องค์ประกอบที่เป็นแอลคาลอยด์กลุ่มไอโซควิโนลินและการแสดงออกของยีนชีวสังเคราะห์ในบัวหลวงที่ถูกทำให้เกิดบาดแผลโดยวิธีกล

นางสาวฐิติรัตน์ มีลาภ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ISOQUINOLINE ALKALOID COMPOSITION AND EXPRESSION OF BIOSYNTHETIC GENES IN MECHANICALLY WOUNDED SACRED LOTUS

Miss Thitirat Meelaph

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biomedicinal Chemistry
Department of Biochemistry and Microbiology
Faculty of Pharmaceutical Sciences
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Thesis Title	ISOQUINOLINE ALKALOID COMPOSITION AND EXPRESSION OF BIOSYNTHETIC GENES IN MECHANICALLY WOUNDED SACRED LOTUS
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ฐิติรัตน์ มีลาภ : องค์ประกอบที่เป็นแอลคาลอยด์กลุ่มไอโซควิโนลินและการแสดงออกของยีนชีวสังเคราะห์ในบัวหลวงที่ถูกทำให้เกิดบาดแผลโดยวิธีกล (ISOQUINOLINE ALKALOID COMPOSITION AND EXPRESSION OF BIOSYNTHETIC GENES IN MECHANICALLY WOUNDED SACRED LOTUS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. วันชัย ดีเอกนามกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ภญ. ดร. สรกนก วิมลมั่งคั่ง, หน้า.

ไอโซควิโนลินแอลคาลอยด์เป็นสารขั้นทุติยภูมิที่พบในพืชหลายชนิด รวมถึงบัวหลวง (*Nelumbo nucifera* Gaertn.) ใบของบัวหลวงมีองค์ประกอบของไอโซควิโนลินที่ให้ฤทธิ์ทางชีวภาพหลักสองชนิด คือ nuciferine และ *N*-nornuciferine มีรายงานถึงเอนไซม์ที่เกี่ยวข้องกับวิถีชีวสังเคราะห์ของสารสำคัญกลุ่มนี้ ได้แก่ NCS 6OMT CNMT และ WRKY TFs การศึกษาครั้งนี้ได้ทำการวัดปริมาณการสะสมของสารสำคัญและระดับการแสดงออกของยีนที่กล่าวมาในข้างต้น ในใบของบัวหลวงที่ถูกทำให้เกิดบาดแผลโดยวิธีกลและในชิ้นส่วนบัวหลวงที่เก็บจากแหล่งธรรมชาติ เพื่อศึกษาและเปรียบเทียบหน้าที่ของยีนที่มีผลต่อการสะสมสารสำคัญ ผลการศึกษาพบว่า ยีนเป้าหมายที่เลือกมาทำการศึกษาแสดงอัตลักษณ์ของกลุ่มยีนเดียวกันที่มีการรายงานในพืชชนิดอื่น และการแสดงออกของยีน *NCS CNMT* และ WRKY TFs (*NNU_24385*) ยังเพิ่มขึ้นสอดคล้องกับการสะสมสารสำคัญ อีกทั้งลำดับการแสดงออกของยีนบ่งชี้ว่า CNMT เข้ามามีบทบาทในวิถีชีวสังเคราะห์ของสารสำคัญก่อน 6OMT ในบัวหลวงที่เก็บจากแหล่งธรรมชาติ พบว่า มีการแสดงของยีน *6OMT* และ *NCS* สูงที่สุดในเนื้อเยื่ออ่อนและแก่ ตามลำดับ โดยมี WRKY TFs (*NNU_24385*) แสดงออกอย่างโดดเด่นในเนื้อเยื่อของบัวทุกส่วนที่นำมาศึกษา เป็นที่สังเกตว่า ระดับการแสดงออกของยีน *CNMT* เพิ่มขึ้นอย่างมีนัยสำคัญสอดคล้องกับการสะสมสารสำคัญ โดยเฉพาะ nuciferine ในใบบัวหลวงที่มีบาดแผล ซึ่งพฤติกรรมการแสดงออกของยีน *CNMT* อาจจะแสดงถึงความสำคัญของยีนนี้ในการเพิ่มการสะสมของสารสำคัญ จากการศึกษาสรุปได้ว่า *CNMT* และ WRKY TFs (*NNU_24385*) มีการแสดงออกที่โดดเด่นในสภาพการเกิดบาดแผลในใบบัวหลวง นอกจากนี้ ยังพบว่า บัวหลวงกลุ่มที่ให้ดอกสีชมพู (Rosem Plenum) มีปริมาณการสะสามสารสำคัญค่อนข้างสูง โดยเฉพาะในใบอ่อน บัวกลุ่มนี้อาจจะเป็นตัวแทนที่ดีในการพัฒนาเพื่อเพิ่มผลผลิตสารสำคัญในบัวหลวงในท้องตลาดต่อไป

ภาควิชา	ชีวเคมีและจุลชีววิทยา	ลายมือชื่อนิสิต .....
สาขาวิชา	ชีวเวชเคมี	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา	2560	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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THITIRAT MEELAPH: ISOQUINOLINE ALKALOID COMPOSITION AND EXPRESSION OF BIOSYNTHETIC GENES IN MECHANICALLY WOUNDED SACRED LOTUS. ADVISOR: ASSOC. PROF. WANCHAI DE-EKNAMKUL, Ph.D., CO-ADVISOR: ASST. PROF. SORNKANOK VIMOLMANGKANG, Ph.D., pp.

Isoquinoline alkaloids, a plant secondary metabolites present in many plant species including lotus (*Nelumbo nucifera* Gaertn.). Lotus leaf contains high amount of bioactive nuciferine and *N*-nornuciferine. A set of enzyme including NCS, 6OMT and CNMT and WRKY transcription factors (WRKY TFs) has been reported to have corresponding function in the biosynthesis isoquinoline alkaloids. Thus, we performed quantitative analysis on expression level of these corresponing genes and on nuciferine and *N*-nornuciferine content in lotus leaf using mechanical wounding method. Sequence analysis clearly showed that all the targeted genes possess the conserved region belongs to their protein families. The accumulation of compound correlated well with expression of *NCS*, *CNMT* and one WRKY TFs (*NNU_24385*) in the wounded leaf. Pattern of gene expression suggested that CNMT played a role in the biosynthetic pathway before 6OMT. In normal condition, *6OMT* and *NCS* showed the highest transcript level in young and mature tissues of wild Thai lotus organs, respectively, while *NNU_24385* was dominant WRKY TFs in all subjected organs. Interestingly, expression of *CNMT* in the wounded leaf showed a well relationship with compound accumulation unlike the normal leaf; this may suggest an important role of *CNMT.* To conclude, *CNMT* and *WRKY TFs (NNU_24385)* played a dominant role in response to mechanical wounding in lotus leaf. Moreover, Rosem Plenum contain high level of compounds. Thus, this group may be a good source for the development on BIA production in commercial lotus.

Department:	Biochemistry and Microbiology	Student's Signature .....
		Advisor's Signature .....
Field of Study:	Biomedicinal Chemistry	Co-Advisor's Signature .....
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Lastly, I praise to God and give thanks to my family for the faith they have given to me at the beginning.

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CHAPTER I
Introduction

Lotus (*Nelumbo nucifera* Gaertn), an aquatic perennial plant (Bera et al., 2011), is used as an important raw material of folk medicine in Thailand and widely distributed throughout Asia and Oceania (Nakamura et al., 2013). Leaves of lotus, tender rhizomes, and stems are edible and its seeds are rich in protein as well as minerals. Many research studies revealed that lotus plants provide several bioactive compounds; terpenes, phenolics, flavonoids, and alkaloids (Akinjogunla et al., 2010; Nakamura et al., 2013)

The analysis of organic compounds (secondary metabolites) in lotus plant using High Performance Liquid Chromatography (HPLC) leads to the discovery of its biological active compounds and adds economic value of lotus. The determination of all major alkaloids in *N. nucifera* leaves shows that lotus leaves and petal accumulate high amount of benzylisoquinoline alkaloids (BIAs) including *N*-nornuciferine, *O*-nornuciferine, nuciferine, and roemerine (Chen et al., 2013; Deng et al., 2016; Do et al., 2013). In addition, lotusine, liensinie, isoliensinine, and neferine are major compounds found in leaf sap, seed, embryo, and tender rhizome (Zheng et al., 2010). Nuciferine and *O*-nornuciferine are the most abundant in seed-producing cultivars and some in flower-producing cultivars (Chen et al., 2013; Do et al., 2013; JI et al., 2014).

The pathway leading to the formation of many BIAs in various alkaloid-producing plant species have been successfully elucidated with identification on the corresponding genes (Balandrin et al., 1985; Deng et al., 2016; Liscombe and Facchini, 2007). Generally, Simple BIAs derived from tyrosine via a complex array of biosynthetic enzymes, such as norcoclaurine synthase (*NCS*) (Vimolmangkang et al., 2016) and methyltransferase; *O*-methyltransferase and *N*-methyltransferase and then downstream enzymes subsequently generate a specific BIA structure (Staniek et al., 2013). Based on their chemical structure, BIAs are divided into three categories; monobenzylisoquinoline-type alkaloids or simple BIAs, aporphine-type alkaloids and bisbenzylisoquinoline-type alkaloids (Deng et al., 2016).

Plants containing BIAs have been investigated for their pharmaceutical values, such as analgesics morphine and codeine from *Papaver somniferum*, berberine from *Coptis japonica* and antibacterial agent sanguinarine from *P. somniferum and Eschscholzia californica*. Similarly, lotus containing BIAs has also been reported to possess interesting biological activities. For example, aporphine-type nuciferine and *N*-nornuciferine exhibit anti-diabetic (Sakuljaitrong et al., 2013), anti-HIV (Do et al., 2013), and melanogenesis inhibitory activities. (Nakamura et al., 2013). Though their chemical structure and potential bioactivity have been discovered but their biosynthetic pathway and key enzymes are still undocumented. Thus, it is interesting to study on this and indicate the key genes for metabolic control under the desire of high BIA production.

Moreover, wild Thai lotus is genetically separated from other wild lotuses from USA and China which represented different ecotypes of *N. nucifera* (Hu et al., 2012; Mei et al., 2013) and most of them are subjected to study on Micropropagation and bioactivity but the knowledge on their qualitative and quantitative distributions are still not consistent. To increase the value of Thai lotus and to facilitate pharmacological study which requires high level of alkaloid production, it is necessary to determine alkaloid level and to investigate individual alkaloid distribution throughout Thai lotuses.

In this study, we conducted quantitative analysis of two major BIAs; nuciferine and *N*-nornuciferine and investigated the expression of some corresponding genes, including *NCS*, *CNMT*, *6OMT* and the WRKY family of TFs using mechanical wounding method in lotus leaf. We also subjected two organs from different developmental stages of wild Thai lotus and commercial lotus under the purpose of pharmaceutical usage and simultaneously improve the production of BIAs in this lotus plant. Our findings could be helpful for developing lotus varieties with high level of desired BIAs.

CHAPTER II

Literature review

2.1 Isoquinoline Alkaloid

Alkaloids constitute; a low-molecular weight, nitrogenous containing compounds, found in many plant genera, is one of the three vast majority of plant natural products; isoprenoids, phenylpropaniods and alkaloids. More than 21,000 different alkaloid structures are currently known and present in about 20% of plant species, and also have been targeted to study on their properties and bioactivities The potent biological activity of some alkaloids has also led to their exploitation as pharmaceuticals, stimulants, narcotics, and poisons (Staniek et al., 2013).

Alkaloids are classified base on their chemical structure and geographical distribution. They have been described function as following (ศรีตุลารักษ์, 2553).

1) Alkaloid acts as the defense of plant against herbivores. The bitterness expressed by alkaloid is able to protect a whole plant from herbivores
2) Alkaloid is produced by detoxification system in plant
3) Alkaloid acts as a plant regulator
4) Alkaloid is nitrogen supplementary source for plant metabolism
5) Alkaloid acts as the defense of plant against pathogens

Alkaloid derived from amino acids including ornithine, lysine, phenylalanine, *L*-histidine, anthranilic acid, nicotinic acid and tyrosine. Tyrosine takes an important role as a precursor in biosynthetic pathway of isoquinoline alkaloids. Isoquinoline alkaloid contains isoquinoline nucleus (Figure. 1), a core structure derived from tyrosine, and form the small to the large scale structure. Regarding to the chemical structure, isoquinoline alkaloid can be divided into 5 categories; 1) Simple tetrahydroquinoline alkaloids 2) Benzyltetrahydro-isoquinoline alkaloids (BIAs) 3) Phenethylisoquinoline alkaloids 4) Monoterpenoid tetrahydro-isoquinoline alkaloids and 5) Amaryllidaceae Alkaloids. To date, Many BIAs with potentially biological activity were found to present in many plant families such as Nelumbonaceae but the knowledge on their biosynthetic pathway are still lack. Study on the corresponding genes in BIA-producing plant species would add value to the plants and fulfill the knowledge gap,

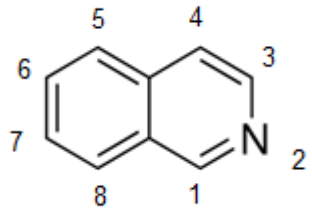


Figure 1 Isoquinoline nucleus

2.2 Biosynthesis of benzyltetrahydroisoquinoline alkaloids (BIAs)

The biosynthesis of BIAs begins with the condensation of dopamine and 4-hydroxyphenylacetaldehyde by norcoclaurine synthase (*NCS*) to yield the trihydroxybenzylisoquinoline alkaloid (*S*)- norcoclaurine, a central-branch intermediate of BIAs, 2 prior steps are involved; 1) L-tyrosine converted to tyramine and to dopamine through *ortho*hydroxylation, decarboxylation and oxidation reaction respectively 2) L-tyrosine converted to 4-hydroxyphenylpyruvic acid and 4-hydroxyphenyl acetaldehyde through deamination and decarboxylation reaction, respectively (Stadler et al., 1987; Stadler et al., 1989). (*S*)-norcoclaurine is converted to (*S*)-reticuline by a 6-*O*-methyltransferase (*6OMT*) (Facchini, 2001b; Frick and Kutchan, 1999), an coclaurine *N*-methyltransferase (C*NMT*) (Frenzel and Zenk, 1990), a P450 hydroxylase (Pauli and Kutchan, 1998), and a 4'-*O*-methyltransferase (*4'OMT*) (Frenzel and Zenk, 1990) couple with methyltransferase reactions; the SAM-dependent 6-*O*- and 4'-*O*-methyltransferases (*6OMT* and *4'OMT*, respectively) (Facchini, 2001a; Facchini, 2001b) (Figure. 2). Base on their chemical structure, benzyltetrahy-droisoquinoline alkaloids are classified into 5 categories; 1) simple benzylisoquinolines 2) bisbenzyltetrahydro-isoquinolines 3) aporphinoids 4) protoberberine and 5) morphinan alkaloids. Alkaloid-producing species have been subjected to study on alkaloid composition.

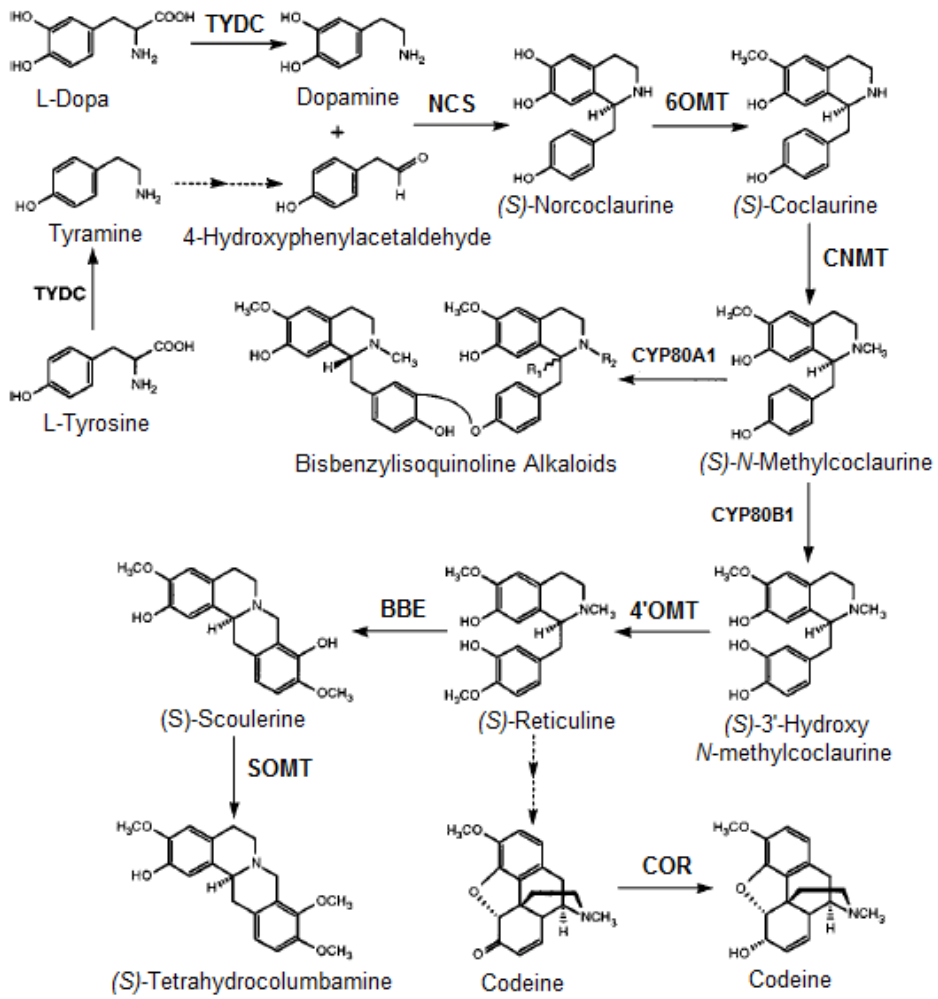


Figure 2 Reactions catalyzed by enzymes involved in benzylisoquinoline alkaloid biosynthesis. TYDC, tyrosine/dopa decarboxylase; NCS, norcoclaurine synthase; 6OMT, norcoclaurine 6-O-methyltransferase; CNMT, coclaurine-N-methyltransferase; 4'OMT, 3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase; CYP80A1, berbamunine synthase, CYP80B1, (S)-N-methylcoclaurine 3'-hydroxylase; BBE, berberine bridge enzyme; SOMT, scoulerine N-methyltransferase; COR, codeinone reductase. (Facchini, 2001b)

and reported on their chemical structures for example bisbenzylisoquinoline alkaloids, such as berbamunine and tubocurarin; protoberberine alkaloids, such as berberine and palmatine (Hashimoto and Yamada, 1994); morphinan alkaloids, such as morphine and codeine (Facchini and Bird, 1998); benzophenanthridine alkaloids, such as sanguinarine and macarpine (Kutchan et al., 1991). The well-known BIA alkaloids derived from (*S*)-reticuline are antibiotic berberine found in *C. japonica* and antibiotic sanguinarine found in *P. somniferum* and *E. californica.*

Simple benzylisoquinoline alkaloids are benzylisoquinoline containing small substituent molecules such as methoxyl group and hydroxyl group. Substituent molecule typically present on the C-6 and C-7 position of isoquinoline nucleus, whereas the benzene ring, presented on the C-11, C-12 and C-13 position, such as reticuline type presenting substituent at C-11 and C-12 position, and coclaurine presenting substituent at C-12 position on the benzene ring. The common structures of simple BIAs are 1,2,3,4-tetrahydro derivatives, some of which may have an aromatic ring, such as papaverine alkaloid found in opium resin (*P. somniferum*), a member of Papaveraceae (Inui et al., 2007). They are also found in Ranunculaceae, Berberidaceae, Menispermaceae and Nelumbonaceae. Likewise, aporphiniods form the bond which is the linkage between C-8 and C-10 position on simple benzylisoquinolines. Apophinoids are found in Annonaceae, Hermandiaceae, Hernandiceae, Magnoliaceae, Monimiaceae, Menispermaceae Ranundaceae (Facchini, 2001a; Facchini, 2001b) and Nelumbonaceae (Deng et al., 2016).

