Inheritance of Floral Pigmentation in *Aerides japonicum*

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ABSTRACT

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 and the SCAR marker system to identify the floral pigmentation on the early stage of breeding. The pigment of sepals and labellum of *A. 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. *A. 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 F1 of *A. japonicum* and F1 of *A. japonicum* alba segregated essentially as a 1:1 ratio in the progeny. UBC351, UBC375, and UBC39 specific primer pairs were selected as useful markers to distinguish *A. japonicum* from *A. japonicum* alba in early stage of breeding. The identification of SCAR marker, UBC351U720 primer, showed the segregation of crossing between *A. japonicum* and *A. japonicum* alba essentially as a 1:1 ratio.

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). 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 intergeneric hybrids), and different genera (intrageneric 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).

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

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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. Also, in *Aerides japonicum*, is found the variation such as alba 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. However there has been not much documented about inheritance of pigmentation in *A. japonicum* and ways of producing alba yet.

In this study the inheritance of floral pigmentation in *A. japonicum* by the cross hybridization through the second generation, anthocyanins in the flower and SCAR marker system to identify the floral pigmentation on the early stage of breeding.

MATERIALS AND METHODS

Flower color in *Aerides japonicum*

Anthocyanins in the labellum and sepals of flowers in *A. japonicum* (Fig. 1, left) and *A. japonicum* alba (Fig. 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 (Beckman DU-64, USA). *A. japonicum* alba is one of variations of *A. japonicum* which has not reddish pigment on sepals and labellum.

Cross hybridization to produce alba variation

Cross hybridization was carried out for the matching as follows:

(1) *A. japonicum* × *A. japonicum*

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

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

After the six months of pollination, seeds were germinated in Hyponex medium

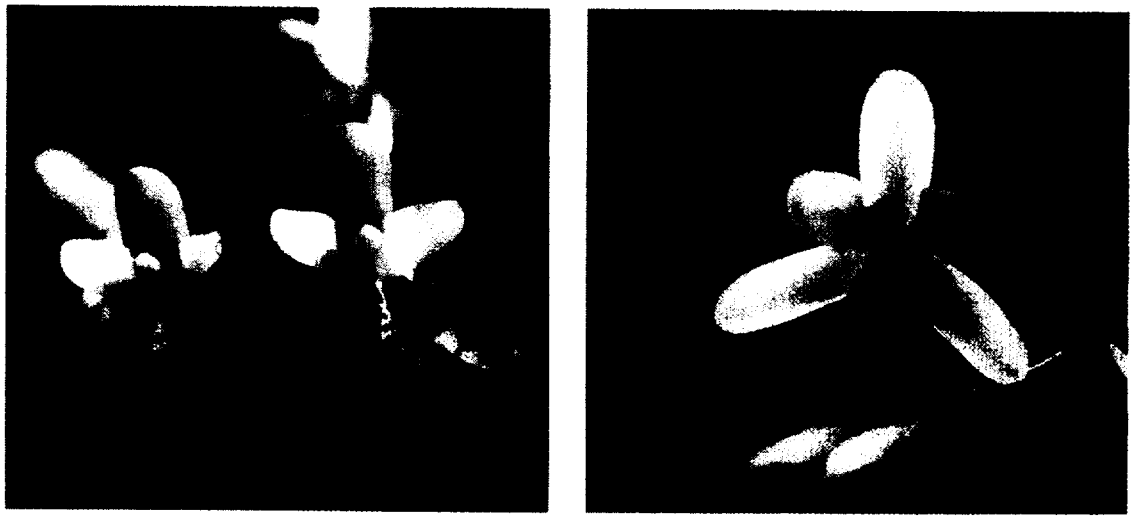

Fig. 1. Flowers of *A. Japonicum* (left) and *A. japonicum* alba (right).

(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 35±2℃ under cool-white fluorescent light (1.5 klux) from 800 to 2400 hours in darkness from 2400 to 800 hours. Young seedlings were hardened in greenhouse, and induced flowering.

Genomic DNA isolation

The genotypes used for making the SCAR marker system to detect the floral pigmentation in the early stage of breeding were *A. japonicum*, *A. japonicum* alba, and F1 seedlings of hybridization between them. Genomic DNA was isolated from *A. japonicum* using PVP, SDS, and chloroform extraction (Kim et al., 1997).

