Transformation of Butterfly Pea (*Clitoria ternatea*) *F3'5'H* (Flavonoid-3',5'-Hydroxylase) Gene into Patumma (*Curcuma alismatifolia*) 'Blue Moon'

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Abstract

Patumma (*Curcuma alismatifolia*) is a Zingiberaceae family with beautiful colors and needs to the market. There is one of the flower bulbs that make a high income. Patumma bulb was exported to foreign countries. When compared with the types of cut flowers and popularity, the improvement on a new appearance, value-added and competitive of Patumma, was necessary. In this research, the progress of Patumma 'Blue Moon' to blue shade was studied. The isolation, cloning, and characterization of flavonoid-3',5'-hydroxylase (*F3'5'H*) genes of butterfly pea (*Clitoria ternatea*), encoding an enzyme involved in the coloration of plant blue pigment, was the focus. The *F3'5'H* gene was isolated from butterfly pea (*Clitoria ternatea* and transferred to retarded shoot of Patumma 'Blue Moon' using *Agrobacterium*-mediated transformation technique. The results show that the *F3'5'H* genes were inserted into Patumma genome and expressed in the transcription phase. The study on Patumma 'Blue Moon' coloration found that transformed Patumma is bluer than non-transformed Patumma 'Blue Moon', but purple. All obtained results can be concluded that we successfully transformed *Clitoria ternatea F3'5'H* gene into Patumma and these genes could be expressed and changed the color of Patumma. Based on the findings, the knowledge to improve the color of flowers using genetic engineering is possible to be applied to improve other flowering plants to increase value and opportunity in the market.

Keywords: gene related to blue pigment biosynthesis; butterfly pea *F3'5'H* gene; *Curcuma alismatifolia*; plant transformation; blue flower

1. Introduction

In nature, blue flowers are rare. Most blue flowers have no economic value, such as the flower of butterfly pea, Asiatic dayflower. Therefore, blue is rare in economic flowers as this color is associated with organic molecules in alkaline conditions. The most popular and economical flowers in the international markets,

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such as rose, carnation, petunia, chrysanthemum, and tulip, are mostly pink and purple shades (Tanaka *et al*. 2005). Actual blue flowers are challenging to produce often expensive to come by. So far, there are not so many blue flowers having been developed. Blue rose and carnation is in fact not true blue, but pale purple. Their prices are about 600-1,000 Bath per flower for rose and 1,200 Bath per 20 flowers for carnation. At the Asia Pacific Orchid Conference 11th, the blue Phalaenopsis was shown. Since the novel blue flowers are relatively high value, many researchers are interested in studying the biosynthetic pathway of blue pigment in flowers.

For the pigment synthesis in flowers, it is derived from transformation of dihydrokaempferol to delphinidin. For the biosynthetic pathway, the anthocyanins (pelargonidin for yellow, cyanindin for red and delphinidin for blue) are biosynthesized from the malonyl- CoA derived from Kreb's cycle. They are transformed to dihydrokaempferol by chalcone synthase (CHS), chalcone isomerase (CHI), and flavonoid- 3- hydroxylase (F3H) respectively. There are three key enzymes involved in the formation of yellow, red, and blue pigment, including flavonoid- 3'- hydroxylase (F3'H), flavonoid- 3', 5'- hydroxylase (F3'5'H), and dihydroflavonol-4-reductase (DFR). For the blue pigment, it is derived from transformation of dihydro- kaempferol to delphinidin. There are three key enzymes involved in the formation of blue pigment, dephinidin, including flavonoid- 3'-hydroxylase (F3'H), flavonoid- 3',5'- hydroxylase (F3'5'H), and dihydroflavonol-4-reductase (DFR). They are many researches about these enzymes and genes that regulated enzymatic function and application of these genes for plant improvement (Tanaka *et al*., 2005; Togami *et al*. 2006). They are interested in genes such as *F3'5'H* and *DFR* because many flowers don't have them, or the gene expression is very low.

F3'5'H controls the synthesis of flavonoid-3',5'- hydroxylase that transforms dihydroquercetin into dihydromyricetin. The transgenic purple rose that is derived from the expression of the viola *F3'5'H* gene. So, the color of the flower will be turned to purple (Katsumoto *et al.* 2007). Wherever the pink lobelia is wild- type, and the blue one is transgenic lobelia derived from expression of lisianthus *F3'5'H* gene, the color of the resultant flower will be turned to deep blue (Kanno *et al*., 2003).

