Proceedings of International Conference on Biodiversity: IBD2019 (2019); 116 - 121

Proposal to reduce anthocyanin-deficient banana *Musa siamensis* to a *M. rubra* variety

Wandee Inta, Panida Kongsawadworakul, Unchera Viboonjun, Paweena Chuenwarin, Paweena Traiperm and Sasivimon Chomchalow Swangpol*
Department of Plant Science, Faculty of Science, Mahidol University, Ratchathewi District, Bangkok, Thailand
*Corresponding author e-mail: sasivimon.swa@mahidol.edu

Abstract: *Musa rubra* Wall. ex Kurz and *M. siamensis* Häkkinen & Rich. H. Wallace are small wild banana species with erect inflorescences of orange and yellow bracts, respectively. While *M. rubra* is commonly found in North-East India, Myanmar to Western Thailand, *M. siamensis*, described from a specimen collected in Eastern Thailand bordering Cambodia, is known only in cultivation. Striking characters shared by both *M. rubra* and *M. siamensis* include extended rhizome, shape and texture of inflorescence bracts, morphological characters of flowers and smooth-surface seeds. Bract coloration caused by changes in cellular anthocyanin contents and revealed by expression levels of genes involved e.g. dihydroflavonol 4-reductase (*DFR*) is the lone recognized difference between the two taxa. It is, therefore, proposed here to reduce this yellow-bract taxon to *M. rubra* var. *siamensis*. These and other bananas with a wide range of colorful bracts are useful as proper models for genetics, biochemistry, cytology and biotechnology in flavonoid biosynthesis pathway.

Keywords: Chek Meas banana, Leucoanthocyanidin, *Musa laterita* Cheesman, *Rhodochlamys*, Thai Gold banana

Introduction

Ornamental banana species possess attractive bract colors such as orange, pink, bright red, purple, and also, though less frequently found, yellow and green. Among these colorful taxa, we have suspected that two species in the *Rhodoclamys* sections, *Musa rubra* Wall. ex Kurz with orange bracts and *M. siamensis* Häkkinen & Rich. H. Wallace with yellow ones, are conspecific.

The *Musa* genus has been classified into sections by Cheesman in 1947. Two out of his four sections, *Musa* and *Rhodoclamys*, both having basic chromosome number x=11, are proved later by the genetic evidences to be very closely allied (Cheesman 1947, Wong et al. 2002). They possess less glaucous, rarely or never polished, non- or less overlapping inflorescence bracts, often dorsiventrally compressed, sometimes subglobose to irregular angulate seeds, though the two sections are distinguished by different rachis directions; *Musa* is with hanging to horizontal inflorescences and *Rhodoclamys* with erect ones.

Musa rubra was first discovered by Wallich in Burma (Myanmar) and grown in Calcutta Botanical Gardens at least since as earlier as 1845 (Voigt, 1845; Hooker, 1895). It was later described and published in 1867 by Kurz from specimens he collected by himself. Afterward, Cheesman (1949) separately described a banana grown from seeds also collected from that country as *M. laterita*, however this name is recently declared as a synonym of *M. rubra* by Joe et al (2016).

In 2007, Häkkinen and Wallace described *M. siamensis* from a specimen collected in 2002 in Eastern Thailand. It has been called "Chek Meas" and used as ornamental plants by the Cambodians for a long time and later commercialized by Thai nursery industry under the name "Thai Gold." This species is closely related to *M. rubra* by having similar itinerant rhizomes, smooth-surface seeds, and molecular and cytogenetic evidences (Häkkinen and Wallace 2007; Čížková et al. 2015).

