolate complexes organised in two oligonuclear clusters (Otvos and Armitage, 1980) which are biologically unique (Vallee and Maret, 1993).

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The anthocyanin biosynthetic pathway is remarkably conserved among flower plants. Anthocyanins share a common biosynthetic origin and core structure, with species-specific decoration of the core by hydroxylation, methylation, sugar addition or acylation. These modifications result in the diverse red, blue and purple colors in the vacuoles of flowers, fruits and leaves. Despite the extraordinary chemical diversity of anthocyanins, these cytoplasmically synthesized molecules

Table 1.3. *Aerides japonicum* flower related genes of suppression subtractive hybridizaton (SSH) generated by BLAST analysis.

No.	Genes of suppression subtractive hybridization	cDNA length of cloning (bp)	ORFs (bp)	Genbank Accession No.
1	Non specific lipid transfer protein precursor	439	32-388	AF198157
2	Lipid transfer protein LPT III	363	31-363	AF198160
3	Phospholipid transfer protein precursor	297	62-297	AF198164
4	Phospholipid transfer protein	657	42-311	AF198162
5	Metallothionein-like protein	360	13-132	AF198158
6	DNA binding protein	307	9-307	AF198159
7	Cytoplasmic aldolase	527		AF198161
8	Profilin mRNA	403		AF198163
9	Dormancy-associated protein	342	79-342	AF198165
10	Flavodoxin gene	455	102-455	AF198166
11	Plasma membrane intrinsic protein	470		AF198167
12	Phospholipid transfer protein mRNA	697	65-427	AF198168
13	3-hydroxy-3-methylglutaryl-CoA-synthase	342	27-342	AF198169
14	Germin-like protein gene	255		AF198170
15	Glutathione S-transferase	164		AF198171
16	Pollen specific mRNA	253		AF198172

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sp|O22319|MT2_MUSAC METALLOTHIONEIN-LIKE PROTEIN TYPE 2

Score = 65.6 bits (157), Expect = 5e-11

Identities = 27/39 (69%), Positives = 29/39 (74%)

```
Query: 1   MILGFAPAKGGMEGFEMAGGSENGCKXGSNCTCDPCTCK 39
           MI+G AP KG  E  E A GS+NGCK GSNCTCDPC CK
Sbjct: 40  MIMGVAPQKGHFEELETAAGSDNGCKCGSNCTCDPCNCK 78
```

Fig. 1.12. BLAST analysis of metallothionein-like protein obtained by suppression subtractive hybridization in *Aerides japonicum* flower.

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gb|AF002692.1|AF002692 *Solanum commersonii* glutathione S-transferase, class-phi (GST1) mRNA

Score = 38.2 bits (19), Expect = 1.2

Identities = 22/23 (95%)

```
Query: 104 tatgtattgtgttatggctcttg 126
Sbjct: 273 tatgtattgtgtaatggctcttg 251
```

Fig. 1.13. BLAST analysis of glutathione S-transferase obtained by suppression subtractive hybridization in *Aerides japonicum* flower.

are all ultimately localized to the vacuole (Marrs, 1995). It is result from that anthocyanins are toxic substances that could be harmful to the cell. To cope with this potential problem, anthocyanins are stabilized and detoxified by transport to the vacuole. Recent evidence indicates that glutathionation and active transport of the conjugate by a glutatione S-X pump play a critical role in this process (Marrs et al., 1995) (Fig. 1.13). They showed that the *Bz2* gene of maize encodes a type Ⅲ glutathione S-transferase (GST). *Bz2* mutants lacking this activity accumulate anthocyanins in the cytosol, conferring a tan-bronze phenotype. In this step, several kinds of lipid transfer proteins act on a acceptor to DNA binding protein (Mol et al., 1999) (Fig. 1.14, 1.15, 1.16, 1.17).

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There have been reported that anthocyanins are increased by abscisic acid (Coombe and Hale, 1973; Inaba et al., 1976; Kataoka et al., 1982; Han 1997). ABA appears to act as the dormancy-inducing hormone, as indicated by an observed correlation between levels of endogenous ABA and the physiological state of seed (Taiz and Zeiger, 1991). A role of ABA in dormancy is also indicated by the observation that exogenous ABA prevents germination and induces dormancy (Zeevaart and Creelman, 1988). In this study, dormancy-associated protein (Fig. 1.18) and germin-like protein gene (Fig. 1.19) were found in *Aerides japnicum*, which indicated that they may be related to

sp|P19656|NLTP_MAIZE NONSPECIFIC LIPID-TRANSFER PROTEIN PRECURSOR (LTP) (PHOSPHOLIPID TRANSFER PROTEIN) (PLTP)

>gi|82711|pir||A31779 phospholipid transfer protein 9C2 precursor - maize

>gi|168576 (J04176) phospholipid transfer protein precursor [*Zea mays*]

Score = 53.5 bits (126), Expect = 2e-07
Identities = 28/76 (36%), Positives = 42/76 (54%), Gaps = 1/76 (1%)

```
Query: 2  ALLAMTFIAINTVESAVPCSEVAIKFVSCVGYAQTRQPDAS-GVLQGVKHLNAKAITTDD 60
          A++A+  +A  T E+A+ C +VA     C+ YA+ +    S G   GV+ LN  A TT D
Sbjct: 13 AVVALVLLAAATSEAAISCGQVASAIAPCISYARGQGSGPSAGCCSGVRSLNNAARTTAD 72

Query: 61 RRTVCYCISSLAQQVT 76
          RR  C C+ + A  V+
Sbjct: 73 RRAACNCLKNAAAGVS 88
```

sp|P10976|NLTP_SPIOL NONSPECIFIC LIPID-TRANSFER PROTEIN PRECURSOR (LTP) (PHOSPHOLIPID TRANSFER PROTEIN) (PLTP)

>gi|170117 (M58635) lipid transfer protein [*Spinacia oleracea*]

>gi|228406|prf||1803519A lipid transfer protein [*Spinacia oleracea*]

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Score = 53.1 bits (125), Expect = 2e-07
Identities = 27/74 (36%), Positives = 38/74 (50%), Gaps = 1/74 (1%)

```
Query: 2  ALLAMTFIAINTVESAVPCSEVAIKFVSCVGYAQTRQPDASGVLQGVKHLNAKAITTDDR 61
          A+L    +A    E+ + C  V+ K   C+GY +   P   G   G+K LNA A TT DR
Sbjct: 12 AVLLCIVVAAPYAEAGITCGMVSSKLAPCIGYLKGG-PLGGGCCGGIKALNAAAATTPDR 70

Query: 62 RTVCYCISSLAQQV 75
          +T  C C+ S A  +
Sbjct: 71 KTACNCLKSAANAI 84
```

Fig. 1.14. BLAST analysis of lipid transfer protein precursor obtained by suppression subtractive hybridization in *Aerides japonicum* flower.

gb|AAB70540.1| (AF017360) lipid transfer protein LPT III [*Oryza sativa*]

Score = 73.0 bits (176), Expect = 8e-13

Identities = 42/98 (42%), Positives = 56/98 (56%), Gaps = 1/98 (1%)

```
Query: 4   ATARGLVMALLAMTFIAINTVESAIPCSRWHSVRKLRRLPQHGGQMP-AACCRGVKQLKA 62
            A A+ +++AL+A   +A       AI C + +S           GG  P AACC GV+ LKA
Sbjct: 2   ARAQLVLVALVAALLLAAPHAAVAITCGQVNSAVGPCLTYARGGAGPSAACCNGVRSLKA 61

Query: 63  KAITTDDRRTVCYCIKSLAQQVDGLNQNLLANIPGKCG 100
            A TT DRRT C C+K+ A+ + GLN    A+IP KCG
Sbjct: 62  AASTTADRRTACNCLKNAARGIKGLNAGNAASIPSKCG 99
```

gb|AAB70539.1| (AF017359) lipid transfer protein LPT II [*Oryza sativa*]

Score = 73.0 bits (176), Expect = 8e-13

Identities = 40/101 (39%), Positives = 55/101 (53%), Gaps = 1/101 (0%)

```
Query: 1   MARATARGLVMALLAMTFIAINTVESAIPCSRWHS-VRKLRRLPQHGGQMPAACCRGVKQ 59
            MARA   + +   A+    +T  +AI C + +S V    P+ G    AACC GV+
Sbjct: 1   MARAQLVLVALVAAALLLAGPHTTMAAISCGQVNSAVSPCLSYPRGGSGPSAACCSGVRN 60

Query: 60  LKAKAITTDDRRTVCYCIKSLAQQVDGLNQNLLANIPGKCG 100
            L + A TT DRRT C C+K++A  + GLN    A+IP KCG
Sbjct: 61  LNSAASTTADRRTACNCLKNVAGSISGLNAGNAASIPSKCG 101
```

Fig. 1.15. BLAST analysis of lipid transfer LPT III obtained by suppression subtractive hybridization in *Aerides japonicum* flower.

gi|1498597 (U66105) phospholipid transfer protein [*Zea mays*]

Score = 101 bits (248), Expect = 3e-21

Identities = 54/116 (46%), Positives = 71/116 (60%), Gaps = 2/116 (1%)

```
Query: 3   SATARGLVMALLAMTFIAINTVESAIPCSEVAIKLVSCVGYAQHGGQMPAA-CCRGVKQL 61
           SA A  +V A+L +   A  T E+AI C +V+  +  C+ YA+. G  P+A CC GV+ L
Sbjct: 6   SAVAVAVVAAVLLLA-AAATTSEAAITCGQVSSAIAPCLSYARGTGSGPSASCCSGVRNL 64

Query: 62  KAKAITTDDRRTVCYCIKSLAQQVGGLNQNLLANIPGKCGVDIGYPISLSVDCSKV 117
           K+ A T  DRR  C C+K+ A+ V GLN     A+IP KCGV I Y IS S DCS+V
Sbjct: 65  KSAASTAADRRAACNCLKNAARGVSGLNAGNAASIPSKCGVSIPYTISTSTDCSRV 120
```

Fig. 1.16. BLAST analysis of phospholipid transfer protein precursor obtained by suppression subtractive hybridization in *Aerides japonicum* flower.

