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RESEARCH ARTICLE

Agricultural Biotechnology

Does an R2R3-MYB transcription factor affect anthurium spathe colour variation via regulation of vacuolar pH?

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Abstract: The anthurium is a popular cut flower worldwide having red, pink, coral, white, green, or brown spathes. There is a constant demand for new flower colours in the commercial market. Flower colour in plants is mainly determined by anthocyanins. Understanding anthocyanin variation and other factors affecting anthurium spathe colour is important for genetic engineering approaches. Therefore, our objectives were to assess the factors affecting colour variation of selected commercially available cut flower anthuriums and to determine the associated regulatory networks and transcription factors (TFs). Nineteen commercial cut flower anthurium cultivars were selected for this purpose. The colour of the spathe surface, anthocyanin location, anthocyanidin type and vacuolar pH were recorded. Anthocyanin associated Gene Network Model generation and analysis were carried out. The CIELAB colourimeter procedure indicated the colour variation among the selected 19 cultivars in terms of colour type, colour intensity, chroma, and hue angle. The location of anthocyanin was limited to mesophyll and epidermal cells. Cyanidin was detected in tested anthurium cultivars as the main anthocyanidin. The pH gradient in pigment extracts indicated a variation with a range of 4.6 to 4.94. The gene pathways of anthocyanin biosynthesis and transport were associated with that of the vacuolar pH/H^+ pump according to Gene Network Model. Three pathways were regulated by an R2R3-MYB transcription factor. Although, cyanidin was the only pigment in all the tested cultivars, different pH levels by *R2R3-MYB* regulated V-H^+ synthase was suggested to be the cause of the high colour variation in addition to the anthocyanidin type and location. Our results indicate the application of *R2R3-MYB* transcription factor genes for desirable vacuolar pH maintenance in genetic engineering of the blue anthurium in the future.

Keywords: Anthocyanidin, anthurium, gene pathways, spathe colour, R2R3-MYB transcription factor, vacuolar pH.

INTRODUCTION

The spathe of the anthurium, *Anthurium andraeanum* (Hort) is important as a cut flower. Introduction of interspecific hybrids had contributed to a significant increase in anthurium production in the world (Kamemoto & Sheffer, 1978; Kamemoto & Kuehnle, 1997; Henny, 1999; Elibox & Umaharan, 2008). Red, green, orange, purple, and white are the common spathe colours of commercial varieties (Jayaprada & Geekiyanage, 2017). There is a constant global demand for novel spathe colours (Puchooa, 2005). The genetic basis of spathe colour is determined by two major genes named *M* and *O* in conventional breeding. The *O* gene confers recessive epistatis over *M*; thus red/pink spathes result due to *MmOo, MmOO, MMOo*, and *MMOO* genotypes. Orange-Coral spathes are by *mmOo* and *mmOO* genotypes, and *mmoo*, *Mmoo,* and *MMoo* produce white-spathed phenotypes (Gopaulchan *et al*., 2014). In addition, several other factors influence the colour variation and understanding the factors leading to

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variation in spathe colour is of great importance in the breeding of new commercial cultivars in the future.

Colours of plants are determined by pigments such as chlorophylls, aurones, anthocyanins, carotenoids, and betalains. The major pigment in the spathe of anthurium is anthocyanin which is a product of the flavonoid biosynthesis pathway (Osorio-Guarín *et al*., 2021). Anthocyanidins are aglycone products of anthocyanins. The anthocyanin biosynthesis pathway is conserved among plants. It has been intensively studied, and the main anthocyanidins, pelagonidin-3-glucoside, cyanidin-3-glucoside, and delphinidin-3-glucoside, which are responsible for the colour variation, are already established (Holton & Cornish, 1995; Morita & Hoshino, 2018; Saigo *et al*., 2020). The early steps of the anthocyanin biosynthesis pathway are catalyzed by structural genes for the enzymes *chalcone synthase*, *chalcone isomerase*, and *flavanone-3-hydroxylase*, whereas the later steps are mediated by the structural genes of *dihydroflavonol-4-reductase*, *anthocyanin synthase*, and *flavonoid-3-O-glucosyltransferase* (*3GT*). In higher plants, the anthocyanin biosynthesis pathway is reportedly regulated by an MBW complex comprising transcription factors of MYB, basic helix-loop-helix (bHLH) domains and W40D repeats (Gonzalez *et al*., 2008; Albert *et al*., 2014). Among them, MYB transcription factors which are the v-myb avian myeloblastosis viral oncogene homologue proteins, play a major role in the regulatory network of anthocyanin biosynthesis (Yan *et al.,* 2021). According to Kobayashi *et al*. (2002), the *UDP glucose flavonoid-3-O-glucosyltransferase* (*UFGT*) gene in anthocyanin pathway is up-regulated by the *VlmybA2* gene from grape. Geekiyanage *et al*. (2007) and Geekiyanage (2010), reported that *VlmybA* genes could induce dark brown seed coats in transgenic tobacco and Arabidopsis in addition to the intense purple pigmentation, in leaves and flowers, suggesting that *VlmybA* genes not only regulated the *UFGT* gene for anthocyanidins but also the genes for proanthocyanidin in seed coat.