Color variation of banana male inflorescence bracts is of aesthetic value for ornamental purposes and undoubtedly intended to attract pollinators. Among other factors, anthocyanin components and combinations are stated as the cause of this diversity (Kitdamrongsont et al. 2008, Kongsawadworakul et al. 2016). Several anthocyanin pigments, genes and biosynthetic pathways have been known to pay roles. Though it is rare for a single species to contain genes encoding the entire spectrum of flower colors (Ahmed et al. 2014), bract colors of related Musaceae species widely range from orange to pink and deep purple and could be useful in the study of anthocyanin biosynthesis. Identification of anthocyanins by high performance liquid chromatography (HPLC), mass spectrometry (MS), and tandem mass spectrometry (MS/MS) revealed six major derivatives in Musaceae, i.e. delphinidin-3-rutinoside, cyanidin-3-rutinoside, petunidin-3-rutinoside, pelargonidin-3-rutinoside, peonidin-3-rutinoside and malvidin-3-rutinoside. It was found that bracts of *M. rubra* contain only non-methylated anthocyanin, i.e., delphinidin-3-rutinoside, and cyanidin-3-rutinoside. On the other hand, in *M. acuminata* with yellow bracts and *Ensete glaucum* (Musaceae) with green bracts, no anthocyanin could be detected. It was suspected that anthocyanin biosynthetic pathway in these anthocyanin-deficient bananas may have been obstructed at leucoanthocyanidin-to-anthocyanidin step or prior (Kitdamrongsan et al. 2008, Figure. 1). One of the enzymes involving in this pathway is dihydroflavonol 4-

reductase (*DFR*) which catalyzes the production of flavan-3,4-diols (leucoanthocyanidins) via the reduction of colorless dihydroflavonols (Tian et al. 2017).

Apart from collected morphological data and investigation of floral morphological characters, we studied expression levels of *DFR* from specimens of the two taxa with two different bract colors, one of which is suspected to be a mutant of the other. The results of this study would clarify taxonomic statuses of these bananas and will be beneficial in genetic resource selection for breedings and future studies on morphology, molecular biology and phylogenetics. These bananas with colorful bracts would be proper models in research involving flavonoid biosynthesis pathway and evolution of bract color variation in plants.

Materials and Methods

Two accessions of rare *M. siamensis* Häkkinen & Rich. H. Wallace from cultivations were investigated along with three accessions of *M. Rubra* Wall. ex Kurz collected from two natural populations and a market.Three accessions of *M. Acuminate* Colla subsp. *siamea* N.W. Simmonds and two accessions of *M. ornata* Roxb. were also examined for comparison (Table 1). Morphological features were investigated and photographed in the fields. Bracts were collected and stored in ice box and refrigerator before transferring to deep freezer and kept at -80°C until used. Mature flowers from male inflorescences were preserved in 70% ethanol for morphological study.

Anthocyanin extraction and analysis

Frozen bract samples were ground to a fine powder with a mortar and pestle. The powdered bracts (0.2 g) were extracted with 5 ml of methanol:HCL (99:1, v/v) at 4°C in darkness overnight. The extracts were centrifuged at 10,000 g for 15 min at 4°C to precipitate the debris. The clear supernatant was transferred to a new tube, and the absorption of the extracts at 530 and 657 nm was determined. Total anthocyanin content was calculated according to the method of Mehrtens et al. (2005) using the following formula modification: QAnthocyanins = A530-0.25xA657, where QAnthocyanins is the amount of anthocyanins and A530 and A657 are the absorption values at the indicated wavelengths. The obtained data were the mean of two independent replicates.

Expression analysis by quantitative real-time PCR (qRT-PCR)

The qRT-PCR analysis was performed with the ABI-7500 real-time PCR machine (Applied Biosystem). Twenty µl of PCR reactions containing 1 µl of 5-fold cDNA dilution, 0.4 µM of each primer, 0.2 mM dNTP mix, 2 mM MgCl2, 0.8 U of Platinum Taq DNA polymerase (Invitrogen) and 1000-fold dilution of SYBR green I (Sigma) in 1X PCR buffer. The Ct (cycle threshold) data were determined using default threshold settings. The relative expression was calculated as $2^{-\Delta Ct}$ where ΔCt = (Ct of candidate gene – Ct of internal control actin). The analyses were performed in triplicate.

