Flower color inheritance of *Rhynchostylis gigantea* (Lindl.) Ridl.

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Submission: 30 April 2019 Revised: 3 August 2020 Accepted: 11 August 2020

ABSTRACT

Rhynchostylis gigantea has been subjected to a conventional breeding program in order to determine genetic inheritance of flower color. Generally, there are three varieties with four different flower patterns, i.e., white, white with red−pink spots, white with red blotches and red. The pure red is called *R. gigantea var. rubrum* Sagarik whereas the pure white is called *R. gigantea var. harrisonianum* HolH., and the white with red−pink spots or blotches is called *R. gigantea*. Recently, another color, orange−peach, has been developed. The objectives of this study were to determine genetic inheritance and conduct chemical component analysis of flower color in *R. gigantea*. Chemical analysis of all colors was conducted to identify major color components of the flowers using liquid chromatography−mass spectrometry (LC−MS). Three major components, cyanidin, peonidin and delphinidin were found in the red, white with red blotches and white with red−pink spots forms, whereas pelargonidin was found only in the orange−peach flowers. Anthocyanins were not found in the white color flower. Hybridization was carried out in order to determine color inheritance in these four−color forms: white, white with red blotches, white with red−pink spots and red. Twenty crosses of intraspecific hybridization of *R. gigantea* were made for F1−progeny production and progeny segregation in which color inheritance was analyzed. A Mendelian genetic analysis was designed to identify the major genes controlling these traits and to evaluate allelic and linkage relationships. In this study, three major genes have been proposed to govern color inheritance in *R. gigantea*. The *C* gene is responsible for the cyanidin accumulation which gives red−pink coloration, and the *P* gene for the expression of peonidin accumulation which gives red coloration, while the *D* gene for the delphinidin accumulation which gives purple coloration of flowers, and it might promote solid−red color accumulation. The *CCPPDD* and *ccppdd* genotypes gives solid−red and pure white flower forms respectively, while *C−P−D−* and *C−P−dd* gives white with red−pink spots or blotches flower forms, whereas the orange−peach color might be derived from other species or hybrids, but not through hybridization within *R. gigantea* varieties.

Keywords: Anthocyanin, epiphytic orchid, genetic inheritance, LC−MS, reciprocal cross

Thai J. Agric. Sci. (2020) Vol. 53(4): 178−191

INTRODUCTION

Rhynchostylis gigantea is one of the most popular and ecologically important orchids in Thailand and worldwide (Kuanprasert, 2005; Thammasiri, 2016). There are several flower colors and color patterns on petals, sepals and lips, including pure white and solid magenta−red flower forms (Fuchs, 2006) that make it attractive. According to the difference in flower colors, it can be divided into three main varieties. *R. gigantea* (Lindl.) Ridl. has white with red−pink spots flowers. *R. gigantea* var. *harrisonianum* HolH. has pure white flowers. The last variety is *R. gigantea* var. *rubrum* Sagarik having pure dark−red color flowers (Kuanprasert, 2005). However, white with red−blotches flower form is also called *R. gigantea* (Lindl.) Ridl. This orchid is a major source of potted plants in the flower market. To date, more than four varieties or flower color forms have been improved by intraspecific hybridization for novel cultivar/variety production (Thammasiri, 2016).

Flower colors represent the accumulation of pigment compounds. Three main pigments in plants are chlorophylls, carotenoids and anthocyanins. Anthocyanins are groups of flavonoid glycosides constituting the major color pigments in flower and fruit. Anthocyanins are synthesized along with flavonoid biosynthesis through a series of enzymatic reactions that convert chalcone into three major anthocyanidin types. Cyanidin is a reddish−purple (magenta) pigment. It is the major pigment in plants and other red−colored flowers and some fruits. Delphinidin appears as a reddish−blue or purple pigment in flowers. The blue hue of flowers is due to the delphinidin pigment. Whereas pelargonidin gives an orange hue to flowers and red to some fruits and berries (Tanaka *et al*., 2008; Khoo *et al*., 2017). Anthocyanin pigment analysis has been carried out in some orchids, e.g., *Dendrobium* Pramote (Saito *et al*., 1994), *Dendrobium* × Icy Pink 'Sakura' (Kuehnle *et al*., 1997), *Vanda* hybrids (*Vanda teres* × *Vanda hookeriana*), *Rhynchostylis retusa* and *Aerides multiflora* (Junka *et al*., 2011). The major pigments of red to purple flowers of Vandaceous orchids have been identified as cyanidin, delphinidin and pelargonidin (Junka *et al*., 2011).

Flower color inheritance was studied in other orchids such as *Dendrobium* regarding inbreeding effects, reciprocal crosses, androgenesis and the genetics of some characters (Kamemoto and Amore, 1990). A similar study was carried out in *Phalaenopsis* (Chen and Chen, 2011). Basic knowledge of the inheritance of the flower color traits or flower color patterns of some species or hybrids is not well−illustrated for some breeding programs, especially that of *R. gigantea*.