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gi|1498597 (U66105) phospholipid transfer protein [*Zea mays*]

Score = 68.7 bits (165), Expect = 8e-12

Identities = 32/75 (42%), Positives = 49/75 (64%), Gaps = 1/75 (1%)

```
Query: 1   MARSTASMAVVCIVSFLLVSGVFREASGTITCGQVVSTLTPCISYIRGDSTLPQ-TCCSG 59
           MAR+ +++AV  + + LL++      +   ITCGQV S + PC+SY RG  + P  +CCSG
Sbjct: 1   MARTQSAVAVAVVAAVLLLAAAATTSEAAITCGQVSSAIAPCLSYARGTGSGPSASCCSG 60

Query: 60  VKKLNALASTSPDRQ 74
           V+ L + AST+ DR+
Sbjct: 61  VRNLKSAASTAADRR 75
```

Fig. 1.17. BLAST analysis of phospholipid transfer protein obtained by suppression subtractive hybridization in *Aerides japonicum* flower.

gi|2995990 (AF053746) dormancy-associated protein [*Arabidopsis thaliana*]

>gi|2995992 (AF053747) dormancy-associated protein [*Arabidopsis thaliana*]

Score = 78.0 bits (189), Expect = 1e-14

Identities = 42/74 (56%), Positives = 54/74 (72%), Gaps = 7/74 (9%)

```
Query: 1   MVLLDKLWDDVLAGPHPDKGLGKLRGKISSKALVINPPVGEGESSGKVYQRSSSLPA--- 57
           MVLL+KLWDDV+AGP PD+GLG+LR KI+++ + I   +GEG SS  V  RS ++PA
Sbjct: 1   MVLLEKLWDDVVAGPQPDRGLGRLR-KITTQPINIR-DIGEGSSSKVVMHRSLTMPAAVS 58

Query: 58  --TPSTPITPVTPR 69
             TP+TP TP TPR
Sbjct: 59  PGTPTTPTTPTTPR 72
```

Fig. 1.18. BLAST analysis of dormancy-associated protein obtained by suppression subtractive hybridization in *Aerides japonicum* flower.

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gb|AF170550.1|AF170550 *Arabidopsis thaliana* germin-like protein 7 (GLP7) gene

Score = 36.2 bits (18), Expect = 4.1

Identities = 21/22 (95%)

```
Query: 107  tgaatatacatattcaacccaa 128
Sbjct: 1594 tgaatatacatatttaacccaa 1615
```

Fig. 1.19. BLAST analysis of germine-like protein gene obtained by suppression subtractive hybridization in *Aerides japonicum* flower.

anthocyanins. Alleman and Kermicle (1993) reported that R gene regulates the timing and tissue-specificity of anthocyanin deposition during maize development. The patterns of somatic variegation of these mutants, resulting from excision of Ds, define a spectrum of phenotypes ranging from sparse to dense variegation. The sparsely variegated mutants produce few germinal revertants but relatively many stable null derivative alleles; densely variegated mutants produce many germinal revertants and few stable null derivatives.

The enzyme 3-hydroxy-3-methylgrutaryl-coenzyme A (HMG-CoA) plays a key role in the synthesis of various sterols and isoprenoids in eykaryotic cells. Strerols are structural components of plasma membranes, whereas terpenoids are involved in various functions including respiration, glycosylation and signal transduction (Goldstein and Brown, 1990). The isoprenoid pathway of plants is characgterized by its diversity, which leads to the synthesis of various molecules such as phytohormones (gibberellins and abscisic acid) and secondary metabolites (aromatic terpenoids and phytoalexins) (Montamat et al., 1995). There are two alternative biosynthetic pathways for ABA. In the indirect pathway, ABA is derived from a 40-carbon compound, violaxanthin. In the direct pathway, ABA is derived from a 15-carbon precursor, farnesyl pyrophosphate which are derived from mevalonic acid. In the mevalonate pathway, the conversion of acetyl-CoA to HMG-CoA is

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mediated by HMG-CoA synthase (Fig. 1.20) (Taiz and Zeiger, 1991). The conversion of HMG CoA to mevalonic acid by HMG CoA reductase and the regulation of the genes encoding this enzyme have been implicated in the network that controls isoprenoid biosynthesis in higher plants (Learned and Connolly, 1997).

Many biosyntheses of secondry metabolites by plant cell suspensions are enhanced by light irradiation (Bjöerk, 1986). Light acts as a signal for inducing production, different from photosynthesizing microbes utilizing light as energy. Anthocyanin production depends on light intensity (Kurata et al., 2000). Anthocyanin is synthesized through a dozen reaction steps, where several enzymes including chalcone synthase (CHS), the first enzyme for anthocyanin production, are activated by light, following several-hour-phase (Feinbaum adn Ausubel, 1988). Falvodoxins (Fig. 1.21) are a group of small flavoproteins which function as low-potential one-electron carries and contain a non-covalently bound flavin mononucleotide (FMN) cofactor (Mayhew and Tollin, 1992), which are related to photosystem I and electron transfer.

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All the biochemical processes that determine form and function (phenotype) of plants are the result of information encoded within the DNA sequence of the genome and the interaction of that information with the environment. This information is converted into biochemical

emb|CAA65250| (X96386) 3-hydroxy-3-methylglutaryl-CoA-synthase [*Pinus sylvestris*]

Score = 196 bits (494), Expect = 3e-50

Identities = 92/103 (89%), Positives = 98/103 (94%)

```
Query: 1   MAFCTELEDVISMSLTVVTSLLEKYQIDPKQIGRLEVGSETVIDKSKSIKTWLMRIFEEY 60
           M FCT+LEDVISMSLT VTSLLEKY+IDPKQIGRLEVGSETVIDKSKSIKTWLM IFE+
Sbjct: 47  MTFCTDLEDVISMSLTAVTSLLEKYEIDPKQIGRLEVGSETVIDKSKSIKTWLMHIFEKC 106

Query: 61  GNTDIEGVDSTNACYGGTAALFNCVNWVESSSWDGRYGLVVCT 103
           GNT+IEGVDSTNACYGGTAALFNC+NW+ESSSWDGRYGLVV T
Sbjct: 107 GNTEIEGVDSTNACYGGTAALFNCINWIESSSWDGRYGLVVAT 149
```

Fig. 1.20. BLAST analysis of 3-hydroxy-3-methylglutaryl CoA synthase obtained by suppression subtractive hybridization in *Aerides japonicum* flower.

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pdb|1FVX| *Clostridium* Beijerinckii Flavodoxin Mutant: G57n Oxidized

Score = 24.7 bits (52), Expect = 3.8

Identities = 11/31 (35%), Positives = 15/31 (47%)

```
Query: 28 LEQRLLMRLIEEPSELLKGSSLCYVGSFSWG 58
          LE+      IEE S  + G  +   GS+ WG
Sbjct: 61 LEESEFEPFIEEISTKISGKKVALFGSYGWG 91
```

Fig. 1.21. BLAST analysis of flavodoxin gene obtained by suppression subtractive hybridization in *Aerides japonicum* flower.

activity and the resulting macromolecular structure of the plant through biosynthesis of specific enzymes or proteins via transcription and translation. In other words, all of the morphology and physiology of plants is based on metabolic processes, and in turn, all of the metabolic processes are the result of the conversion of genetic information into the enzymes and proteins that control metabolism (Salisbury and Ross, 1992).

Complex multicellular eukaryotes differentiate irreversibly so that different cell types express a different profile of genes. Genes that are expressed are usually associated with swollen chromatin. Proteins found in active regions of the genomes show characteristic modification. The primary sequences in the noncoding regions of eukaryotic DNA contains (1) signals for the binding of RNA polymerase, (2) gene regulatory proteins, (3) transcription starts sites, and (4) potential coding regions for small peptides. In addition to signals for regulatory protein binding in the immediate vicinity of the polymerase binding site, there are other signals, which bind at some distance from the promoter, that have a major influence on gene expression. A mumber of unique regulatory problems must be confronted in higher plants (1) the arrangement of DNA in the nucleus must be efficiently disposed so that those few genes (probably less than 1%) that are expressed in any particular cell type are accessible, (2) as the embryo develops, new genes are

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expressed and already expressed gene in some cases are turn off. Development must be precisely timed and spatially organized so that different cell types are produced in the appropriate numbers and orientation relative to one another, and (3) finally, in the developing and the adult organisms, intercellular communication is essential to coordinate the activities among different cells (Zubay, 1993).

These results obtained from SSH in this study did not show exact genes controlling anthocyanin biosynthesis such as flavonoid-3',5'-hydroxylase in petunia (Shimada et al., 1999) and dihydroflavonol 4-reductase in *Forsythia* flower (Rosati et al, 1998). However, these may be suggested (1) genes related to anthocyanins and (2) relative possession of specific genes in quantity between *Aerides japonicum* and *A. japonicum alba*.

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In conclusion, these results suggest that the pigment of *Aerides japonicum* flowers is a cyanidin-based anthocyanins, and expressed by some genes related to anthocynin biosynthesis such as glutathione S-transferase, dormancy-associated protein, lipid transfer proteins, germin-like protein gene and 3-hydroxy-3-methylglutaryl-CoA synthase. They are inherited through two hybrids dominantly. To obtain *A. japonicum alba* massively, it needs to cross between *A. japonicum alba* themselves and propagate by seeds in vitro.

CHAPTER 2 Chimeral Variegation through Mutagenesis of Korean Native *Cymbidium*

ABSTRACT

The present works were performed on *Cymbidium goeringii* and *C. kanran* in relation to chimeral variegations. In this study, mutagenesis for inducing various linear chimeras, microscopic observation, and characteristics of genetic relationship to chimeras using RAPD analysis were also investigated.

Survival rates of *Cymbidium* rhizome treated with the mutagen were declined as time elapsed, while the percent of mutant occurrence was increased. 3,000 mg/L EMS for 2 hours was regarded to be the level of LD_{50}. Cuttings into pieces of rhizome were shown as a higher percentage of variegated shoots than the control, and 500 ㎛ thickness was the least size to permit survival and variegation.

When rhizome appeared to be lighter in color (greenish to light green, yellowish, and whitish), greater extent of variegated variants could be expected than respective opposites in the ordinary green. There

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was a positive correlation between the chlorophyll content of rhizomes and that of leaves. Number of stomates per unit area was less in the variegated portion than the normal.

Primarily, mutated rhizomes induced margined (white- and yellow-) and medioalbinated (white- and yellow-) leaves. During the period of subculture, an albino was obtained from white-margined leaves by the replacement of layers, while a normal green leaf was obtained from yellow-margined, white-/yellow-medioalbinated leaves.

RAPD analysis showed mutually identical DNA pattern between induced chimera and normal green plant restored by replacement of layers.

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INTRODUCTION

The genus *Cymbidium* is native to southeastern Asia and found in Korea, Chana and Japan (Arditti, 1992). C. goeringii and C. kanran are very popular in Korea because they have specific characteristics. The former blooms in early spring and the latter is in autumn or winter. Cultivation of this geneus inculding *C. goeringii* and *C. kanran* have been known to originated mostly from those collected from natural

habitats. Especially, variegated cymbidums are very favorite and too expensive because they are rare in nature. Thus it is need to produce artificially and massively.

Ethyl emthansulfonate (EMS) is probably one of the most effective chemical mutagens for use on higher plants. It gives a high percentage of mutation with little damage to chromosomes.

RAPD has been used in several taxonomic studies at the specific level and genetic relationship. In comparison with others, RAPD procedure is less expensive, faster, requires a smaller amount of DNA (0.5 to 50.0 ng), does not involve the use of radioisotope, and requires less skill to operate. Because of these advantages, RAPDs have proven to be useful in genotype identification and gene mapping.