Result & Discussion

Musa acuminata is quite different from other species in this study. It has pendent rachis and fruits in two rows and was previously grouped in the *Musa* section (Cheesman 1947) apart from the *Rhodoclamys* with erect type of inflorescence and fruits in one row, which *M. ornata*, *M. rubra* and *M. siamensis* belong to. Besides inflorescence bract colors, the latter three species are distinct in several aspects. Similar to most banana species in the *Musa* genus, *M. ornata* has suckers and angular rough seeds. On the other hand, *M. rubra* and *M. siamensis* possess short pseudostem of 1-1.5 m with extended rhizomes and subglobose seeds with smooth seed surface. These characters are unique and rarely found among the members of the *Musa* genus. Though *M. itinerans* Cheesman also has long travelling rhizome, its pseudostems are vigorous at 4-6 m tall. Moreover, with close investigation of floral morphology, it was found that the flowers of *M. rubra* and *M. siamensis* having median inner tepal with acuminate apex and wing apex as long as apex or nearly so (Figure 3C and D, arrow), are quite similar comparing to *M. ornata* which have median inner tepal with attenuate apex and no wing apex present in neither *M. ornata* nor *M. acuminata* (Figure 3A and B). These distinct characters were compiled in key to species as stated below.

Key to Species based on Morphology

Herbs small or large; perennial, monocarpic, monoecious. Underground stem a rhizome or corm, commonly suckering. Pseudostems clump, erect, formed by closely clasping leaf sheaths. Leaves spirally arranged, leaf blade oblong to lanceolate. Inflorescence erect to pendulous. Bracts spirally arranged, usually with bright color. Flowers basal male sterile, terminal female sterile; perianth in 2 whorls with 3 outer tepals and 2 inner ones united into a compound tepal, 5-lobed; adaxial inner tepal free. Stamens 5; Pistil 1; ovary inferior, 2 rows of ovules in each loculus. Stigma 3-lobed to clavate, or capitate. Fruit a fleshy berry. Seed surface smooth or rough.

1. Pseudostems less than 1.5 m high, inflorescence erect, fruits in one row.
   2. Rhizomatous, male bract orange red or yellow, median inner tepal apex acuminate, seed surface smooth……..…….. ***M. rubra, M. siamensis***

2. Suckering, male bract pink, median inner tepal apex attenuate, seed surface rough……..…….. ***M. ornata***

1. Pseudostems more than 1.5 m high, inflorescence pendent, fruits in two rows…… ***M. acuminata***

Anthocyanin content

The analysis revealed the correlation of total anthocyanin contents and color variation of male inflorescence bracts. The total anthocyanin level was highest in the red purple bract and, in parallel, sample of the yellow bracts yielded low anthocyanin contents (Figure 4A).

Expression analysis of DFR gene by qRT-PCR

Preliminary real-time RT-PCR analysis showed that the anthocyanin biosynthetic gene, *DFR*, expressed differently in the banana bracts of distinct colors, i.e. low or undetectable in *M. siamensis* (Figure 4B). This low or undetectable level of *DFR* gene in *M. siamensis* is consistent with the absence of *DFR* gene expression in white pomegranate (Zhao et al., 2015). This result suggested that *DFR* may be one of the main factors responsible for anthocyanin accumulation in the bract of banana species.

Previous researches on molecular biology of wild bananas which included *M. rubra* and *M. siamensis* stated that they are closely related. Čížková et al. (2015) reported that the two species share the same number of 45S rDNA and 5S rDNA loci and were grouped in the same cluster. Later, Christelová et al. (2017) constructed a dendrogram based on the results of SSR analysis and revealed that *M. ornata* formed a distinct separate cluster distantly positioned from the other *Rhodochlamys* entries which *M. rubra* and *M. siamensis* belong to. In addition, information on distribution areas of the two species hints their position in the classification. The fact that *M. siamensis* is rare and has never been found in natural habitats by the authors, while *M. rubra* distributes widely from North-East India, Myanmar to Western Thailand indicates that the former could be an unusual mutant of the latter. From these findings on morphology and molecular biology, we propose that *M. rubra* and *M. siamensis* are conspecific and reduce taxonomic status of *M. siamensis* to *M. rubra* Wall. ex Kurz var. *siamensis* (Häkkinen & Rich. H. Wallace) Swangpol & Inta.

Conclusion

Several characters including pseudostem heights, inflorescence positions, and number of fruit row, can be used to distinguish *M. acuminata* subsp. *siamea*, *M. ornata* and *M. rubra*. On the other hand, *M. rubra* and *M. siamensis* are quite similar and only difference is their inflorescence bract colors. The data obtained from this study indicated the relation of this color modification and the expression of gene involved in anthocyanin biosynthetic pathway. Further investigations are underway to study the expressions of the other structural genes and its regulators including in different banana species. This information will ease the understanding of molecular genetic background of the color differences in the banana bracts and further studies on co-evolutionary aspects with pollinators.