Patumma or Siam tulip (*Curcuma alismatifolia*) is a member of Zingiberaceae family with beautiful colors and market needs. There is one of the flower bulbs that make a high income. Patumma bulb was exported to foreign countries. Compared with the other types of cut flowers and popularity, the improvement on a new appearance, value-added, and competitiveness of Patumma was necessary. The flower's colors of Patumma have been developed through conventional breeding, but successfully bred flowers with blue pigment. Patumma 'Blue Moon' is the one of Patumma that has light blue bracts. Previously research, we found that all anthocyanin biosynthetic genes of 'Blue Moon' were expressed. They are capable to develop blue Patumma.

This research aimed to study cloning and characterization of F3'5'H gene related to flower blue pigments biosynthesis and to develop blue color in Patumma 'Blue Moon' using *Agrobacterium* transformation. Further, the information can be the guideline of Thai commercial flower to improve to using genetic engineering.

2. Materials and Methods

2.1 Plant materials

In this study, the micropropagation of Patumma 'Blue Moon' was modified by Topoonyanont *et al.* (2002). The retarded shoots of Patumma 'Blue Moon' were induced from inflorescence the shoot that cultured on MS medium (Murashige and Skoog, 1962) that supplemented with 10 mg/L of 6-benzyl amionopurine (BA), 0.1 mg/L indole-3-acetic acid (IAA), 30g/L sucrose, and 8 g/L agar, pH 5.8 and cultured under tissue culture condition. Then, retarded shoot was established.

The retarded shoots of Patumma 'Blue Moon' from tissue culture condition were used. The shoots were cut into 1 cm length segment and cultured on MS medium supplemented with a combination of various concentrations of TDZ, IAA, and IMA. All media were supplemented with 30 g/L sucrose and 8 g/L agar, pH 5.8, and were cultured at 25 ºC and 16 hours photoperiod. Six weeks later, the shoot formation and differentiation of each treatment were investigated.

2.2 Kanamycin resistance of Patumma 'Blue Moon'

The retarded shoots of Patumma 'Blue Moon' from tissue culture condition were used. The retarded shoots were cut into 1 cm length segment, were cultured in multiplication medium supplemented with five Kanamycin concentrations, 0, 50, 100, 150, or 200 mg/L. All media were supplemented with 30 g/L sucrose and 8 g/L agar, pH 5.8. The cultures were maintained in a tissue culture room at 25 ºC and 16 hours photoperiod. Six weeks later, the shoot formation and differentiation of each treatment were investigated.

2.3 Gene Cloning and Plasmid construction

This research is investigated *F3'5'H* genes from butterfly pea (*Clitoria ternatea*). We start with primer design. The primer used for amplifying the interested gene was designed from gene coding in GenBank database using oligo explorer and analyzed the properties by oligo analyzer software. Then, mRNA was extracted from the young petal of butterfly pea using easy-RED^{TM} Total RNA Extraction Kit for liquid sample (Intron Biotechnology, Korea), the first-strand cDNA was synthesized by RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA) to using oligo (dT) primer and amplified the *F3'5'H* gene by 5' and 3' RACE PCR technique.

While the *F3'5'H* gene was inserted into pTZ57R plasmid (Fermentas, USA), the DNA sequence was investigated using dye terminator sequencing (1st BASE Laboratories Sdn Bhd, Malaysia) and compared with by gene in Genbank database using BLAST. Then,

F3'5'H gene was subcloned into pStart, plant transformation binary vector, pArtblue. The pArtblue plasmid was transformed to *Agrobacterium tumefaciens* strain LBA4404 used in Patumma 'Blue Moon' transformation.

2.4 Gene transformation

The retarded shoots of Patumma 'Blue Moon' were cut into 1 cm length segment, and then they were cultured for 3 days in contact with multiplication medium supplemented with 100μM acetosyringone (AS). The *A. tumefaciens* harboring pArtblue plasmid was cultured in Luria-Bertani (LB) medium containing 50 mg/L kanamycin, 50 mg/L rifampicin at 25 ºC, 120 rpm for 3 days. It was centrifuged at 6,000 rpm for 15 min, and the pellet was resuspended in a multiplication medium supplemented with 100 μM AS.