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The present works were performed on *Cymbidium goeringii* and *C. kanran* in relation to its chimeral variegation. In this study, the mutagenesis for inducing various linear chimeras by the alkylating agent, ethyl methanesulfonate (EMS), the morphological characteristics by microscopic observation, phenomena of replacements during the period of subculture, and characteristics of genetic relationship of chimeras using randomly amplified polymorphic DNA (RAPD) analysis were also investigated.

MATERIALS AND METHODS

(1) Mutagenesis for inducing variegated mutants

Ethyl methanesulphonate treatment

Rhizomes of *Cymbidium goeringii* and *C. kanran* were selected to be an uniform size with 1.0 cm and prepared a water bath so that the EMS treatment can be performed under constant temperature conditions. EMS treatments were carried out under the following treatment conditions:

Concentration	3,000 mg/L solution
Temperature	30-35℃ (in water bath)
Time	0.5～4 hours

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A thorough post-wash in sterile water after the EMS treatment was carried out to have full control over post-treatment effects (Mikaelsen, 1961).

Cuttings of mutagen treated rhizomes

The EMS-treated rhizomes were cut by vibratome with 100, 250, 500, 750, and 1,000 ㎛ thickness, respectively and cultured in vitro. As a control, 1 cm rhizomes (which are not cut) were used.

Twenty replications were set per treatment with ten samples per replication. Aseptic condition was established during the procedures.

In vitro culture

Basal culture medium consisted of Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) supplemented with 0.1 mg/L NAA, 0.1 mg/L BA, 1 g/L polyvinylpyrrolidone, and 40 g/L sucrose (basal medium). The medium was adjusted to pH 5.7 prior to autoclaving and gelled with 2.5 g/L gelrite (So et al, 1998). Cultures were maintained in a growth chamber at 25±2℃ under cool-white fluorescent light (1.5 klux) from 0800 to 2400 $_{HR}$ and in darkness from 2400 to 0800 $_{HR}$. Numbers of chlorophyll chimeras and the degree of variegation were determined after shoots appeared. Chimeral arrangements were determined by comparing the patterns of variegation with strains studied by Miller et al. (1980) and with those of other known plastome mutants (Tilney-Bassett, 1986;).

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Relation between rhizome color and the degree of variegation of seedlings

To elucidate the relation between rhizome color and the degree of variegation of seedlings obtained from EMS treatments, a numerical color density scale was assigned by optical panel, corresponding to the

green coloration of rhizomes as follows (Fig. 2.1):

0 : Albino, completely white

1 : White rhizome striped with green

2 : Yellowish rhizome dotted with green

3 : Greenish rhizome with some yellowish par.

4 : Green rhizome

(2) Morphological characteristics of variegated mutants

Chlorophyll contents of chimeral variegated leaves and rhizomes

Plant materials

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Examination of chlorophyll content was conducted to elucidate relationship between the chlorophyll content of rhizome and variegation of shooting. Plant materials were obtained from healthy leaf and rhizome of albino, white-medioablinated, yellow-medioalbinated, white-margined, yellow-margined, and normal green leaf and rhizome of *Cymbidium goeringii* and *C. kanran*, respectively (Fig. 2.2).

Plant tissue storage

0.2 g (fresh weight) samples were placed into light proof test tube with 10 mL *N,N*-dimethylformamide (DMF), and established the 12

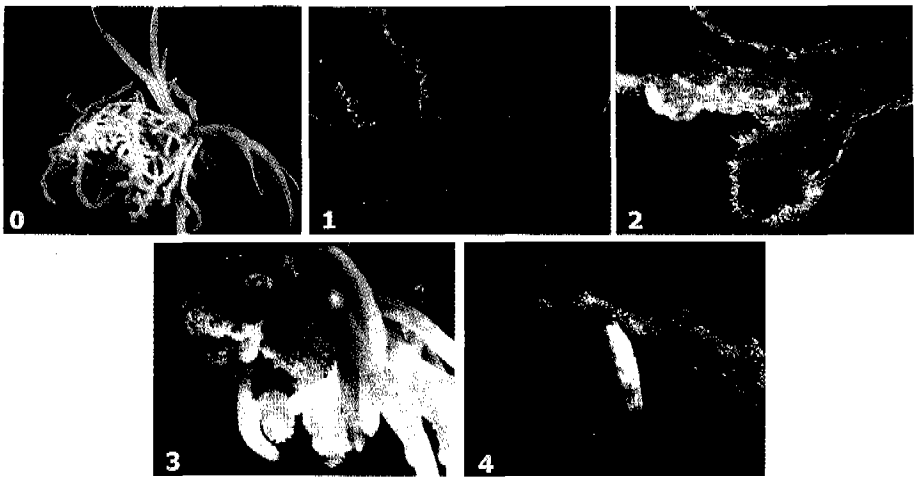

Fig. 2.1. Color scoring of rhizome used for estimating the occurrence of variegated shooting of *Cymbidium*.
0 : Albino
1 : White rhizome striped with green
2 : Yellowish rhizome dotted with green
3 : Greenish rhizome with some yellowish part.
4 : Normal green rhizome

Fig. 2.2. Linear chimeral leaves and rhizome of *Cymbidium*. →

treatment combinations, each treatment contained five replications. After 2 days later (at 5℃), each tube were shaken upright on a voltex and read absorbance at 664.5 nm for chlorophyll a and at 647 nm for chlorophyll b in 1.00 cm cuvettes on the Pharmacia Biotech spectrophotometer. The simultaneous equations necessary for quantifying Chl *a*, Chl *b*, or total Chl in DMF in the absence of other chlorophyllous pigments are: Chl a = $12.70A_{664.5}$ - $2.79A_{647}$; Chl b = $20.70A_{647}$ - $4.62A_{664.5}$; total Chl = $17.90A_{647}$ + $8.08A_{664.5}$, where A = absorbance in 1.00 centimeter cuvettes and Chl = milligrams per liter (Inskeep and Bloom, 1985).

Characteristics of number of stomata between medioalbinated and normal green leaves

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Microscopic observation was carried out on the lower (abaxial) surface of leaf to show the difference of number of stomata between normal green and medioalbinated leaves.

Fig. 2.2. (continued) From left,
Albino shoot and white rhizome of *Cymbidium goeringii.*
White-medioalbinated shoot and rhizome of *Cymbidium goeringii.*
Yellow-medioalbinated shoot and yellowish rhizome of *Cymbidium kanran.*
White-margined shoot and green striped white rhizome of *Cymbidium goeringii.*
Yellow margined shoot and yellowish green rhizome of *Cymbidium kanran.*
Normal green shoot and green rhizome of *Cymbidium goeringii.*

Microscopic observation of variegated leaves

Various variegated leaves of *Cymbidium* which are periclinal plastid chimeras included cultivars from 2 species (Table 2.1). These chimeras were stable in that they present a repeated, similar pattern of green and white tissue along the length of a shoot. It was determined the make-up of each of these chimeras by microscopic examination of transections of leaf in free hand and vibratome. The convention of designating the outer apical layer L-I, the second layer L-II, etc., was followed. The genetic potential for normal chloroplastid development was indicated by G and lack of normal potential by W since the most common mutation was a suppression of chlorophyll development resulting in white tissue.

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(3) Genetic characteristics of variegated mutants using the randomly amplified polymorphic DNA (RAPD) analysis

Plant materials

Leaves and/or rhizomes of *Cymbidium goeringii* and *C. kanran* that differently express in the same clones were used for explaining the

Table 2.1. The genetic potential for chloroplast development of the apical layers of some *Cymbidium* plastid chimeras.

Phenotypic expression	Apical layer		
	L-I	L-II	L-III
Cymbidium goeringii alba	W[z]	W	W
Cymbidium goeringii white-margined	W	W	G
Cymbidium goeringii white-medioalbinated	W	G	W
Cymbidium goeringii green	G	G	G
Cymbidium kanran yellow-margined	G	W	G
Cymbidium kanran yellow-medioalbinated	G	G	W
Cymbidium kanran green	G	G	G

[z] G indicates potential for formation of chloroplasts. W indicates no chloroplast potential.

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genetic difference between them such as albino and white-margined leaf, yellowish-medioalbinated and green leaf, respectively (Table 2.2).

DNA isolation

Genomic DNA was isolated from plant materials using PVP, SDS, and chloroform extraction (Kim et al., 1997). The DNA samples had $OD_{260}/ OD_{280} \fallingdotseq 1.8$ and $OD_{260}/OD_{230} \fallingdotseq 1.8 \sim 2.0$.

Randomly amplified polymorphic DNA (RAPD)

Reaction mixtures in 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 0.1% Triton X-100, 37.5 μg/ml BSA and 1.5mM $MgCl_2$ contained 0.018 units/μL Taq DNA polymerase (ABGENE, England); 100 μM dNTP mix (dATP, dTTP, dGTP, and dCTP); 0.2 μM primer; and about 5 to 10 ng total DNA in a final volume of 20 μL, respectively. Reaction mixture was overlayed with mineral oil (Sigma Co., USA), mixed and centrifuged for 2 min at $2 \times 10^3 \times$g. RAPD primers were obtained from the University of British Columbia (Table 2.3).

Amplification of genomic DNA was performed in a PCR system PTC-100 thermal controller (MJ Research, INC., USA) programmed for 5 min at 95℃ (initial denaturation) and 45 cycles of 15 sec at 94℃ (denaturation), 60 sec at 37℃ (annealing), 90 sec at 72℃ (extension), followed by 10 min at 72℃ (final extension). Reaction products were

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Table 2.2. The 6 accessions represented cymbidiums (*Cymbidium goeringii* and *C. kanran*) variegated type and scientific name used for RAPD analysis.

No.[z]	Variegated type	Scientific name
1	Albino	*Cymbidium goeringii*
2	White-margined	*Cymbidium goeringii*
3	Yellowish-medioalbinated	*Cymbidium goeringii*
4	Green	*Cymbidium goeringii*
5	Yellowish-medioalbinated	*Cymbidium kanran*
6	Green	*Cymbidium kanran*

[z] No. 1 and 2, No. 3 and 4, and No. 5 and 6 are from the same clones, respectively.

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Table 2.3. The list of 50 random primers used in RAPD for chimeral variegated *Cymbidium goeringii* and *C. kanran*.

No.[z]	Sequences	GC content (%)
301	5'-CGGTGGCGAA-3'	70
305	5'-GCTGGTACCC-3'	70
306	5'-GTCCTCGTAG-3'	60
307	5'-CGCATTTGCA-3'	50
308	5'-AGCGGCTAGG-3'	70
309	5'-ACATCCTGCG-3'	60
310	5'-GAGCCAGAAG-3'	60
314	5'-ACTTCCTCCA-3'	50
315	5'-GGTCTCCTAG-3'	60
316	5'-CCTCACCTGT-3'	60
318	5'-CGGAGAGCGA-3'	70
319	5'-GTGGCCGCGC-3'	90
320	5'-CCGGCATAGA-3'	60
321	5'-ATCTAGGGAC-3'	50
324	5'-ACAGGGAACG-3'	50
325	5'-TCTAAGCTCG-3'	50
326	5'-CGGATCTCTA-3'	50
327	5'-ATACGGCGTC-3'	60
329	5'-GCGAACCTCC-3'	70
330	5'-GGTGGTTTCC-3'	60
331	5'-GCCTAGTCAC-3'	60
335	5'-TGGACCACCC-3'	70
337	5'-TCCCGAACCG-3'	70
339	5'-CTCACTTGGG-3'	60
340	5'-GAGAGGCACC-3'	70

[z] Accession number of UBC(the University of British Columbia) primer set.