Acknowledgement

The projects led by S.C.S. has been supported by Mahidol University and the Thailand Research Fund (TRF) (RSA56_80033), Biodiversity-based Economy Development Office (Public Organization- BEDO) and the Faculty of Science, Mahidol University, Bangkok, Thailand.

References

Ahmed NU, Park JI, Jung HJ, Yang TJ, Hur Y, Nou IS. 2014. Characterization of dihydroflavonol 4-reductase (*DFR*) genes and their association with cold and freezing stress in *Brassica rapa*. Gene 550(1): 46-55.

Cheesman, E. E. 1947. Classification of the bananas III. The genus *Musa* L. Kew Bulletin 106-1 17.

Christelová, P., De Langhe, E., Hřibová, E, Čížková, J., Sardos, J., Husakova, M., Van den houwe, I., Sutanto, A., Kay Kepler, A., Swennen, R., Roux, N., Dolezel, J. 2016. Molecular and Cytological Characterization of the Global *Musa* Germplasm Collection Provides Insights into the Treasure of Banana Diversity. Biodiversity and Conservation 26 (4): 801-824.

Čížková, J., Hřibová, E., Christelová, P., Van den Houwe, I., Häkkinen, M., Roux, N., Swennen, R. and Dolezel, J. 2015. Molecular and Cytogenetic Characterization of Wild *Musa* Species. PLOS ONE 10(8): e0134096. https://doi.org/10.1371/journal.pone.0134096

Häkkinen, M. and Wallace, R. H. 2007. *Musa siamensis*, a new *Musa* species (Musaceae) from S.E. Asia. Folia Malaysiana 8:61-70.

Hooker, J.D. 1895. *Musa rubra*. In. Curtis's Botanical Magazine. V. 121, London: L. Reeve & Co., England. Table 7451.

Joe, A., Sreejith, P.E. and Sabu, M. 2016. Notes on *Musa rubra* Kurz (Musaceae) and reduction of *M. laterita* Cheesman as conspecific. Taiwania 61: 34-40.

Kitdamrongsont, K., Pothavorn, P., Swangpol, S., Wongniam, S., Atawongsa, K., Svasti, J. and Somana, J. 2008. Anthocyanin composition of wild bananas in Thailand. Journal of Agricultural and Food Chemistry 56: 10853-10857.

Kongsawadworakul, P., Viboonjun, U., Chuenwarin, P., Traiperm, P., & Swangpol, S. C. 2016. Expression of anthocyanin biosynthetic genes in banana bracts. In ThammaAbstracts of the First International Symposium on Tropical and Subtropical Ornamentals (TSO2016). 7-9 March 2016, Krabi, Thailand, p. 44.

Liew, C.-F., Loh, C.-F., Goh, C.-J. and Lim, S.-H. 1998. The isolation, molecular characterization and expression of dihydroflavonol 4-reductase cDNA in the orchid, *Bromheadia finlaysoniana*. Plant Science 135(2): 161–169, doi:10.1016/S0168-9452(98)00071-5.

Mehrtens, F., Kranz, H., Bednarek, P. & Weisshaar, B. 2005. The Arabidopsis transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiology 138: 1083-1096.

Nu, A., Park, J.I., Jung, H.J., Yang, T.J., Hur, Y. and Nou, I. S. 2014. Characterization of dihydroflavonol 4-reductase (DFR) genes and their association with cold and freezing stress in *Brassica rapa*. Gene 550(1): 46-55.

Quattrocchio, F.; Verweij, W.; Kroon, A.; Spelt, C.; Mol, J.; Koes, R. 2006. PH4 of Petunia is an R2R3 MYB Protein That Activates Vacuolar Acidification through Interactions with Basic- Helix- Loop- Helix Transcription Factors of the Anthocyanin Pathway. Plant Cell 18: 1274–1291.

Tian, J., Chen, M. C., Zhang, J., Li, K.T., Song, T. T., Zhang, X., Yao, Y.C. 2017. Characteristics of dihydroflavonol 4-reductase gene promoters from different leaf colored *Malus* crabapple cultivars. Horticultural Research. 4:17070. doi: 10.1038/hortres.2017.70.