Bisbenzylisoquinoline alkaloid compose of 2 structures of benzylisoquinoline alkaloid (*N*-methylcoclaurine) joined by phenolic oxidative coupling reaction generating ether bridge between the molecules. This alkaloid group is mostly found in Menispermaceae, Berberidaceae, Ranunculaceae, Monimiaceae and Lauraceae. The well-known bisbenzylisoquinoline alkaloid is tubocurarine, found in *Chondrodendron tometosum* Ruiz et Pavon; a member of Menispermaceae, which has been used in conjunction with an anesthetic to provide skeletal muscle relaxation during surgery or mechanical ventilation (Gautrelet et al., 1933).

The important enzymes involved in the early step of biosynthetic pathway of BIAs including NCS, CNMT and 6OMT are shown in figure 2. BIAs shares the first common step, where isoquinoline backbone is generated by *NCS* (Inui et al., 2007) Consequently, *6OMT* and *CNMT* conduct methylation reaction by transferring methyl group from methyl donor, *S*-adenosyl-L-methionine (SAM), to the isoquinoline nucleus of tetrahydrobenzylisoquinoline alkaloid coclaurine. SAM-dependent N-methyltransferases occur in diverse metabolic pathways and are common in plant specialized metabolism, mostly associated with alkaloid biosynthesis (Choi et al., 2002). BIAs strongly require these enzymes and methylation reaction for the production of BIAs (Inui et al., 2007).

2.3 The study of genes involved in biosynthetic pathway of BIAs

BIAs-producing species has been targeted to study on the function of genes and enzymes related to BIAs production using transgenic plants and harboring biotechnology to study activity within plant cells. Gene expression phenotype in heterozygous carrier can prove function of the predicted genes, as a consequence, the transgenic plant becomes the most powerful tool to study cell activity and elucidate alkaloid biosynthetic pathway in BIAs-producing species (Table 1) (Facchini, 2001a; Facchini, 2001b).

In 2002, *NCS* was isolated and characterized in *Thalictrum flavum* ssp. Glaucum (Samanani and Facchini, 2002) . Meanwhile, Kum-Boo Choi has reported the result of gene cloning and characterization on *CNMT* in *C. japonica* (Choi et al., 2002) Later, in 2007, Inui has reported the result of overexpression of gene related to *6OMT* of *C. japonica* in *E. californica.* This finding also suggest that methyltrasferases in BIAs biosynthetic pathway are involved in rate-limiting step (Inui et al., 2007) In 2009, *CNMT*-related genes were Isolated from *E. californica, Papaver bracteatum,* and *T. flavum cell* for elucidating enzyme function (Liscombe et al., 2009). In addition, BIAs are found in approximately 20 percent of all plant species including a-well known remedy in Asia *N. Nucifera* Gaertn (Deng et al., 2016). Several bioactive BIAs in *N. nucifera* are listed in Table 2. The result of amino acid sequence alignment of motif A domains of the *S*-adenosyl-L-methionine methyltransferase in

C. japonica and cyclopropane-fatty acyl phospholipid synthase in *Mesorhizobium loti* indicated that motif A is a conserved sequences motif a in plant *S*-adenosyl-L-methionine-dependent methyltransferases (Choi et al., 2002) (Figure. 3).

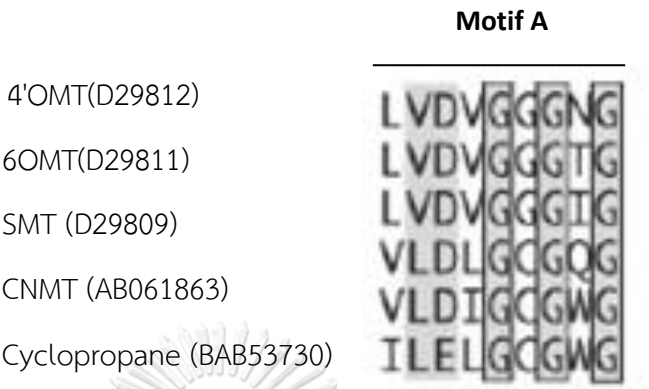


Figure 3 Amino acid sequence alignment of motif A of *S*-adenosyl-L-methionine methyltransferase. *C. japonic*a; *S*-adenosyl-L-methionine:3'-hydroxy-N-methyl-coclaurine (D29812), S-adenosyl-L-methionine norcoclaurine 6-*O*-methyltransferase (D29811), *S*-adenosyl-L-methionine scoulerine 9-*O*-methyltransferase (D29809), coclaurine *N*-methyltransferase (AB061863) and cyclopropane-fatty acyl phospholipid synthase in *M. loti* (BAB53730).

Table 1 Enzymes related to BIAs production

Enzyme	Function	Species	Type
TYDC	Tyrosine decarboxylase	*P. somniferum*	Pyridoxal-5'-phospahate-dependent decarboxylase
6OMT	Norcoclaurine 6-*O*-methyltransferase	*C. japonica*	*S*-Adenosyl-L-methionine-dependent *O*-methyltransferase
4'OMT	3'-Hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase	*C. japonica*	*S*-Adenosyl-L-methionine-dependent *O*-methyltransferase
CYP80A1	Berbamunine synthase	*Berberis stolonifera*	P450-dependent monooxygenase
CYP80B1	*N*-Methylcoclaurine 30--hydroxylase	*E. californica* *P. somniferum*	P450-dependent monooxygenase
BBE	Berberine bridge enzyme	*E. californica* *P. somniferum* *B. stolonifera*	Flavinylated oxidoreductase
SOMT	Scoulerine-9-*O*-methyltransferase	*C. japonica*	*S*-Adenosyl-L-methionine-dependent *O*-methyltransferase
COR	Codeinone reductase	*P. somniferum*	Aldo/keto reductase

Table 2 BIAs in *N. Nucifera*

Organ	Compound	Effects	Structure	Reference
Stem	Nuciferine *N*-Nornuciferine	Anti-diabetic and Anti-HIV activity	(1) (2)	(Deng et al., 2016; Sridhar and Bhat, 2007)
Seed	Neferine Isoliensinine Liensinine Lotusine	Inhibit Bacteria growth, Anti-HIV activity, Anti-arrhythmic action, and Inhibits platelet aggregation Inhibit Bacteria growth Anti-HIV activity Anti-HIV activity Anti-hypertension, Anti-arrhythmia, anti-myocardial ischemia, synergistic antitumor, inhibiting hypertrophic scar and increasing insulin sensitivity	(8) (6) (7) (3)	(Akinjogunla et al., 2010; Sridhar and Bhat, 2007; Yang et al., 2012) (Sridhar and Bhat, 2007; Yang et al., 2012) (Rai et al., 2006) (Kashiwada et al., 2005) (Kashiwada et al., 2005)

Table 3 (cont.) BIAs in *N. Nucifera*

Organ	Compound	Effects	Structure	Reference
Leaf	Nuciferine Liensinine Isoliensinine	Anti-diabetic and Anti-HIV activity Anti-HIV activity Inhibit Bacteria growth Anti-HIV activity	(1) (7) (6)	(Do et al., 2013; Duan and Jiang, 2008; Sridhar and Bhat, 2007) (Kashiwada et al., 2005; Nguyen et al., 2012)
Flower	Nuciferine, *N*-methyla similobine (-)-Lirinidine	Inhibit melanogenesis	(1) (4) (5)	(Nakamura et al., 2013)
Rhizome	A whole extract	Antioxidative capacity	-	(Hu and Skibsted, 2002)

2.4 Role of WRKY transcription factors in BIA biosynthetic pathway

BIA corresponding genes have been reported that they are regulated by specific WRKY transcription factors (WRKY TFs) which are able to be simultaneously induced in response to biotic and abiotic stresses such as drought and wound (Phukan et al., 2016). Currently, many WRKY TFs have been isolated and there are only 2 identified WRKY1 from *C. japonica* and *Catharanthus Roseus* which have been documented that they are involved in berberine and catharanthine production, respectively (Schluttenhofer and Yuan, 2015). Moreover, there is an evidence suggesting that wound induced WRKY TF1 subsequently regulating BIA pathway in *P. somniferum* (Mishra et al., 2013). Similarly, WRKY TF1 from *C. japonica* induced the biosynthetic gene expression of berberine production which shared common pathway with various BIAs (Phukan et al., 2016; Suttipanta et al., 2011). Thus, it is necessary to study on the role of WRKY TFs along with BIA corresponding genes mentioned above in wounded lotus leaf.

2.5 The use of mechanical wounding method in plant

Plants have evolved the defense mechanisms to respond to wound and pathogen infection by releasing endogenous molecules from wounded area. They play a role as Damage-Associated Molecular Patterns (DAMPs) which subsequently activate plant innate immune system and the expression of defense-related genes (Mishra et al., 2013). Defense response mediated by wounding is similar to those

mediated by DAMPs and microbe-associated molecular patterns (MAMPs) which indicates that mechanical injury shares similar manner in defense with herbivores and insects. Thus, the mechanical wounding has become a powerful method to study cellular activity in plants (Rehrig et al., 2014).

2.6 Alkaloid extraction, separation, and detection

2.6.1 Alkaloid extraction

Alkaloid, a nitrogenous compound, is a complex cyclic structure. Most of them are alkaline, and become salt when combined with acid. Alkaloids which have an alkaline mostly exist in organic salt form, such as citrate, oxalate, tartrate and succinate, whereas some are in inorganic salt form, such as berberine and morphine sulfate. There are other forms of *N*-oxide or alkaloid glycosides. Different plant families share either similar or different alkaloid structures; however, the same parent nucleus or the similar structure of alkaloid coexist in the individual plant (JI et al., 2014). To get the high amount of alkaloid production from plant, the extraction of water or acidic water method is employed. The organic acid of alkaloids salt is replaced by inorganic acid salt resulting in increasing its solubility. Acid extraction method normally uses 0.1% to 1% sulfuric acid, hydrochloric acid, or acetic acid. The advantage of acidic extraction is changing alkaloid molecules into small molecule organic acid salts, increasing its solubility in water, and it is relatively simple (JI et al., 2014; Silva et al., 1998; ศรีตุลารักษ์, 2553) (Figure 4).

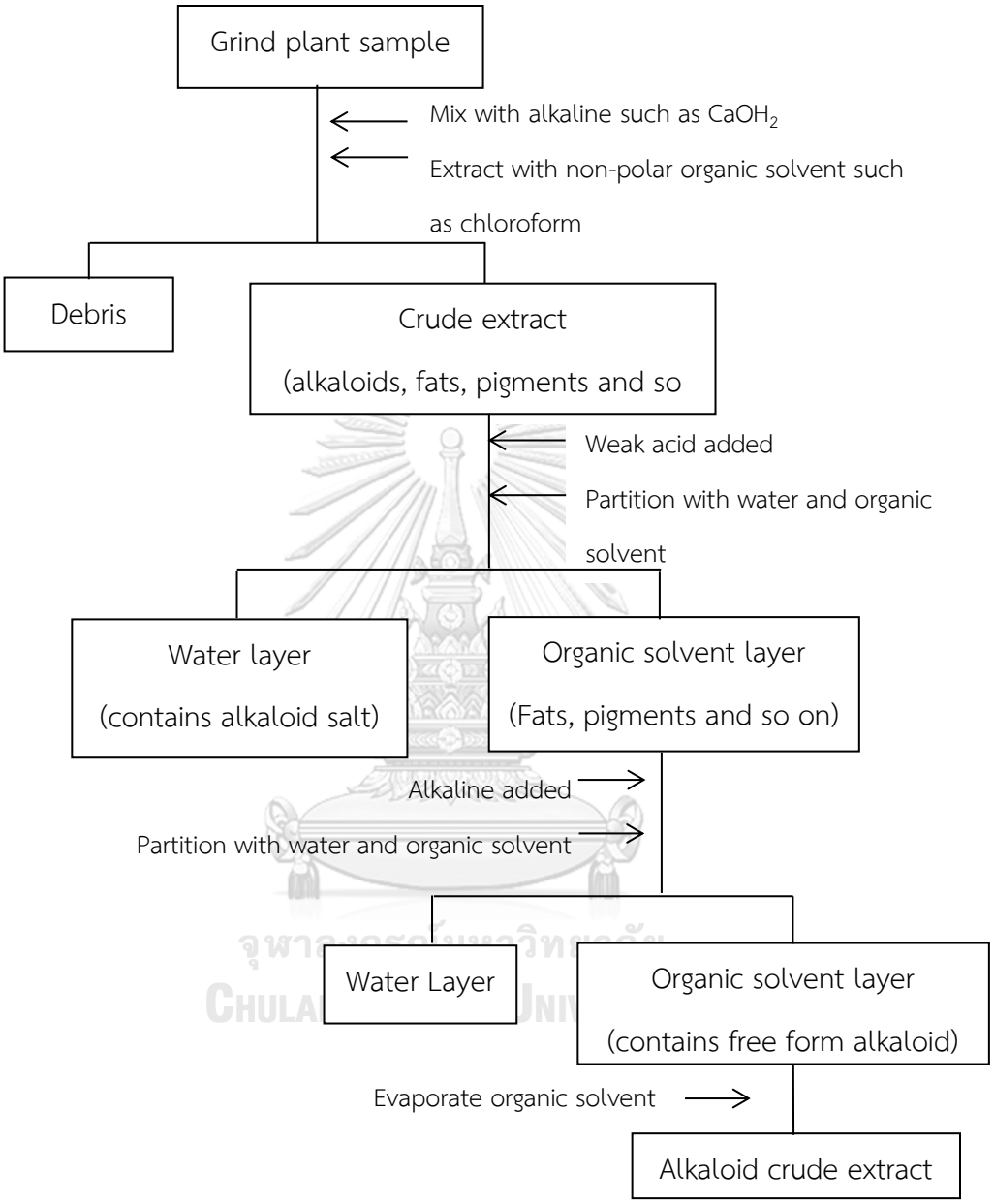


Figure 4 Flow chart of alkaloid extraction method

2.6.2 Alkaloid separation

Separation of an individual alkaloid from the alkaloid extract obtained from a certain plant material employing well-known separation techniques, such as, high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). HPLC separation system has good performance, high sensitivity, fast analysis and ability in separating mixed alkaloids which are difficult to be separated by other chromatography. Mobile phase consisting of methanol (MeOH), acetonitrile, water, and ammonium acetate (CH_3COONH_4) has been widely employed for the separation of alkaloids performed in C18 column (Petruczynik, 2012) (Table 3). TLC is also used for qualitative and quantitative analysis of plant natural products and also the study of the biosynthesis of a certain alkaloid group, for example BIA group of which TLC system is already developed (Table 4). TLC is particularly well suited for checking the processes of synthesis as well as for establishing the progress of reactions and testing of products in pharmaceutical preparations. The presence of alkaloids in drugs of abuse and their metabolites in biological system such as urine and blood has also been tested by means of TLC (Silva et al., 1998; ศรีตุลารักษ์, 2553).

2.6.3 Alkaloid detection

To visualize and detect alkaloid on TLC separation system, Dragendorff's reagent is utilized. Dragendroff's reagent is a solution of potassium bismuth iodide prepared from basic bismuth nitrate ($Bi(NO_3)_3$), tartaric acid, and potassium iodide

(KI). In alkaloid extract, the nitrogen in the alkaloids combines with the heavy metal atom (BiL4) in Dragendorff's reagent to form ion pairs and produce an insoluble reddish precipitate (Khatun et al., 2014). Also, UV detectors are used in HPLC system to detect and identify analytes in the sample. BIAs are usually detected at 254 and 280 nm. The absorbance is maximum (λ_{max}) is 272 nm (Deng et al., 2016).

Table 4 Guideline of the selective developing solvent used for the separation of alkaloids in reverse-phase high performance liquid chromatography systems.

Mobile phase Oraganic solvent A	*Mobile phase Oraganic solvent B*	*Mobile phase Oraganic solvent C*
Dichloromethane Chloroform Diethyl/isopropyl ether, Tetrahydrofutan, or Ethyl acetate	Methanol or Isopropanol	Ammonia, Diethylamine or Triethylamine (1% of the mobile phase)

Table 5 Examples of the most popular chromatographic systems for TLC of the BIA groups.