The displayed colour depends not only on the pigment present, but also on several factors including vacuolar pH (Rodríguez *et al*., 2020) and the location of pigment (Chen *et al*., 2019). Vacuolar pH had been considered as a major factor in studies to change the flower colour of floricultural crops such as hydrangea, petunia, morning glory, orchids, and roses (Noda, 2018; Okitsu *et al*., 2018; Sangeetha Priya, 2020). In petunia, the inheritance of flower colour is attributed to the combined effect of anthocyanidin pigmentation and vacuolar pH (Berardi *et al*, 2021). In morning glory, flower colour varies from reddish purple buds to blue flowers with an increase in vacuolar pH from 6.6 to 7.7 (Morita & Hoshino, 2018). The wide variation of spathe colour among many commercially available cultivars of anthurium has not been completely explained.

The objectives of the study were to characterize selected anthurium cultivars in terms of spathe colour, vacuolar pH, location of pigments on spathe and pigment type, and to determine the effects of pH regulatory genes on the anthocyanin biosynthesis pathway through Gene Network Models. Therefore, we selected nineteen anthurium cultivars of different colours and checked the effects of pH increase externally on pigment colour *in vitro*. Secondly, types of anthocyanidins in selected *Anthurium andraeanum* accessions were determined. Finally, associated gene pathways were extracted to justify the influence of the above factors on colour variations and to decipher the most influential genes through Gene Network Models.

MATERIALS AND METHODS

Materials

Paradise, Maxima, Tulip Summer, Sharren, Maxima Erigancia, Flabia, Losa green, Eternum, Tropic Night, Maxima, Sabia, T. Sopink, Maxima Berdin, Karisht, T. Arura Pink, and Torenda cultivars with colourful spathes and Wind, Okinawa, Kisstouche, and Maxima Berdin cultivars with green spathes were selected for the study (Figure 1). These cultivars are cut flower anthuriums, which are commercially available in Japan. The study was carried out in the Faculty of Agriculture, Kagawa University, Japan and the Department of Agricultural Biology, Faculty of Agriculture, University of Ruhuna, Sri Lanka.

Colour assessment of spathe surfaces

Nineteen anthurium cultivars were subjected to colour assessment of spathe surfaces. The lightness (L*) and chromatic components a* and b* of CIELAB standards of the spathe were measured by a colourimeter (NR-12B; Nippon Denshoku Industries Co., Ltd., Tokyo, Japan). The L* represents the degree of darkness and brightness, a* represents the degree of green and red, and b* represents the degree of blue and yellow. In the CIELAB, the colour coordinates C^* and H^* were calculated based on the following equations: $C^* = (a^{*2}+b^{*2})\ 1/2$ and $H^* = \tan^{-1}(b^*/a^*)$, according to Punangkrit *et al*. (2018).

Figure 1: The commercially available anthurium cultivars collected from Japan for the pigment analysis. (a) Wind, (b) Paradise, (c) Okinawa, (d) Maxima, (e) Tulip Summer, (f) Sharren, (g) Maxima Erigancia, (h) Flabia, (i) Losa green, (j) Eternum, (k) Kisstouche, (l) Torenda, (m) Maxima, (n) Sabia, (o) T. Sopink, (p) Maxima Berdin, (q) Karishth, (r) T. Arura Pink, (s) Tropic Night

Observation of pigment locations in the spathe

Wind, Kissotouch, Maxima, Berdin, Paradise, Sabia, Sharren, Losa Green, Eternum, Torenda, Karishth, Tropic Night, and Flabia cultivars were selected to represent the total colour variation. Pieces of 0.5 cm × 0.5 cm from the middle of the spathe were dipped in 5% agar longitudinally. Spathe pieces were picked with agar using a scalpel. The pieces were put in the micro slicer. The thickness of a longitudinal cross section was 125 µm. The longitudinal cross sections were observed under an enhanced light microscope (Olympus, Japan) to locate the pigments on the spathe.

Sample preparation for high performance liquid chromatography (HPLC)

Paradise, Maxima, Tulip Summer, Sharren, Maxima Erigancia, Flabia, Losa green, Eternum, Tropic Night, Maxima, Sabia, T. Sopink, Maxima Berdin, Karisht, T. Arura Pink, and Torenda cultivars with colourful spathes were selected for the analysis. Green colour cultivars of Wind, Okinawa, Kisstouche, and Maxima Berdin with chlorophyll were not used for the analysis. Five grams from each sample were placed in a test tube. Five milliliters of 5% formic acid in methanol (v/v) were added to each sample. The extraction solution was filtered, and used for HPLC analysis.