In *Dendrobium*, it was proposed that *C* and *P* genes govern flower color. In dominant and recessive epistasis, the *C* gene in the dominant form (*C*−) repressed the *P* gene regardless of its genotypic form to produce the white flower form. In duplicate recessive epistasis, the homozygous recessive form suppressed color formation (Kamemoto *et al*., 1999). In *Cattleya*, the presence of both *C* and *P* genes is important for the production of red or red−purple flower forms (Hurst, 1925), which is similar to genes controlling *Dendrobium* coloration (Vajrabhaya and Vajrabhaya, 1996). The study of the chemical components of anthurium spathes assisted in determining genetic inheritance. There were two major genes controlling spathe color (Kamemoto and Kuehnle, 1996). Hence, the focus of this study was clarification of the genetic inheritance of flower characteristics of *R. gigantea*. The objectives of this study were to determine genetic inheritance and conduct chemical component analysis of flower color in *R. gigantea*.

MATERIALS AND METHODS

Plant Materials

Four flower color forms in three varieties of 4−year−old *R. gigantea* plants were chosen according to flower color pattern, i.e., 1) white (White group N155A; compared with RHS color chart), 2) white with red−pink spots (Red−purple group N57C), 3) white with red blotches (Red−purple group 58A) and 4) red (Red group 46B) as parental plants of

intraspecific hybridizations. For white with red−pink spots and white with red blotches flower forms, they are the same variety. Recently, orange−peach color has been found and claimed this new cultivar of *R. gigantea*. The flower of this cultivar was used for chemical analysis.

Hybridization

Twenty crosses and reciprocal crosses of intraspecific hybridization of four *R. gigantea* flower color forms (Figure 1) were made. Pollination was performed to make seed pods. For experimental design, there were three replications (one plant per replication) per flower color forms, and each replication had five flowers for hybridization and seed germination. All pollinated flowers yielded seed pods. Three 10−month−old seed pods of each cross were harvested and subjected to *in vitro* seed germination.

Seeds and Seedling Maintenance

Seeds developed to form protocorms and then seedlings. Ten to 12−month−old seedlings were transplanted and grown in a 60% shaded house equipped with a mini−sprinkler irrigation and fertigation system. Plants were watered twice a day for 45−60 min at 08.30 a.m. and 04.00 p.m. to maintain 60−80% relative humidity, at a growing temperature of 25−35°C.

Data Collection and Genetic Inheritance Analysis

Progenies were grown for 3−4 years. Flower colors of progenies derived from each cross were assessed and recorded. Flower characteristics were grouped according to flower color and pattern. Hypotheses of flower color inheritance were determined. An analysis of variance was performed using Fisher's Protected Least Significant Difference (PLSD) to make pair−wise comparisons (StatView version 5.0). The chi−square test was carried out to test the observed segregation ratio of progenies versus different flower forms: white × white, white × color and color × color. Crosses against expected ratios were based on the number of genetic models according to the research of Kamemoto *et al.* (1988). The chi−square test was performed according to the results of flower color component identification.

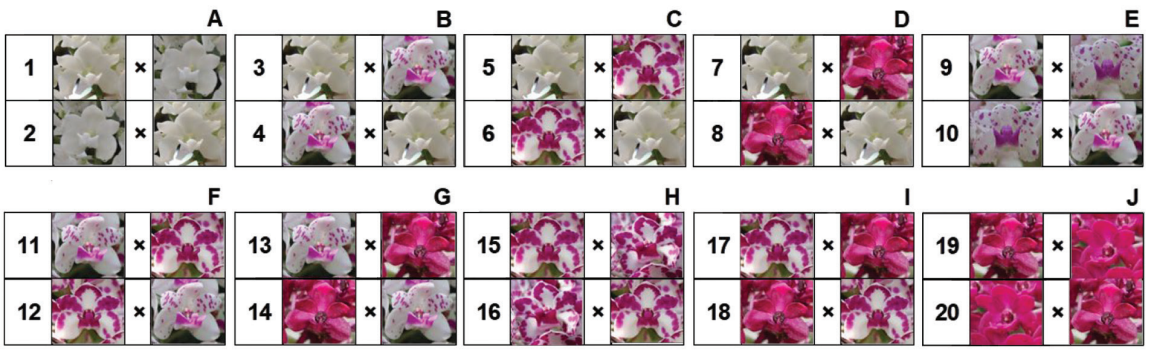

Figure 1 Intraspecific hybridization involving four varieties of *R. gigantea* flower forms. White × white (A), white × white with red-pink spots (B), white × white with red blotches (C), white × red (D), white with red-pink spots × white with red-pink spots (E), white with red-pink spots × white with red blotches (F), white with red-pink spots × red (G), white with red blotches × white with red blotches (H), white with red blotches × red (I), red × red (J). Reciprocal crosses of each pair were also made

Flower Color Analysis

In this experiment, the orange−peach flower form was added, because this flower color form is a new cultivar of *R. gigantea,* which is very interesting to anthocyanin pigments analysis comparing other flower color forms of *R. gigantea*. Five flowers from each inflorescence of different types of *R. gigantea* flowers were subjected to liquid chromatography–mass spectrometry (LC−MS) analyses following the methods of Junka *et al.* (2011) which was modified from the method of Rodriguez−Saona and Wrolstad (2005) and Goodman *et al.* (2004).