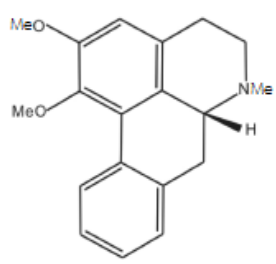
Compounds	*Adsorbent*	*Solvent system*
Benzylisoquinoline	Silica gel	Chloroform: methanol: diethylamine: ammonium hydroxide (8 : 2 : 2 : 0.5) Benzene: acetone: ammonium hydroxide (15 : 15 : 1) Chloroform: toluene: methanol:acetone: ethyl acetate: ammonium hydroxide (270 : 30 : 80 : 30 : 3)
Aporphine	Silica gel	Cyclohexane : ethyl acetate (3 : 2) Cyclohexane : acetone (9 : 1) Petrol ether : acetone (7 : 3) Chloroform : methanol (9 : 1)

2.7 *Nelumbo nucifera*

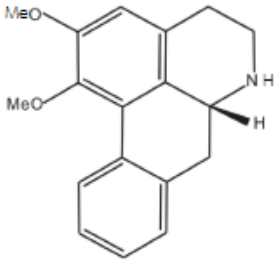
2.7.1 Plant description

Lotus (*N. nucifera* Gaertn), a perennial aquatic plant, are consumed throughout Asia, especially in South East Asia. Lotus belongs to Nelumbonaceae family. All parts of *N. nucifera* have been used for various medicinal purposes in oriental medicine. In particular, the leaves and stems are known for diuretic and astringent properties, and used to treat fever, sweating and strangury (Duan and Jiang, 2008; Kashiwada et al., 2005). Nowadays, 600 lotus cultivars has been reported and divided into 2 species according to geographical area; 1) *N. nucifera* Gaertn are widely distributed throughout Asia, mostly found in India, China, and Thailand; 2) *N. lutea* (Willd.) Pers are from North America. *N. nucifera* is also called Chinese lotus since it originated from China and has been cultivated long time ago as an aquatic crop (Shou et al., 2008). They are classified into three types based on usage; seed producing cultivars, flower producing cultivars, and rhizome producing cultivars (Do et al., 2013). Chinese and Thai lotus provides several potent bioactive compounds derived from BIAs such as anti-diabetic, anti-HIV activity agents, and so on. The chemical structures of bioactive BIAs from lotus were listed in Figure 5. Though the composition and bioactivity of alkaloids in Thai lotus are revealed, little is known about quantity of BIAs among Thai lotuses. Thus, the quantitative and qualitative study on BIAs among Thai lotuses would facilitate a pharmaceutical

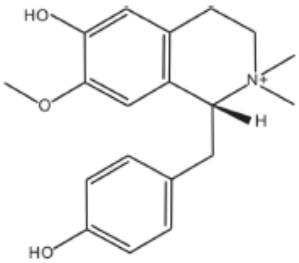
study with a high production of an individual alkaloid and economically added value on Thai lotus.



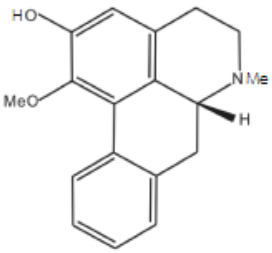
(1) Nuciferine



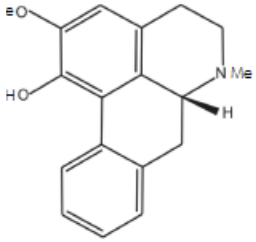
(2) *N*-Nornuciferine



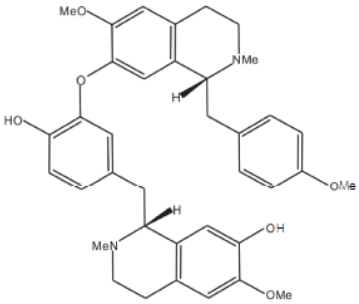
(3) Lotusine



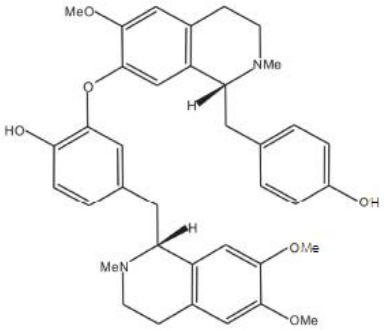
(4) *N*-methylasimilobine



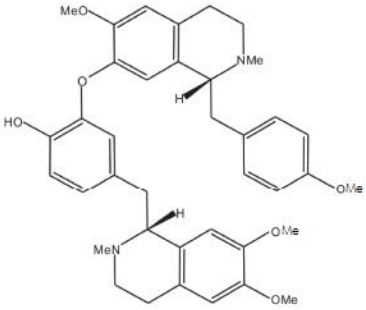
(5) (-)-Lirinidine



(6) Isoliensinine



(7) Liensinine



(8) Neferine

Figure 5 Structurally diverse BIAs found in lotus. The chemicals are labeled with number and their corresponding names are shown in Table 2

2.7.2 BIAs in lotus and their bioactivity

Terpenes, phenolics, flavonoids, and alkaloids are bioactive metabolites found in lotus plant. The chemical structure analysis of each compound is conducted by high performance liquid chromatography-mass spectrometry (HPLC-MS) and further study on their bioactivities (Akinjogunla et al., 2010; Sridhar and Bhat, 2007). Alkaloids, the most abundant bioactive metabolites in lotus, are highly accumulated in leave. Major alkaloids belong to BIAs group; the simple benzylisoquinoline or aporphine structure appears to be essential (Nakamura et al., 2013).

2.8 Hypothesis

2.8.1 Isoquinoline alkaloid content are different among Thai lotus plant

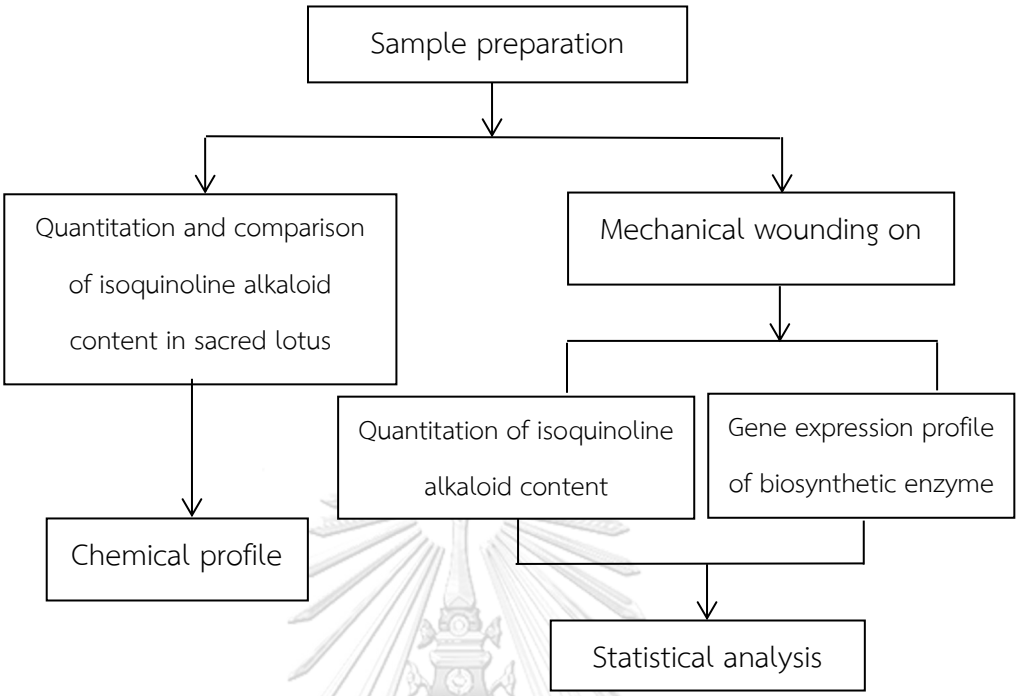
2.8.2 Expression of biosynthetic genes are increased in mechanically wounded sacred lotus.

2.9 Objective

2.9.1 Determine isoquinoline alkaloid content in non-wounded and mechanically wounded sacred lotus

2.9.2 Study on the relationship of isoquinoline alkaloid content and biosynthetic gene expression in mechanically wounded sacred lotus

2.10 Conceptual framework



CHAPTER III

Materials and methods

3.1 Plant materials

Wild lotuses were collected from different provinces in Thailand and cultured at Rajamangkala University of Technology Tawan-ok, Chonburi Thailand. A total of 9 wild Thai lotuses were divided into 3 groups based on their characteristics. Group I was *N. nucifera* Gaertn. 'Album Plenum' or Magnolia lotus. It has at least 30 white petals with oval shape and the petals diameter expands to 12-15 cm at the blooming stage. Its yellow stigma and petal-like stamen will turn to pale green at senescent stage. Group II was Rosem Plenum lotus (Figure 6). This group is pink flower-producing lotus. Rosem Plenum lotus has similar morphology as Album Plenum lotus. It has many petaloid staminodes with white anther, pale pink filament, and stigma. The third group was Bua Khem Chin. This group has quite small flower compared with the other two groups. It has maximum 20 petals with spiky tip and strictly expands its petal diameter up to 6 cm at blooming stage. The pink, white, and orange petals are commonly found. It has small number of complete yellow stamen where the white appendage located at the top of its anther. The lotus morphology was retrieved from The Botanical Garden Organization, Ministry of Natural Sources and Environment (http://www.qsbg.org/webBGO/database.html). The commercial lotus was purchased from an urban market in Bangkok area. All wild and

commercial lotuses were subjected to BIA analysis and gene expression profiling in young and mature stages of leaf and petiole. The commercial lotus was only used for mechanical wounding analysis. Samples were collected and frozen in liquid nitrogen and then stored in a -80 °C freezer until use.

Figure 6 Morphology of 3 groups of wild Thai lotus flowers.

3.2 Total RNA extraction

Total RNAs were purified from lotus tissues using RNAprep Pure Kit (Tiangen, China). Fresh tissue was thoroughly ground in liquid nitrogen immediately with a mortar and pestle. The ground sample was put in 1.5-ml tube containing 500 µl buffer SL (ß-mercaptoethanol was added to buffer SL before use) and then mixed vigorously. The mixture was centrifuged for 2 min at 12,000 rpm and the lysate was transferred to an RNase-Free Filter Columns CS placed in a 2 ml collection tube, and further centrifuged for at least 2 min at 12,000 rpm. The flow through was carefully transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. The 0.4 volume pure ethanol (170 µl) was added into the

cleared lysate, mixed immediately by pipetting and loaded the mixture to RNase-Free Spin Column CR3 placed in a 2 ml collection tube; it was again centrifuged for 15 sec at 12,000 rpm. The flow-through from this step was discarded. 350 µl of buffer RW1 was then added into the spin column CR3 and centrifuged for 15 sec at 12,000 rpm; the flow-through was discarded. After this step, DNase was employed to remove the entire contaminated DNA by adding the DNase I working solution (80 µl) directly to the center of spin column CR3, and placed on the benchtop (20-30°C) for 15 min (Preparation of DNase I working solution: added 10 µl DNase I stock solution to 70 µl Buffer RDD). After 15 min, 350 µl of buffer RW1 was added into the spin column CR3, and centrifuged for 15 sec at 12,000 rpm; the flow-through was discarded. Add 500 µl of buffer RW to the CR3 spin column (Ethanol is added to buffer RW before use). Close the lid gently, centrifuge for 15 sec at 12,000 rpm; the flow-through was discarded (repeat this step once). To dry the spin column membrane, it was given an extra centrifuged period for 2 min at 12,000 rpm. The spin column CR3 was placed in a new 1.5 ml collection tube. RNase-Free water was directly added to the spin column membrane, placed it in room temperature for 2 min and centrifuged for 1 min at 12,000 rpm to elute the RNA. The eluted RNA sample were diluted 20 times in sterile ultrapure water and sampling 200 ul into cuvette to measure RNA purity and quantify RNA concentration by spectrophotometer under UV light 260/280 nm. The absorbance unit under 260 nm

was used to calculate total RNA concentration (ng/ul). The concentration retrieved from this equation (Haimes and Kelley, 2010);

Total RNA (ng/ul) = absorbance unit × dilution factor

× 40 (an RNA extinction coefficient)

Samples were placed on ice to avoid RNA degradation and then 500 ng total RNA was subjected to cDNA synthesis which was immediately performed within 1 hour.

3.3 cDNA Synthesis

The cDNAs were prepared using the ProtoScript® II Reverse Transcriptase (New England Biolabs). The reaction mixture containing 500 ng template RNA, 2 mM Oligo18 and nuclease free water up to 12.5 µl, was incubated at 65°C for 5 min. The reaction was mixed with 1x ProtoScript buffer, 0.4mM DTT, 200U ProtoScript II RT, 2mM dNTP mix, 40U RNase Inhibitor, and Incubated at 42°C for 1 hour. Reaction was inactivated at 65°C for 20 min for downstream PCR application. cDNA samples were prepared for relative gene expression analysis using semi-quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).

3.4 Searching of genes related to BIA biosynthesis

Based on the previous report on the biosynthetic enzymes of isoquinoline alkaloid in *C. japonica* and the chemical structures of nuciferine and *N*-nornuciferine which containing methyl residue at *O* and *N* position on the isoquinoline nucleus,

this information suggested that 6OMT and CNMT are required in the biosynthetic of these nuciferine and *N*-nornuciferine. NCS is also related because it is a biosynthetic enzyme involved in early step of biosynthetic pathway and has been reported to have high transcript level which related to the accumulation of total alkaloid in Chinese lotus leaf (Vimolmangkang et al., 2016). Moreover, the WRKY1 transcription factor from *C. japonica* was also used in this study. *CjWRKY1* has been documented that it has a crucial role in the production of berberine, a major isoquinoline in *C. japonica* (Schluttenhofer and Yuan, 2015). Thus, the putative gene sequences corresponding to NCS, CNMT, 6OMT, and WRKY transcription factors in lotus were employed in this study. To retrieve the putative genes encoding NCS, a total of 5 putative NCS isolated from cDNA of *N. nucifera* were retrieved from GenBank database. They were name as NnNCS1, NnNCS3, NnNCS4, NnNCS5 and NnNCS7. Their GenBank accession number are as follows; ANI26411, ANI26412, ANI26413, AND61511, AND61512. Similarly, to retrieve the putative genes encoding 6OMT, CNMT, and WRKY TFs, the previously reported genes from *C. japonica* (Choi et al., 2002; Phukan et al., 2016; Sato et al., 1994) were used to search for the closely related sequences using the sequence homology method to search in the lotus database available at http://lotus-db.wbgcas.cn/ (Wang et al., 2015). The mRNA sequences were retrieved from the database and used to design the primers for gene expression profile analysis.

3.5 Phylogenetic tree analysis

All nucleotide sequences were submitted to Expasy translate-tool (http://web.expasy.org/translate/) to predict their amino acid sequences. The deduced amino acid sequences of all target genes in this study and known BIA-related genes from alkaloid-producing plant species were aligned with MUSCLE alignment and they were further analyzed for phylogenetic relationship using in MEGA7 program (Kumar et al., 2016). The resulting data matrix was analyzed using equally weighted maximum parsimony (MP). Phylogenetic tree was constructed using Maximum likelihood method based on the JTT matrix-based model. The topology with superior log likelihood value was selected. The bootstrap consensus tree was inferred from 1,000 replicates.

3.6 Mechanically wounding method

Three plants of the commercial lotus were employed for mechanical wounding experiment (biological replications). Each plant was set for 3 treatments as follows: 1) control leaf, 2) wounded leaf, and 3) non-wounded leaf. The two abaxial sites which divided by midrib of a single leaf were set for treatment number 2 and 3; while control leaf was solitary. Mechanical wounding was performed by making several long parallel lines on the wounded site using sterile surgical blade No.21 (Skidmore instruments, England). The wounded and non-wounded leaves were collected for the following observation days; day 0, 2, 3, 4, and 7 while the control leaves were collected on day 0 and day 7 only. The fully opened shooting leaf with

control size of 11 cm x 13 cm was chosen for all treatments. Samples from various observation days were separately prepared for the analysis of BIA content using HPLC method and the relative gene expression using qRT-PCR method. (Figure 7). The relationship between gene expression and BIA accumulation was further discussed.

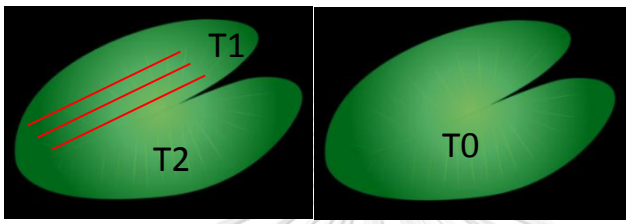


Figure 7 Graphical picture indicating 3 treatments performed on lotus leaf for mechanical wounding experiment.

3.7 Expression profile of BIA corresponding gene using semi-quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR assay was performed in BIO-RAD T100™ Thermal Cycler (BIO-RAD). The assay was set in a total volume of 10 µl reaction mixture (Table 5) with the presence of i*Taq*™ Universal SYBR® Green Supermix (BIO-RAD), which is able to generate fluorescent signal by binding DNA to yield DNA-dye-complex. This complex absorbs blue light (**λ**max = 497 nm) and emits green light (**λ**max = 520 nm). The specific primers for a group of target genes which designed from their consensus sequences were listed in Table 6. The amplification program was set as shown in Figure 8 described in the previous study (Vimolmangkang et al., 2016). Lotus actin

gene (Accession no. XM_010267617) was used as a constitutive control. The samples were conducted in triplicate and the Ct value was used to measure relative gene expression following this equation.

$$\textbf{Relative expression} = 2^{\Delta CT}$$

Where; $\Delta C_T = C_T$ Reference gene - C_T Target gene

Note: C_T is cycle number at which detectable fluorescent signal is achieved

Table 6 The components of PCR reaction for qRT-PCR

Component	Amount
2× iTaq™ Universal SYBR® Green Supermix(BIO-RAD)	5 µl
Forward and reverse primer	0.2 µM each
cDNA template	500 ng
Sterile water	upto10 µl

Table 7 The list of primers for qRT-PCR

Target genes	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR product size (bp)	Template
NCS	TGCCTGCTGACGATATTTGGG	GTGCCGACCGTTCCATCAC	121	Consensus sequence from 5 isoforms of NCS (Vimol-mangkang, Deng et al. 2016)
CNMT	TGCCATCAAGACCGACTTACCAAAG	GATCTGTGCCCTCTCACAGTACAG	175	*NNU11880*
6OMT	TCCGATGTGCCATTGAGCTGG	GTAACGCATTAACCTGTGCAAGTG	134	Consensus sequence from 4 isoforms of putative 6OMT
ACTIN	GGTGCTGAGTTCGTCGTAGA	TGGGAATGATGTTGAAGGAA	120	XM_010267617
WRKY1	ATAAGGCTGTTGGAGCAAGAGT	CTGTGGATCGTCTGCATCTCT	191	NNU_11881RA
WRKY2	GACACAGCATCTGTCACTCATG	GACGTCCCCTGACAACATGAA	211	NNU_05136RA
WRKY3	CGGTGAAGAACAGTCCAAACC	GTTGTAATAAACCACACACGGGC	161	NNU_09891RA
WRKY4	TCTGCAGAAGCCACTCTTGT	GAATATCTTCTGTGCTCCGCGT	190	NNU_01372RA

Table 8 (cont.) The list of primers for qRT-PCR

Target genes	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR product size (bp)	Template
WRKY5	GAAACAATCCACCTGGGTTCTTAGG	ACGTGGCTTCTTGTTTGAAAAGC	190	NNU_22208RA
WRKY6	ATCTTAATGACACCAGTCCAGCT	CTGATTTTCTTCTGATCACCCTTCTTA	166	NNU_02028RA
WRKY7	AGTCTCACTTCCAACAACAGC	GATCATCATATCCGAAGGAACGACA	188	NNU_05834RA
WRKY8	GGCGCCGATGCAATTGAC	CGTGGTCTCTGATAAAAGAAGTTGC	173	NNU_13849RA
WRKY9	GCCGGAAAATTCGCCAATCAA	GACAGTGGTGTTGAGTGCC3	190	NNU_24385RA
WRKY10	CATGAGATGGAGGAGCTCACC	CGACGAAGCTCATGTCCAGAC	169	NNU_12194RA

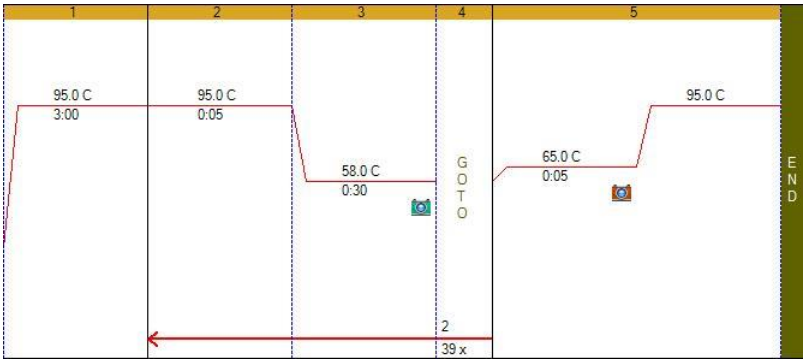


Figure 8 The amplification program for qRT-PCR

3.8 Identification and measurement of alkaloid content by HPLC method

Alkaloid extraction and separation method were described in the Figure 9. Sample separation and detection were performed by HPLC-DAD (Shimadzu, Japan) using a TOSOH® C18 column (4.6 mm × 150 mm, 3.5 μm). *N*-nonuciferine and nuciferine are two major BIAs which classified in aporphine-type alkaloid so they were targeted for the study of changes in both non-treated and wounded lotus leaf. According to the report on the similarity of aporphine-type alkaloid standard curve, *N*-nornuciferine and nuciferine content were considerably determined against the linear standard curve of nuciferine standard (Yuanye Biotechnology Shanghai, China) (Figure 10 A).