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Table 2.3. (continued)

No.[z]	Sequences	GC content (%)
341	5'-CTGGGGCCGT-3'	80
342	5'-GAGATCCCTC-3'	60
345	5'-GCGTGACCCG-3'	80
346	5'-TAGGCGAACG-3'	60
347	5'-TTGGCGAACG-3'	60
349	5'-GGAGCCCCCT-3'	80
350	5'-TGACGCGCTC-3'	70
351	5'-CTCCCGGTGG-3'	80
355	5'-GTATGGGGCT-3'	60
356	5'-GCGGCCCTCT-3'	80
358	5'-GGTCAGACCT-3'	80
359	5'-AGGCAGACCT-3'	60
360	5'-CTCTCCAGGC-3'	70
361	5'-GCGAGGTGCT-3'	70
362	5'-CCGCCTTACA-3'	60
363	5'-ATGACGTTGA-3'	40
364	5'-GGCTCTCGCG-3'	80
366	5'-CCTGATTGCC-3'	60
368	5'-ACTTGTGCGG-3'	60
369	5'-GCGCATAGCA-3'	60
370	5'-TCAGCCAGCG-3'	70
372	5'-CCCACTGACG-3'	70
373	5'-CTGAGGAGTG-3'	60
374	5'-GGTCAACCCT-3'	60
377	5'-GACGGAAGAG-3'	60

[z] Accession number of UBC(the University of British Columbia) primer set.

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resolved by electrophoresis in 1.4% agarose gels and visualized by ethidium bromide staining. Discrete and visible DNA segments on the gels were recorded by the particular size based on a sized 1 Kb DNA marker (GIBCO BRL/Life Technologies,Gaithersburg, MD, USA). The RAPD PCR analysis for each condition was at least twice.

Analysis of RAPD profiles

Polymorphic bands were scored as 1 (band present) or 0 (band absent). Data generated from RAPD analysis was analyzed using the Nei similarity index (Nei and Li, 1979), according to the following equation: Similarity = $2N_{XY}/(N_X+N_Y)$, where N_{XY} = number of scored amplification fragments with the same molecular weight shared between genotypes X and Y; N_X = number of scored amplification fragments in genotype X, and N_Y = number of scored amplification fragments in genotype Y. A phenogram was constructed based on the similarity matrix data by applying unweighted pair group method with arithmetic averages (UPGMA) cluster analysis using the NTSYS program (Exeter Software, Setauker, N.Y.).

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RESULTS AND DISCUSSION

(1) Mutagenesis to induce a variegated mutant

Genetic mosaics can occur spontaneously or be induced by mutagens. In either case, mutant cells can be present in organs, such as petals or leaves, or in the shoot apical meristem. When present in the meristem, the mutant cell lines can continue to contribute to organs as they develop (Marcotrigiano and Morgan, 1988). In this study, the survival rates of *Cymbidium* rhizome treated with 3,000 mg/L EMS for 0, 30, 60, 120, 180, and 240 minutes were declined as time elapsed (Fig. 2.3). 3,000 mg/L EMS for 2 hours represented a lethal dosage 50 (LD_{50}) (Fig. 2.3). The percent of mutant occurrence was increased as time elapsed. In cutting treatments to increase the effect of mutagen, cut into pieces of rhizome resulted in a higher percentage of variegated shoots than without cutting as a control (Table 2.4). The first reason that the vigor of normal sectors which are not mutated may be stronger than that of mutated part. The second, the larger size of not mutated sectors are distributed per unit area, the more normal shoots occur. The studies carried out to estimate the size of the mutated sectors in barley (Eriksson, 1965) that showed small sectors appeared frequently, especially following EMS treatment. In this study, it is suggested that 500 ㎛ thickness is the least size to permit survival and variegation.

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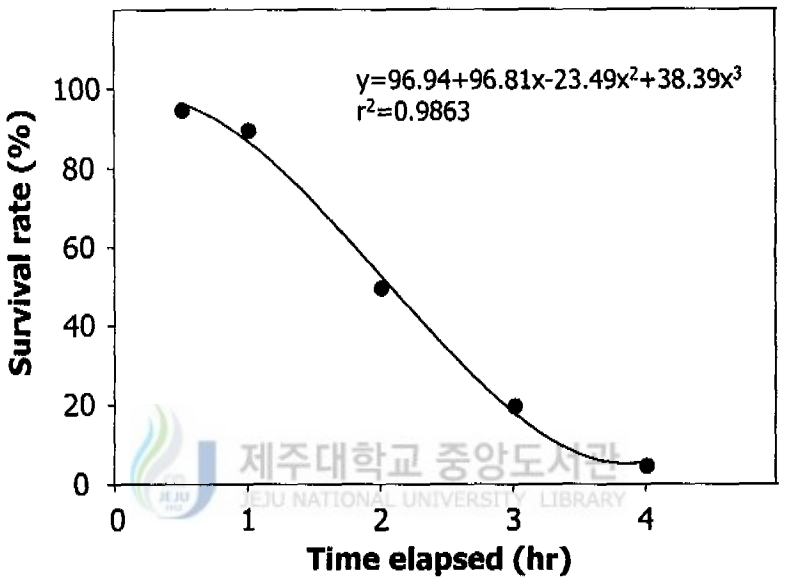

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Fig. 2.3. Relation between survival rate and time elapsed after 3,000 mg/L EMS treatment of *Cymbidium* rhizomes.

Table 2.4. The effect of cuttings of the EMS treated rhizome on the degree of variegation of seedlings (%).

Thickness (㎛)	Time elapsed (hour)				
	0.5	1.0	2.0	3.0	4.0
100	0	0	0	0	0
250	0	0	5	0	0
500	0	10	50	10	0
750	0	15	60	15	0
1,000	0	20	70	10	10
Control (1 cm)	0	10	30	20	10

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Lee et al. (1998) reported the induction of chloropyll deficient mutant plant of *Cymbidium kanran* by EMS treatment. They cultured rhizomes in a liquid growth medium containing 0.2% EMS for three weeks.

In this study, it is suggested that 3,000 mg/L EMS for 2 hour treatment and cutting into piece of rhizome ranging from 500 to 1,000 ㎛ are effective in inducing variegated cymbidiums.

(2) Morphological characteristics of variegated mutants

When rhizome appeared to be lighter in color (greenish to light green, yellowish, and whitish), greater extent of variegated variants could be expected than respective opposites in the ordinary green (Fig. 2.4). According to chlorophyll analysis, the lighter colors of leaves and rhizomes, the less the content of chlorophyll (Fig. 2.5, 2.6). There was a positive correlation between the content of rhizomes and that of leaves (Fig. 2.7). These results suggest that variegation of shoots may be occurred from the mutated rhizome in which genetic mosaics was taken place by a chloroplast DNA mutation. Leaf variegation is a chimera that usually involves chlorophyll production (Vajrabhaya, 1977). The amount of chlorophyll varies from are to area. In one case the

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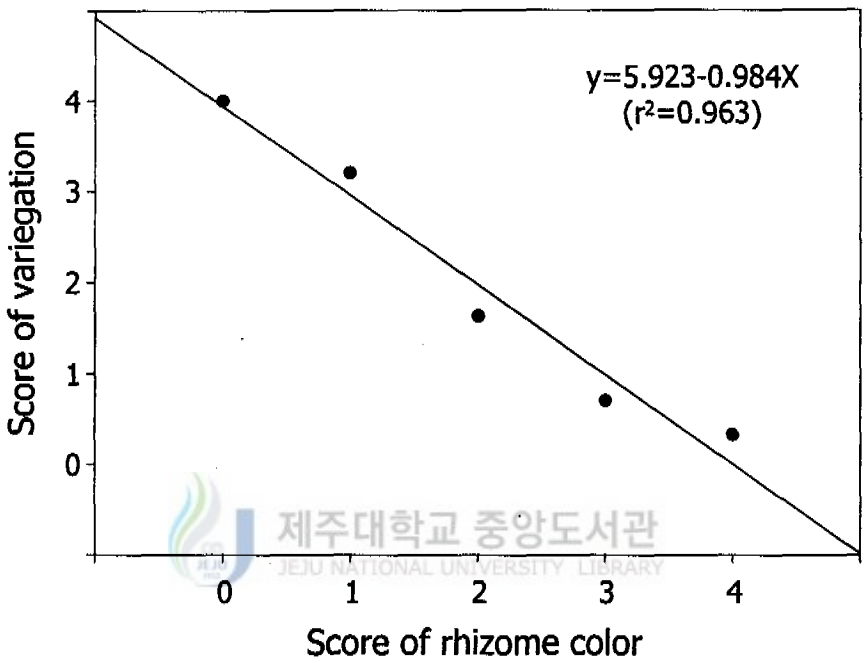

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Fig. 2.4. Relationship between the score of rhizome color and degree of variegation in *Cymbidium*.

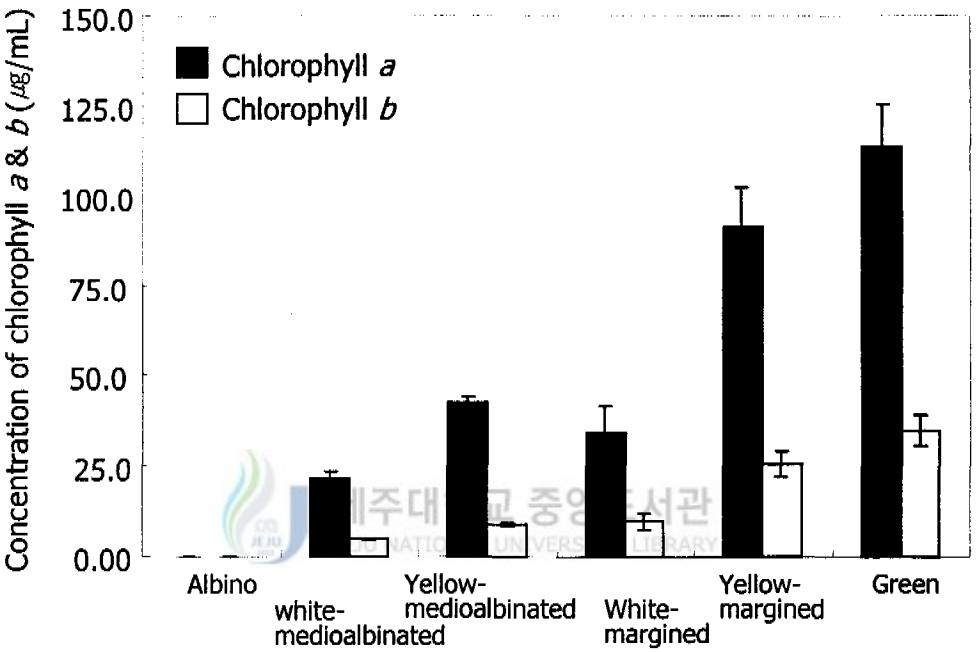

Fig. 2.5. Comparison of chlorophyll content of leaves according to the chimeral variegation of *Cymbidium*. Vertical bars represent SE of mean.