In case of the infection, retarded shoot segments were incubated in *Agrobacterium* suspension and were shaken at 100 rpm for 15 min at room temperature. After the retarded shoot segments were transferred to co-cultivation medium (the multiplication medium supplemented with 100 μM AS) and were cultured in dark conditions for 3 days. Then, the segments were washed with sterile water containing 250 mg/L cefotaxime to remove the excess *A. tumefaciens* and then cultured on a selection medium (the multiplication medium supplemented with 100 mg/L kanamycin and 250 mg/L cefotaxime). The regenerated shoot was separated into an individual culture on a selection medium but none cefotaxime containing. After the selected Patumma 'Blue Moon' was rooted, they were transferred into the soil and culture in a closed system.

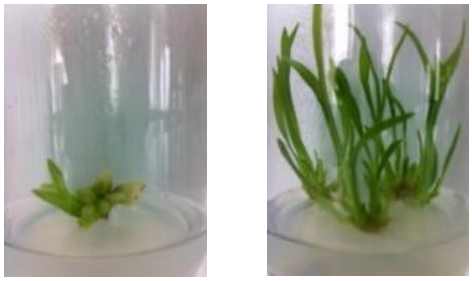
2.5 Analysis of transgenic Patumma 'Blue Moon'

The integration of *F3'5'H* gene in transgenic Patumma 'Blue Moon' was analyzed using PCR. Total genomic DNA and mRNA were extracted from bract of Patumma 'Blue Moon'. The expected product of PCR and reverse transcription polymerase chain reaction (RT-PCR) were amplified using *F3'5'H* specific primer to investigate gene integration and expression, respectively. As the sequences of the primer were as follows: F3'5'H_F, 5'-TTCAA TCCAGAGAGGTTTAT-3'; F3'5'H_R, 5'-TTAAAA CATTAATCCCAGGT-3'. PCR conditions were as follows: 2 min at 94 ºC, 35 cycles of 1 min at 94 ºC, 1 min at 47 ºC, and 1 min at 68 ºC, ending with 10 min at 68 ºC.

Then, the stability of *F3'5'H* gene in transgenic Patumma 'Blue Moon' was studied using phenotypic and genotypic observation of transgenic Patumma 'Blue Moon' in T_0, T_1, T_2, and T_3 generation. The bulb of each generation of transgenic Patumma 'Blue Moon' was planted into the soil and culture in a closed system. After that, total genomic DNA and mRNA of the bracts of transgenic Patumma 'Blue Moon' were extracted for studying gene integration and expression.

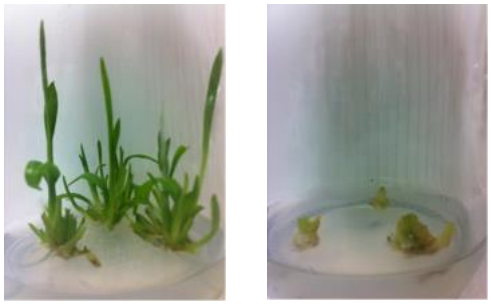
3. Results and Discussion

3.1 Optimization of multiplication of Patumma 'Blue Moon'



(A) (B)

Figure 1 Retarded shoot of Patumma ‘Blue Moon’ was cultured on (A) initiation medium (MS + 10 mg/L BA + 0.1 mg/L IAA) and (B) multiplication medium (MS + 0.5 mg/L TDZ + 0.1 mg/L IAA + 4 mg/L IMA)



(A) (B)

Figure 2 Growth and shoot formation of Patumma ‘Blue Moon’ on media supplemented with different concentrations of kanamycin after 6 weeks. (A) 0 mg/L, (B) 100 mg/L

For multiplication of Patumma ‘Blue Moon’, the retarded shoots was established from inflorescence cultivation for six weeks to induced by incorporation of plant hormones, IAA and BA, in MS media at 0.1 and 10 mg/L, respectively. Then, the multiplication of retarded shoot was induced by incorporation of TDZ, IAA and IMA, in MS media at 0.5, 0.1 and 4 mg/L, respectively. The retarded shoots were healthy. As the result of plant hormones combination can be used for induction the growth of ‘Blue Moon’ after plant transformation. (Figure 1)

