MOLECULAR CLONING AND EXPRESSION ANALYSIS OF CONSTANS-LIKE 2 IN *Mucuna bracteata* DC. IN DIFFERENT TISSUES AND AT LOW TEMPERATURE

By

MISFAHULHAIRAH BINTI SHARIFF

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MOLECULAR CLONING AND EXPRESSION ANALYSIS OF CONSTANS-LIKE 2 IN *Mucuna bracteate* DC. IN DIFFERENT TISSUES AND AT LOW TEMPERATURE

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The flowering signals were perceived by angiosperms for the transition from vegetative to reproductive state at their apical tissue. The transition was triggered by environmental conditions and the internal regulation of various genes. Plants are capable of integrating environmental changes such as photoperiod and temperature into their developmental program. *Mucuna bracteata* is a legume originated from North India and is planted as a cover crop in the oil palm and rubber plantations in Malaysia. This legume is able to grow well locally but unable to produce flowers. Non-flowering *M. bracteata* plants may not perceive the flowering signals to initiate the reproductive phase due to environmental differences. This study is the first report on the cloning of a putative flowering gene, *CONSTANS-LIKE 2* (*COL2*) from the leaves of *M. bracteata*. In this study, the *MbCOL2* sequence with the length of 1335bp was successfully identified and cloned. MbCOL2 protein (335 amino acid residues) consisted of two BBOX domains, a CCT domain and a VP motif. The bioinformatic analysis on the unpublished transcriptome data showed that 13 COL protein members were present in the *M. bracteata* genome which had been classified into Groups 1, 2 and 3. To understand the molecular regulation of this gene in locally grown *M. bracteata*, the expression analysis of *MbCOL2* in different tissues of *M. bracteata* seedlings and in response to low temperature was carried out using real time PCR. *MbCOL2* expression in all selected tissues; leaf, shoot apical meristem and stem of 2-month, 6-month and 12-month old seedlings showed that *MbCOL2* was expressed at the early stage of the plant growth. However, a higher abundance of *MbCOL2* expression was detected in the leaf tissues as reported previously in other reference species. Meanwhile, the expression of *MbCOL2* was increased by low temperature. However, the actual molecular mechanism of the flowering initiation of locally grown *M. bracteata* is still

unknown. In the future, identification and expression profiling of various flowering genes can be performed to further understand the flowering induction in *M. bracteata*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Master Sains

PENGKLONAN MOLEKUL DAN ANALISIS PENGEKSPRESAN CONSTAN-LIKE 2 *Mucuna bracteata* DC. DALAM TISU BERBEZA DAN PADA SUHU RENDAH

Oleh

MISFAHULHAIRAH BINTI SHARIFF

Julai 2021

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Angiosperma mengesan isyarat pembungaan untuk peralihan daripada fasa vegetatif ke fasa pembiakan pada tisu apikalnya. Peralihan ini dicetus oleh keadaan persekitaran dan regulasi dalaman pelbagai gen. Tumbuhan mampu mengintegrasikan perubahan persekitaran seperti fotokala dan suhu ke dalam program pengembangannya. *Mucuna bracteata* adalah sejenis kekacang penutup bumi yang berasal dari India Utara dan ditanam sebagai tanaman penutup bumi di ladang kelapa sawit dan getah Malaysia. Kekacang ini mampu tumbuh dengan baik secara tempatan tetapi tidak mampu menghasilkan bunga. Tumbuhan *M. bracteata* yang tidak berbunga mungkin tidak mengesan isyarat pembungaan untuk memulakan fasa pembiakan kerana perbezaan persekitaran. Kajian ini adalah laporan pertama tentang pengklonan gen berbunga, *CONSTANS-LIKE 2 (COL2)* daripada daun *M. bracteata*. Dalam kajian ini, jujukan *MbCOL2* dengan panjang 1335bp telah berjaya dikenal pasti dan diklon. Protein MbCOL2 (335 asid amino) terdiri daripada dua domain BBOX, domain CCT dan motif VP. Analisis bioinformatik pada data transkrip yang belum diterbitkan menunjukkan bahawa terdapat 13 ahli protein COL dalam genom *M. bracteata* yang telah diklasifikasikan kepada Kumpulan 1, 2 dan 3. Untuk memahami peraturan molekul gen ini dalam *M. bracteata* yang ditanam secara tempatan, analisis ekspresi *MbCOL2* dalam tisu berbeza anak benih *M. bracteata* dan sebagai tindak balas kepada suhu rendah telah dijalankan menggunakan PCR masa nyata. Ekspresi *MbCOL2* telah dikenal pasti dalam semua tisu terpilih; daun, pucuk meristem apikal dan batang anak pokok berumur 2 bulan, 6 bulan dan 12 bulan menunjukkan bahawa *MbCOL2* telah terekspresi pada peringkat awal pertumbuhan tumbuhan. Ekspresi *MbCOL2* yang lebih tinggi telah dikesan dalam tisu daun seperti yang dilaporkan sebelum ini dalam spesies rujukan lain. Sementara itu, jujukan *MbCOL2* meningkat dengan suhu rendah. Walau bagaimanapun, mekanisma sebenar permulaan proses pembungaan *M. bracteata* yang ditanam tempatan masih