To construct the standard curve, a series of concentration of nuciferine standard was prepared. Absolutely 1 mg nuciferine standard was dissolved in 1 ml extraction buffer. To create 1 mg/ml nuciferine standard stock solution. It was subsequently used for a serial dilution stock as follows; 20, 40, 60, 80 and 100μg/ml. The extraction buffer and separation condition were conducted following flow chart as shown in Figure 9. Each standard concentration was injected 3 times within the same day. HPLC chromatogram of each standard was recorded under 272 nm and column temperature was controlled at 30°C; peak 1 and 2 are corresponding to *N*-nornuciferine (RT= 10.494 min) and nuciferine (RT=13.151 min) (Figure 10 B).

Grind fresh sample in liquid nitrogen

Add 5ml extraction buffer (0.3 M HCl–methanol, 1:1, v/v)

Sonicate for 30 min at room temperature

Separation condition

Mobile phases:

A (triethylamine–water, 1:1000, v/v), B (CH_3CN)

The gradient elution:

0–15 min, 40–80% B

15–18 min, 80% B

18–19 min, 80–40% B

19–25 min, 40% B.

Flow rate: 0.8 mL/min.

Oven: 30°C

Detection: 272nm

Filtrate the crude with 2µm sterile filter before injection

Collect the crude at 11000g for 10 min (repeat extraction step).

Figure 9 Flow chart showing alkaloid extraction method and HPLC separation condition.

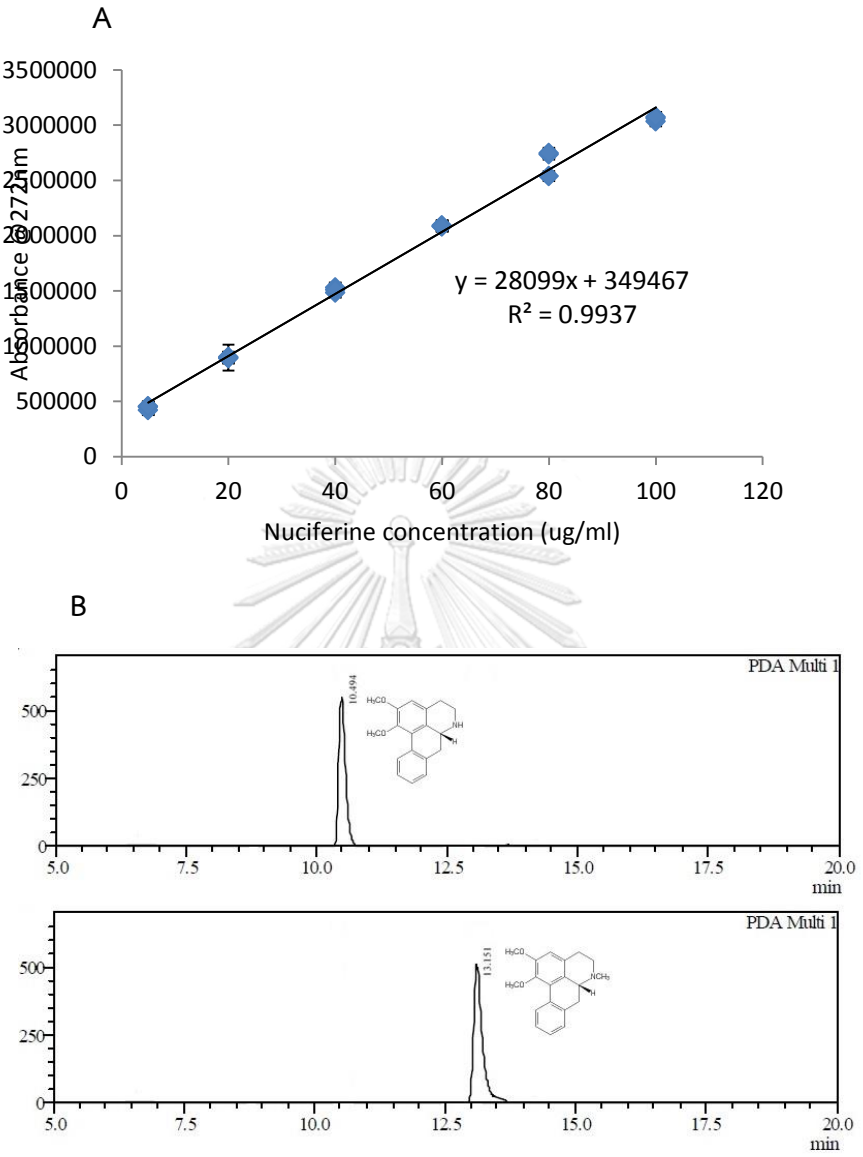


Figure 10 Nuciferine standard calibration curve and chromatograms of standards *N*-nornuciferine and nuciferine. (A) Linearity of calibration curve. Each standard concentration (X-axis) was conducted in triplicate. The absorbance unit (Y-axis) was recorded under 272 nm. (B) HPLC chromatogram; peak 1 and 2 were corresponding to *N*-nornuciferine (RT= 10.494 min) and nuciferine (RT=13.151 min).

3.9 Validation of HPLC assay

To check HPLC repeatability (Papadoyannis and Samanidou, 2004), the peak areas of five sequential injection repeats of 60 ug/ml nuciferine standard were assessed. The relative standard deviations (RSD) of the peak areas and inter-day injection repeats were 0.26%. The recoveries of alkaloid assay were determined by standard addition method. Briefly, nuciferine alkaloid standard at various concentration including 5, 20, 40, 60, 80, and 100ug/ml was dissolved in extraction buffer and then subjected to alkaloid extraction and HPLC analysis (Figure 9). Their recoveries were 65.75%, 97.40%, 103.15%, 103.07%, 103.44%, and 96.38%, respectively. According to the acceptance recovery percentages range between 80-110%, the results suggested that the HPLC system used was reliable (Taverniers et al., 2004).

3.10 Statistical Analysis

Triplicates of each alkaloid standard and samples were subjected to statistical significance analysis using SPSS software ver.21 with one-way analysis of variance (ANOVA) method, and significant difference was defined at $p < 0.05$.

CHAPTER IV
Results

4.1 Retrieval of genes encoding biosynthetic enzymes and transcription factors (NCS, CNMT, 6OMT and WRKY TF)

From the BLAST analysis result of lotus genome against *CjCNMT* (Accession no. AB061863) and *Cj6OMT* (Accession no. D29811), lotus has one putative sequence of *CNMT* (*NNU_11880*); arbitrarily named as NnCNMT1, and 4 sequences of *6OMT* (*NNU_19035, NNU_03165, NNU_03166, and NNU_23168*); arbitrarily named as Nn6OMT1, Nn6OMT2, Nn6OMT3 and Nn6OMT4. For WRKY TFs related to the regulation of BIA biosynthesis, 60 putative *WRKY TF* sequences were found and chosen to construct phylogenetic trees. The retrieved lotus sequences were translated to the deduced amino acid sequences and blast against the NCBI database to identify their related sequences of other alkaloid-producing plant species to ensure that the sequences selected from the lotus database were possibly encode the targeted genes. All selected putative nucleotide sequences from lotus were listed in Table 7.

The putative 6OMT, CNMT and BIA-related WRKY-TFs were used to construct the phylogenetic tree and used for amino acid sequence alignment. The result suggested that four of 6OMTs and CNMT share close relationship to different known methyltransferases. 6OMT was grouped in the branch of Cj6OMT and CjSMT

(Accession no. D29809) while CNMT shared more close relationship to CjCNMT (Figure 11). Besides, the deduced amino acid sequence alignment of methyltransferase showed that they possess a conserved protein sequence motifs of AdoMet methyltransferase superfamily (SAM or AdoMet-MTase) which presented in CjCNMT (BAB08004) and 4 putative 6OMT indicated by the motif A, B and C of a conserved *S*-adenosylmethionine (*SAM*)-binding domain located at the C-terminal end containing three conserved glycine residues (Figure 12) (Choi et al., 2002; Inui et al., 2007). It was observed that all the CNMT-corresponding sequences and *N. nucifera* CNMT (NnCNMT1) possess motif A which present in CjCNMT (BAB71802) alone (Morishige et al., 2000) (Figure 13)

The deduced amino acid sequences of five *N. nucifera* NCS (NnNCS) in bold possess a Glycine-rich loop, the ligand binding domain of Bet V1 protein family which are also presented in known NCS of other plant species (Figure 14). This protein family is one of the ubiquitous PR-10 family of plant pathogenesis-related proteins providing ligand binding activity for a hydrophobic compounds for example hormones or antibiotics (Radauer et al., 2008)..

A total of 60 putative WRKY TF sequences were obtained from BLAST result of lotus genome against *CjWRKY1* which has been described its function as the transcription factor for the expression of berberine biosynthetic gene (Phukan et al., 2016). The molecular phylogenetic analysis revealed that the 10 sequences

highlighted in yellow showed the closest evolutionary relationship to *WRKY TF* in alkaloid-producing plant species including *WRKY TF* in *P. somniferum* (Accession no. AFK73557.1), *WRKY TF1* in *C. japonica* (Accession no. BAF41990.1), and *WRKY TF1* in *Catharanthus roseus* (Accession no. ADT82685.1) (Figure 15) Thus, they were selected for qRT-PCR analysis (Table 10). A total of 10 selected *WRKY* TFs were then observed the presence of WRKY TF conserved region as shown in Figure 16. The β-sheet WRKY DNA binding domain/s (DBD) is highlighted. WRKYGQK and WRKYGKK which is a primary motif DNA binding domain were presented in 8 sequences (Mishra et al., 2013; Phukan et al., 2016; Xie et al., 2005). In contrast, the other two putative *WRKY TFs* (*NNU_11881RA* and *NNU_05136RA*) possess WKKY anomaly of the primary WRKYGKK motif of DBD (Phukan et al., 2016). Many WRKY anomy were also found; WRRY, WSKY, WKRY and WVKY (Xie et al., 2005). Though, they share close relationship to WRKY domain sequences but they deem to encode premature protein.

Table 9 A list of putative BIA-related gene retrieved from coding sequences of *N. nucifera*

Gene code	Sequences (5'-3')
Putative lotus *CNMT* genes	
NNU11880	ATGGATGCGTTGATCCAGGTACCATACGATGCAACTGTACGTTTAATGCT GTCGTCTCTCGAGCGTAACCTCCTCCCCGACGTCGTCATAAGGAGGCTC ACGCGGCTGCTGTTGGCTAGCCGTCTTCGTTGGGGATACAAGCCGTCCT CTCAACTCCAACTTTCTGATCTTCTCCAATTTGTTCACTCGCTAAAAGAT ATGCCCATTGCCATCAAGACCGACTTACCAAAGTCCCAACATTATGAATT ACCCACTTCCTTCTTCAAGCTGGTTTTAGGGAAGAATCTCAAATACAGCT GCTGTTACTTCCTTGACAAGTCAAGCACCTTAGAGGATGCAGAGAAAGCT ATGCTGGAGCTGTACTGTGAGAGGGCACAGATCAAAGATGGCCAATCTG TGCTTGATGTTGGTTGTGGCTGGGGATCATTGTCCTTGTATATTGCACAA AAGTTTTCTAGCTGCAGGATAACAGGGATTTGCAATTCAAAGACACAGAA AGCATATATAGAGGAGCAATGTAGGGAACTGAAGCTGCAAAATGTGGAG ATCATTGTTGCAGATATCAGCACTTTTGAAATGGAGGCATCATTTGATAG GATTTTATCCATAGAAATGTTTGAACACATGAAGAACTACAAGGCACTTC TTAATAAGATATCAAAATGGATGAAGGAGGATAGCCTCCTTTTATTTAAC TACTTCTGCCATAAAGCATTTGCTTACCACTTTGAGGACAAGAATGAAGA TGACTGGATTACCAGGTACTTCTTCACTGGAGGGACAATGCCTGCTGCAA ACCTTCTCCTCTATTTCCAGGATGATGTTTCTGTTGTCAACCATTGGCTT GTAAATGGGAACCATTATGCAAGAACAAGTGAGGAGTGGCTTAAAAGAA TGGACCAGAACATGGCTTCTATTAAGCCAATAATGGAGTCAACTTATGGC AAGGATTCCGCTGTTAAGTGGACTGCCTATTGGCGTACATTCTTCATCTC AGTGGCAGAACTGTTTGGCTATAACAATGGAGAAGAATGGATGGTTGCA CTGTTCCTATTCAAGAAAAAAATAAATTAA

Putative lotus *6OMT* genes	
NNU_19035	ATGGAAAATCAGAAGGAAGTTCAAGCAGCCGAGGCTAAAATCTGGAATT TCGTCTATGGCTTTGCCGACACTTTAGTCCTCCGATGTGCCATTGAGCTG GGTATTGCAGACATAATCCATAAGCAGGGAGAACCCTTGACGCTCTCTGA ACTGGGGGCTCAAATTCCTCTGAAGTCGGTCAACACCGACCACTTGCAC AGGTTAATGCGTTACTTGGTGCACATGAAGCTCTTCACCAAGGAAACCCT AGATGGCGAAGCTCGATATGGGCTGGCTCCACCGGCTAAGTTGCTTGTA AAATGGTGGGAGGACAAGGGCTTGGCGTCAATCATATTTGGGATCACTG ACAAGGATTTCATAGCACCCTGGCACCATCTCAAGGATAGCTTGGCCGG CGATGGCGAGGAGACAACTTTTGAGAAGGTGTTAGGGAAGAGCATATCG ACATACATGGCTGATCATCTGGAGAAGAGTATGTTGTTCAATGAATCAAT GGTTCATGATACCAGGCTCTTCACATCAGTCTTGATTCAAGACTTCAAGG ATGTATTCCAAGGAATTAAGTCGTTGGTGGATGTTGGTGGAGGCTCTGGA ACTGACATGGGAGCCATTGCCAAGGCCTTTCCCCACCTAAAATGTACAAT TTATGGTCTACCTCATGTCATTGCCGACTCCCCTGATTACCCTGAGGTCG ACCGGATTTCAGGCGACATGTTCAAACACATTCCCAGTGCCGATGCCATC TTATTGAAGTGCATCCTCCATTACTGGGGTGATGGTCAATGCATTGAAAT TCTAAAGAGATGCAAAGAATCAGTGCCTAGAGAGGGTGGAATAGTTATC ATCGCCGACGCAGTAGTAGATTTGGAATCTAAGCATCCCTACTTAACAAA AACTTTACTAAGCACGGATTTGGACATGATGCTCAACACTGGAGGAAAAG AGAGGACTGAGGCAGAATGGAAGAAGCTTTTTAATGCTGCAGGGTTCCC TGCATATAAGATTACACATGTAGCTGACGTTGAGTACTCTGTAATTGAGG CCTATCCTTATTAG
NNU_03165	ATGGAAATTCAGAAGGAAGTTCAAGCAGCCGACGTTGAAATCAGGAAAT TCGGTTATGGCTTTGCCGACATTTTAGTCATCCGATGTGCCATTCAGCTC GGAATTGCAGACATAATCCATAAGCAGGGGGAACCCTTGACGCTCTCTG AACTGGAGGCTCAAATTCCTGTGAAACCGGTCAACACCGATCACTTGCAC AGGTTAATGCGTTACATGGTGCACATGAAGATCTTCACCAAGGAAACCCC TGATGGCGAAGAACGATATGGGCTGGCTCCACTGGGTAAGTTCCTTGTA AATGGGTGGGACAGGAACATGGTGTCAGCCATATTAGCGGTCACTGACA AGGATTTCATGGTACCCTGGTACCGTCTCAAGGATAGCTTGGTCGGCGA