3.2 Kanamycin resistance of Patumma ‘Blue Moon’

Resistance of Patumma ‘Blue Moon’ to various concentrations of kanamycin (0, 50, 100, 150, 200 mg L^{-1}) was studied. Kanamycin was chosen as the selective agent in this study because the binary vectors pArtblue contained the neomycin phosphotransferase gene that Patumma ‘Blue Moon’ derived this gene able to resistant to kanamycin. Six weeks later, the retarded shoot cultured on a medium supplemented with kanamycin at the concentration level over 100 mg/L could not grow. All shoots of Patumma ‘Blue Moon’ cultured on MS medium supplemented 0 and 50 mg/L kanamycin can grow (Figure 2, Table 1). Thus, the concentration of kanamycin at 100 mg/L was the suitable concentration for transformed leaves of Patumma ‘Blue Moon’ selection after gene transformation.

3.3 Gene cloning and plasmid construction

Isolation and cloning of *Clitoria ternatea* (*CtF3'5'H*) cDNA were performed. The PCR products were 1,572 bp and encoded a polypeptide of 523 amino acid residues. We were successfully cloned and sequenced (Figure 3). The nucleotides and amino acid sequence of *CtF3'5'H* were 100 % similarity to *Clitoria ternatea* F3'5'H mRNA (Ac no. AB185900.1).

Then, *CtF3'5'H* was subcloned into pStart, plant transformation vector, and this gene was driven by ubiquitin promoter, as pArtblue plasmid (Figure 4). This plasmid (pArtblue) also has neomycin phosphotransferase (nptII) gene as a selectable marker gene driven by nopalin synthase (nos) promoter. This plasmid was transferred into *Agrobacterium tumefaciens* strains LBA4404.

Table 1 Shoot formation of Patumma 'Blue Moon' that was cultured on medium supplemented with various concentration of Kanamycin for 6 weeks.

Kanamycin (mg/L)	No. of retarded shoot/explant*	No. of leaf/explant*	No. of root/explant*
0	6.4±0.50^{a}	3.6±0.24^{a}	2.4±0.24^{a}
50	1.4±0.24^{b}	2.6±0.24^{b}	1.4±0.24^{b}
100	0.0±0.00^{c}	0.0±0.00^{c}	0.0±0.00^{c}
150	0.0±0.00^{c}	0.0±0.00^{c}	0.0±0.00^{c}
200	0.0±0.00^{c}	0.0±0.00^{c}	0.0±0.00^{c}

*Values represent means±SE means followed by the same letter within columns are not significantly different ($p \leq 0.05$) using Duncan's new multiple range test (DMRT).