Confirmation of the presence of anthocyanidins

The filtered spathe extract was injected into HPLC, in which chromatograms were developed with two LC-20AT pumps (Shimadzu Co. Ltd., Japan), a CTO-10ASvp column oven (Shimadzu Co. Ltd., Japan) equipped with ODS-3 columns (3.0 mm I.D. × 50 mm + 3.0 mm I.D. × 250 mm; GL Science Co. Ltd., Japan) and an SPD-10MAvp detector (Shimadzu Co. Ltd.) set at 530 nm. The column temperature was maintained at 40^0C. A linear gradient elution for 40 min from 25 to 85% solvent B (0.1% trifluoroacetic acid, 20% acetic acid (CH_3COOH) and 25% CH_3CN in H_2O, v/v) in solvent A (0.1 % trifluoroacetic acid in H_2O, v/v) was employed as the solvent system, and the flow rate of 0.4 mL/s was maintained.

Acid hydrolysis of anthocyanidin extracts

For the identification of anthocyanidin type, the same amount of 6 M hydrochloric acid was added to the above spathe extract, and the mixture hydrolysed for 120 min at 90 ^{0}C. The acid hydrolysates were passed through a membrane filter and applied to a Sep-Pak C18 cartridge (Waters). Aglycones (anthocyanidins) trapped on the cartridge were washed with distilled water to eliminate the water-soluble hydrophilic contaminants, and then eluted with small amounts of 5% formic acid (HCOOH) methanolic solution.

Identification of the anthocyanidin type

The filtered elutes were injected into HPLC, in which chromatograms were developed with two LC-20AT

pumps (Shimadzu Co. Ltd.), a CTO-10ASvp column oven (Shimadzu Co. Ltd.) equipped with Cosmosil 5C18 AR-2 columns (4.6 mm I.D. × 50 mm + 4.6 mm I.D. × 250 mm; Nakarai Tesque Co. Ltd., Japan) and an SPD-M10Avp detector (Shimadzu Co. Ltd.).The column temperature was maintained at 40 ^{0}C. A mixed solvent of 45% solvent A (1.5% H_3PO_4 in H_2O, v/v) and 55% solvent B (1.5% H_3PO_4, 20% Acetic Acid (CH_3COOH) and 25% CH_3CN in H_2O, v/v) was employed, and a flow rate of 1.0 mL/s was maintained. The retention time of each peak of each accession was compared with the chromatograms of the standards. As the standards, Cyanidin, Pelargonidin, and Delphinidin were used.

Measurement of vacuolar pH of spathe samples

Five grams of spathe samples were crushed using mortar and pestle. The crushed sample was dipped in 5 mL of distilled water overnight. The pH was measured in the final extract. The pH of pigment extract in distilled water was assumed to be representing the vacuolar pH (Verweij *et al.*, 2008).

Adjustment of the pH of the pigment extract

Five grams of spathe pieces were dipped in 5 mL of glacial acetic acid overnight for total pigment extraction of the spathe. In order to assess the colour change with increased pH, the pH of the glacial acetic acid extracts was increased by adding 10 M NaOH. After the establishment of the reaction, the pH was measured. The change in colour in contrast to the control was observed and recorded.

Gene network model generation

Information on genes involved in anthocyanin biosynthesis, trafficking, and vacuolar pH regulation was collected from KEGG (*https://www.genome.jp/kegg/pathway.html*), and STRING (*https://string-db.org/*) databases and from the literature. The network table files were created. Network data were imported into the Cytoscape interface. Interaction parameters were defined by specifying columns of data containing the Source Interaction, Target Interaction, and Interaction Type. Network models were generated using Cytoscape 3.8. In the generated network, nodes (or vertices) represented enzyme genes, while edges represented relationships of interactions. The generated network was analysed to decipher gene interactions. Key transcription factors involved were identified.

RESULTS AND DISCUSSION

Insignificant influence of anthocyanidin type on anthurium spathe colour variation

The nineteen anthurium cut flower cultivars selected had a significant variation in terms of colour intensity, Chroma (C*) and hue angle (H*). According to the CIELAB procedure of colour measurement "L*" with a lower number (0-50) indicates darkness, and a higher number (51-100) indicates brightness. The "a" scale indicates the state of green versus red where a positive number indicates red and a negative number indicates green. Moreover, the "b" scale indicates the state of blue versus yellow where a positive number indicates yellow, and a negative number indicates blue. The "C*" and "H*" describe chroma and the hue angle respectively. The hue angle describes the relative amounts of redness and purpleness where a value around 240 represents blue and a value around 300 represents purple. The purple colour cultivar "Flabia" had a hue angle of around 347, red, brown, pink and orange cultivars had the lowest hue angles residing within a range of 20-40. This confirmed that colours determined through the naked eye coincide with the CIELAB standards. Chroma meant the purity or intensity of the colour. The colours of low chroma are considered to be "weak" while those of high chroma are considered to be "highly saturated" or "strong" when colour is measured using Hunter L, a, b versus CIE L*a*b*. In our collection, red cultivars gave higher chroma ranges of 60-75, indicating the presence of high anthocyanin content in the spathe (Table 1).

The three most common anthocyanidins of delphinidin-3-glycoside for blue and purple colour, cyanidin 3-glucoside for brick red and magenta colour and pelargonidin-3-glycoside for orange and red colour were used as standards in HPLC (Figure 2; Table 2a). Despite the high colour variation and saturation among the cultivars, HPLC identified mainly one anthocyanidin type in them, which was cyanidin (Figure 2; Table 2b).