For individual anthocyanins, the purified anthocyanin method was followed with the method of Junka *et al.* (2011) which was modified from the method of Rodriguez−Saona and Wrolstad (2005). Frozen samples were finely homogenized with acidified methanol (0.01% HCl) and then filtered through Whatman paper No.1 until colorless. The sample was transferred to a separatory funnel. Two volumes of chloroform were added to the sample and gently mixed a few times. The samples were kept overnight at 4°C until a clear partition between the two phases appeared. The upper phases were transferred to a boiling flask. The methanol was removed via rotary evaporation at 40°C and the sample was adjusted to a known volume with acidic deionized water (0.01% HCl). The extracted anthocyanins were flowed through a C18 minicolumn (Waters Sep−Pak®) after which, the absorbed anthocyanin samples were washed twice with acidic distilled water (0.01% HCl) and then with ethyl acetate. The purified anthocyanins were eluted by acidic methanol (0.01% HCl). The remaining methanol was removed by flushing with nitrogen gas until nearly dried. The anthocyanin compounds were separated and detected using a Bruker Daltonics Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) connected to an Agilent 1100 HPLC system (Agilent Technologies) equipped with a binary pump and a variable wavelength detector. The components were separated with a Hypersil Gold C18 column (150 mm in length × 4.6 mm in i.d., the particle size of 3 µm).

The total anthocyanin content (TAC) was determined by the pH−differential method following the methods of Junka *et al.* (2011) and Rodriguez−Saona and Wrolstad (2005) with slight modification. The frozen plant materials of five flower color forms of *R. gigantea* were ground to a fine powder and mixed with 0.01% HCl in methanol. The samples were then sonicated until colorless. The supernatant was recovered after centrifugation at 12,000 rpm for 30 min at 4°C, and the anthocyanin content was measured using the pH−differential technique (Junka *et al.*, 2011). Potassium chloride buffers (0.025 M KCl, pH 1.0) and sodium acetate (0.4 M $CH_3CO_2Na{\cdot}3H_2O$, pH 4.5) were used for examining the monomeric anthocyanin contents. A mixture of 900 µL of either pH 1.0 or pH 4.5 buffer and 100 µL of the extracted sample was incubated for 15 min at room temperature (25°C) and then measured by spectrum scanning (320−700 nm) with a UV−visible spectrophotometer (Shimadzu®, Japan) controlled by the UV−Probe program (Shimadzu®, Japan). The absorbance of the diluted sample was calculated as follows:

$$A = (A_{\lambda\ vis-max} - A_{700nm})_{pH\ 1.0} - (A_{\lambda\ vis-max} - A_{700nm})_{pH\ 4.5}$$

The anthocyanin pigment concentration in the sample was calculated using the following formula:

$$\text{Anthocyanin pigment (cyanidin−3−glucoside, mg/L)} = (A \times MW \times DF \times 10^3)/(\varepsilon \times l)$$

where A is the absorbance of the diluted sample, MW is the molecular weight of cyanidin−3−glucoside (cyd−3−glu) which is 449.2 g/mol, DF is dilution factor, 10^3 is a factor for conversion from g to mg, ε is 26,900 molar extinction coefficient in $L \times mol^{-1} \times cm^{-1}$ for cyd−3−glu and l is pathlength in cm. TAC was then calculated and expressed in mg cyanidin/kg fresh weight.

RESULTS AND DISCUSSION

Flower colors of all progenies were assessed and recorded, they could be divided into four groups: 1 = white, 2 = white with red−pink spots, 3 = white with red blotches and 4 = red flower forms. The flower color variations of progenies of white with red−pink spots and white with red blotches groups were segregated into five discrete characteristics with color patterns having different spots or blotches on sepals and petals (Figure 2).

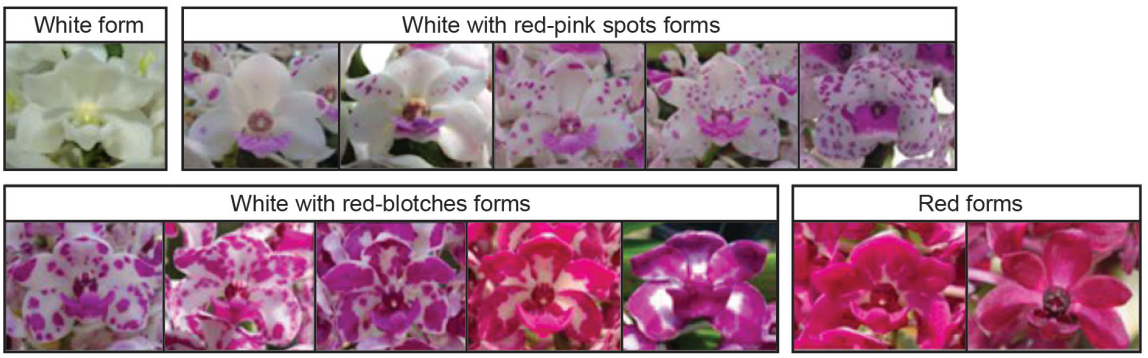

Figure 2 Segregation of progeny phenotypic characteristics involving four groups of *R. gigantea* flower forms: white, white with red-pink spots, white with red blotches and red