ATGTTCCTTCTAAGAGAAATTGGGGTATCAATTTTGATCTTCATGATCACCCATCTTGTGA
TTCGTTTAGTTCTGAAAGAGAAGGAACAACGGAAACTTCCACCAGGGCCAAAAGGTTGG
CCAATTGTGGGTGCACTGCCTCTAATGGGAAGCATGCCCCATGTCACACTCTCAGAAAT
GGCTAAAAAATATGGACCTGTTATGTACCTTAAAATGGGCACAAACAACATGGCTGTAGC
ATCTACTCCCTCTGCAGCTCGTGCATTCCTCAAAACCCTTGACCTTAACTTCTCCAATCG
CCCCCCAAATGCTGGGGCAACTCACTTAGCTTATGATGCCCAGGACATGGTGTTTGCTG
ATTACGGATCTAGGTGGAAGTTGCTTAGAAAACTAAGCAACTTACACATGCTTGGAGGAA
AGGCTCTTGAAGAATGGTCACAAGTTAGAGAGATTGAGATGGGGCACATGCTTCGTGCA
ATGTACGATTGTAGTGGTGGCGGTGACGGCAACAACGACAATGATGGCAACAAGAAAAA
GGGTACTCGTCATGAGCCTATTGTGGTGGCTGAAATGTTAACATACGCGATGGCCAACA
TGATAGGTCAAGTGATCTTGAGCCGTCGTGTATTCGAGACAAAGGGTTCGGAATCGAAC
GAGTTTAAGGACATGGTGGTTCAGCTCATGACCGTTGCTGGCTACTTTAACATTGGTGAT
TTTATTCCCTTTTTGGCTCGCTTCGACCTCCAAGGCATCGAGCGTGGCATGAAAACTTTG
CATAACAAGTTCGATGTTTTGTTGACGACGATGATTCATGAGCATGTGGCTTCTGCTCAT
AAACGAAAGGGTAAACCTGATTTCTTGGATGTTCTCATGGCTCATCATACCAACGAGTCT
CATGAACTGTCGCTCACCAACATCAAAGCACTCCTCTTAAATCTATTTACTGCAGGCACA
GATACATCATCAAGTATCATAGAGTGGGCACTAGCAGAGATGTTGATAAACCCAAAAATC
ATGAAGAAAGTGCATGAGGAAATGGACAAAGTGATAGGCAAGGATAGAAGGCTAAAAGA
ATCCGACATAGAAAATCTCCCTTACTTGCAGGCAATTTGCAAAGAGACATATAGAAAGCA
CCCATCAACGCCACTCAACTTGCCTAGAATCTCATCCCAAGCATGCCAAGTGAATGGCT
ACTACATCCCAAAGAACACTAGGCTTAGTGTCAACATCTGGGCCATTGGAAGAGACCCT
AATGTGTGGGAGAACCCTTTGGAGTTCAATCCAGAGAGGTTTATGGGTGCCAATAAGAC
TATTGATCCACGTGGGAATGATTTTGAGCTCATTCCATTTGGTGCTGGGAGAAGGATTTG
TGCTGGGACAAGGATGGGGATTGTGTTGGTTCAATACATTTTGGGCACTTTGGTACATT
CCTTTGATTGGAAGTTACCAAATGGTGTTGTGGAGTTGAACATGGAAGAGACTTTTGGC
CTTGCTTTGCAGAAAAAGATACCACTTTCTGCTTTGATTACCCCTAGGTTGCCCCCAACT
GCTTACAATGTTATTAATTCCTAA

(A) Nucleotide sequence

MFLLREIGVSILIFMITHLVIRLVLKEKEQRKLPP
GPKGWPIVGALPLMGSMPHVTLSEMAKKYGP
VMYLKMGTNNMAVASTPSAARAFLKTLDLNFS
NRPPNAGATHLAYDAQDMVFADYGSRWKLLR
KLSNLHMLGGKALEEWSQVREIEMGHMLRAM
YDCSGGGDGNNDNDGNKKKGTRHEPIVVAEM
LTYAMANMIGQVILSRRVFETKGSESNEFKDMV
VQLMTVAGYFNIGDFIPFLARFDLQGIERGMKTL
HNKFDVLLTTMIHEHVASAHKRKGKPDFLDVLM
AHHTNESHELSLTNIKALLLNLFTAGTDTSSSIIE
WALAEMLINPKIMKKVHEEMDKVIGKDRRLKES
DIENLPYLQAICKETYRKHPSTPLNLPRISSQAC
QVNGYYIPKNTRLSVNIWAIGRDPNVWENPLEF
NPERFMGANKTIDPRGNDFELIPFGAGRRICAG
TRMGIVLVQYILGTLVHSFDWKLPNGVVELNME
ETFGLALQKKIPLSALITPRLPPTAYNVINS

(B) Amino acid sequence

Figure 3 Nucleotide (A) and amino acid (B) sequence of *CtF3'5'H*

3.4 Plant transformation

After transformation of Patumma 'Blue Moon', the retarted shoots were grown on selection media and elongation media (Figure 5). Then, the transgenic plant of Patumma was planted in the soil, and gene integration and expression of *CtF3'5'H* gene were analyzed. The results found that the transformed Patumma 'Blue Moon' with *CtF3'5'H* genes (No. 1-7) were found by expected fragment, 1,572 bp, whereas non-transformed 'Blue Moon' was no fragment (Figure 6). The results indicated that the genes were integrated into genome of 7 transformed Patumma 'Blue Moon' and expressed in mRNA level. When the flowers of Patumma 'Blue Moon' were investigated, the results showed that the bract color of *CtF3'5'H* transgenic Patumma 'Blue Moon' was purple, whereas the wild-type was white (Figure 7). After flower color of *CtF3'5'H* transgenic Patumma 'Blue Moon' was observed for 3 generations, as the result of all generation of *CtF3'5'H* transgenic Patumma 'Blue Moon' has purple bracts (Figure 8). These results indicated that *CtF3'5'H* genes were permanent integration in the genome.