Table 1: Colour phenotypes, CIELAB parameter values, vacuolar pH and anthocyanin locations of selected anthurium cultivars

Cultivar	Phenotype	L	a*	b*	C*	H*	Vacuolar pH	Anthocyanin Location
Wind	Green	56.19	-15.66	28.28	28.52	82.52	4.94	Mesophyll
Kisstouch	Green	54.6	-8.33	43.27	44.06	100.9	4.94	Mesophyll
Maxima Berdin	Green	58.62	-8.28	22.57	22.64	85.49	4.94	Mesophyll
Paradise	Brown	28.81	38.4	13.01	40.55	18.72	-	Mesophyll
Okinawa	Brown	35.39	14.59	13.74	20.04	43.3	-	-
Maxima	Pink	69.89	18.14	15.22	23.67	40	4.63	-
Maxima Ergancia	Pink	64.57	19.2	19.06	27.06	44.79	4.63	-
Sabia	Pink	45.94	56.2	16.81	58.66	16.65	4.63	Lower Epidermis
T. Sopink	Pink	66.63	26.49	10.49	28.49	21.6	4.75	-
Aurora Pink	Pink	72.26	14.33	2.96	14.63	11.67	4.75	-
Tulip Summer	Orange	62.71	24.79	14.06	28.5	29.55	4.73	-
Sharren	Red	41.24	60.57	34.7	69.8	29.81	4.73	Lower Epidermis
Losa Green	Red	58.8	38.8	12.19	40.67	17.44	4.73	Lower Epidermis
Eternum	Red	52.13	47.7	22.52	52.74	25.27	4.77	Lower Epidermis
Torenda	Red	40.08	67.03	27.02	72.27	21.96	4.77	Mesophyll and Lower Epidermis
Karishth	Red	33.41	53.45	31.68	62.13	30.65	4.77	-
Tropic Night	Red	23.61	29.72	9.09	31.08	17.01	4.77	Lower Epidermis
Flabia	Purple	47.08	43.43	-9.55	44.47	347.59	4.94	Mesophyll
Maxima	White	83.72	-1.55	18.89	18.95	94.69	4.6	-

Variation in pigment location and pH as factors determining colour variation

Anthocyanins are accumulated in the vacuoles. There are reports on vacuolar pH and pigment location as important factors in flower colour variations in several species (Zhang *et al*., 2021). Consequently, the vacuolar pH and pigment localization in spathes of selected anthurium cultivars were assessed using spathe cross sections consisting of epidermal and mesophyll layers (Figure 3). The anthocyanins in pink, red, orange and brown cultivars were mainly localized in the epidermal layer of the spathes. The anthocyanins of the purple cultivar were mainly localized in the mesophyll cells of the spathes (Figure 3; Figure 4; Table 1).

The tested anthurium cultivars exhibited a pH variation among different colours within a range of 4.6 to 4.94. Purple cultivars had the highest pH of 4.94 followed by red and white cultivars of pH of 4.77 and 4.6, respectively. Avila- Rostant *et al.* (2010), who reported a variation of spathe colours and vacuolar pH among tested cultivars, had screened the germplasm for lower vacuolar pH to be utilized in genetic engineering for blue anthurium. Athough the pH values of Table 1 in the current work were not similar to the previous study (Avila- Rostant *et al*., 2010), the effect of colour groups such as purple, red and white were consistent with the variation of vacuolar pH. Furthermore, the red cultivars with high chroma values had more anthocyanin saturation as mentioned in the above section. Based on CIE L*a*b* system, the negative values for a* indicated the green cultivars as previously reported by Gopaulchan *et al*. (2015). The red and purple cultivars were of higher pH over 4.77 (Table 1). Therefore, it is suggested that spathe colour and anthocyanin saturation has a relationship with higher vacuolar pH. To confirm that purple colour spathes were associated with higher pH, 10 M NaOH was added to the pigment extracts of red and orange cultivars to increase the pH *in vivo*. The pigment extracts turned purple at a pH of around 4.8 (Figure 5). According to a previous study, the vacuolar pH adjustment to a higher pH level of 5.7 - 6 had given rise to more purplish and bluish flowers in soy bean (Sundaramoorthy *et al*., 2020). The above reports are consistent with our current results suggesting that dark colours appear at higher pH.