White × White Crosses

The cross between white and white flower forms and reciprocal crosses of *R. gigantea* (Figure 1A) produced progenies having uniform phenotypes, all white flower forms (Table 1), which was similar to the result of anthurium spathe color (Kamemoto and Kuehnle, 1996). Spathe color of anthurium is controlled by two major genes when white spathe was crossed with white spathe, all the progenies were white (Kamemoto and Kuehnle, 1996). This indicated that the gene controlling the flower color inheritance of *R. gigantea* progeny is in the homozygous form. In *Dendrobium* when white flowers were crossed with white flowers, the progenies showed white and colored flowers. This indicated that there was complementary gene action (Kamemoto and Amore, 1990), which was the result of genes controlling *Dendrobium* hybrid flower color (Vajrabhaya and Vajrabhaya, 1996). In *Cattleya*, the presence of two major genes is important for the production of red or red−purple flower forms when white flowers were crossed with white flowers, the progenies showed all colors or white and colors (Hurst, 1925). The results indicated that genes controlling the flower color inheritance of *Dendrobium and Cattleya* progenies were in heterozygous forms, the genes which control the white color was recessive form.

White × Color Crosses

The cross between white and colored flowers and reciprocal crosses were made as shown in Figures 1B−1D. The progenies of these crosses produced four patterns of flower characteristics (Table 1). The offspring of white × white with red−pink spots, white × white with red blotches and reciprocal crosses segregated into three discrete groups: white, white with red−pink spots and white with red blotches. While the offspring of white × red and reciprocal cross segregated into four discrete groups: white, white with red−pink spots, white with red blotches and red (Table 1). In addition, the white flower form occurred less frequently in progenies from the crosses between white × red, white × white with red blotches and white × white with red−pink spots and reciprocal crosses. Because several color patterns of progenies were produced and controlled by multiple pairs of color genes.

Table 1 Segregation of flower color characteristics and phenotypic ratios of *R. gigantea* progenies for crosses of white × white, white × color, color × color and red × red flower forms (reciprocal crosses)

Hybridization crosses		Segregation of progenies					Expected ratio	χ^2	*P*
Female	**Male**	**W**	**W−spts**	**W−blts**	**R**	**Total**			
White × White									
W1	W2	798	0	0	0	798	1:0:0[a]		
W2	W1	602	0	0	0	602	1:0:0[a]		
White × Color									
W	W−spts	18	670	97	0	785	1:63:0[b]	0.099	<0.50
W−spts	W	16	702	120	0	838	1:63:0[b]	0.418	<0.50
W	W−blts	8	304	430	0	742	1:63:0[c]	0.287	<0.50
W−blts	W	11	398	466	0	875	1:63:0[c]	0.466	<0.50
W	R	10	64	580	44	698	1:6:53:4[d]	0.961	<0.90
R	W	14	78	836	52	980	1:6:53:4[d]	0.442	<0.90
Color × Color									
W−spts1	W−spts2	90	901	16	0	1,008	1:63:0[e]		
W−spts2	W−spts1	102	1,023	20	0	1,147	1:63:0[e]		
W−spts	W−blts	12	420	508	0	940	1:63:0[f]		
W−blts	W−spts	13	410	550	0	973	1:63:0[f]		
W−spts	R	0	11	863	26	900	0:63:1[g]		
R	W−spts	0	12	846	32	890	0:63:1[g]		
W−blts1	W−blts2	0	67	725	75	867	0:63:1[h]		
W−blts2	W−blts1	0	78	832	96	1,006	0:63:1[h]		
W−blts	R	0	0	908	92	1,000	0:63:1[i]		
R	W−blts	0	0	837	87	924	0:63:1[i]		
Red × Red									
R1	R2	0	0	0	730	730	0:0:1[j]		
R2	R1	0	0	0	1,045	1,045	0:0:1[j]		

Note: W = white, W−spts = white with red−pink spots, W−blts = white with red blotches, R = red