RAPD reaction and construction of SCAR marker

Reaction mixtures is consisted of 0.018 units/μL Taq DNA polymerase (ABGENE, England), 100μM dNTP, 0.2 μM primer, and about 5 to 10 ng DNA in a final volume of 20 μL, respectively. RAPD primers were obtained from the University of British Columbia (UBC) (Table 1).

PCR 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 ℃, 60 sec at 37 ℃, 90 sec at 72 ℃, followed by 10 min at 72 ℃. The PCR products were electophoresed in agarose gels, isualized by ethidium bromide staining, and then were cloned into pGEM-T Easy vector (Promega Co.).

Table 1. the list of 30 random primers and their sequences used in RAPD for construction of sequence-specific primers of *A. 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.

PCR for sequence characterized amplified regions (SCARs) marker

PCR using putative SCARs markers was performed in a PTC-100 thermal cycler (MJ Research Inc.). PCR reaction conditions were 95 ℃ for 5 min and 30 cycles at 94℃ for 30 sec, 50 to 65 ℃ for 2 min and 72℃ for 2 min.

RESULTS AND DISCUSSION

Flower color in *A. japonicum*

A. japonicum alba produces flowers without

red stripes and spots on sepals and lip. The spectrum showed that *A. japonicum* alba did not have any anthocyanin (Fig. 2). *A. japonicum* produces flowers with red stripes on a cream sepal and red spots in a white lip. The spectrum showed that almost all of color is due to anthocyanin (peaked at about 524 nm). Other studies indicated that this anthocyanin was probably a cyanidin-based anthocyanin.

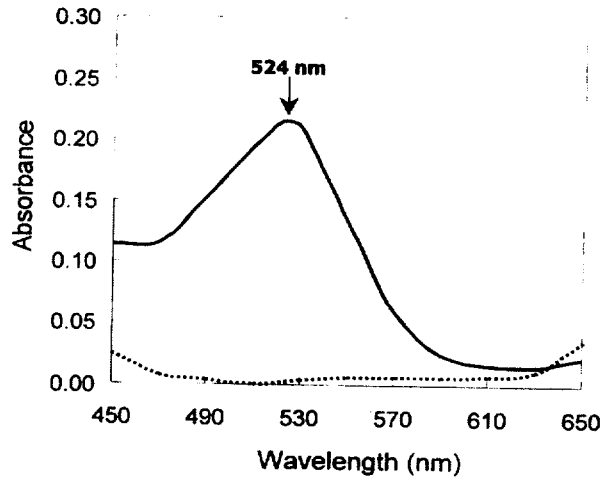

Fig. 2. Spectrum from sepals and labellum of *A. japonicum* (solid line) and *A. japonicum* alba (dotted line)

Cross hybridization to produce alba variation

Variations within species are not unusual among orchids since they are usually cross-pollinated plants. *A. japonicum* (a *R*_, red colored sepals and lip) when crossed with *R*_ red produced only red colored progeny, presumably of the *R*_ genotype. *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 cross between F1 of *A. japonicum* and F1 of *A. japonicum* alba produced 286 colored and 237 alba hybrids, essentially a 1:1 ratio (Fig. 3 and 4).

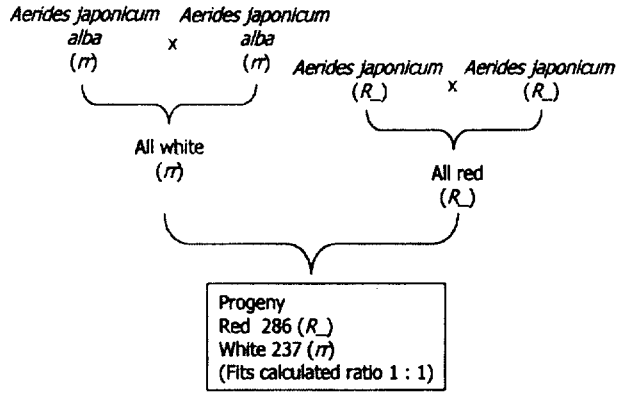

Fig. 3. Pedigree of the progeny of *A. japonicum* with assigned hypothetical genotypes.