In this study, the resultant transformed Patumma 'Blue Moon' indicated that purple flower colors are primarily due to overexpression of flavonoid-3',5'-hydroxylase (*F3'5'H*) gene for anthocyanin biosynthesis. They are capable of developing blue color, but itself is purple in neutral. As a review of Yoshida *et al*. 2009, the biosynthesis pathway of blue color development in flower petals have other factors such as the presence of co-pigments and the vacuolar

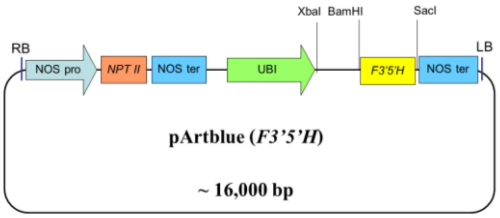
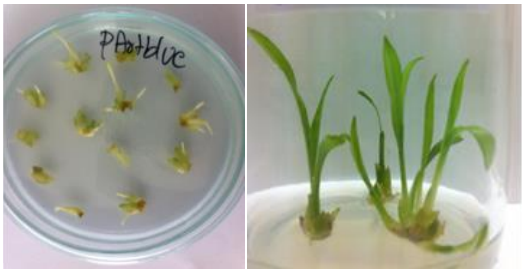


Figure 4 Plasmid map of pArtblue



(A) (B)

Figure 5 Growth and shoot formation of Patumma 'Blue Moon' after transformation on selection media (A) and elongation media (B) that transformed with *CtF3'5'H* gene.

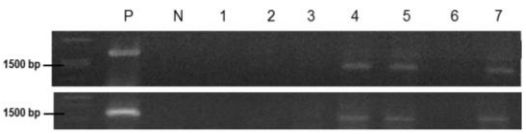


Figure 6 PCR (above) and RT-PCR (below) analysis of transformed Patumma 'Blue Moon'. Lane 1, λ/*Pst*I marker; Lane P, plasmid pArtblue as a positive control (*CtF3'5'H*); Lane N, non-transformed Patumma 'Blue Moon' as a negative control; Lane 1-7, *CtF3'5'H* transformed Patumma 'Blue Moon', PCR and RT-PCR products of the expected size were 1,572 bp.

(A) (B)

Figure 7 Flowers of Patumma 'Blue Moon' that transformed with *CtF3'5'H* (A) and non-transformed 'Blue Moon' (B).

pH also affects flower color. The resultant transgenic petals of potted Patumma 'Doitung Mini Ruby' that was transformed with dihydroflavonal 4- reductase (*DFR*) gene (Chundet *et al*. 2014) and cytochrome b_5 (*Ctyb*$_5$) genes (U- kong *et al*. 2017), produced anthocyanins, their magenta color was more brilliant than wild- type by expressing the genes. According to Araki *et al*. (2008), the highly expressed *F3'5'H* gene of *Commelina communis* was transfected to a white moth orchid having a blue flower with co- pigments gene, a gene encoding a dihydroflavonol 4-reductase (*DFR*) of Torenia or Gerbera; a gene encoding a flavanone 3- hydroxylase; and a gene encoding an anthocyanidin synthase and expressing the genes, whereby a blue moth orchid can be produced and having blue flowers. Therefore, the transformation of the gene encoding co-pigments and the vacuolar pH in flower petal are useful for altering and diversifying flower color.

(A) (B) (C) (D) (E)

Figure 8 Flowers of Patumma 'Blue Moon' that transformed with *F3'5'H* gene in T_0 (A) T_1 (B) T_2 (C) T_3 (D) generation and non-transformed 'Blue Moon' (E).

4. Conclusion

All results were indicated that we successfully isolated and cloned *CtF3'5'H* gene from Butterfly pea (*Clitoria ternatea*). The *CtF3'5'H* gene that transformed into Patumma 'Blue Moon' was successfully integrated by genome and expressed in mRNA level. Then, the genes related to the coloration of flower, co-

pigments, and the vacuolar pH in a flower petal, are further studied. The additional results could be applied to Thai flower improvement.

5. Acknowledgements

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