	GGGGACAGCTTTTGAGAAGGCGTTAGGGAAGACCATATGCGAATGCATG GCTGATCATCCGGAGAAGAAAAAGCCCTTCAATGAAGCAATGGCTTGTG ATACGACCAGGCTCCTCACATCAGCCTTGATTCAAGACTGCAAGGATTTA TTCCAAGGAATAATGTCGTTGGTGGATGTTGGTGGAGGCACTGGAACTG CCATGAGAGACATTGCCAAGACCTTTCCCCACCTAAAATGTACAATTTAT GATCTACCTCATGTCATTGCCGACTCCCCGGATTACCCTGAGGTCGACCG GATTGCAGGCAACATGTTCAAACACATTCCTAGTGCCGATGGCATCTTGT TGAAGTGCATCCTCCATGACTTGGGTGACCGTCAATGCATTGAAATTCTA CAGCGATGCAAAGAATCAGTGCCTAGAGAGGGTGGAAAAGTTATCATCG TCGACATAGTACTAGATCCGGAATCTACGGATCCCTTAACAAAGGCCAGA TTAAGGTTGGATTTGGACATGATGGTCTACACTGGAGGAAAAGAGAGGA GTGAGGCAGAATGGAAGAAGCTTTTGAATGCTGCAGGGTTCCCTCGATA TAAGATTTTACATATAGCTGCCGTTCAATCTGTAATTGAGGCCTATCCTT ATTAG
NNU_03166	ATGGAAATTCCGAAGGAAGTTCAAGCTGACGAGGTTGAAATCTGGAAATT CGGATATGACTTTGCCGACACTTTAGTCCTCCGATGTGCCATTGAGTTCG GTATTGCAGACATAATCCATAAGCAGGGAGAACCCTTGACGCTCTTTGAA CTGGGGGCTCAAATTCCTGTGCAACCAGTCAACACCGATCACTTGCACA GGTTAATGCGTTACATGGTGCACATGAAGATCTTCACCAAGGAAACCCTA GGTGGCGAAGAACAATATGGGCTATCTCCACACGGTAAGTTCCTTGTAAA AGGGTGGGACAAGAGCATGGCGTCAGCCATATTAGCGATCACTGACGAG GATTTCTTTGCACCCTGGCACTGTCTCAAGGATGTCTTGGCCGGCGAGG GGACAGCTTTTGAGAAGGCGTTAGGCAAGAGCATATGGGCATACGTGGC TGATCATCCGGAGAAGAATAAACTCTTCAATGAAGTAATGGCTTGTGATA CCAGTTTCATCACATCAGTCTTGATTCAAGACTGTAAGGATGTATTCCAA GGAATAAAGTCGGTGGTGGATGTTGGTGGAGGCACTGGAACTGCCATGA GAGACATTGCCAAGGCCTTTCCCCACCTAAAATGTACAATTTATGATCTA CCTCATGTCATTGCCGACTCACCTGATTACCCTGAGGTCGACCGGATTGC AGGCGACATGTTCAAACACATTCCTAGTGCCGATGCCATCTTATTGAAGT GGATCCTCCATGATTGGGATGATGGTGAATGTATTGAAATTCTAAAGCGA TGCAAGGAATCAGTGCCTAGAGAGGGTGGAAAAGTTATCATCGTCGACA

	TAGTACTAGATCCGGAATCTAAGGATCCCTTAACAAAGGCTAGATTAAGG TTGGATTTGGACATGATGGTCTACACTGGAGGAAAAGAGAGGAGTGAGG CAGAATGGAAGAAGCTTTTGAATGCTGCAGGGTTCCCTGGATATAAGATT TTACATGTAGCTGCCGTTCAATCTGTAATTATGGCCTATCCTTATTAG
NNU_23168	ATGGAAATTCAGAAGGAAGGTCAAGCAGCGGCGGCTAAAATCTGGAAAT TCGTTTATGGCTTTGCCGACTGTTTAGTCCTCCGATGTGCCATTGACCTC GGAATTGCAGACATAATCCATAAGCAGGGAGAACCCTTGACGCTCTCTG AACTGGGGGCTCAAATTCCTGTGCAACCGGTCAACACCGATCACTTGCA CAGGTTAATGCGTTACTTGGTGCACATGAAGATCTTCACCAAGGAAACCC TAGATGGCGAAGCACGATATGGGCTGGCTCCACCGGCTAAGTTCATTGT AAAAGGGTGGGACAAGAGCATAGTGTCAATCATATTAGTGGTCACCGAC AAGGATTTCATGGCACCCTGGCACTGCCTCAAGGATAGCTTGTGCGGCG AGGGGACAGCTTTTGAGAAGGCGTTAGGGAGGAGCATATGGACATACAT GGCTGATCATCCGGAGAAGAATAAGCTCTTCAATGAAGGAATGGCTTGT GATACCAAACTCCTCATATCAGCCTTGGTTCAAGACTGCAAGGATTTATT CCAAGGAATAATGTCGTTGGTGGATGTTGGTGGAGGCACTGGAACTGCC ATGAGAGCCATTGCCAAGGCCTTTCCCCACCTAAAATGTACAATTTATGA TCTACCTCATGTCATTGCCGACTCCCCTGATTACCCTGAGGTCGACCGGA TTGCAGGCGACATGTTCAAACACATTCCTAGTGCCGATGCCATCTTATTG AAGTGCATCCTCCATGACTGGGATGATGGTGAATGCATTGAAATTCTAAA GCGATGCAAGGAATCAGTGCCTAGAGAGGGTGGAAAAGTTATCATCGTC GACATAGTAGTAGATTTGGAATCTAAGCATCCCTTAACAAAGACTAGACT AAGCTTGGATTTGGACATGATGGTCACCACTGGAGGAAAAGAGAGGACT GAGGCAGAATGGAAGAAGCTTTTGAATGCTGCAGGGTTCCCTGTATTTAA GATTACACATATATCTGCCGTTCAATCTGTAATTGTGGCCTATCCTTATT AG

Selected lotus WRKY TFs based on the phylogenetic analysis	
NNU_11881	ATGACTCCGGCAATAGCAGCAGCACTCAAGCTGAAGGGACTAGCAGGAG TAACATGCAAGCGACGGGGAACTAATAAGGCTGTTGGAGCAAGAGTTGT ATTTAGAACAAAAACCGAGCTAGATATTATGGACGACGGCTTCAAGTGGA AGAAGTATGGGAAGAAGATGGTGAAGAACAGACCATTTCCAAGGAACTA CTACAGGTGTTCGGTGGAAGGATGCCCAGTGAAGAAGAGAATAGAAAGA GATGCAGACGATCCACAGCACGTGATAACGACATACGAAGGCACCCACA ACCACGAAAGCCCCTCGGCCTGA
NNU_05136	ATGAATATTGCCCAGAATTTGTCTATCGATATCGAGATGTCTAATCGTCA TCCGACTCCAGCCGGAATTTCACCGGAAAAAATCTCCCATCATCTCCTAA ACTTCGAACTTCCCGACTACCTGGACTTGAACCAATGGTTTGAAGGAGAC ACAGCATCTGTCACTCATGGATCTTCTAATCAGGATTTGATCCTTCCAAT GCCCAACATCGTCGGTTCCGGCAAGAATGGCAGTCAAGCTGAAGTCTCT AGCCCGAGCAACATGCAATGTTACTTTCTTCTGTGTGCAGCTTTGGAATT TTCTTTCTTTTCTGTATCAATTAAAACTGGTATTTGTTTTCATGTTGTCAG GGGACGTCGTAGTCGAACCAGAAATTCCGTTCTGGCTAGAGTTGCATTCA GAATAAAAACCGAGAAAGATATCTTAGACGATGGATTCAAGTGGAAGAA GTATGGAAAGAAGATGGTGAAGAACAAACCATATCCAAGGAACTACTTCC GGTGTTCAGTTGAAGGATGTCCAGTTAAGAAGAGAATAGAAAGAGATGC AGACGACCCACGCCATGTGATAACTACATATGAAGGCACCCATAACCAC GAAAGCCCCTTTTCCTGA
NNU_09891	ATGGATGTAGGCTCCAGAGTTGCATTCAGAACCAAATCTGAGCTTGAGGT CATCGACGATGGATTTAAATGGAGAAAGTACGGGAAGAAGACGGTGAAG AACAGTCCAAACCCGAGGAATTACTATCGCTGCTCAAGTGGAGGATGCA ATGTGAAGAAGAGAGTGGAAAGAGACCGTGAGGACTCGAGGTATGTGAT AACGACGTATGAGGGTGTGCACAATCATGAAAGCCCGTGTGTGGTTTATT ACAACGAAATGCCATTAATGGTTCCTAGTGGATGGACTTTGCAAGCTTCA CATTCACACTCATCATCCTCTTGA
NNU_01372	ATGGAAGGAGAAGCCCCACCACTACTGCCACCATTGTCGTCCCACAATA ACCCATCTTACATCTTGACACCCTCACTTGCATCCACGTCATTGCACCCT CCTCTTCTTTATCAACCTTATAACCTGCTGCAAGGTTCCAATATCCTACC

	AGACATCGACTGGGTTAGCCTCCTCTCTTCGCCATTTGGATTTGGCGATC TGCAGAAGCCACTCTTGTCGAATGCAGATGTGACAACTAGAACTGGAAAT AAAGCCGAAGATGAAAAGAGTGGTAAAGATAAGGTAAAATCGAGCAGGA TGAAGAAGGCAAGTCGGCCGAGGTTTGCGTTCCAGACGCGGAGCACAGA AGATATTCTCGATGATGGTTACCGCTGGAGGAAATACGGGCAGAAAGCT GTGAAGAACAGCAACTTTCCCAGGAGTTATTATCGCTGCACGCATCATAC ATGCAATGTGAAGAAGCAGGTTCAACGACTGTCAAAGGACACAAGCATC GTCGTGACAACATATGAAGGCATACACAACCATCCATGTGAGAAACTAAT GGAGAGTTTGAGTCCTCTTCTGAAGCAAATACAGTTCCTCTCT
NNU_22208	ATGGACATGGAGAACTACCCAATACTCCTCTCCTCTTCATCATCATCATC GTTAGCAACCGCTATTCCATTCTCATCTAACATGGTGACTTCTCATGTTA TTAACCATCTTCATGGAAACAATCCACCTGGGTTCTTAGGATTGAAGTCG GAGATGGATACCCCACTTAGCTCCGACGACTTTACAACCACCCTTCCTCA GATTCAGAGCTTTGGTGGGCCTAAAAATGAGATGAAACTAGGTATCAAAA AGGGGGAGAAGAAGATTAGAAAGCCCAGATATGCTTTTCAAACAAGAAG CCACGTCGATATACTTGATGATGGATATCGATGGAGGAAATATGGCCAAA AGGCTGTGAAGAACAACAAATTTCCTCGAAGCTACTATCGGTGTACGCAC CAAGGATGCAACGTGAAGAAGCAAGTTCAACGGCTATCCAAAGATGAAG GAATTGTGGTGACAACCTACGAAGGGATGCATACCCATCCTATTGAGAA GTCTACCGACAACTTTGAACACATCTTGAATCAGATGCAAATCTATTCTG CCTTTTAG
NNU_02028	ATGGAGAACTATTCAATACTCTTCCCGTGTTCATCATCATCGTCGGCAGC AGTAGCTGTTCCATTCTCCTCAAACATGGCAAATTCTCGTATTTTTGCTG ATCTTAATGACACCAGTCCAGCTGGGTTCTTAGGATTGAAGACGGAGAC GGATGCACATGCACCACGTTCAGATGTTAAAACCCTTCTTCAGAATGAAA GCTTTGGCCGGCCTAAAAGTGAAACGAAGCTCGGTATCAATAAGAAGGG TGATCAGAAGAAAATCAGAAAACCCAGATATGCTTTTCAAACAAGAAGCC AGGTCGATATACTTGATGATGGATATCGATGGAGGAAATATGGGCAAAA GGCTGTGAAGAACAACAAATTTCCTCGGAGCTATTATCGATGTACGCATC AAGGATGCAATGTCAAGAAGCAAGTTCAACGCCTATGCAAAGATGAAGG AATCGTCGTGACAACCTACGAAGGGATGCATACTCATCCAATTGAGAAAT

	CTACGGACAACTTCGAACACATTCTGAGTCAGATGCAAATCTATGCTTCC TTTTAG
NNU_05834	ATGGAGAAGAAAGAGACAACAATGGAGACAGATAATTCGATCGGAGCTA CGACATTTTCCGATCAGATTCCAACCACTTTCTCTTTATCCAGCATCTTT GACATGTCCTGTGAAGGTGAAAAAGGCTCTTTAGGCATCATGGATTTATT GGGCATCCAAGATTTCACTCCTTCTATATTCGATTTGCTACAGCAACCGT CGACGCTACTACCACCATCACCACCACCGCCACTACCACCGACGTCATC ACTTCCGGAGTCGTCTGAGGTGTTGAATTTGCCAGCAACACCCAACTCTT CTTCGATTTCATCATCATCGACTGAAGCAGCAAATGATGAACAGACCAAA GCAGTGGAAGAGGAGGAGCAGGAGAAGACTAAGAAGCAGCTGAAACCCA AAAAAAAGAACCAGAAACGGCAGAGAGAACCGAGATTTGCTTTCATGAC AAAGAGCGAGGTCGATCATCTGGAAGACGGGTACAGATGGAGAAAGTAT GGACAAAAAGCTGTGAAAAATAGCCCTTTTCCAAGGAGCTACTATCGTTG CACCAGTGCCACATGCGGTGTGAAGAAGCGAGTGGAGAGATCATCAGAT GATCCTTCCATTGTCGTGACAACGTACGAAGGCCAGCACACACATCCAA GCCCAGTAATGCCTCGTGGAAGCTCCACCGGAATCTCTTCGGATTCCGG CAGCTACGGTGCGGCCTTTGCCATGCCAATGCAATTGACGCAGTCTCACT TCCAACAACAGCAACAACAACAACAACCCCATTTCCACAACTTACCACCT TTGAATTTTAATTCTAATATTTCTTCGTCTCCTACTTTTGTACAAGAGAGA CGATTTTGCACTTCAGCAGCTTCCTTCCTTAGAGATCATGGCCTTCTTCA AGATGTCGTTCCTTCGGATATGATGATCAAAAAGGAGTAG
NNU_13849	ATGGAGACAGAGAATTCCATTGGAGCAGCAGTTACGGCGTTTTCGGATC AGATTCCCACCAACTTCGCTTTATCCAGCATCTTCGACACGCCTTTCGGA GGTGAAAAATGGTCTCTAGGATTCATGGATTTGTTGGGAGTCCAAGATTT TACCCCATCCATGTTCGATTTACTACAGCAACCTTCGATGCCATCACCGC CACCCATAGTGTCAGTCGGGGAGTACTCCTCCGATATATTGAATTTGCCT GCAACGCCCAACTCTTCTTCCATTTCGTCATCATCGACTGAAGCAGCAAA TGATGAACAGTCTAAAGCAGTGGAAGAGGAGGAGCAGGAGAAGACTAAG AACCAACTGAAATCCAACAAGAAGAATAAAAAACGGCAGAAAGAGCCGA GATTTGCTTTCATGACGAAGAGCGAGGTTGATCATCTGGAAGACGGGTA

	TAGATGGAGAAAGTACGGGCAAAAAGCAGTAAAGAACAGCCCCTTTCCG AGGAGCTACTACCGTTGCACCACTGCCACATGTGGTGTGAAGAAGAGAG TGGAGAGATCGTCGGATGATCCGACCATTGTCGTGACAACGTACGAAGG TCAGCACACACATCCGAGCCCTGTAATGCCTCGTGGAATCTCCACCGGA ATCTCTCCGGATTCCAGCAGCTACGCTGCGGCCTTCGCCATGGCGCCGA TGCAATTGACGCAACCTCATCATGACTTTCAAGAACAGCAGCAACCCTAT TTCCATAACTTATTACCACCTTCTCTCAACTATAATTCTAGTGTTTCTTTG GCTCCTACTTTCGTACCAGAGAGACGTTTTTTCACTTCTGCAACTTCTTT TATCAGAGACCACGGCCTTCTTCAAGACATAGTCCCTTCGGATATGAGAA GTGCTTAG
NNU_24385	ATGGGGACAGTGGAATCTCCGTGGCCGGAAAATTCGCCAATCAACCGAA GAAAGGCGATCAACGAGCTTGTCCGTGGCCGTGAATTTACGACCCAGCT TCAAATTATCCTCCGGAATCCCCTCGGAGGTCATGGATCCGTGTCGGCG GAAGACCTCCTCCCAAAAATCTTAACATCGTTCACTGAGGCTATTGCGGC ACTCAACACCACTGTCGAATCCGGGGAGGTTTCCCAGAATCCGGCGAGT ACCCATGTAAGTTCGCCCAGTTGCGGTGACCGGGCGACCGAGGATTCCG GTGAGAGCAAAAAGTCTACGGTTCTCAAGGATCGCAGAGGAAGTTATAA GCGAAGAAAGTTGTTGCAGACATGGACAAAGCTCAGCGCTACTCCGATC GATGACGGCCGTGCGTGGCGGAAATACGGCCAGAAAGTGATCCTCAACT CCAAATACCCAAGGAACTACTACAGGTGTACCCACAAGAACGATCAAGG CTGCCAAGCAACCAAACAGGTCCAACAAACCGAAGACAACCCGCCCATG TATCGGACCACATACATAGGCGATCACACATGCATAGACATGTCAAAGGC TCCCCGATTCCTCCTGGATTCTATCCACAACAACGCTTTTGTGCTCAGCT TTGAACCCGAAGCTCCGAGGAAGCAAGACCAACCCCACCCCTTTTTCTCC TGCTTTTCTCCCTCAATAAAACAGGAATGCAAAGAGGTGGAGATCCCGAG TGACCCGACCCACAAGCAATGTTCATCGGAATACCATCTTTCCCACGATG AAATCACATTCGGATCGTCCTGCCCCACAACAGTATTGCCGTCGACACCC GGGTCGGATCACGGGGATGTGATCTCGGGCGTGTACTCAGGTAGCACCA GCTCTCGCAGTCTGGACAATTATTTTGTGGGGATGAGTGACTTTGATGAC GTATTCCACTTTGAAGAGGACATCTTTCAGGTTTCAATGTAA

NNU_12194	ATGGACACAACTGTTGAATCTCCGTGGCCGGGAAATTTCTCTATTGATCG GAAAAGGCTGATCAACAGTCTCGTGCGAGGCCGTGAACTCACAACCCAA CTTCAAACTATCCTCTGCAATCGCCTCGGACATGAGGATGGGTCCTTGTC CACCGAAGATCTCCTCCCAAGAATCTTACGATCCTTCACCGAGGCTATTT CTGCACTCAAGTCCGCTGACTCCGGAGAGGTCTGTCAGAACCCGTCGAG TACGAATGTGAGTTCGCCCAGTTGTGATGGCCCAAGGACGGAAGATTCC GGCGAGAGCAGGAAGTCTCCAGCAGTCAAGGATCGTAGAGGAGATTATA AGAGAAGAAAGGTTTCTGAGACATGGACAAAGATCACTCCCACTCCCATT GACGACGGCCGTGCGTGGCGAAAATACGGCCAAAAAGTGATCCTCAATT GCAAATACCCAAGGAACTATTATAGGTGCACTCACAAAAACGACCAAGG ATGTGCAGCAACCAAACAAGTACAGCAAATTGAAGATGACCCACCAAAG TATCGAACTATATACAAGGGCCAGCACACATGCAAAGATATATCAAAGCC CCCCCAATTCATCATGGACTCTACCCATACAGACTCCTCCTCCTTCGTTC TAAGCTTTCAGTCAGACTCAGACGCTCCAATAACTAACCAGCAGCAGCTC CAGGAGCACCATCCCTTCTTCTCTTCCTCTTTCCCCCCAATAATAAAACA GGAATGCCAGGAAGAAATCCCAAACCATGAGATGGAGGAGCTCACCCAC AACCAGCAATCTTCATCACAATATTTTCTGCCATCTGTCCCTACATCAGC GTTGCCATCCACACCCGGGTCTGATCATGGGGATGTGATTTCGGGCGTC TACTCGTGTAGCACCAGCTCTCACAGTCTGGACATGAGCTTCGTCGGTGA CTTTGATGATGTTTTCCATTTTGATGATGATGACTTTTTTCCGGTTTAA