[a] All white

[b] White : white with red−pink spots + white with red blotches : red

[c] White : white with red−pink spots + white with red blotches : red

[d] White : white with red−pink spots : white with red blotches : red

[e] White : white with red−pink spots + white with red blotches : red

[f] White : white with red−pink spots + white with red blotches : red

[g] White : white with red−pink spots + white with red blotches : red

[h] White : white with red−pink spots + white with red blotches : red

[i] White : white with red−pink spots + white with red blotches : red

[j] All red

Color × Color Crosses

Crosses of *R. gigantea* involving white with red-pink spots × white with red-pink spots (Figure 1E), white with red-pink spots × white with red blotches (Figure 1F), white with red-pink spots × red (Figure 1G), white with red blotches × white with red blotches (Figure 1H) and white with red blotches × red flower forms (Figure 1I) and reciprocal crosses were conducted. Segregation of flower color characteristics from the crosses between white with red-pink spots × white with red-pink spots and white with red-pink spots × white with red blotches flower form and reciprocal crosses produced different phenotype of progenies which could be divided into three discrete groups: white, white with red-pink spots and white with red blotches, but the red flower form did not occur (Table 1). While the crosses between white with red-pink spots × red and white with red blotches × white with red blotches and reciprocal crosses produced progenies with different phenotypes of flower color which could be divided into three discrete groups: white with red-pink spots, white with red blotches and red, but the white flower form did not occur (Table 1). Whereas the cross of white with red blotches × red flower form and reciprocal crosses produced progenies having different phenotypes of flower colors which could be divided into two discrete groups: white with red blotches and red, but white and white with red-pink spots flower forms did not occur (Table 1). In addition, these results might have been due to transgressive segregation related to flower color formation of *R. gigantea* involving multiple genes controlling flower color characteristics, especially white with red-pink spots and white with red blotches flower forms. These results indicated that white with red-pink spots and white with red blotches were heterozygous forms that produced several patterns of flower colors in progenies.

Red × Red Crosses

The cross between red and red flower forms and reciprocal crosses of *R. gigantea* (Figure 1J) produced progenies having red coloration (Table 1). This indicated that genes controlling red color were in the dominant homozygous form, whereas white color was in the recessive homozygous form. Interestingly, in the cross of red × red flower forms and reciprocal crosses, not all progenies were solid−red. Red with white at the base of petals and sepals the same as white with red blotches was also produced (Figure 2). This might be related to temperature effect on anthocyanins accumulation of flower color. Anthocyanin biosynthesis occurs better when temperature is low (Jiafu *et al*., 2013).

Chemical Analysis

Flower color analysis using LC−MS was conducted. The results showed that the total anthocyanin contents (cyanidin−3−glucoside) of white with red-pink spots, white with red blotches, red and orange-peach flower forms were detected at 17.70, 166.77, 234.90 and 137.57 mg/kg fresh weight, respectively, while the white flower form was not detected (Table 2). This result indicated that anthocyanin is a group of pigments which are important to the flower color accumulation of *R. gigantea*.

Table 2 Total anthocyanin content of five flower forms of *R. gigantea*

Flower color forms of *R. gigantea*	Total anthocyanin content (cyanidin−3−glucoside) (mg/kg fresh weight)
White form	−
White with red−pink spots form	17.70[c]
White with red blotches form	166.77[b]
Solid−red form	234.90[a]
Orange−peach form	137.57[b]
F−test	**
CV (%)	4.22

Note: [a,b,c] Means with different superscripts in the same column are significantly different (P < 0.01), ** Significantly different at P < 0.01

Flower color patterns and characteristics of all progenies from intraspecific hybridization among four varieties of *R. gigantea* could be divided into four groups: white, white with red−pink spots, white with red blotches and red. Anthocyanin derivative analysis of *R. gigantea* flowers was conducted in this research. The verification of LC−MS could be confirmed with the HPLC results of Sumaythachotphong *et al.* (2017), the results showed that the anthocyanin derivatives in sepals and petals of *R. gigantea* flowers were cyanidin, peonidin, delphinidin, cyanin, callistephin, keracyanin, kuromanin and pelargonidin which were found in colored flower forms, but not in the white flower form. There were three major components of anthocyanins in *R. gigantea* flowers which were cyanidin, peonidin and delphinidin (Figure 3). This result was similar to anthocyanin analysis in *Oncidium* Gower Ramsey and *Oncidium* Sharry Baby (Chiou and Yeh, 2008).

The different types of flower color had different pigments. Chemical components of the white with red-pink spots flower form were cyanidin and peonidin. For the white with red blotches flower form, chemical components were cyanidin, peonidin, delphinidin and callistephin and for the solid−red flower form were cyanidin, peonidin, delphinidin, cyanin, callistephin, keracyanin and kuromanin. While the white flower form had no anthocyanins. Whereas the orange-peach flower form contained pelargonidin and delphinidin (Figure 3).