Voigt, J.O. 1845. Musaceae. In: Hortus Suburbanus Calcuttensis. Calcutta: Bishop's College Press. pp. 578–579.

Wong, C., Kiew, R., Argent, G., Set, O., Lee, S. K., Gan, Y. Y. 2002. Assessment of the validity of the sections of *Musa* (Musaceae) using AFLP. Annals of Botany 90: 231-238.

Zhao, X., Yuan, Z., Feng, L., & Fang, Y. 2015. Cloning and expression of anthocyanin biosynthetic genes in red and white pomegranate. Journal of Plant Research 128: 687-696.

Table 1. List of banana accessions in this study**.**

No.	Acc. No. SS & JS	Species	Source/Origin
1	001	*Musa acuminata* Colla subsp. *siamea* N.W. Simmonds	Phetchabun
2	247	*Musa acuminata* Colla subsp. *siamea* N.W. Simmonds	Kanchanaburi
3	300	*Musa acuminata* Colla subsp. *siamea* N.W. Simmonds	Nakhon Nayok
4	449	*Musa ornata* Roxb.	Bangkok [1]
5	542	*Musa ornata* Roxb.	Chiang Mai [2]
6	352	*Musa rubra* Wall. ex Kurz	Mae Hong Son
7	420	*Musa rubra* Wall. ex Kurz	Mae Hong Son
8	516	*Musa rubra* Wall. ex Kurz	Bangkok [1]
9	524	*Musa siamensis* Häkkinen & Rich.H.Wallace	Bangkok [1]
10	624	*Musa siamensis* Häkkinen & Rich.H.Wallace	Bangkok [2]

[1] Cultivated clones purchased from a plant market.
[2] Specimens grown as ornamental plants in public parks.

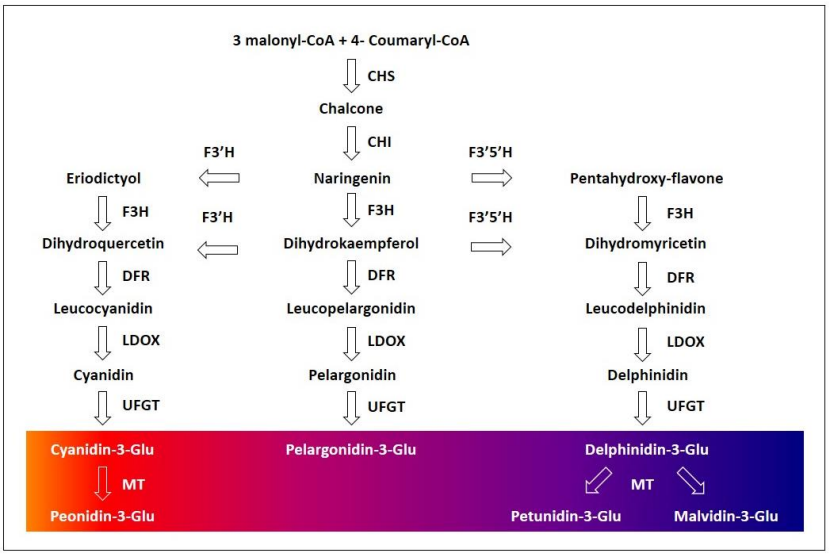

Figure 1. Simplified scheme of the flavonoid biosynthesis pathway modified from that of petunia (Quattrocchio, 2006).