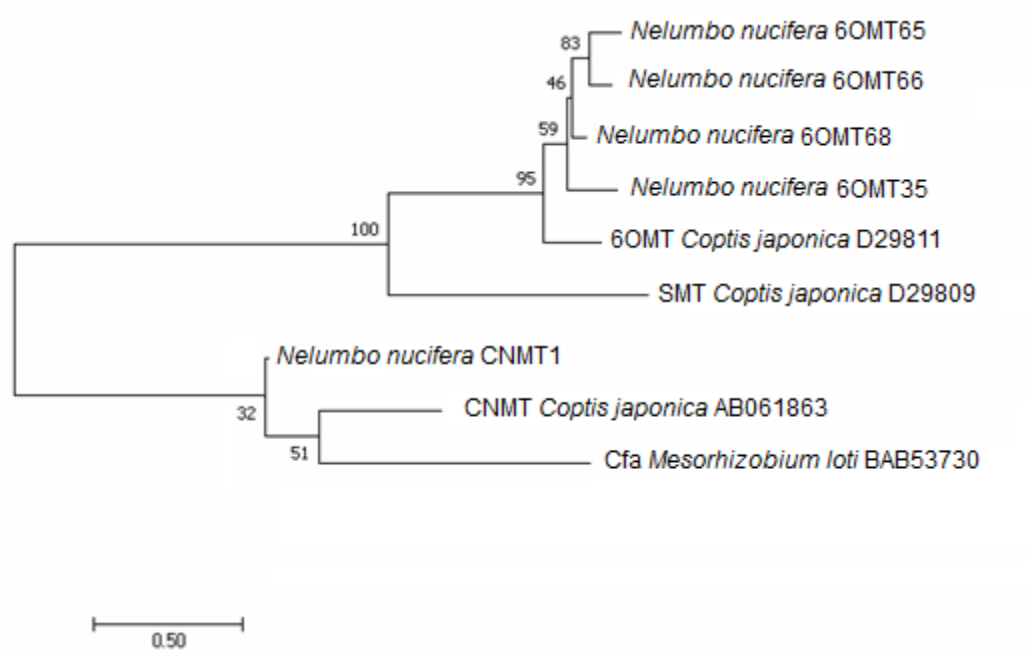


Figure 11 Unrooted dendrogram of deduced amino acid sequences of 6OMT and CNMT from lotus genome analyzed with plant S-adenosyl-L-methionine-dependent methyltransferases (SAM).

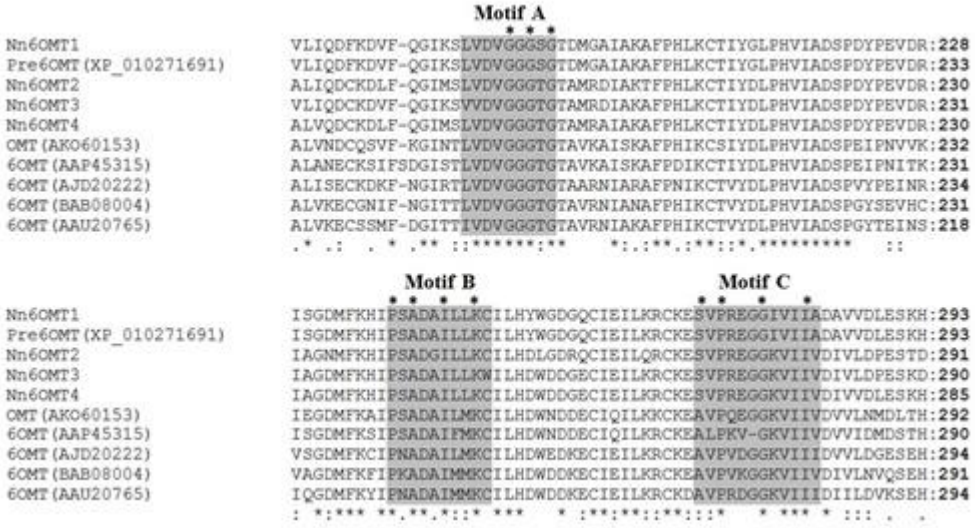


Figure 12 The deduced amino acid sequence alignment of *N. nucifera* 6OMT (Nn6OMT1-4) and the known 6OMT sequences retrieved from GenBank database. The sequence motif A, B and C of plant *S*-adenosyl-L-methionine methyltransferase (SAM) present in the targeted 6OMT. XP_010271691; predicted (*RS*)-norcoclaurine 6-O-methyltransferase-like *(N. nucifera).* AKO60153; *S*-adenosyl-L-methionine O-methyltransferase (*P. somniferum*). AAU20765; (*S*)-norcoclaurine 6-*O*-methyltransferase (*T. flavum* subsp. Glaucum). AJD20222 norcoclaurine 6-O-methyltransferase (*Sinopodophyllum hexandrum*). AAP45315; S-adenosyl-L-methionine norcoclaurine 6-O-methyltransferase (*P. somniferum*). BAB08004; S-adenosyl-L-methionine norcoclaurine 6-O-methyltransferase (*C. japonica*).

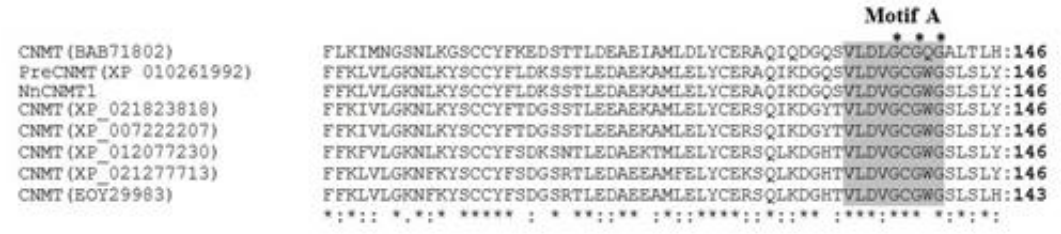


Figure 13 The deduced amino acid sequence alignment of *N. nucifera* CNMT (NnCNMT) and the known CNMT sequences retrieved from GenBank database. The sequence motif A of plant S-adenosyl-L-methionine methyltransferase (SAM) present in the targeted CNMT (NnCNMT1). XP_010261992; predicted (*S*)-coclaurine N-methyltransferase-like (*N. nucifera*). XP_012077230; (*S*)-coclaurine N-methyltransferase (*Jatropha curcas*). XP_021823818; (*S*)-coclaurine N-methyltransferase-like (*Prunus avium*). XP_021277713; (*S*)-coclaurine N-methyltransferase (*Herrania umbratica*). XP_007222207; (*S*)-coclaurine N-methyltransferase (*Prunus persica*). EOY29983; S-adenosyl-L-methionine-dependent methyltransferases superfamily protein isoform 1 (*Theobroma cacao*). BAB71802; coclaurine *N*-methyltransferase (*C. japonica*).

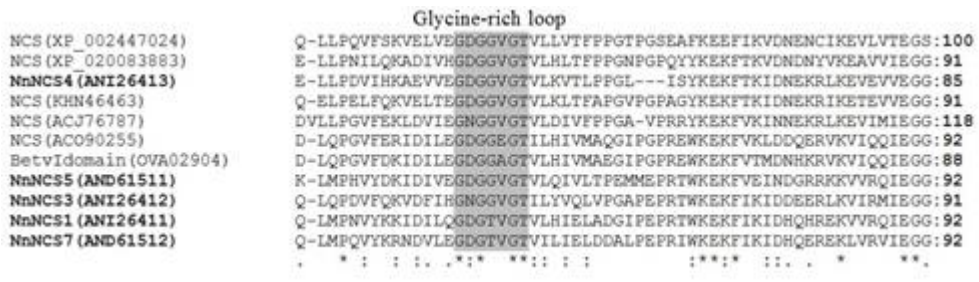


Figure 14 The deduced amino acid sequence alignment of *N. nucifera* NCS (NnNCS) and the known NCS sequences retrieved from GenBank database. Five targeted NCS in bold present a Glycine-rich loop, the ligand binding domain of Bet V1 protein family. ACO90255; pathogenesis-related (PR)-10-related norcoclaurine synthase-like protein (*E. californica*), OVA02904; Bet v I domain (*Macleaya cordata*), XP_020083883; *S*-norcoclaurine synthase 2-like (*Ananas comosus*), KHN46463; *S*-norcoclaurine synthase (*Glycine soja*), ACJ76787; *S*-norcoclaurine synthase 2 (*Argemone mexicana*) and XP_002447024; *S*-norcoclaurine synthase (*Sorghum bicolor*).

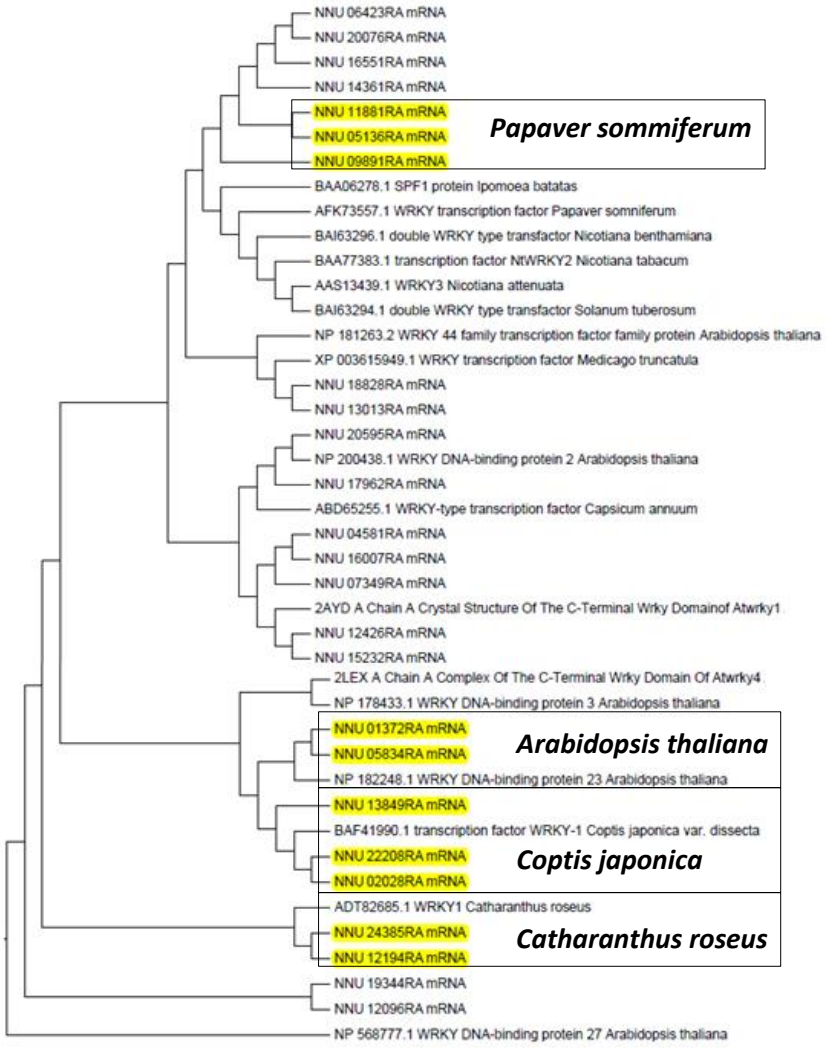


Figure 15 Unrooted dendrogram of deduced amino acid sequences of 60 WRKY TF sequences retrieved from lotus genome. The sequences were analyzed with secondary metabolite-related WRKY TFs retrieved from GenBank including *P. somniferum, A. thaliana, C. japonica,* and *C. roseus.* The 10 selected *WRKY TFs* sequences were highlighted.

Figure 16 The deduced amino acid sequence alignment of *N. nucifera* WRKY TFs and the known WRKY TFs sequences retrieved from GenBank database. A total of 10 putative WRKY TF sequences retrieved from blast analysis result of lotus genome with CjWRKY1. The putative WRKY TFs possess the β-sheet WRKY DNA binding domain/s (DBD) which located by the line while the other highlighted areas present the zinc-finger motif. Asterisks indicate conserved amino acid residues.

4.2 Relative gene expression and chemical contents in mechanically wounded lotus leaves

The previous work revealed that mechanical wounding on lotus leaves triggered high production of alkaloid compared with control group (Deng et al., 2016). This leads us to discover the change of related gene behind this mechanism and determine the relationship between BIA production and the related gene expression of NCS, CNMT, 6OMT, and WRKY TFs. To address such question, a group of related genes in early step of BIA biosynthetic pathway (Facchini, 2001b), including 7 isolated sequences of *NCS* , putative *CNMT*, and *6OMT* sequences retrieved from the lotus database were employed. Since some of the gene families have more than one member, the selected consensus regions were then used for primer design and further performed quantitative gene expression analysis using real time PCR technique. It has been known that nuciferine and *N*-nornuciferine are major compounds found in lotus leaf; therefore, they were measured their accumulation in wounded and control lotus leaves.

The plant materials including commercial and wild lotuses were nurtured in the pond located at Faculty of Pharmaceutical Science, Chulalongkorn Univeristy (Figure 17A). To get rid of unwanted factors which could generate the wound to the studied plant, fishes were first removed out of the pond and pesticide was applied in the culture area before placing lotus pot into the pond. The growth of the plant was

observed for 2 months before starting this experiment. The sign of normal growth was seen as the producing of pink flower and shooting leaf (Figure 17B). According to the guideline from The Botanical Garden Organization, Ministry of Natural Sources and Environment (http://www.qsbg.org/webBGO/database.html), the commercial lotus used in this study was characterized as Rosem Plenum lotus, a pink-flower producing varieties (Figure 17C). The fully expanded lotus leaf was used to perform mechanical wounding on the abaxial site of the leaf. Each pot was set for the wounding experiment as described above in the method section. After wounded, leaf samples were extracted for alkaloids and quantified *N*-nornuciferine and nuciferine using HPLC. HPLC chromatograms demonstrated a clear separation of *N*-nornuciferine (RT=10.494 min) and nuciferine (RT= 13.151 min) in the control leaf, the non-wounded, and wounded leaf when comparing to the standard compounds (Figure 18). The same leaf materials were employed to study the level of relative gene expression.

To discuss the relationship between gene expression level and BIA accumulation, all designated primers for qRT-PCR were tested with lotus cDNA by conventional PCR method before setting up reaction for qRT-PCR as follows; 1 cycle of Initial denaturation at 95°C for 3 min; 35 cycles of denaturation 95°C for 30 min followed by annealing 60°C for 40 sec and extension 72°C for 1min; 1 cycle of finale extension at 72°C for 5 min; Infinity hold at 4°C. Subsequently, cDNA was then used

for qRT-PCR reaction. The cDNA of lotus leaf collected from all treatments and days was individually estimated the level of relative gene expression using SYBR Green I dye. To validate this method, the specificity of designated primers toward target genes was observed on their related melting curve. The melting curve indicated the specific temperature which each amplified gene requires degrading the double-strand DNA. Thus, a group of melting curves retrieved from the reactions performed by individual designated primer was overlapped. The size of target genes is between 100-200 bp and the corresponding melting temperature is ranged between 75-85°C. The melt peak analysis showed that DNA fragments amplified from lotus cDNA of the control, wounded, non-wounded leaf and wild Thai lotus organs shared the same peak manner suggesting that the designed primers were specific to each target gene (Figure 19). Accordingly, the relative gene expressions were considerably measured. The C_T value and used to estimate the level of relative expression and together discussed with HPLC result.

The expressions of BIA related genes were compared between the wounded and control leaves. Our target genes displayed different patterns of gene expression during the observation period. We found that *NCS* has the highest relative expression compared with *CNMT* and *6OMT* in the wounded leave during most of the observation day. The expression of *NCS* increased greatly on day 2 after wounding and maintained its expression in relatively high level until day 7. It was also observed that the expression of *CNMT* exhibited the significant striking up on day 2 and

drastically decreased on day 3 while the expression of *6OMT* was gradually increased and considerably high on day 7 after wounding. Based on this result, *NCS* and *CNMT* played a dominant role on day 2 of which *CNMT* increased over 2 folds compared with the control group (Figure 20). Apart from the structural genes, the WRKY TFs also showed the promising relationship in this mechanism. Among the selected 10 *WRKY TFs*, *NNU_22208* was undetectable in our tested tissues. Thus, a total of 9 putative *WRKY* sequences were furthered studied. We found that five putative *WRKY TFs* have predominant expression level among the nine which are *NNU_09891*, *NNU_02028*, *NNU_05834, NNU_24385* and *NNU_12194.* On day 0, their relative expression striking up over 10 fold compared with the control group. Two putative *WRKY TFs* which are *NNU_09891* and *NNU_24385* were recognized to have the most response to mechanical wounding. Their transcript levels were fluctuated during an observation days and also shared similar expression pattern with *NNU_12194*, a minor transcript observed in this study while other *WRKY TF* candidates maintained their transcript level and displayed small changes throughout a week (Figure 21). The accumulation of 2 major BIA which are *N*-nornuciferine and nuciferine were estimated in the control and wounded lotus leaf (Figure 22). The sum content (ug/g dry weight) of BIAs slightly increased on day 2 and reached the highest level on day 3 and again slightly decreased on day 4. Finally, it remained the similar level on day 7. We also separately quantified an individual BIA targeted in this study; it was found that the content of nuciferine presented in the control leaf and the wounded leaf was higher

than *N*-nornuciferine. After wounded, the accumulation level of *N*-nornuciferine slightly increased and reached the highest level on day 3 which is about 2 times higher than its level in the control leaf and gradually increased until day 7 with the lowest content among observation days. Conversely, nuciferine was prior increased about 2 times on day 2 compared with its level in the control leaf and day 0 and remarkably reached the highest level on day 3. Nuciferine shared the accumulation level parallel with *N*-nornuciferine but the wounded leaf relatively remained high level of nuciferine content on day 7 after wounding compared with Day 0. Overall, 2 targeted compounds increased in the early day after wounded and slightly increased after day 3 (Figure 22A). HPLC chromatogram indicated a clear separation of *N*-nornuciferine and nuciferine indicated by different retention time showing peak 1 and 2 are corresponding to *N*-nornuciferine (RT= 10.494 min) and nuciferine (RT=13.151 min) (Figure 22B)

Based on this result, relative expression of biosynthetic genes correlated with the accumulation of aporphine-types alkaloids as shown in Figure 22B. The sum of alkaloid content on day 2 and day 3 was correlated with the increase of the *NCS* and *CNMT* expressions while 6OMT seemed to have late response on the alkaloid accumulation. HPLC analysis showed the majority in increasing of nuciferine content in the wounded leaf collected from all the observation days compared with *N*-nornuciferine content. Particularly, the accumulation level of nuciferine increased faster than *N*-nornuciferine as shown on day 2 which related to the significant

increase of total BIA content. *WRKY TFs* seemed to be more sensitive over the structural genes because it rapidly increased on day 0 with the outstanding increase of *NNU_24385*. To conclude, the order of BIA-corresponding gene expression in the wounded leaf are ranged as follows; WRKY TFs (*NNU_24385)*, *NCS*, *CNMT* and *6OMT*. The relative expression level of the target genes showed most correspondence to the accumulation of nuciferine.