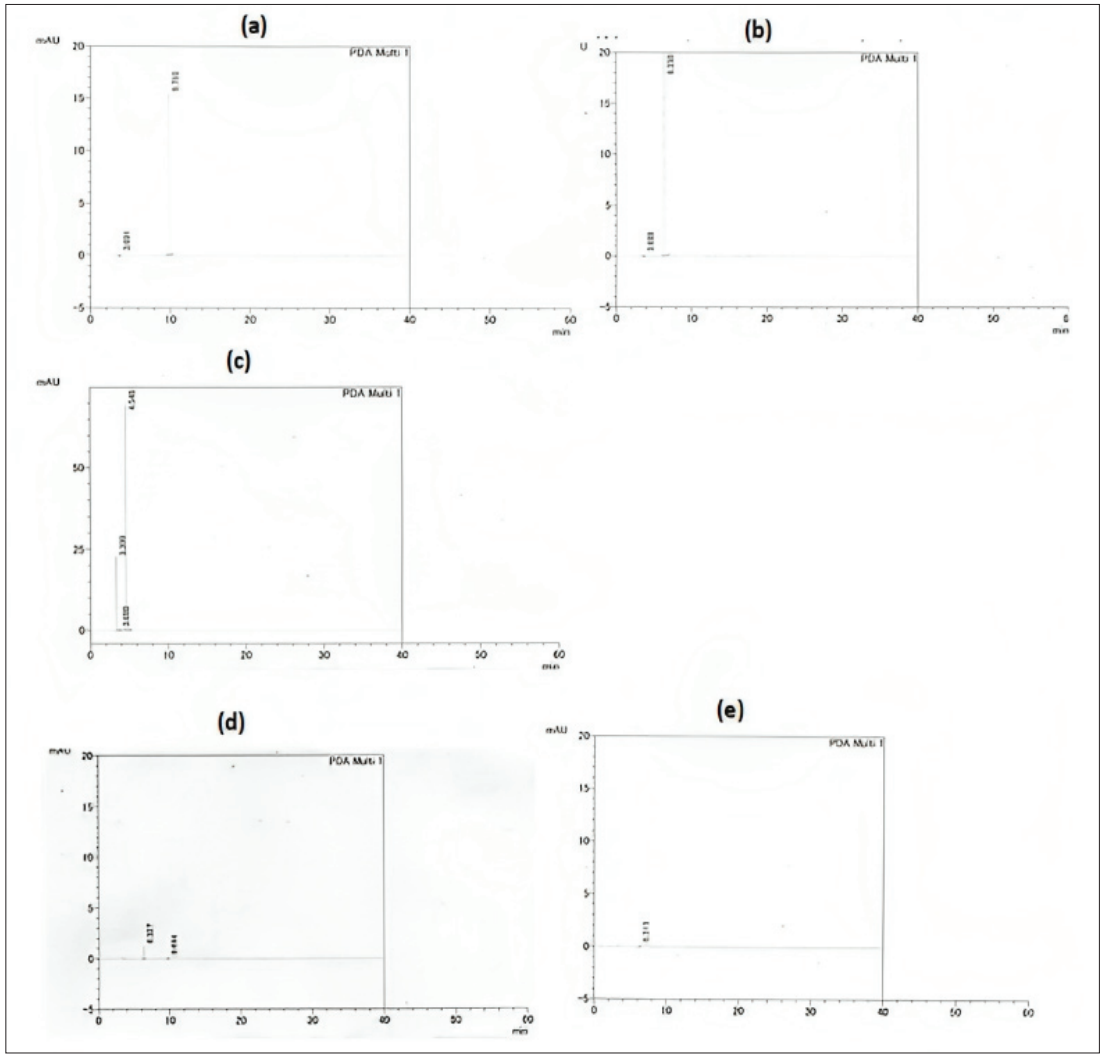

Figure 2: HPLC chromatograms for standard anthocyanidins, (a) Pelargonidins, (b) Cyanidins, (c) Delphinidins; For tested cultivars, (d) Maxima with cyanidins and pelargonidins, (e) T. Arura Pink with cyanidins

The fate of dihydrokaempferol in the anthocyanin biosynthesis pathway is crucial for the production of blue colour. Dihydrokaempferol is converted to dihydromyricetin through the *flavonoid-3',5'-hydroxylase* (*F3'5'H)* gene. Higher pH in the vacuole is favourable for this reaction. Noman *et al*. (2017) had reviewed the procedures of transgenic blue rose and chrysanthemum production, where the introduction of *F3'5'H* gene is highlighted for accumulation of delphinidin for blue colour.

Table 2a: The standards of anthocyanidins used in HPLC

Retention time (min)	Anthocyanidin type
6.350	Cyanidin (Cy)
9.790	Pelargonidin (Pg)
4.546	Delphinidin (Dp)

Higher values for vacuolar pH had been recorded for selected blue flowers in a previous study of ours (Jayaprada & Geekiyanage, 2016). The increase of pH in pigment extract of above selected blue flowers with 0.1 M NaOH had resulted a darker blue colour. However, the pigment extracts of current work must not contain the required genetic pathway including *F3'5'H* gene for the reaction. Therefore, identification of anthurium cultivars with higher vacuolar pH is important in future breeding for blue anthurium with an introduced *F3'5'H* gene.