Fig. 4. A photograph showing the flower pigmentation and segregation according to the different hybridization. (Top) *A. japonicum* alba x *A. japonicum* alba. (Middle) *A. japonicum* x *A. japonicum*. (Bottom) *A. japonicum* alba x *A. japonicum*.

Therefore the pigmentation of sepals and labellum in *A. japonicum* is controlled by a single dominant gene (*R*) and flower color in this species due to cyanidin-based anthocyanins. The result suggests that the pigment of *A. japonicum* flowers is a cyanidin-based anthocyanins and may be inherited through two hybrids dominantly.

SCAR marker for detecting the floral pigmentation

The sequences of the 20-mer SCAR primers obtained by the RAPD primer extension are given in Table 2. SCAR primers were synthesized and used for PCR. PCR amplification with the selected UBC351, UBC375, and UBC396 specific primers was performed using the DNA extracted from *A. japonicum* and *A. japonicum alba* (Fig. 5). The 720 bp fragment of UBC351 primer and the 2,030 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 primer 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. 5). These results suggest that UBC351, UBC375, and UBC396 specific primer pairs can be useful marker to distinguish *A. japonicum* from alba.

Table 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

[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.

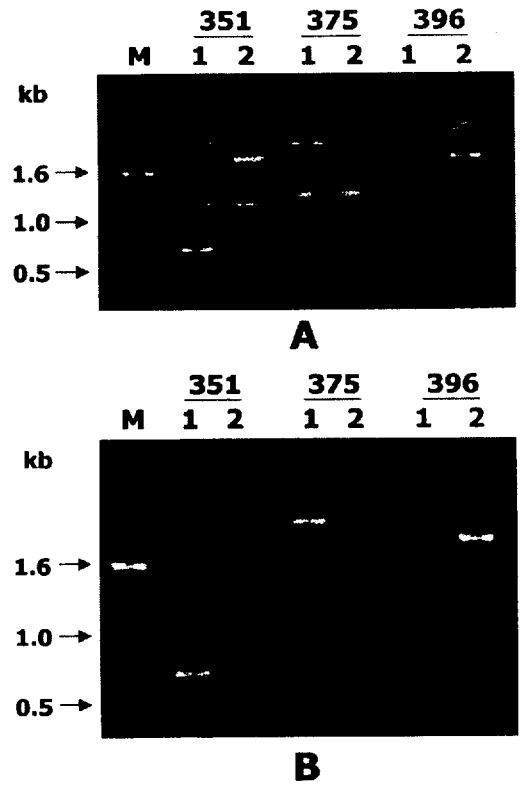

Fig. 5. DNA profiles obtained from *A. japonicum* (lane 1) and *A. japonicum* alba (lane 2) with the primers UBC 351, UBC 375, and UBC 396. M: 1 kb DNA ladder, A: RAPD profiles, B: Selected bands as a marker

The identification SCAR marker for progenies of cross between *A. japonicum* and alba obtained with UBC351U720 primer (Fig. 6). The 720 bp fragments was recovered in 9 of 14 randomly selected progenies and totally 31 of 50 randomly selected progenies, essentially a 1:1 ratio.

The primer pairs of UBC351U720 were made from the decamer, UBC351 primer, and produced the sequence-specific primers according to their size. In lane of Fig. 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. Thus this result could be indicated that the amplified bands with UBC351U720 specific primer may be located in the different alleles.

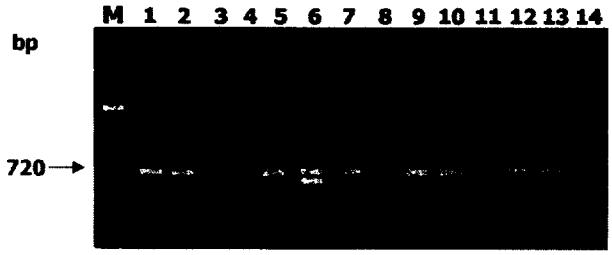

Fig. 6. Amplification products obtained with sequence-specific primers UBC351U720. Lanes are randomly selected progeny DNA which are crossed between *A. japonicum* and *A. japonicum* alba.

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 *A. japonicum* flowers pigment may be controlled by a single dominant gene.

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