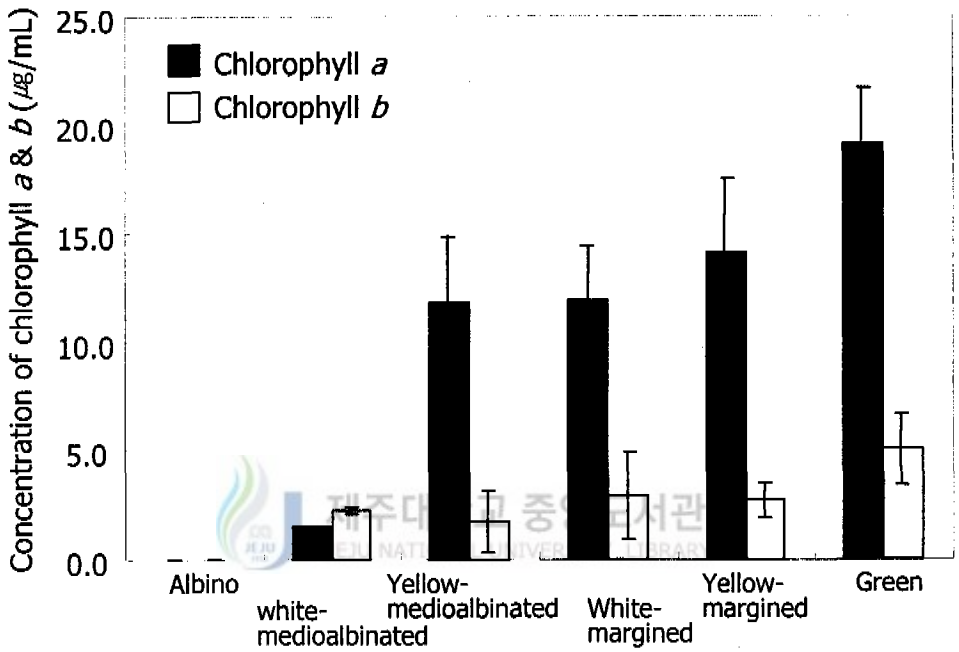

Fig. 2.6. Comparison of chlorophyll content of rhizomes according to the chimeral variegation of *Cymbidium*. Vertical bars represent SE of mean.

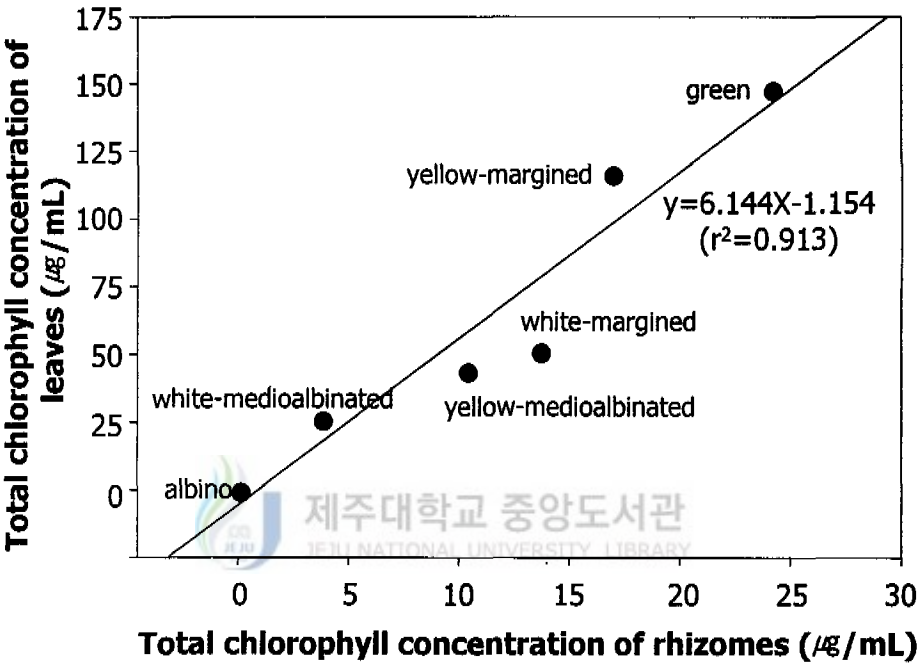

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Fig. 2.7. Relationship between total chlorophyll content of rhizomes and leaves of chimeral variegated *Cymbidium*.

variegation of *Brassocattleya* xLanguedoc 'Singapore Welcome' was not limited to leaves and stem but extended to the flowers as well, which were striped purple and white. This means that cells in the mutated regions were unable to produce enough chlorophyll or other pigments. A plant chimera is a specific type of genetic mosaic (individuals with cells of different genotype) in which the genetically dissimilar cells are present in the shoot apical meristem, where they continue to give rise to the cells that form the body of the plant (Marcotrigiano, 1997). The cells of one color are clonally related, so that each colored layer, streak, or patch is descended by successive cell divisions from the original cell in which the color change occurred. Chimeral variegation patterns are related directly to cell division planes, and patch (clone) size and shape is related to the duration and rate of cell division, and, to a lesser extent, to differences in the magnitude of cell elongation.

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Number of stomata per unit area (1.0 ㎟) was less in the variegated portion (see Fig. 2.2, 2.16, 2.21) than the normal in *Cymbidium goeringii* and *C. kanran* (Fig. 2.8, 2.9). Number of stomata per unit area of *C. goeringii* was more than that of *C. kanran.* This may be due to the difference of species between them. Gas exchange (mainly transpiration and uptake of carbon dioxide) between the atmosphere and the interior of the leaf occurs through the stomata (Taiz and Zeiger, 1991). Their number and distribution patterns can vary

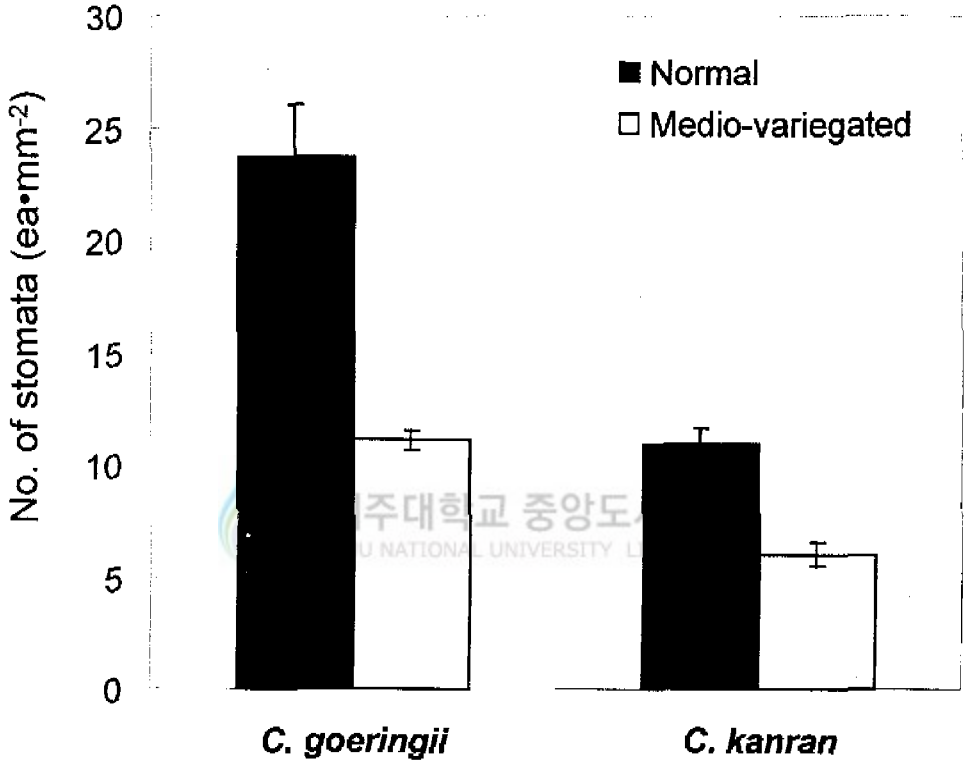

Fig. 2.8. Comparison of the number of stomata in normal and chimeral medio-variegated leaf of *Cymbidium goeringii* and *C. kanran*. Vertical bars represent SE of mean.

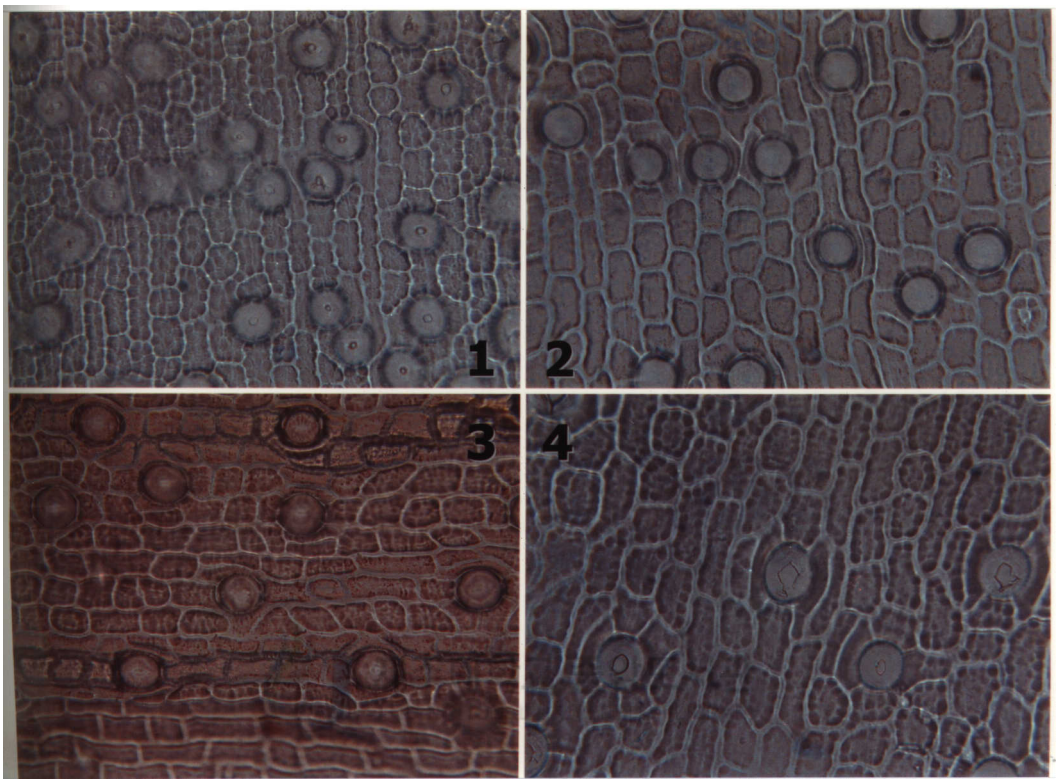

Fig. 2.9. Distribution of stomata on the lower (abaxial) surface of the leaf.