In addition, the expression pattern of the major *WRKY TFs* fluctuated between day 0 - 3. These results may indicate some limitation in BIA production controlled by WRKY TFs on the wounded leaf because there was an evidence revealed the significant increase of BIA in the non-wounded site of Chinese lotus leaf (Deng et al., 2016). Consequently, it is important to observe the activity in the neighbor tissues for the better understanding.

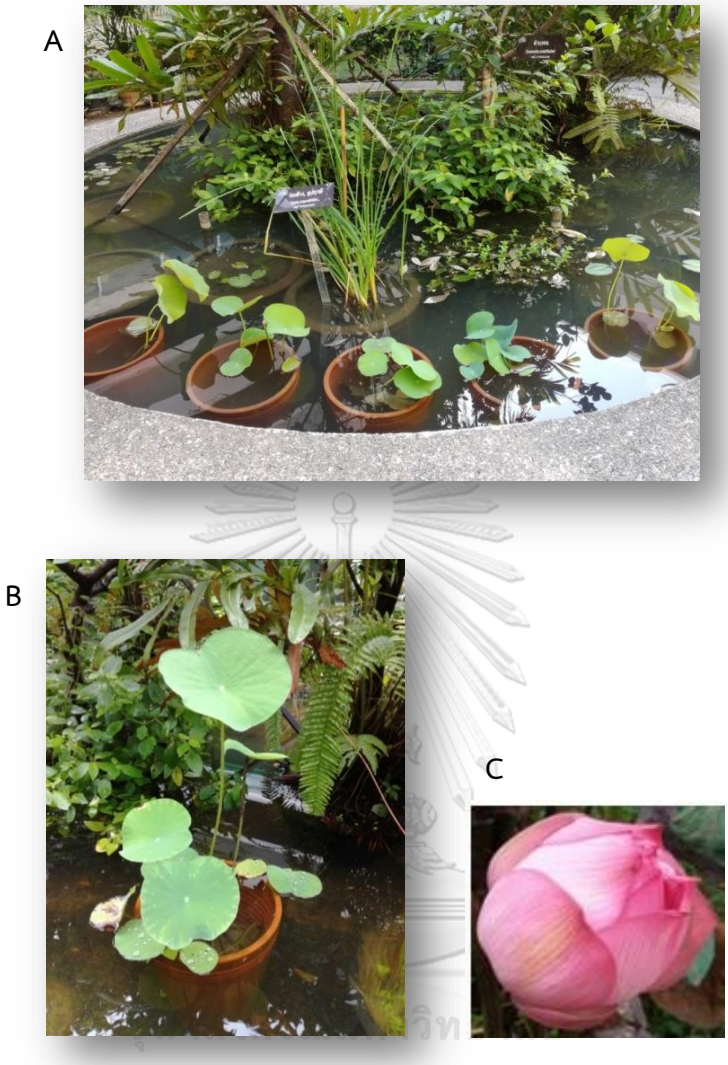


Figure 17 Plant materials used in this study. (A) A lotus culture area. (B) A lotus shooting leaf in mature stage of commercial lotus indicating a normal growth of lotus plant. (C) A pink flower of commercial lotus.

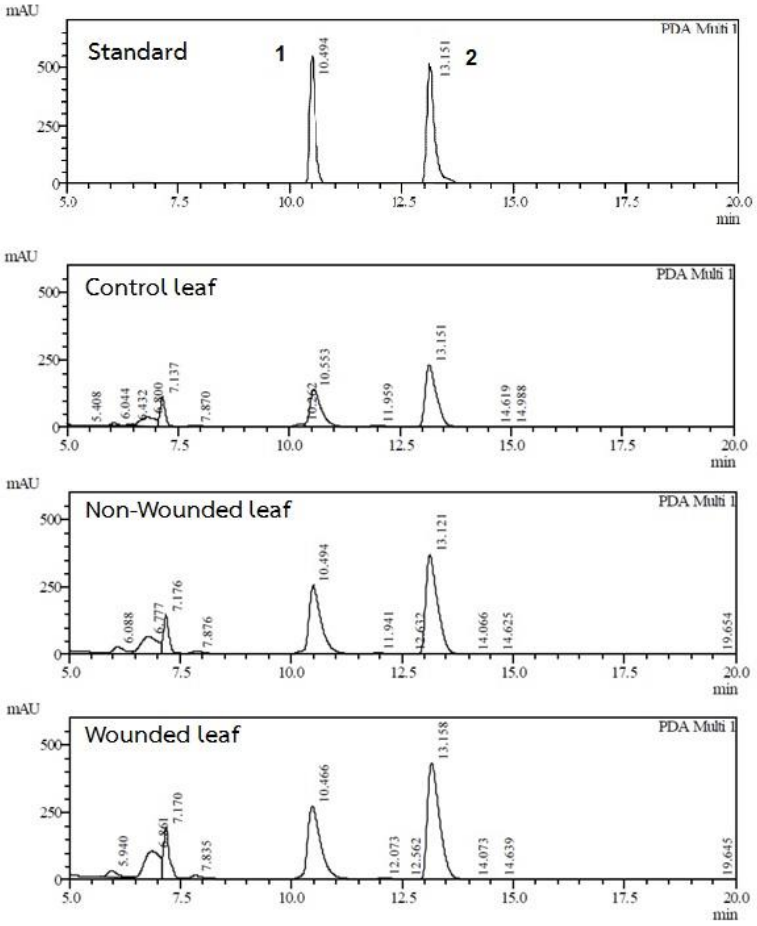


Figure 18 HPLC chromatogram demonstrated the separation on major BIA in lotus leaf. The absorbance unit (Y-axis) was recorded under 272nm and column temperature was controlled at 30°C. The identical retention time (X-axis) locate peak 1 and 2 which are corresponding to *N*-nornuciferine (RT=10.494 min) and nuciferine (RT= 13.151 min) in the two commercial standards, the control leaf, the non-wounded, and wounded leaf.

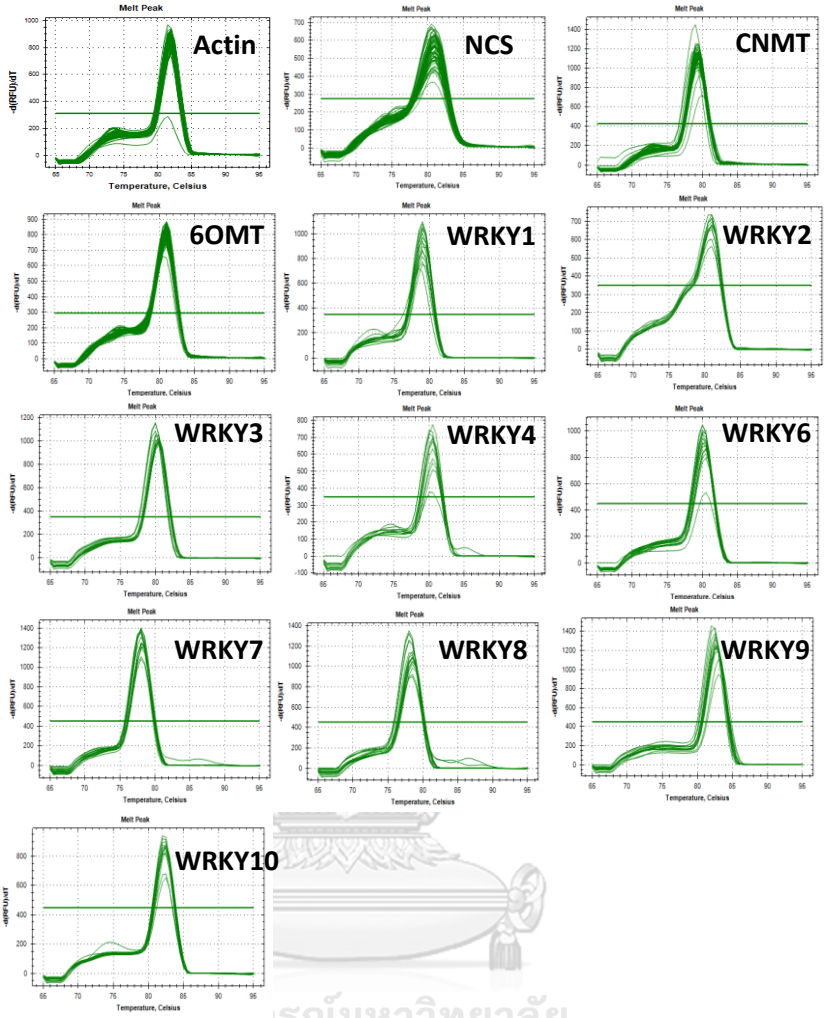


Figure 19 The overlap melt peak retrieved from qRT-PCR analysis. Several melt peaks from all treatments performed by the individual designated primers shared the common temperature for melting curve analysis (X-axis).

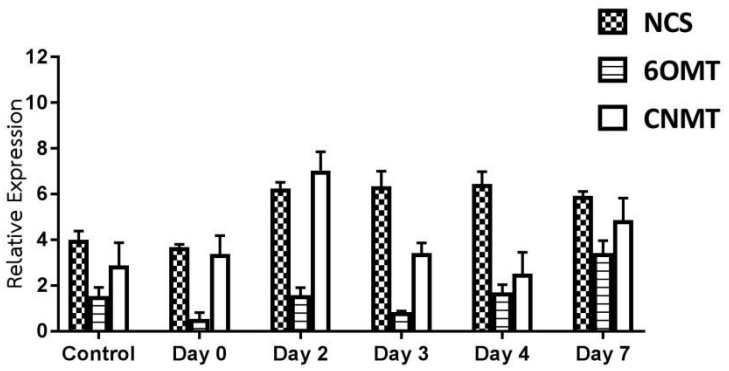


Figure 20 qRT-PCR analysis of wounded lotus leaf collected throughout one week. Bar graphs show the relative expression of *NCS*, *CNMT* and *6OMT* from *N. nucifera*.

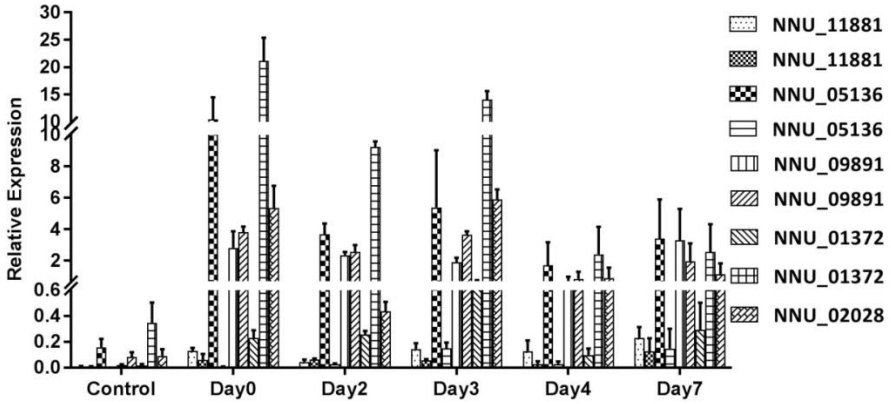
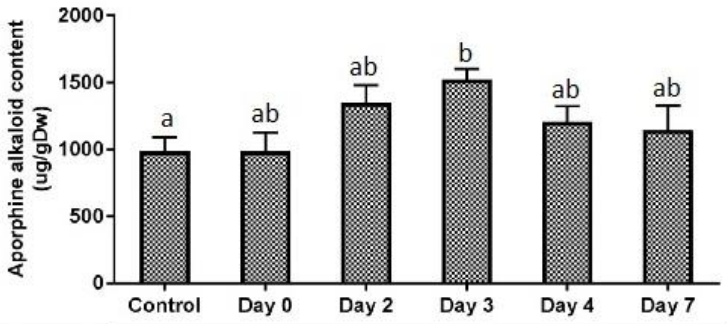


Figure 21 qRT-PCR analysis of wounded lotus leaf collected throughout one week. Bar graphs show the relative expression of 10 putative *WRKY TFs* from *N. nucifera.*

A



	Control	Day 0	Day 2	Day 3	Day 4	Day 7
N-nornuciferine	324.94	373.30	395.82	528.87	276.32	158.46
Nuciferine	523.51	626.87	995.35	1008.90	899.67	781.66
%RSD	0.09	0.07	0.10	0.20	0.10	0.06

B

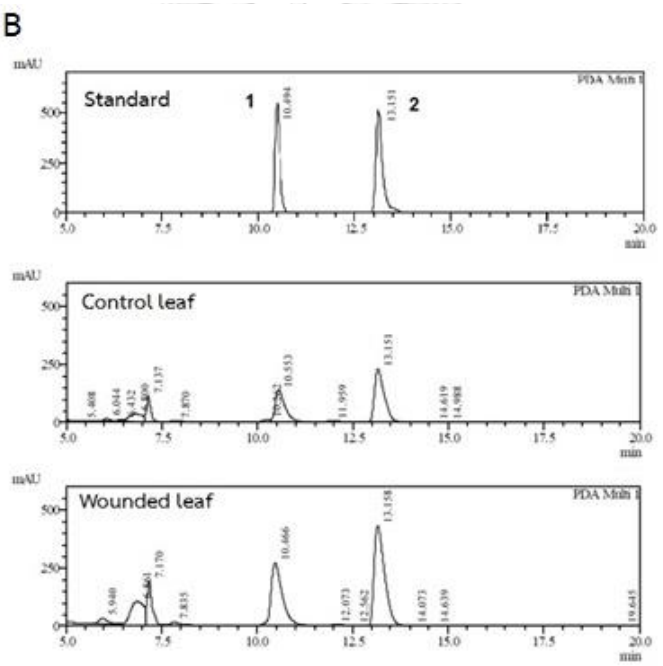


Figure 22 HPLC analysis of wounded lotus leaf collected throughout one week. (A) The sum of nuciferine and *N*-nornuciferine content in the wounded lotus leaf. (B) HPLC chromatogram indicated 2 peaks corresponding to *N*-nornuciferine and nuciferine, respectively. Error bars show the SD of the mean. The different lowercase letters (a and b) indicate the significant difference at $p < 0.05$; Mean value in group a and b is significant difference.

4.3 Comparison of relative gene expression and chemical contents in mechanically wounded lotus leaves and undamaged lotus leaves

Wound response occurs in both wounded site (local response) and non-wounded site (systematic response) which are generated by hormones such as jasmonic acid or typical signaling of wounding such as cell wall-derived oligogalactoronides (OGs) (Savatin et al., 2014). It is important to study the cellular event in non-wounded site. The result from previous study using the same method (Deng et al., 2016) has revealed the significant increasing of particular BIAs in non-wounded site of Chinese lotus leaves including *O*-nornuciferine, anonaine, and nuciferine. Thus, the putative genes which mentioned before were also employed to study the role of BIA-corresponding genes in non-wounded leaf (Figure 23A). Results from the wounded leaf were combined and discussed here.

The transcript level of structural genes and *WRKY TFs* in the non-wounded leaf exhibited massive significance in relative expression level compared with that of the wounded and control leaf (Figure 23B and 24). In the wounded leaf, the structural genes showed variegated gene expression patterns during day 0-4 whereas the similar expression pattern was observed on day 7. In the non-wounded leaf, *NCS* had quite similar transcript level as the control leaf and it remained the low transcript level after wounding until Day 7. *6OMT* had low transcript level in the first 3 days after wounding but it showed the significant increased on day 4 which is

about 5 times higher than its level on day 0 and slightly decreased on day 7. Meanwhile, *CNMT* gradually increased in early day and showed 5 time-striking up on day 3 compared with its level on day 0. Then, the transcript level gradually decreased after day 3 and remained the transcript level until day 7. To conclude, the *NCS* and *CNMT* took the first role in early day after wounding followed by the late collaboration of *6OMT* (Figure 23B).

The transcript level of *WRKY TFs* in the non-wounded leaf apparently displayed enormous increase over the wounded and control leaf. Most of putative *WRKY TFs* in the non-wounded leaf increased in day 0 and seemed to remain their transcript level with the relatively high level throughout the observation days. Five putative *WRKY TFs* observed in the wounded leaf were also found to have the most correlation in this mechanism. *NNU_24385* was again recognized to be the predominant *WRKY TF* with a fluctuated transcript level. It showed the highest transcript level on day 3 and considerably increased until day 7. Briefly, *NNU_24385* was the outstanding one because its expression remarkably increased right after wounding on day 0 and reached the highest level on day 2 in the wounded leaf. Likewise, in the non-wounded site, *NNU_24385* took a major role and its expression significantly increased on day3 (Figure 24).

Total BIA content of in both leaves exhibited different accumulation pattern during observation days. In the wounded leaf, the accumulation level constantly increased with the significant highest level on day 3 and then decreased until day 7.

Unlike, we could observe the fluctuating changes of accumulation level in the non-wounded leaf between days as clearly seen in day 2 and 3. Though the non-wounded leaf was also found to have the significant increase of compound accumulation but they seemed to have more complex inside of the undamaged tissues. Interestingly, relative gene expression of *CNMT* in the non-wounded and wounded leaf seemed to be correlated with its aporphine-type alkaloid accumulation as shown in day 3. Herein, the transcript level of *CNMT* on day 3 of the non-wounded leaf resulted in the highest alkaloid accumulation among the collection day. Similarly, the increased *NNU_24385* on day3 related to the highest accumulation of target compound in the non-wounded sites (Figure 25)

Overall, the expression pattern of all structural genes and *WRKY TFs* in the non-wounded leaf generally related to the compound accumulation in their tissues during an observation day whereas the wounded leaf displayed indirect relation in early day after wounding and seemed to be correlated with the total compound content after day 3. It's worth noting that the increase of *CNMT* and *NNU_24385* on day 3 of the non- wounded leaf correlated well with the highest alkaloid accumulation. Moreover, we combined the order of BIA-corresponding gene expression in the wounded and non-wounded leaf which sharing a common phenomenon; the order of gene expression in response to wounding effect was ranged as follows; WRKY TFs (*NNU_24385)*, *NCS*, *CNMT* and *6OMT*.