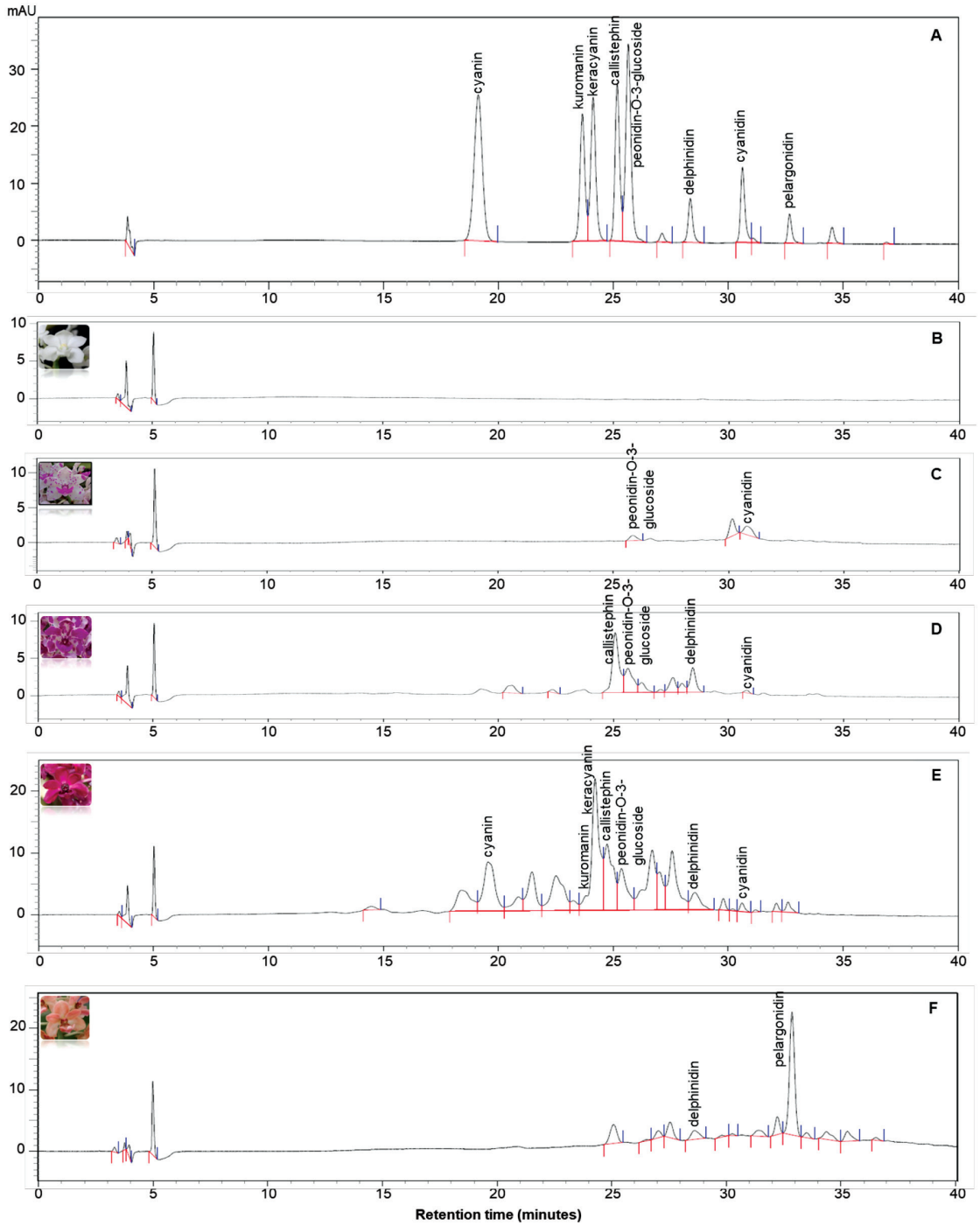

Figure 3 Chromatograms of anthocyanin derivatives from five flower forms of *R. gigantea* pigments which were extracted and analyzed from sepal and petal tissues; Standard (A), white flower form (B), white with red-pink spots flower form (C), white with red blotches flower form (D), red flower form (E), orange-peach flower form (F)

Cyanidin or cyanidin−3−O−glucoside was found in portions of some flowers including white with red−pink spots, white with red blotches and red flower forms (Figure 3). In the white with red-pink spots, peonidin and cyanidin were found (Figure 3C), which were detected at 0.420 and 0.130 mg/100g fresh weight, respectively. For the white with red blotches form, peonidin, cyanidin and delphinidin were found as the main pigments which the contents were detected at 0.500, 0.620 and 0.489 mg/100g fresh weight, respectively, and callistephin was found as the minor pigment (Figure 3D). While in the red flower form, peonidin, cyanidin and delphinidin were found as the main pigments, which were detected at 2.330, 1.010 and 0.719 mg/100g fresh weight, respectively. Callistephin, keracyanin and kuromanin were found as the minor pigments of the red flower form (Figure 3E). Interestingly, cyanin was found only in the red flower form, and was detected at 0.963 mg/100g fresh weight. On the other hand, the main pigments of the orange-peach flower form were found to be pelargonidin and delphinidin, which were detected at 1.200 and 2.340 mg/100g fresh weight, respectively. Pelargonidin was found only in the orange-peach flower form (Figure 3F). The anthocyanin content in *R. gigantea* flowers was similar to that were found in *R. retusa* and *Aerides multiflora* flowers (Junka *et al*., 2008) and strawberry fruit (Durst and Wrolstad, 2005).

The anthocyanin biosynthesis pathway requires at least six enzymes including chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3−β−hydroxylase (F3H), dihydroflavonol−4−reductase (DFR), anthocyanidin synthase (ANS) and flavonoid glycosyltransferase (UFGT) (Tanaka *et al*., 2008), and the three main pathways are divided into three main pigment syntheses (Figure 4).