Table 2b: The anthocyanidins of cultivars detected through HPLC

Anthurium cultivar	Peak number	Retention time (min)	Anthocyanidin type
Paradise	1	6.341	Cyanidin
Maxima	1	6.327	Cyanidin
Maxima	2	9.694	Pelargonidin
Tulip Summer	1	6.347	Cyanidin
Sharren	1	6.338	Cyanidin
Sharren	2	9.697	Pelargonidin
Maxima Erigancia	1	6.331	Cyanidin
Flabia	1	6.330	Cyanidin
Losa green	1	6.324	Cyanidin
Eternum	1	6.321	Cyanidin
Torenda(l)	1	6.326	Cyanidin
Maxima(m)	1	6.322	Cyanidin
Sabia	1	6.314	Cyanidin
T. Sopink	1	6.311	Cyanidin
Karishth	1	6.323	Cyanidin
T. Arura Pink	1	6.313	Cyanidin
Tropic Night	1	6.314	Cyanidin

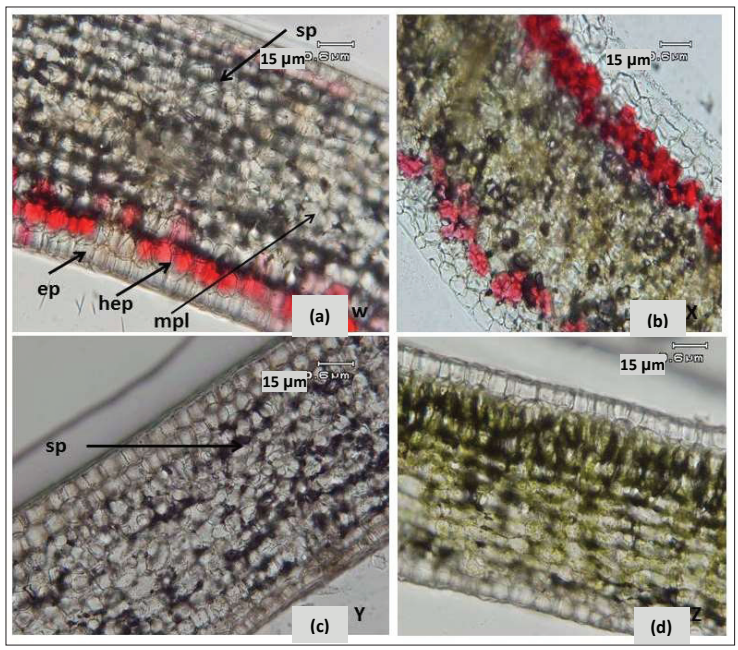

Figure 3: Localization of anthocyanin. (a) in lower epidermal layers, (b) in outermost mesophyll layer and chlorophyll in inner mesophyll cells, (c) (purple cultivar Flabia) in mesophyll cells, (d) chlorophyll in inner mesophyll cells, ep epidermal Layer, hep lower Epidermal layer, mpl mesophyll cells. (Enhanced light Microscope, Olympus, Japan; ×100)

Figure 4: Location diversity of pigments of selected different anthurium cultivars of (a) Wind, (b) Paradise, (c) Sharren, (d) Flabia, (e) Eternum, (f) Torenda, (g) Tropic Night, (h) Kisstouche, (i) Maxima Berdin, (j) Sabia and (k) Maxima Berdin, (l) Middle part of Losa Green, (m) Lobes of Losa Green. (Enhanced light Microscope, Olympus, Japan; ×10)

The location of the pigment was restricted to mesophyll and epidermis. Based on the fact that cyanidin pigment was present in all tested anthurium cultivars, the anthocyanidin type did not determine the colour variation in them. Our findings led us to investigate the genetic basis behind anthocyanidin transport and vacuolar pH, along with anthocyanin biosynthesis towards anthurium spathe colour variation.

Association between factors determining colour variation and anthocyanin regulatory pathways

Mostly the anthurium colour variation is influenced by the expression changes in structural genes such as *CHI, F3H, F3'5'H, F3'H, ANS,* and *CHS* in the anthocyanin biosynthesis pathway (Spelt *et al*., 2002; Collette *et al*., 2004; Avila-Rostant *et al*., 2010; Gopaulchan *et al*., 2014; Osorio-Guarín *et al*., 2021). According to the gene network analysis, spathe colour in anthurium was determined by three major pathways, namely, anthocyanin biosynthesis, anthocyanidin vacuolar transport, and vacuole pH/H^+ pumps in plants (Figure 6). The proposed coordinated mechanism is explained hereafter.

After biosynthesis, the anthocyanidin is transported and stored in vacuoles (Shitan & Yazaki, 2020). Fluctuations in vacuolar pH led to structural changes in the stored anthocyanidins, which result in colour changes. Furthermore, network analysis identified that all these three pathways were regulated by an R2R3-MYB transcription factor. The R2R3-MYB transcription factor fine-tunes the vacuolar pH by regulating the Vacuole-type ATPase (*V-ATPase*) which is the main H^+ concentration controller in the cell vacuole (Sundaramoorthy *et al*., 2020). The different spathe colours in tested cultivars are the result of a combination of cyanidin, which is the end product of anthocyanin biosynthesis, and the R2R3-MYB transcription factor regulating different pH values. R2R3-MYB transcription factors from chrysanthemum are reported to be inducing key structural genes in the anthocyanin pathway (Hong *et al.*, 2019).