1 : Normal green leaf of *Cymbidium goeringii*

2 : Medioalbinated leaf of *Cymbidium goeringii*

3 : Normal green leaf of *Cymbidium kanran*

4 : Medioalbinated leaf of *Cymbidium kanran*

considerably between and within genera and may be related to leaf thickness and adaption to ecological conditions (Arditti, 1992). Stomata are found on virtually all green parts of a plant, especially the leaves and stems. On the leaves, they are typically more abundant on the abaxial surface, with the upper surface having fewer or even none (Mauseth, 1988). Typically, each square millimeter of leaf surface has about 100 stomates, but the number can be ten times that (Ting 1982), with a maximum so far recorded of 2,230 (Howard, 1969). The figures calculated for orchids are within the lower part of this range (40-110/㎟, Singh and Singh, 1974; 8-180/㎟, Avadhani et al., 1982).

Observations of the presence of absence of chloroplasts in epidermal cells and mesophyll cells showed that all the variegated *Cymbidium* obtained from mutagenesis listed in Table 2.1.

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An albino (W-W-W) seedlings (Fig. 2.10) arose from white-margined *Cymbidum goeringii* leaf (W-W-G) (Fig. 2.13). This leaves and rhizomes were not shown any chloroplast in the cells (Fig. 2.11, 2.12). Albino orchids grow slowly and develop into individuals which look very much like normal plants except for the color, but they have never reached maturity. However, these plants should be able to grow indefinitely if supplied with the proper nutrients in vitro.

A white-margined (W-W-G) *Cymbidium goeringii* (Fig. 2.13) was obtained from EMS treated rhizomes (Fig. 2.1-1). "Marginata" is a

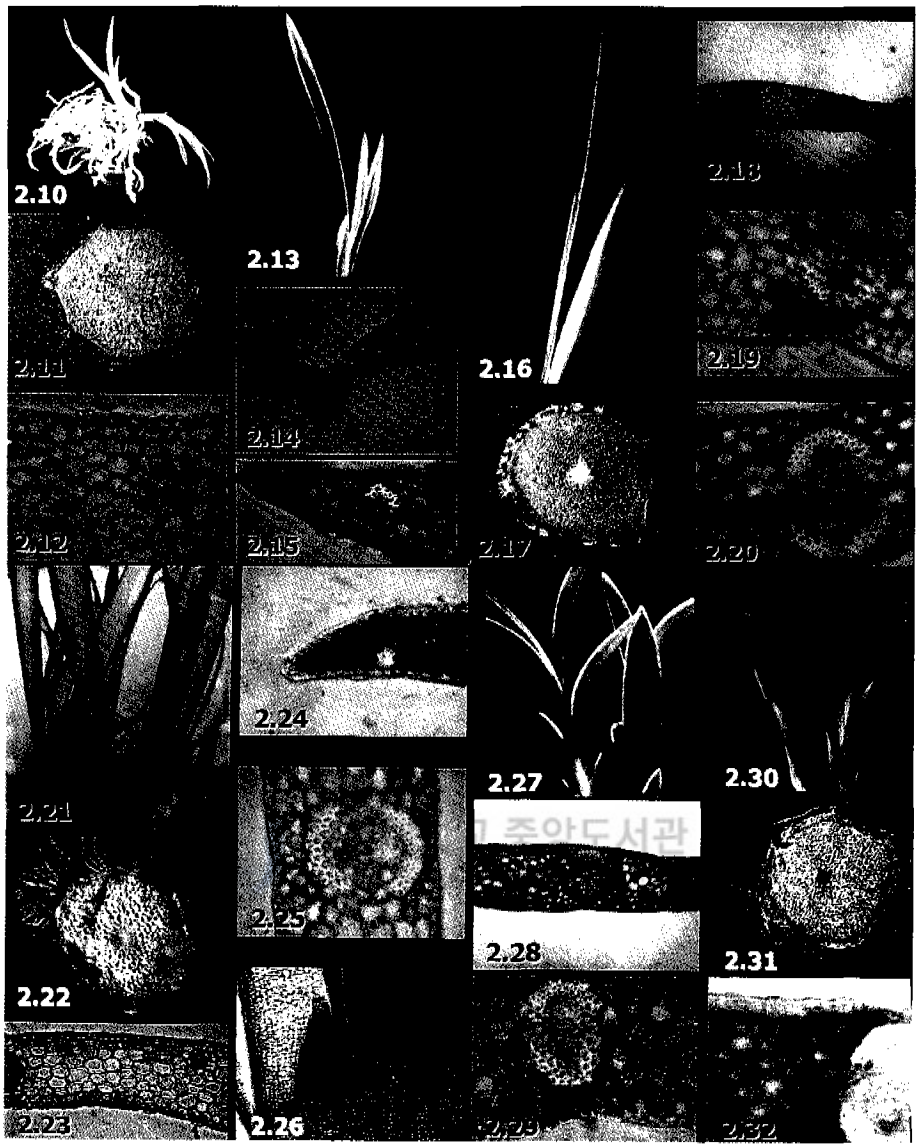

Fig. 2.10-2.12. Albino seedlings. 2.10. W-W-W shoot of *Cymbidium goeringii*, the result of the replacement of the G L-III by W L-II of white-margined leaf (W-W-G). 2.11. Whole section of W-W-W C. goeringii rhizome showing no chloroplasts. 2.12. W-W-W leaf also showing no chloroplasts.

Fig. 2.13-2.15. White-margined leaves. 2.13. Leaf from W-W-G white-margined shoot of *Cymbidium goeringii*. 2.14. Transection of W-W-G *Cymbidium goeringii* leaf showing replacement of G L-III by W L-II. 2.15. Leaf edge of W-W-G white-margined *Cymbidium goeringii* showing lack of chloroplasts.

useful term to indicate a white skinned periclinal chimera without specifying precisely the structure, whether two-layered (WG), three-layered and thick skinned (WWG), or a sandwich (GWG) (Tilney-Bassett, 1986). Chloroplasts were distributed mainly through the mid-vein of leaves (Fig. 2.14), but not the edge of leaves (Fig. 2.15).

A white-medioalbinated (W-G-W) *Cymbidium goeringii* (Fig. 2.16) was occurred in this mutagenesis. The term "medioalbinata" was suggested by Imai (1934, 1935) for which the likely structures would be two-layered (G-W), thre-layered and thick skinned (G-G-W), or a sandwich (W-G-W). Whole section of rhizome showed scarcely scattered chloroplasts, which represented paler yellow (Fig. 2.17). Chloroplasts existed mainly in leaf edge (Fig. 2.18) and scarcely in the central portion of leaf L-II layer. (Fig. 2.20).

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Fig. 2.16-2.20. White-medioalbinated leaves. 2.16. Leaf from W-G-W white-medioalbinated shoot of *Cymbidium goeringii*. 2.17. Whole section of W-G-W *Cymbidium goeringii* rhizome showing lack of chloroplasts. 2.18. Transection of W-G-W *Cymbidium goeringii* leaf showing replacement of W L-III by G L-II. 2.19. Transection of W-G-W *Cymbidium goeringii* leaf showing chloroplasts in G L-II. 2.20. Central portion of W-G-W *Cymbidium goeringii* showing pale yellowish color.

Fig. 2.21-2.26. Yellow-medioalbinated leaves. 2.21. Leaf from G-G-W yellow-medioalbinated shoot of *Cymbidium kanran*. 2.22. Whole section of G-G-W *Cymbidium kanran* rhizome showing yellowish color. 2.23. Transection of G-G-W *Cymbidium kanran* leaf. 2.24. Leaf edge of G-G-W yellow-medioalbinated *Cymbidium kanran* contain chloroplasts. 2.25. Central portion of G-G-W *Cymbidium kanran* showing yellowish color. 2.26. Section through a lateral bud of *Cymbidum kanran* before growth.

A yellow-mediolbinated (G-G-W) *Cymbidium kanran* (Fig. 2.21) was obtained from EMS treated rhizomes (Fig. 2.1-2). This rhizomes were yellowish and whole sectioned surface as well (Fig. 2.22). Chloroplasts existed mainly along leaf edges (Fig. 2.24) and scarcely in the central portion of leaf except L-III. (Fig. 2.25). Fig. 2.26 shows a lateral bud of *Cymbidium kanran* prior to the growth.

A yellow-margined (G-W-G) *Cymbidum kanran* (Fig. 2.27) was obtained from EMS-treated rhizomes (Fig. 2.1-3). Chloroplasts of these leaves were distributed over all of the layers except certain portions (Fig. 2.28, 2.29).

A normal green leaves (G-G-G) also was observed (Fig. 2.30). All of leaves and rhizomes had chloroplasts in all of the three layers (Fig. 2.31, 2.32).

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In tissue culture, occasionally an albino arises, presumably unstable chimeras. Conversely, a normal green plant can arise from a variegated one (Vajrabhaya, 1977). In this study, an albino also arose

Fig. 2.27-2.29. Yellow margined leaves. 2.27. Leaf from G-W-G yellow-margined shoot of *Cymbidium kanran*. 2.28. Transection of G-W-G *Cymbidium kanran* leaf. 2.29. Central portion of G-W-G *Cymbidium kanran* showing greenish color.

Fig. 2.30-2.32. Normal green leaves. 2.30. Leaf from G-G-G normal green shoot of *Cymbidium goeringii*. 2.31. Whole section of G-G-G *Cymbidium goeringii* rhizome showing plenty of chloroplasts. 2.32. Transection of G-G-G *Cymbidium goeringii* leaf showing greenish color in all layers.

from white-margined plant, and a normal green plant arose from white-medioalbinated, yellow-medioalbinated, and yellow-margined plant. This interesting phenomenon may be due to replacement between layers. When cells from an inner layer take over the position of a cell from an outer layer, "displacement" is said to occur while "replacement" occurs when there is a periclinal division of a cell in an outer layer which invades an inner layer (Stewart and Dermen, 1970). In this studies, there seems to be no occurrence of displacement, because white-margined plant did not bring about a normal green plant, and then white-medioalbinated, yellow-medioalbinated, and yellow-margined plant did not an albino as well. Thus, unstable chimeras of mutated *Cymbidum* may be shifted further inward by duplication of an outer layer, or replacement.