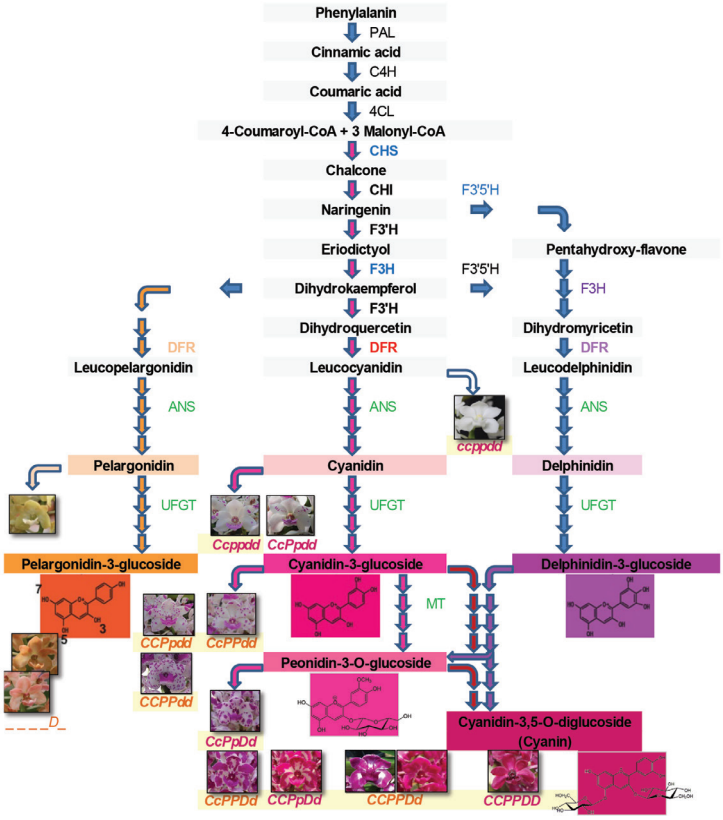

Figure 4 A possible genetic model for intraspecific hybridization of *R. gigantea* based on the genes *C*, *P* and *D*

The results indicated that cyanidin and peonidin were the major pigments in white with red−pink spots, white with red blotches and red flower forms of *R. gigantea*. Whereas in the orange-peach flower form, pelargonidin was found as the main pigment. While delphinidin synthesis resulted in solid-red accumulation in *R. gigantea* flowers (Figure 4). Furthermore, the other pigments were detected. Callistephin, keracyanin and kuromanin were the minor components that enhanced the red-magenta color accumulation of *R. gigantea* flowers. Chemical analysis using LC−MS confirmed the anthocyanin derivatives which were analyzed by HPLC. Sumaythachotphong *et al*. (2017) reported that the red-magenta coloration in *R. gigantea* floral tissues such as sepals, petals and lips were composed of cyanidin, peonidin, delphinidin and cyanin compounds. The minor pigments such as callistephin, kuromanin and keracyanin were responsible for flower color accumulations. In contrast, most of the pigments were not detected in orange-peach flower except for delphinidin. Pelargonidin was found as the major anthocyanin pigment only in the orange-peach flower form.

Acylated cyanidins were the main pigments in the red−purple flowers of *Dendrobium* Pramote (Saito *et al*., 1994), *Phalaenopsis* hybrids (red-purple flowers) (Tatsuzawa *et al*., 1997; Chen, 2009) and the hybrid, *Dendrobium* × Icy Pink 'Sakura', accumulated high pelargonidin and a few cyanidins (Kuehnle *et al*., 1997). *Vanda* hybrids (*Vanda teres* × *Vanda hookeriana*) were found to have anthocyanin and derivatives as major pigment components in flowers. Analysis of *Rhynchostylis retusa* and *Aerides multiflora* (Junka *et al*., 2011) and *Phalaenopsis schilleriana* (Griesbach, 1990) indicated that cyanidin was the major pigment in flowers. Whereas in *Vanda coerulea* flower, cyanidin and delphinidin were found as major flower pigments. Cyanidin was the main pigment in orchid flowers which gave a red-magenta color, while delphinidin gave a purple-blue flower color (Junka *et al*., 2011). Color inheritance of *R. gigantea* flowers was analyzed to determine the different characteristics of flower colors based on the studies of Kamemoto and Kuehnle (1996), Kamemoto *et al*. (1999), Kloos *et al*. (2004) and Elibox and Umaharan (2008). We used a Mendelian genetic analysis which was designed to identify the major genes controlling color patterns and evaluate allelic and linkage relationships.

Segregation of Flower Colors

Three pairs of genes might be responsible for the flower color inheritance of *R. gigantea*. White × white cross produced all white flowers, these genes might be in the homozygous recessive form. For white × color crosses, the crosses of white × white with red−pink spots and white × white with red blotches and reciprocal crosses produced progenies having different phenotypes of white/colors flower forms, which provided a ratio of 1 white : 63 white with red−pink spots and white with red blotches : 0 red. While the cross of white × red and reciprocal cross produced progenies having different phenotypes of white/colors flower forms, which provided a ratio of 1 white : 6 white with red-pink spots : 53 white with red blotches : 4 red (Table 1).