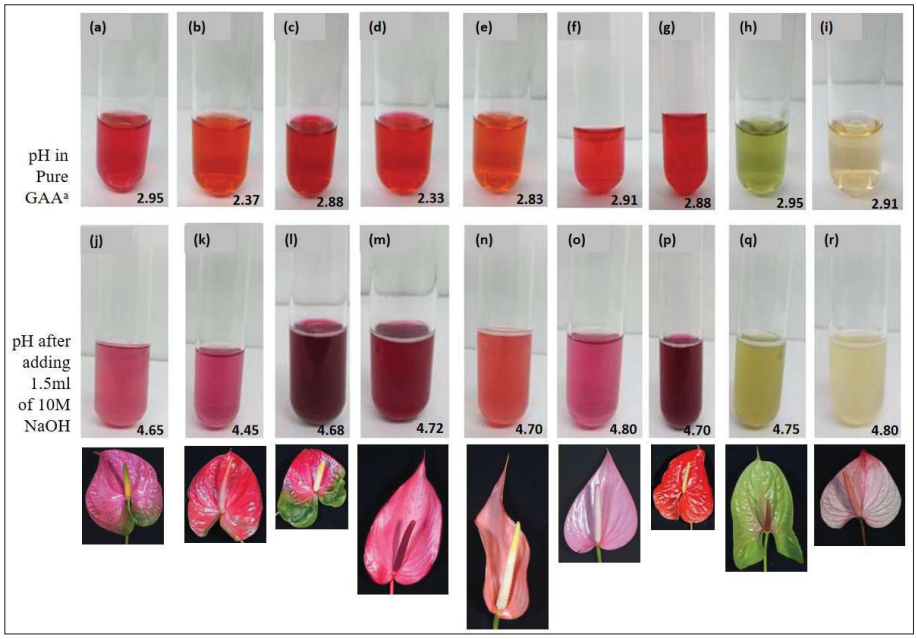

Figure 5: The effect of external change in pH on colour. (a)-(i) Colours of the glacial acetic acid extracts of the cultivars. (a) Losa Green, (b) Eternum, (c) Torenda, (d) Sabia, (e) Tulip Summer, (f) T. Sopink, (g) Karishth, (h) Kisstouche and (i) Maxima, (j)-(r) Colours of the glacial acetic acid extracts after adding 10 M NaOH. (j) Flabia, (k) Eternum, (l) Torenda, (m) Sabia, (n) Tulip Summer, (o) T. Sopink, (p) Karishth, (q) Kisstouche and (r) Maxima. [a]The pH of pure GAA was obtained as 2.73.

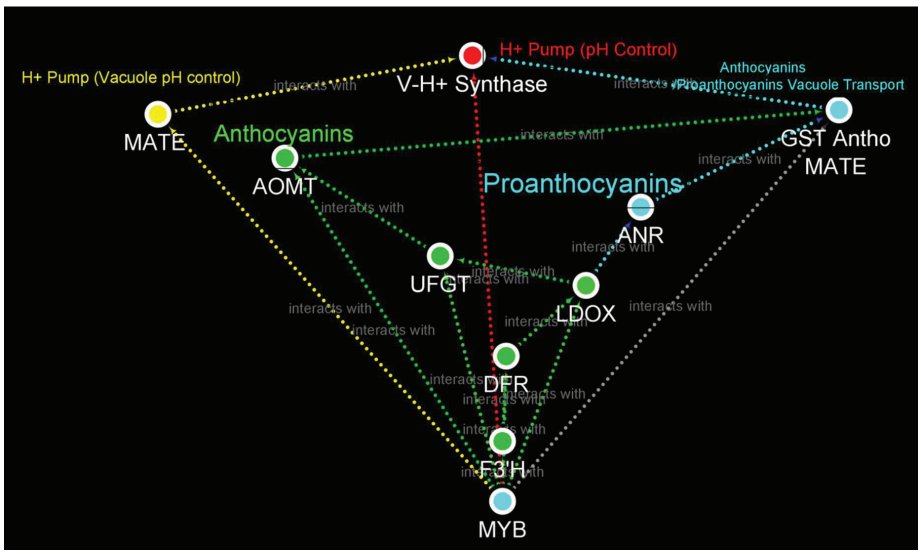

Figure 6: Proposed generalized gene biosynthesis pathway of anthocyanins using Cytoscape 3.8. The *R2R3-MYB* transcription factor gene (*MYB*), Flavanone-3-hydroxylase (*F3H*), Anthocyanidin-3-O-glucosyltransferase (*UFGT*), Leucoanthocyanidin dioxygenase (*LDOX*), Anthocyanin O-methyltransferase (*AOMT*), glutathione S-transferase anthocyanin multidrug and toxic compound extrusion family protein (*GST Antho MATE*), Multidrug and toxic compound extrusion family protein (*MATE*), anthocyanidin reductase (*ANR*), Vacuolar H^+ synthase (*V-H^+ Synthase*), are involved. Each dotted line represents one enzymatic step. Circles represent the Enzyme genes.