tidak diketahui. Pada masa hadapan, pengenalpastian dan pemprofilan ekspresi pelbagai gen berbunga boleh dilakukan untuk memahami lebih lanjut aruhan berbunga dalam *M. bracteata*.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the Master of Science . The members of the Supervisory Committee were as follows:

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LIST OF ABBREVATIONS

α	Alpha
β	Beta
°C	Degree celsius
%	Percentage
A_{260nm}	Optical density at wavelength 260 nanometer
µl	Microliter
µg	Microgram
µm	Micrometer
µmoles	Micromoles
bp	Base pair
cm	Centimeter
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CO	CONSTANS
COL	CONSTANS-LIKE
CTAB	Cetyl trimethylammonium bromide
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
Ft	Flowering Locus T
g	Gram

GSP	Gene specific primer
ha	Hectare
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
kDA	Kilodaltons
kg	Kilogram
L	Litre
LB	Luria-Bertani
LCC	Leguminous cover crop
LiCl	Lithium Choride
M	Molar
ml	millilitre
m	meter
N	Nitrogen
NaCl	Sodium chloride
SAM	Shoot apical meristem
TAE	Tris base, acetic acid and EDTA solution
U	Unit
UPS	Universal primer short
V	Volt
w	Weigh

CHAPTER 1

INTRODUCTION

Oil palm requires large amounts of nutrients to sustain its growth and production, so that high yield levels of oil, 30 tons/ha/yr^{-1} or more can be achieved and maintained. Sustainable oil palm cultivation is gaining popularity in Malaysia. Leguminous cover crop (LCC) has been widely used in oil palm and rubber plantation to maintain soil health. *Mucuna bracteata* is an important LCC, originated in North-Eastern of India. It is distributed in the Indian subcontinent, Thailand, Myanmar, Vietnam, Laos and China (Wilmot-Dear, 1987). In 1991, *M. bracteata* was first introduced to Malaysia by the Golden Hope Plantations Berhad. This legume was first grown in the North Labis Estate, Johor as green manure to cover the inter-row of oil palm plants (Mathews, 1998). The introduction of *M. bracteata* has brought back the interest and emphasis to establish pure legume cover for the oil palm plantation. Preliminary agronomic work has shown that it confers similar benefits to oil palm as the conventional leguminous cover crops, which are more difficult and costly to establish well. Its shade-tolerant attribute enables it to persist under mature palms and continues its roles in soil and water conservation, fertility preservation and atmospheric-fixed nitrogen (N) cycling to the oil palm plants (Chua *et al*., 2007).

In order to compare the amount of LCC needed required per hectare of plantation land, few field studies have been performed. Usually one to two grams per 10 square meters or one to two kilograms of cover crop seeds per hectare is needed to fully cover the planation interrows. However, only around 200 to 300 grams of *M. bracteata* seeds are needed for one-hectare land (Chee, 2007), which is very cost-effective. *M. bracteata* is capable of fixing about 70% of its N requirement and researchers estimated that a net return of 35 kg N ha^{-1} will be produced in a turnover time of 10 weeks with a decomposition rate at 76% (Chiu, 2004). The comparison between *M. bracteata* and *Pueraria phaseoloides* in the conversion of dry weight to fresh weight also showed that *M. bracteata* produces more dry matter than *P. phaseoloides.* The dry green matter conversion of *M. bracteata* and *P. phaseoloides* was 28% and 18%, respectively.