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(3) Genetic characteristics of variegated mutants using the randomly amplified polymorphic DNA (RAPD) analysis

The polymease chain reaction (PCR) has facilitated genetic studies in plants and animals (Erlich et all, 1991; Wolfe and Peters-Van, 1993). One variation of PCR primer results a profile of randomly amplified polymorphic DNA (RAPD). The polymorphisms observed may

result from point mutations, insertions, deletion, and/or inversions (Williams et al., 1990).

There has been investigated the relationship between 3 variegated leaves and their recovered or reversed leaves by replacement, respectively, using RAPD. Each of fifty primers provided clear DNA polymorphic patterns. Amplification products of fifty primers generated polymorphic profiles. A total of 439 bands and 376 polymorphic fragments were generated by 50 primers. The band patterns were unique that each of the same clone, that is, a variegated leaf and their recovered or recovered leaf had same bands (Fig. 2.33).

The Nei estimate of similarity (Nei and Li, 1979) was used to construct a similarity matrix. Similarity values were calculated by scoring total bands for each primer. Similarity values ranged from 0.506 to 0.991 with analysis of total band score (Table 2.5). The phenogram resulting from the UPGMA cluster analysis are shown in Fig. 2.34. There seems to be no difference of genetic relationship between white-margined *Cymbidium goeringii* and albino which were derived from white-margined one by replacement, and the others, too. This result supported that the linear variegation of *Cymbidium* is chimeral variegation by somatic mutation. The most common cause of chimeral variegation is spontaneous mutation in the path way to chlorophyll synthesis or plastid morphology. These mutations are generally

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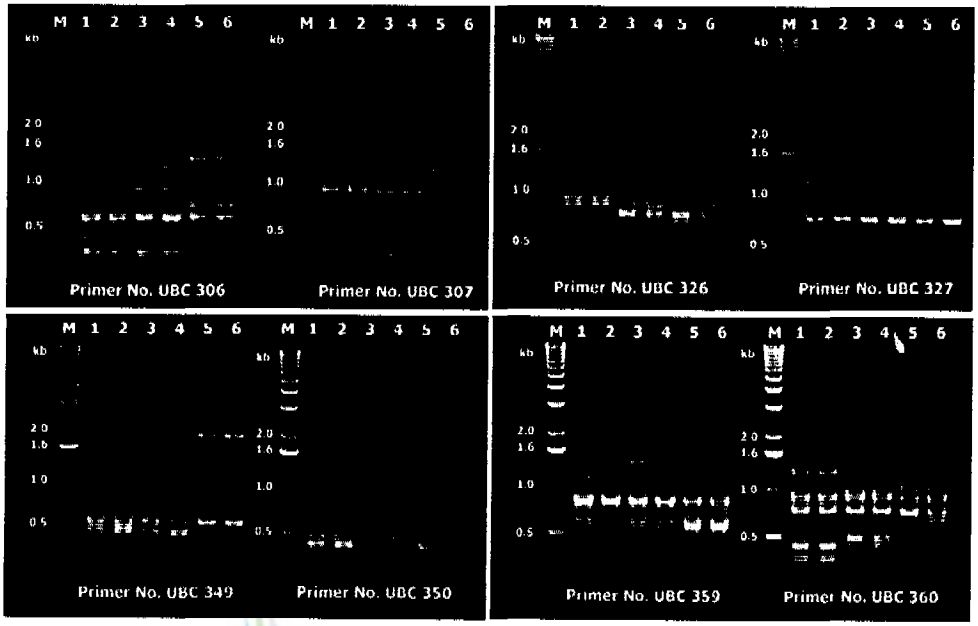

Fig. 2.33. DNA profiles obtained from 6 chimeral leaves of *Cymbidium*. No. 1 and 2, 3 and 4, 5 and 6 are induced from the same clone, respectively.

M : 1kb DNA ladder (GIBCO-BRL, MD.)

1 : Albino (WWW), *Cymbidium goeringii*

2 : White-margined(WWG), *Cymbidium goeringii*

3 : White-medioalbinated (WGW), *Cymbidium goeringii*

4 : Green (GGG), *Cymbidium goeringii*

5 : Yellow-medioalbinated (GGW), *Cymbidium kanran*

6 : Green (WWW), *Cymbidium kanran*

Table 2.5. Similarity matrix generated using Nei's estimate of similarity.

No.[z]	1	2	3	4	5	6
1	1.000					
2	0.991	1.000				
3	0.688	0.690	1.000			
4	0.696	0.699	0.985	1.000		
5	0.506	0.509	0.503	0.506	1.000	
6	0.506	0.515	0.515	0.524	0.958	1.000

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[z] 1 : Albino (WWW), *Cymbidium goeringii*
2 : White-margined(WWG), *Cymbidium goeringii*
3 : White-medioalbinated (WGW), *Cymbidium goeringii*
4 : Green (GGG), *Cymbidium goeringii*
5 : Yellow-medioalbinated (GGW), *Cymbidium kanran*
6 : Green (WWW), *Cymbidium kanran*

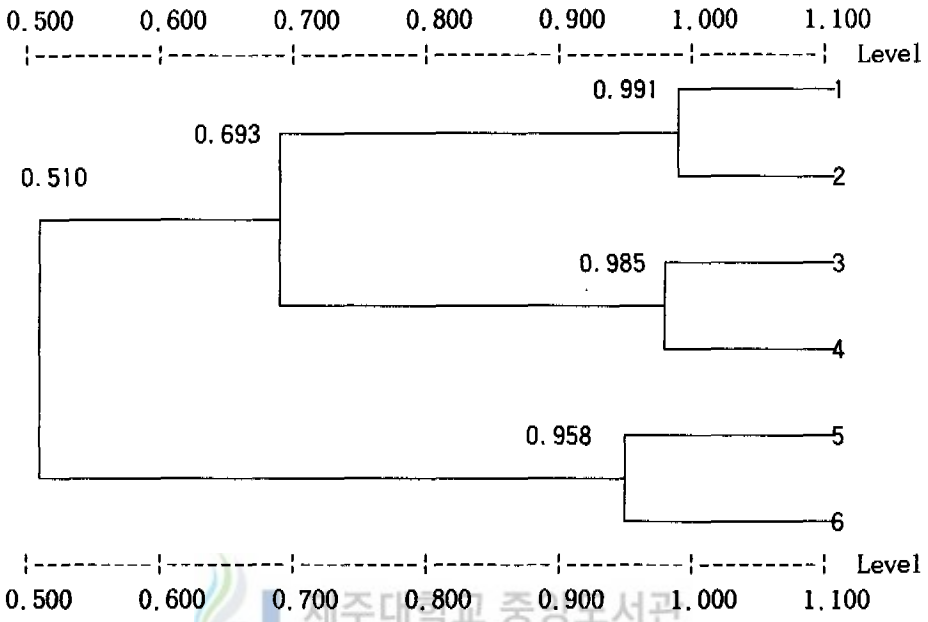

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Fig. 2.34. Phenogram for 6 chimeral leaves of *Cymbidium*. No. 1 and 2, 3 and 4, 5 and 6 are induced from the same clone, respectively.

1 : Albino (WWW), *Cymbidium goeringii*

2 : White-margined (WWG), *Cymbidium goeringii*

3 : White-medioalbinated (WGW), *Cymbidium goeringii*

4 : Normal green (GGG), *Cymbidium goeringii*

5 : yellow-medioalbinated (GGW), *Cymbidium kanran*

6 : Normal green (WWW), *Cymbidium kanran*

chloroplast mutations rather than nuclear mutations as indicated by Marcotrigiano, 1997.

In conclusion, linear variegations of *Cymbidium* are chlorophyll chimeras which are induced by somatic mutation and genetically unstable. They can be easily obtained from the mutagenesis. Because they are not sexually transmitted, they must be propagated asexually in horticultural aspects.

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GENERAL DISCUSSION

Breeders have been somewhat baffled to discover the flower-color inheritance (Lenz and Wimber, 1959). Especially, Flower-color inheritance in *Cattleya*, perhaps the most popular of all orchid genera, has received more attention than in any other member of the Orchidaceae from the early in 1900s. Orchids have been crossed between distinct forms of one species; species within a genus; and different genera (Arditti, 1992), which have been made that few plants surpass the orchids in distribution throughout the world, variability of growth habits, and the magnificent spectrum of colors produced by their flowers and leaves (Arditti and Ernst, 1971; Strauss and Arditti, 1972).

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Variations within species are not unusual among orchids since they are usually cross-pollinated plants (Vajrabhaya, 1977). Color variation such as alba (entirely white) and semi-alba (colored lip, white seplas and petals) may be found within a single species such as *Cattleya* (Mehlquist, 1958; Storey and Kamemoto, 1960). Variation occurring in nature must originate from either asexual or sexual reproduction. Variation such as alba is very important in horticulture. Because it is so rare in nature that the value is very high. Therefore, it is important that we should know the inheritance to produce a new

variety such as alba and mass propagation through cross hybridization.

In this study, the inheritance of flower pigmentation in *Aerides japonicum* was discovered to cause the variation such as alba. Figure 3.1 illustrated the phenomena related to pigmentation of *Aereides japonicum*. The pigment of sepals and labellum in *A. japonicum* was cyanidin-based anthocyanins peated at about 524 nm, and which was controlled by a single dominant gene (R) that proved by cross hybridization through the second generation and SCAR marker system. Results obtained from SSH suggested that there were genes related to biosynthesis of anthocyanins in *Aerides japonicum*.

Mutations are more likely to occur during asexual phases than during sexual ones because the latter are very much shorter. Leaf variegation is a chimera that usually involves chlorophyll production. The amount of chlorophyll varies from area to area, resulting in mottling or striping. A plant chimeras is a specific type of genetic mosaics in which the genetically dissimilar cells are present in the shoot apical meristem, where they continue to give rise to the cells that form the body of the plant (Marcotrigiano, 1997). Genetic mosaics can occur spontaneously or be induced by mutagens. In either case, mutant cells can be present in organs, such as petals or leaves, or in the shoot apical meristem. When present in the meristem, the mutant cell lines can continue to contribute to organs as they develop (Marcotrigiano and