For color × color crosses, the crosses and reciprocal crosses of *R. gigantea* involving white with red−pink spots × white with red−pink spots, white with red−pink spots × white with red blotches, white with red−pink spots × red, white with red blotches × white with red blotches and white with red blotches × red flower forms produced progenies which segregated into different flower patterns. Flower color patterns of the crosses of white with red−pink spots × white with red−pink spots and white with red−pink spots × white with red blotches flower forms and reciprocal crosses produced progenies having different phenotypes of flower colors which provided a ratio of 1 white : 63 white with red−pink spots and white with red blotches : 0 red. While the crosses of white with red-pink spots × red, white with red blotches × white with red blotches and white with red blotches × red flower forms and reciprocal crosses produced progenies having different phenotypes of flower colors which

provided a ratio of 0 white : 63 white with red-pink spots and white with red blotches : 1 red. Whereas, red × red cross produced all red flowers, therefore, these genes might be in a homozygous dominant form (Table 1).

Chemical analysis results provide useful information on the genetic control of color in *R. gigantea* flowers. Since there are three major pigments (cyanidin, peonidin and delphinidin) in *R. gigantea* flowers, there might be three genes as followed: *C* gene for cyanidin, *P* gene for peonidin and *D* gene for delphinidin. In the white flower form, all genes should be recessive as *ccppdd*. Whereas the white with red−pink spots flower forms were controlled with two dominant genes, while the white with red blotches flower forms were controlled by three dominant genes (homozygous or heterozygous dominant). For the white with red-pink spots flower form, the genes should possibly be functional as the following genotypes: *CCPPdd*, *CCPpdd*, *CcPPdd*, *CcPpdd* and *Ccppdd*. For the white with red blotches flower form, the genes should possibly be functional as the following genotypes: *CCPPDd*, *CCPpDd*, *CcPPDd* and *CcPpDd*. While the red flower form might be the result of dominance in all three genes as *CCPPDD* (Figure 5).

In addition, the orange−peach color cannot happen in the main pathway of anthocyanin biosynthesis of *R. gigantea* flowers. There were no *C* and *P* genes in orange−peach color flower form, only the *D* gene. Genetic control in the orange−peach color flower form should be studied in the future.

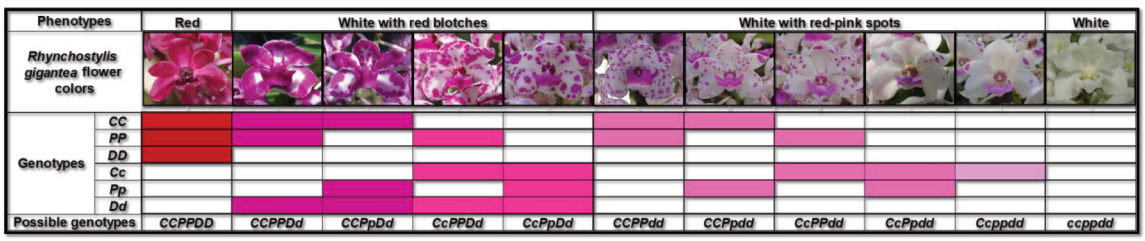

Figure 5 Flower color pattern of each type corresponding to additive gene action of *R. gigantea*

CONCLUSIONS

Hybridization, crosses and reciprocal crosses of *R. gigantea*, were made in order to determine color inheritance of four forms: white, white with red−pink spots, white with red blotches, and red forms. Chemical analysis of all previously existing colors as well as a new color, orange−peach, was conducted to identify major color components of the flowers using LC−MS. Three major color components including cyanidin, peonidin and delphinidin were found in red, white with red blotches and white with red−pink spots forms, whereas pelargonidin was found in the orange−peach flower form. Twenty crosses of intraspecific hybridization of *R. gigantea* were made in order to characterize the flower color of the progeny. It was found that the flower color of the progeny segregated according to Mendelian law. Three genes were proposed to control the flower color of *R. gigantea*. A Mendelian genetic analysis was designed to identify the major genes controlling these traits and to evaluate allelic and linkage relationships. In this study, three major genes have been proposed to govern color inheritance of the four varieties of *R. gigantea*. Flower color pattern of each type corresponded to the additive gene action of *R. gigantea*. We designated the *C* gene is responsible for the cyanidin accumulation which gave red−pink coloration, the *P* gene for the expression of peonidin accumulation which gave red coloration, while the *D* gene is responsible for delphinidin accumulation which produced the purple-

blue coloration of flowers. The *ccppdd* genotype should be responsible for the white flower form. The *C−P−D−* and *C−P−dd* genotypes should result in white with red−pink spots and white with red blotches flowers, and *CCPPDD* should produce the solid−red flower form. On the other hand, the orange−peach color is not the result of anthocyanin biosynthesis. The genetic inheritance of this flower type has not yet been determined.

ACKNOWLEDGEMENTS

This research was partly financially supported by the Center for Agricultural Biotechnology, Postgraduate Education and Research Development Office, Commission on Higher Education, Ministry of Education. The authors would like to thank the Development of Economic Flower Crop: Orchid (DEFCO) project, Department of Plant and Soil Sciences, Faculty of Agriculture, Chiang Mai University, Thailand for the use of plant material. The authors also thank Assoc. Prof. Dr. Sansanee Jamjod for valuable discussion.

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