In term of pest and disease attacks, *M. bracteata* is found to be the most resistant LCC towards all pests and diseases commonly exist in oil palm agroecosystem. It is generally due to the high level of phenolic compound found in the leaf of this legume (Mathews, 1998b). The thick *M. bracteata* covering on the palm trunk and ground also reduce the damage of oil palm plants caused by *Oryctes rhinoceros* beetles from 35% to 7%. The thick cover crop has limited the ability of the beetles to find breeding sites and thus protects the plantation (Chua *et al*., 2007). In addition, this legume has high resistance against nematode (Thankamoni *et al.*, 1989), reduces parasitic

nematode in soil (Kothandaraman *et al*., 1989) and survive with *Ganoderma* infection (Ariffin *et al*., 2003). Despite all the advantages of this species, *M. bracteata* also has few drawbacks. Its fast-growing nature and the ability to regenerate very rapidly, if uncontrolled, can smother the palms, especially the young seedlings. Thus, maintenance of the plantation is still needed to make sure the oil palm plants grow at the optimum pace. The thick cover crop was recommended to be trimmed at knee-level monthly or maintained using a chemical control alternative, which is time-effective to control the rapid growth (Goh *et al*., 2014).

Despite all the advantages of this species, *M. bracteata* also has few drawbacks. Its fast-growing nature and the ability to regenerate very efficiently, if uncontrolled, can smother the palms, especially the young seedlings. Thus, maintenance of the plantation is still needed to make sure the oil palm plants grow at the optimum pace. The thick cover crop was recommended to be trimmed at knee-level monthly or maintained using a chemical control alternative, which is time-effective to control the rapid growth (Goh *et al.*, 2014).

Another drawback of this species is that the locally-grown *M. bracteata* has the non-flowering characteristic. It is almost impossible to produce the flower in Malaysia, although few research had been carried out by growing the plants at hilly regions. Researchers had planted *M. bracteata* in areas with different latitudes and temperature such as Penang Hill in Penang. It has flowered but no seed formation (Chee, 2007). Since *M. bracteata* only flowered under certain environment locally, it is important to study the condition needed for the seeds to be produced. Until now, there is very limited information on the molecular event of *M. bracteata* reproductive growth. The unavailability of the genomic sequence of this legume in the database also causes the difficulty to study the non-flowering properties of this locally grown *M. bracteata*.

Throughout this research, the Zinc-finger transcription factor *CONSTANS* that has a well-established central role in the mechanism of flowering activation was targeted as the gene of interest in this study. Although *CONSTANS-LIKE* (*COL*) genes in other species have also been shown to regulate flowering time, it is not clear how widely this central role in flowering induction is conserved. Orthologs of several *Arabidopsis* genes have been shown to participate in legume photoperiodic flowering, but the possible function of *COL* genes as photoperiodic integrators of the photoperiod response has not yet been thoroughly investigated (Wong *et al*., 2014). Prior to that, *COL* gene from *M. bracteata* needs to be sequenced and characterised in order to investigate the flowering induction in this locally-grown legume. The initiation of flowering is controlled by both genotype and environmental stimuli such as day length, temperature, and light intensity. In a model plant, *Arabidopsis thaliana (A. thaliana*), flowering is stimulated by long photoperiods, and it is therefore categorized as a facultative long day (LD) plant (Rédei, 1962). In some species, flowering is hastened by low temperatures, in which the

process called vernalization and by a high ratio of far red to red light (Bagnall, 1993). In species such as *Arabidopsis*, the flowers are initiated from a population of stem cells, which is collectively known as the shoot meristem. During the transition from vegetative phase to reproductive phase, shoot meristem cells undergo divisions and change the development of leaf primordia into inflorescence primordia, giving rise to both inflorescence and flower meristems (Putterill *et al*., 1995). Signaling cascades are specific to plants in response to stimuli. Light signaling, photoperiodic flowering and regulation of circadian rhythms in *A. thaliana* are controlled by a CCT (CONSTANS, CONSTANS-LIKE, TIMING OF CAB1; TOC1) domain. This domain has been found in 45 *A. thaliana* proteins, which include protein classes that are necessary in several of the signaling pathways. The CCT domain was formerly described as a 43–amino acid region of homologs found in the *A. thaliana*, which were CO, CO-LIKE (COL), and TOC1 proteins (Wenkel *et al*., 2006). In this study, *COL2* gene was successfully identified and cloned, instead of *CO*. Although CO and its close relative COL1 and COL2 exhibit high amino acid sequence similarities, only the CO protein regulates floral induction in Arabidopsis. However, the function of COL family protein members was diversed in different species. In this study, COL2 was hypothesized to be involved in the flowering activation of the locally grown *M. bracteata* and the expression of *COL2* gene is increased when exposed to low temperature.

Hence, the aims of this study were:

1. To identify and clone the sequence of a putative flowering transcript, *CONSTANS-LIKE 2* (*COL2*) from *M. bracteata* leaves;
2. To analyse members of the CONSTANS-LIKE family from transcriptome data obtained from RNA sequencing; and
3. To determine the expression profile of *MbCOL2* in different tissues of locally grown *M. bracteata* seedlings and in response to low temperature.

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