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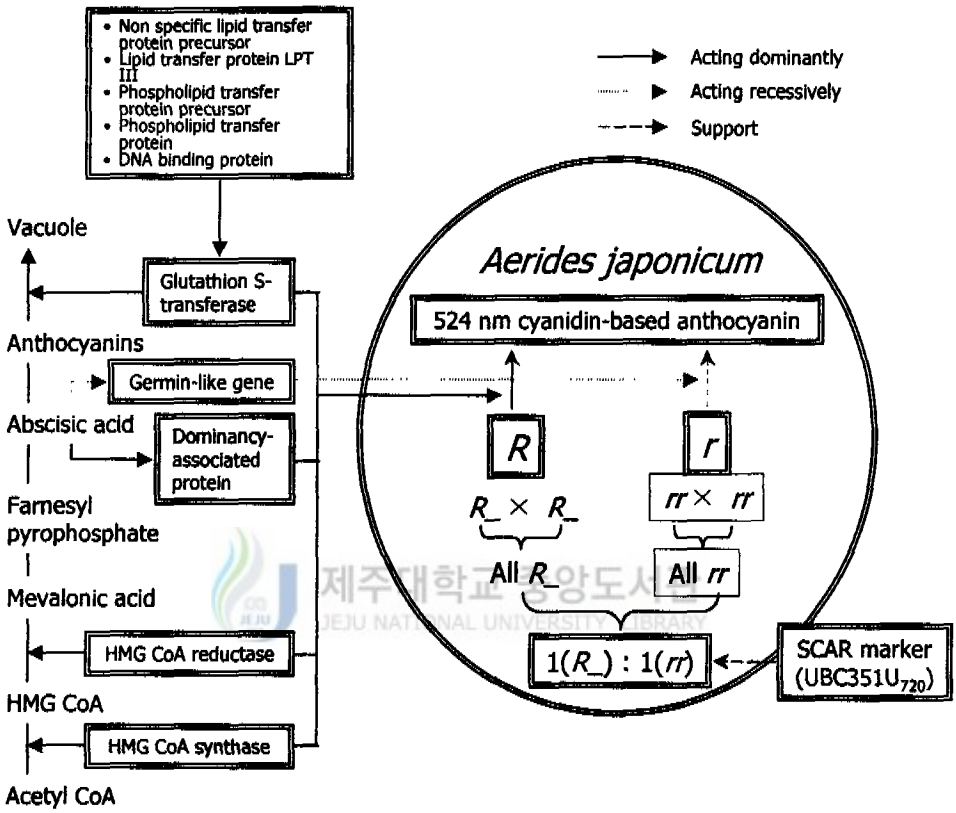

Fig. 3.1. Schematic representation of the inheritance of flower pigmentatioin in *Aeriedes japonicum*.

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Morgan, 1988). In chlorophyll mutation, cells in mutated regions are unable to produce enough chlorophyll. However, all variegated plants are not chimeras (Marcotrigiano and Stewart, 1984). Because noncell lineage variegation patterns can be transmitted sexually from on generation to the next, while chimera can not be propagated by seed (Marcotrigiano, 1997). Periclinal chimeras would never yield variegated offspring, but rather all green or all white seedlings, depending on the genotype of the histogen that produces the eggs (Stewart and Burk, 1970). Therefore, it is most important to know which is the cause of variegated leaves, noncell lineage variegation or chimeral variegation. Because the difference makes a decision about the way of breeding. That is, noncell lineage variegation can be easily bred by cross hybridization and propagated by seeds, while chimeral variegation can be induced by the mutagenesis and propagated asexually. Variegation and chimerism are phenomena with both economic and scientific importance for horticulture and for the study of plant development. Especially in Korea, Japan and China, the vareigated cymbidiums such as *Cymbidium goeringii* and *C. kanran* are invaluable because they are rare in nature.

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In this study, various variegations of cymbidiums were induced by mutagenesis and revealed which are the chimeras or not by RAPD analysis. Figure 3.2 illustrated the phenomena related to variegation of a *Cymbidium* leaf. There are two types in leaf variegation of *Cymbidium*

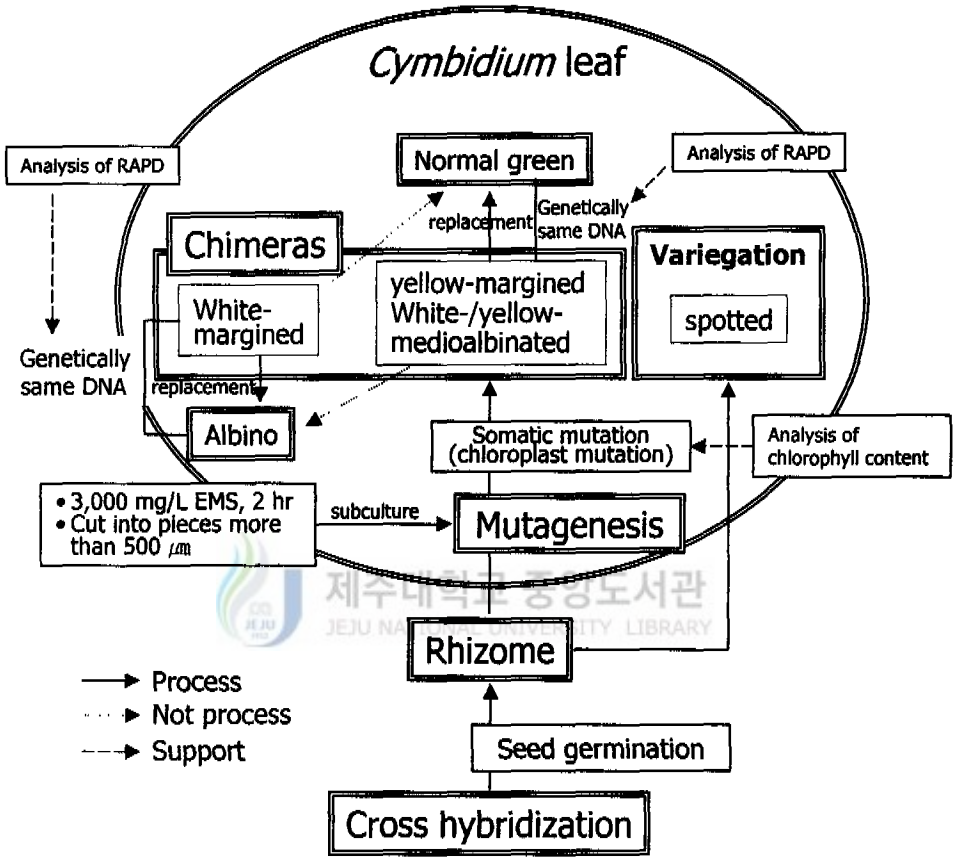

Fig. 3.2. Schematic representation of the chimeral variegation induced by mutagenesis in *Cymbidium*.

showing either striped or spotted variegation. The spotted variegation was transmitted sexually by cross hybridization because they are chimeras, while striped variegation was not. Thus, to obtain striped variegation massively, it is need to mutagenesis and propagation asexually. Chlorophyll mutants were induced by EMS treatment and cutting into pieces more than 500 ㎛. Primarily, mutated rhizomes induced margined (white- and yellow-) and medioalbinated (white- and yellow-) leaves. During the subculture, an albino arose from white-margined leaves by replacement of layers, and a normal green plant arose from yellow-margined, white-/yellow-medioalbinated one. These results suggest that the variegations induced in this study are somehow unstable chimeras. Because RAPD analysis for two of each phenotype which was differentially expressed showed mutually identical DNA pattern.

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In conclusion, because the pigment of *Aerides japoniucum* is controlled by a single dominant gene, *A. japonicum alba* may be obtained from cross between *A. japonicum alba* and *A. japonicum alba*, or cross between recessive genes. To obtain *A. japonicum alba* massively, it needs to cross between *A. japonicum alba* themselves and propagate by seeds in vitro. SCAR marker is available to identification the character in early stage of breeding that will be shorten the

breeding period. These results suggest that it is can be applied *Cymbidium goeringii* which is similar in flower pigmentation to *Aeriedes japonicum*.

In *Cymbidium*, spotted variegations are either induced or spontaneous mutation and genetically stable. Because they are sexually transmitted, they can be easily obtained by seed propagation. While, linear variegations are chlorophyll chimeras which are somatic mutation and genetically unstable. They can be easily obtained from the mutagenesis. Because they are not sexually transmitted, they must be propagated asexually in horticultural aspects.

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나도풍란의 화색유전과 돌연변이육종을 이용한 한국자생 심비디움의 키메라 반입현상에 관한 연구

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(화훼원예학 및 난과식물육종 전공)
지도교수 : 소 인 섭

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적　　요

나도풍란(*Aerides japonicum*)의 화색유전현상을 밝히기 위하여 안토시아닌을 분석하였고, 2세대간에 걸친 교배를 실시하였으며, 육종 초기단계에서 화색을 진단할 수 있는 SCAR marker를 제작하였다. 또한 suppression subtractive hybridization (SSH)를 이용하여 나도풍란 꽃의 유전자를 탐색하였다.

나도풍란의 꽃에 나타나는 붉은색은 최대 흡수파장이 524 nm인 안토시아닌으로, 소심에서는 안토시아닌의 흡수파장이 나타나지 않았다.

교배육종에서 나도풍란 소심종간의 분리비는 모두 소심종이 나타났고, 일반종간의 교배에서는 모두 붉은색을 띤 일반종들이 출현하였다. 각각의 F_1간의 교잡, 즉 일반종과 소심간의 교잡 분리비는 1:1인 것으로 나타났다.

육종 초기단계에서 소심종의 여부를 판별할 수 있는 SCAR marker로는 $UBC351U_{720}$, $UBC375L_{2030}$, $UBC396U_{1832}$ 등이 선발되었으며, 그중 $UBC351_{720}$을 이용하여 나도풍란 일반종과 소심종간의 F_1개체들을 임의로 선별하여 분석한 결과, 1:1의 분리비를 보였다.

Suppression subtractive hybridization (SSH)를 이용한 나도풍란의 꽃 유전자 탐색에서 16개의 새로운 유전자가 발견되어 Genbank에 등록하였다. 탐색된 유전자 중에서 glutathione S-transferase, dormancy-associated protein, 3-hydroxy-3-methylglutaryl-CoA synthase, lipid transfer proteins, germin-like protein gene 등이 안토시아닌의 합성에 관련되는 것으로 나타났다.

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한국춘란(*Cymbidium goeringii*)과 한란(*C. kanran*)의 중투와 복륜과 같은 반입 무늬종을 화화적 돌연변이원을 이용한 돌연변이 육종을 통해 육성하고, 그것들의 형태적인 차이와 RAPD법을 이용한 DNA분석을 통해 반입현상을 규명하였다.

춘란과 한란의 근경에 3,000 mg/L ethyl methanesulfonate (EMS)을 시간별로 처리한 결과, 시간이 경과할수록 생존율이 감소하였고, 변이율이 증가하였으며, 2시간 처리가 LD_{50}로 나타났다. 또한 절단한 근경에서 변이가 보다 많이 나타났는데, 500 ㎛의 두께가 생존 가능한 최소 크기였다.

시각적으로 근경의 색이 옅을수록(흰색-노랑-연두-녹색) 반입이 많이 들어간 변이체가 많이 발생하였는데, 근경의 엽록소 함량과 잎의 엽록소 함량간에는 정의 상관관계가 있었다. 또한 반입된 부분은 녹색의 정상적인 부분보다 단위면적당 기공수가 적었다.

변이를 받은 근경은 1차적으로 백복륜, 황복륜, 중투의 형태를 이룬 개체를 발생시켰고, 이후의 계속적인 계대배양 과정에서 replacement에 의해 백복륜에서 albino 개체가, 황복륜과 중투에서 녹색의 정상적인 개체가 발생되었다. 각각의 영양계에서 서로 다르게 표현된 두 개체간의 DNA는 동일한 것으로 나타났다.

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