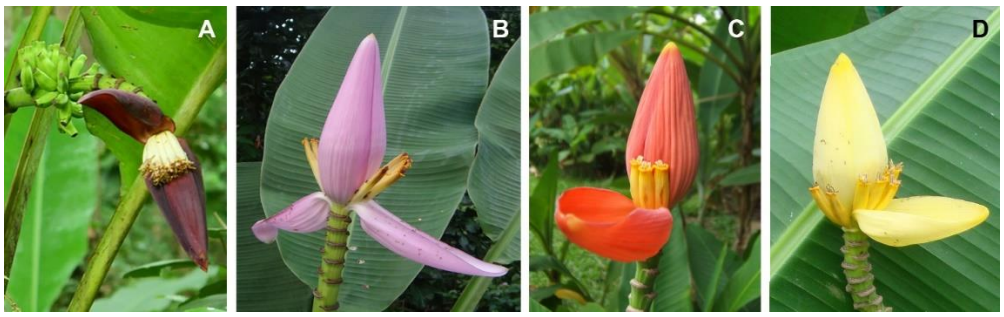

Figure 2. Male inflorescence of *Musa* L. (Musaceae) showing different bract colors; (A) purple, *M. acuminata* Colla subsp. *siamea* N. W. Simmonds, (B) pink, *M. ornata* Roxb., (C) orange, *M. rubra* Wall. ex. Kurz, and (D) yellow, *M. siamensis* Häkkinen & Rich. H. Wallace. Photos not to scale.

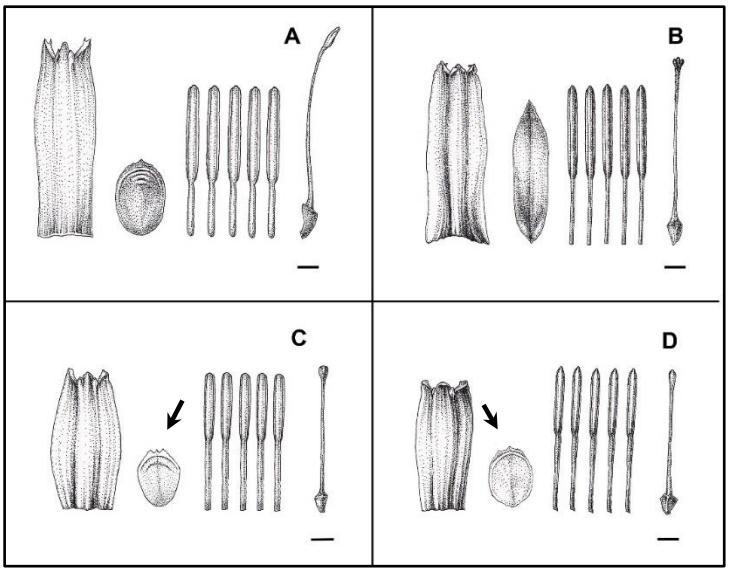

Figure 3. Drawing of floral parts showing, from left to right, a compound tepal, a free tepal, five stamens and a pistil of each taxa (A) *M. acuminata* subsp. *siamea*, (B) *M. ornata*, (C) *M. rubra*, (D) *M. siamensis*. Arrow indicated wing apex. Scale bar = 0.5 cm.

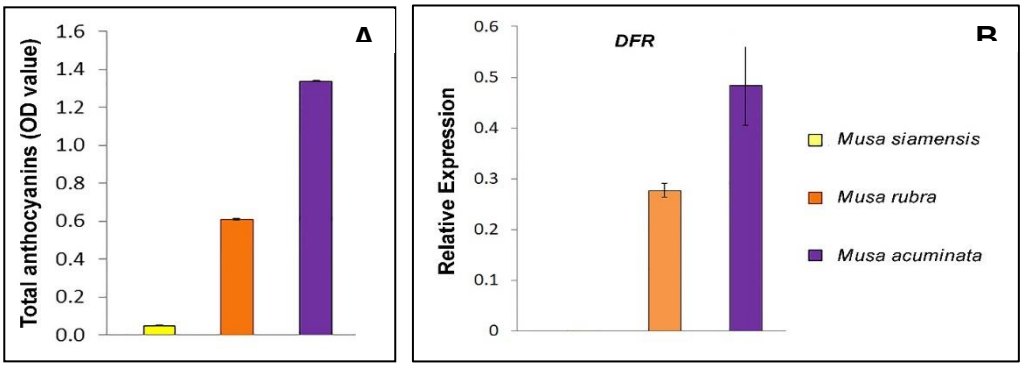

Figure 4. Total anthocyanin content in banana bract (A) and the relative expression pattern of *DFR* genes in bracts of *M. siamensis*, *M. rubra* and *M. acuminata* (B). Notice that relative expression of *DFR* in *M. siamensis* is low or undetectable. Relative expression profiles (means of the normalized expression) were obtained by quantitative real-time PCR analyses. Bars are the standard errors from the means.