The effect of temperature and light on anthocyanin accumulation is regulated by *MYB* genes in the anthocyanin pathway (Azuma *et al.,* 2012). Meanwhile, Wang *et al.* (2022), reported about indirect repressors of anthocyanin biosynthesis from the MYB transcription factor family. The corresponding transcription factors are reported to be unable to bind to the promoters of the target genes and compete with the R2R3-MYB transcription activators by interacting with bHLH transcription factors in MBW complexes (Li *et al*., 2019a). Chen *et al*., 2019 had reported about R2R3-MYB transcription factors with repressive motifs as well, which directly repress the flavonoid biosynthetic genes.

There are previous reports on mutations affecting vacuolar pH regulation leading to bluish flower colour and increased pH of petal homogenates of petunia (Verweij *et al*., 2008; Faraco *et al*., 2014). According to the above study, wild-type (WT) petunia petals carrying cyanidins, displayed red colour at a vacuolar pH of 5.5 when all pH genes were functional (Faraco *et al*., 2014). However, mutations in any of the above genes had increased the vacuolar pH of petals, up to 6.0, resulting in a blue colour (Spelt *et al*., 2002; Faraco *et al*., 2014). Avila-Rostant *et al.,* 2010 had reported the relationship between spathe colour variation and vacuolar pH in anthurium, as green and white cultivars had the highest pH (5.65), while coral, (5.38), red (5.10) and orange (4.5) followed a descending order. However, there are no reports on downstream structural genes that control vacuolar pH in anthurium. Different spathe colours of anthurium were observed from spathes that had cyanidin as the only pigment. According to the pathway analysis an MYB transcription factor is involved with vacuolar pH controlling genes, which opens a new avenue for *MYB* gene function in addition to transcription activation of structural genes of the anthocyanin pathway. Therefore, we would indicate the possibility that different spathe colours resulted due to vacuolar pH manipulation through the MYB transcription factor. Although there are no reports on the identification of downstream structural genes that control vacuolar pH in anthurium, this is one possible scenario that justifies different anthurium spathe colours with cyanidin as the major dominating anthocyanidin type.

R2R3-MYB transcription factors are considered as a key determinant controlling distinct pigmentation patterns throughout the plant (Albert *et al*., 2011). The *R2R3-MYB* genes *AaMYB2* and *AaMYB3* from *Anthurium andraeanum* (Hort.) had positively regulated the anthocyanin biosynthesis genes of *AaF3H*, *AaANS*, *AaDFR,* and *AaCHS* in the spathes and leaves (Li *et al*., 2016; 2019b). In fact, there are reports on increased flower colour expression in transgenic tobacco via ectopic expression of these two R2R3-MYB transcription factors (Li *et al*., 2019). However, there are rare insights on the influence of these R2R3-MYB transcription factors on other influential pathways such as pH manipulation pathways, which affect expression of anthurium spathe colour genes.

Sundaramoorthy *et al*. (2020) had detected that R2R3-MYB was the main transcription factor in controlling vacuole acidification for flower colour changes. Three SNP mutations in an R2R3-MYB transcription factor in soy bean flower had caused fluctuations in vacuolar pH between 5.0-6.1 converting purple petals to blue petals (Sundaramoorthy *et al*., 2020). This is another possibility that explains the colour variations in selected anthurium cultivars.

Generated network models further explained that the R2R3-MYB transcription factor also regulates cyanidin transport genes like *GST Antho MATE* and *MATE*. Cutanda-Perez *et al*. (2009) reported that *VlmybA1* in grapevine is involved in anthocyanin synthesis and transport in transgenic plants. Further, the MdMYB1/10 transcription factor was reported to be affecting the cellular pH and anthocyanin accumulation by regulating different H^+ -pumping and anthocyanidin transport genes in transgenic apples resulting in colour variation in apple calli (Ling-Wang *et al.*, 2010; Hu *et al*., 2016; Li *et al.*, 2019b). In our study, we identified *Antho MATE* as the main anthocyanidin transport gene which is being regulated by R2R3-MYB transcription factor. Differential localization of cyanidin into mesophyll or epidermis in tested cultivars is suggested through the above method of transport regulation. R2R3-MYB transcription factor regulates *V-ATPase* and *MATE* genes*,* which mainly determine spathe colour variation in terms of vacuolar pH and pigment location respectively. Based on the above interpretations, R2R3-MYB transcription factors are among the key regulators of anthurium spathe colour. Therefore, the R2R3-MYB transcription factor will be an effective candidate over structural genes for genetic manipulation of spathe colour. The above assumption could be validated through a genetic transformation of homologous R2R3-MYB transcription factors from other species for novel anthurium colours.

CONCLUSIONS

Selected anthurium cultivars were variable in spathe colour, anthocyanin location, and vacuolar pH, while the main identified anthocyanidin type was cyanidin.

According to the Gene Network Model analysis, the spathe colour, anthocyanin location, and vacuolar pH were controlled by anthocyanin transport, biosynthesis, and vacuolar pH regulatory pathways respectively. An *R2R3-MYB* transcription factor gene was identified as the main regulator of vacuolar pH in the spathe leading to the higher vacuolar pH variation. Therefore, we suggest that different spathe colours resulted due to vacuolar pH manipulation through an R2R3-MYB transcription factor. The R2R3-MYB transcription factor gene could be a potential candidate in colour manipulation via genetic engineering.

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