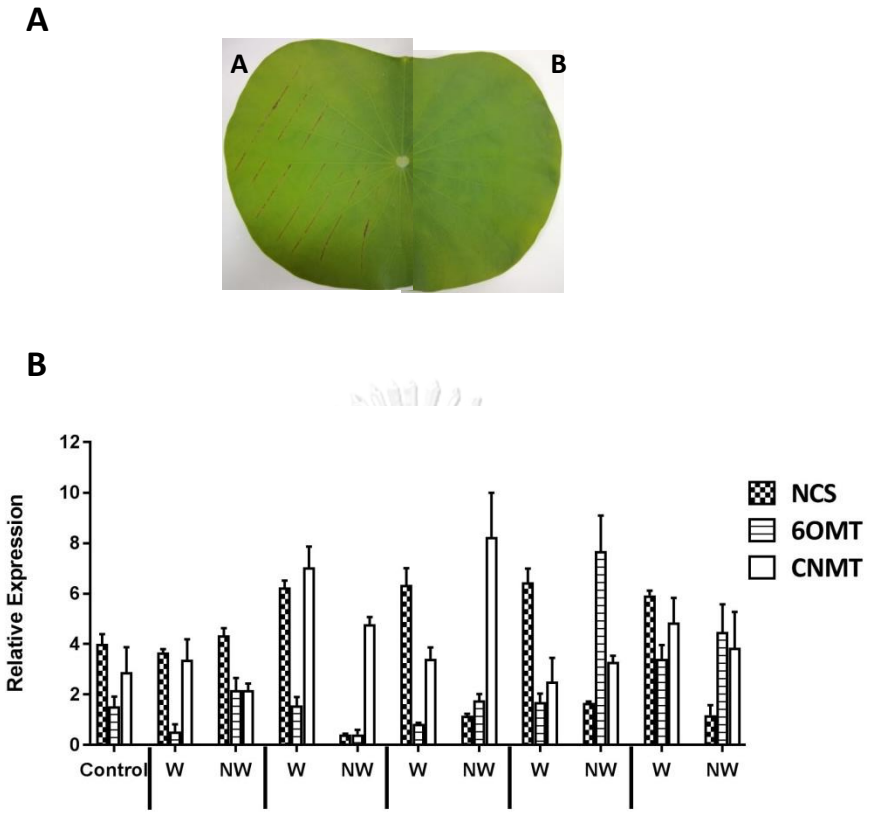


Figure 23 qRT-PCR analysis of wounded lotus leaf collected throughout one week. (A) Picture of lotus leaf. A; the wounded leaf. B; the non-wounded leaf. (B) Bar graphs show the relative expression of *NCS*, *CNMT* and *6OMT* in the control, wounded (W) and the non-wounded leaf. Error bars show the SD of the mean.

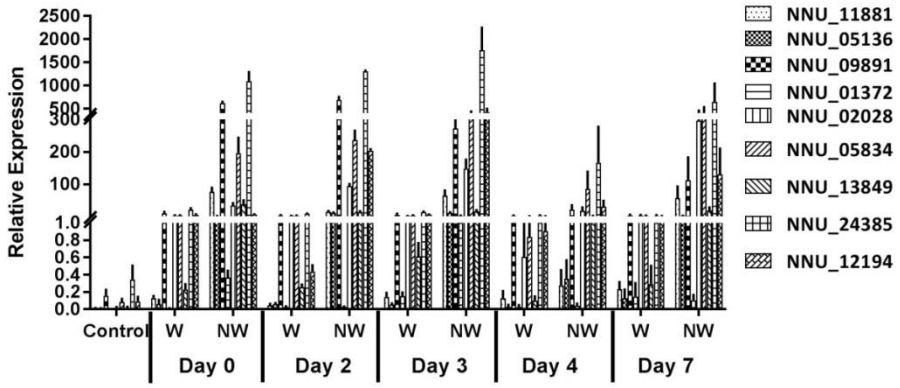
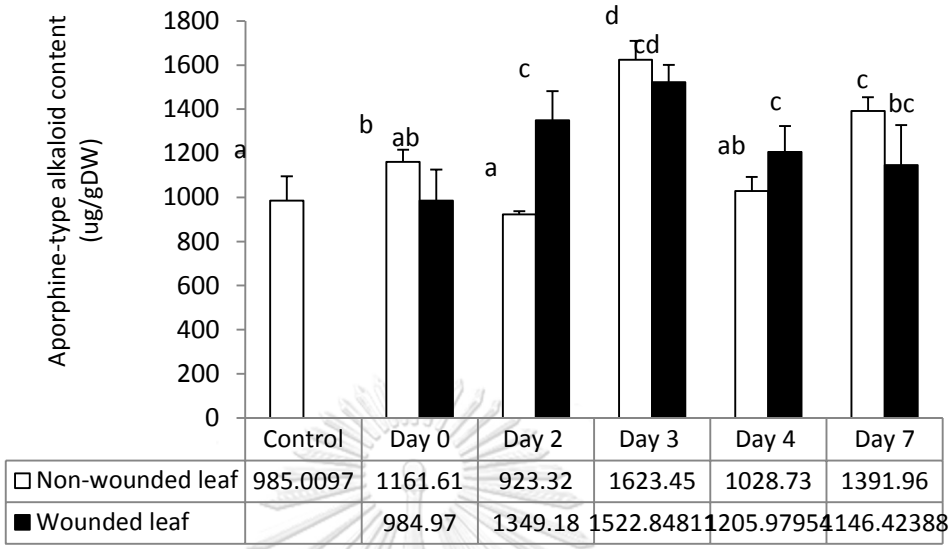


Figure 24 qRT-PCR analysis of wounded lotus leaf collected throughout one week. Bar graphs show the relative expression of 10 putative *WRKY TFs* in the control, wounded (W) and the non-wounded leaf. Error bars show the SD of the mean.



	Control	Day 0	Day 2	Day 3	Day 4	Day 7
□ Non-wounded leaf	985.0097	1161.61	923.32	1623.45	1028.73	1391.96
■ Wounded leaf		984.97	1349.18	1522.8481	1205.9795	4146.42388

Figure 25 HPLC analysis of the wounded and non-wounded lotus leaf collected throughout one week. Bar graph showed the sum of nuciferine and *N*-nornuciferine content in the wounded lotus leaf. BIA content in each leaf is separately showed in the table below bar graph. Error bars show the SD of the mean. The different lowercase letters (a, b, c and d) indicate the significant difference at $p < 0.05$; Mean value in group a, b, c and d is significant difference.

4.4 Comparison of BIA related genes and alkaloid contents in different lotus organs

Lotus accumulates various bioactive BIAs in the tissues. To get a target compound, we need the information about organ source and developmental stage for the specificity. The expression profile and information of a biosynthetic gene which involved in the production of BIAs in different developmental stage of lotus organ would clarify the role of key gene. Here, we elucidated BIA-corresponding gene expression profiles to study the relationship between nuciferine and *N*-norniciferine accumulation and the promissing key genes in two developmental stages of leaf and petiole (Figure 26). In young leaf, *6OMT* showed the large proportion (% relative expression) in all selected lotus whereas *NCS* and *CNMT* remained low transcript level in all subjected sample (Figure 27). Similarly, *NCS* and *CNMT* were not dominant as in young petiole of 5 varieties which were Album Plenum 0 and 1500; Bua Khem Chin 1200, and Rosem Plenum 300 and 600. There were variations in relative gene expression in young petiole but *6OMT* seemed to be dominant transcript in most subjected varieties. We also found that *CNMT* was dominant in young petiole of Bua Khem Chin 1200 (Figure 28). To summarize, the young tissue of lotus, *6OMT* is the most active transcript indicated by the relatively high percentage proportion of its transcript level.

Mature tissues from the same developmental stage were also targeted in this study. In the mature leaf, it clearly showed the highest percentage proportion of *NCS* except Bua Khem Chin 1500, where *6OMT* was highly expressed. *6OMT* and *CNMT* showed the small proportion of relative expression when compared with *NCS* and they are variegated among the varieties. Seven lotus varieties had quite low portion of *6OMT* (Figure 29). Similarly, the mature petiole was found to possess the same manner as the mature leaf. All selected varieties showed the most abundant of *NCS* while the *CNMT* and *6OMT* maintained their relatively low expression level, respectively (Figure 30). It can be concluded that *NCS* is the most active transcript in the young tissues of lotus organs used in this study. Moreover, we found that the relative expression pattern of structural genes in white (Album plenum) and pink (Rosem plenum) flower-producing varieties apparently exhibited similar proportion. Unlike, Bua Khem Chin, expressed various patterns of structural gene expression.

The transcript level of 10 putative *WRKY TFs* was also investigated in lotus organs. *WRKY TF (NNU_24385)* was the major transcript in most subjected young leaf except Bua Khem Chin 300 showing the higher percentage proportion of *NNU_13849.* They seemed to be competitive when compared because some varieties such as Rosem Plenum 300 had quite equal proportion of *NNU_24385* and *NNU_13849* in their young leaf. Five putative *WRKY TFs* including *NNU_09891, NNU_02028, NNU_05834, NNU_24385* and *NNU_12194* are found to be abundant in the young leaf (Figure 31). Young petiole also possessed high percentage portion of *NNU_24385* in

most subjected varieties. *NNU_12194* was found to be a dominant *TF* in some varieties such as Rosem Plenum 600, Rosem Plenum 60, and Bua Khem Chin 300. The five indicated *WRKY TFs* which observed in the young leaf were also found to be a dominant transcript in the young petiole as well (Figure 32). Mature leave of all subjected varieties had the highest percentage proportion of *NNU_24385.* We also observed that *NNU_12194* was the minor transcript as presented in most varieties and it showed quite equal proportion to *NNU_2438* as shown in Album Plenum 900. Three putative *WRKY TFs* including *NNU_05834, NNU_24385* and *NNU_12194* were found to be abundant in the young leaf (Figure 33). Moreover, *NNU_09891, NNU_05834, NNU_24385,* and *NNU_12194* were also found to be abundant in the mature petiole. Here, *NNU_02028,* which showed the small proportion in the subjected organs mentioned before, was found to have higher proportion in some varieties including Album Plenum 1500 and Rosem Plenum 1500 (Figure 34). *NNU_24385* was found to have the highest relative expression level in both young and mature stage of lotus leaf and petiole.

To summarize, it clearly showed that in normal condition, *6OMT* had high percentage of relative expression in young tissue; while, *NCS* took a major role in mature tissues. *NNU_24385* was the predominant *WRKY TF* in all tissues and developmental stages. Each gene deems to take a different role in different developmental stages of lotus.

BIA content in each organ were also estimated and compared between the commercial lotus and wild Thai lotus. The result of chemical analysis showed that BIA content in tissues from young and mature stages of two organs of nine lotus varieties were ranging between 1.37-5.10 mg/g DW (Table 8). Young tissues of Rosem Plenum had the highest aporphine-type alkaloid accumulation compared with other wild varieties and the commercial lotus. Generally, the alkaloid content in Rosem Plenum group was outstanding in young leaf (Figure 35). Similarly, the BIA content was predominant in petiole of Rosem Plenum 600, 300, and Bua Khem Chin 1200 (Figure 36). In mature tissues, the commercial lotus showed the highest accumulation which was similar to some wild varieties. Mature leaf of commercial lotus, Rosem Plenum 300 and Bua Khem Chin 1200 were the first three samples with high aporphine-type alkaloid content (Figure 37). Similarly, Rosem Plenum 600 and the commercial lotus had the highest accumulation in mature petiole (Figure 38). Overall, the young leaf and the mature leaf are rich source of the targeted compounds while the petiole in both stages presented a low accumulation except for the two varieties; Rosem Plenum 600 and 300, respectively. Rosem Plenum group and the commercial lotus provided high content in the subjected tissues.

The expression profile of target genes and compound accumulation were also investigated in the commercial lotus cultured in normal condition. The structural genes in young tissue of commercial lotus demonstrated similar gene expression pattern as in wild lotus. In commercial lotus, the relative gene expression

of *CNMT* was quite low and equal in all tissue; *NCS* highly expressed in most tissue especially in mature leaf; while, *6OMT* was variable among the tissues and dominant in young leaf. In petiole, *NCS* and *CNMT* was a dominant gene but aporphine-type alkaloid content is considerably low when compared with two stages of leaf. The commercial lotus and Thai wild lotus shared similar gene expression pattern of *6OMT* and *NCS* in young and mature leaf, respectively (Figure 39A). Their relative gene expression also related to the accumulation of aporphine-type alkaloid. In addition, total BIA content in the vegetative tissues of commercial lotus is dramatically high unlike; their reproductive tissues including pink petal and staminode contain a trace amount of these major aporphine-type alkaloids (Figure 39B).

4.5 Role of BIA related gene in normal condition and abiotic stress condition

The result of BIA related gene profile and the mechanical wounding experiment suggest that the behavior of BIA related genes in wild Thai lotus and wounded lotus are different. *CNMT* maintain its low transcript level in normal condition as shown in all subjected organs of wild Thai lotus. Interestingly, *CNMT* exaggeratedly increased its transcript level under abiotic tress condition in the early day after mechanical wounding. This shows its sensitivity and important role in wound defense mechanism. Though *NCS* and *6OMT* show their high level of relative expression in normal condition, but they play a less role in this response when compared with the behavior of *CNMT* in wounded lotus leaf. The role of *WRKY TF9* (*NNU_24385)* which controls the transcription of all structural genes in this study also

highlighted. It behaved as similar as *CNMT*; its transcript level massively increased more than 10 times in biologically active tissues. It showed even more sensitivity compared with the transcription level of all structural genes in this study.

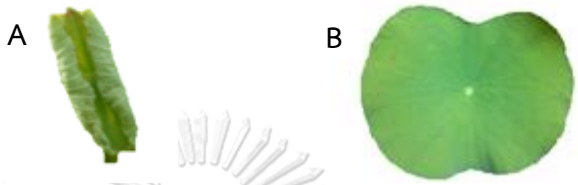


Figure 26 Lotus organs collected from different developmental stages. (A) Young stage leaf with folded leaf margin. (B) Mature stage leaf with fully-opened leaf margin.

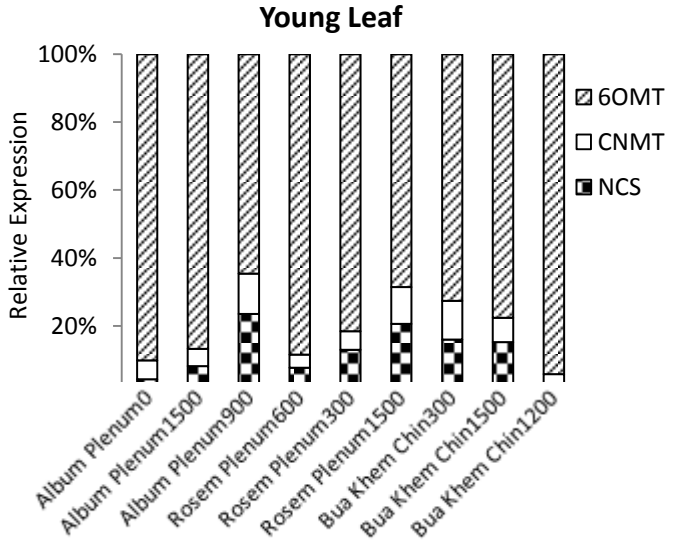


Figure 27 The expression profiles (percent proportion) of putative structural genes in young leaf of the 9 wild lotus varieties.

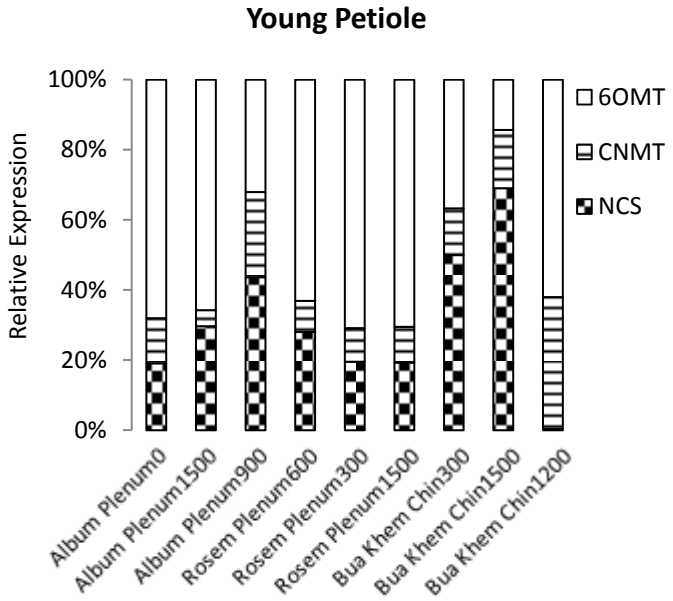


Figure 28 The expression profiles (percent proportion) of putative structural genes in young petiole of the 9 wild lotus varieties.

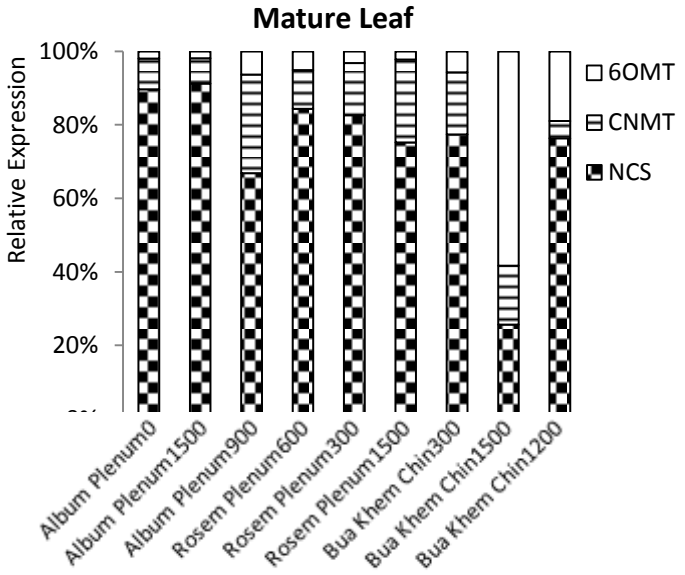


Figure 29 The expression profiles (percent proportion) of putative structural genes in mature leaf of the 9 wild lotus varieties.

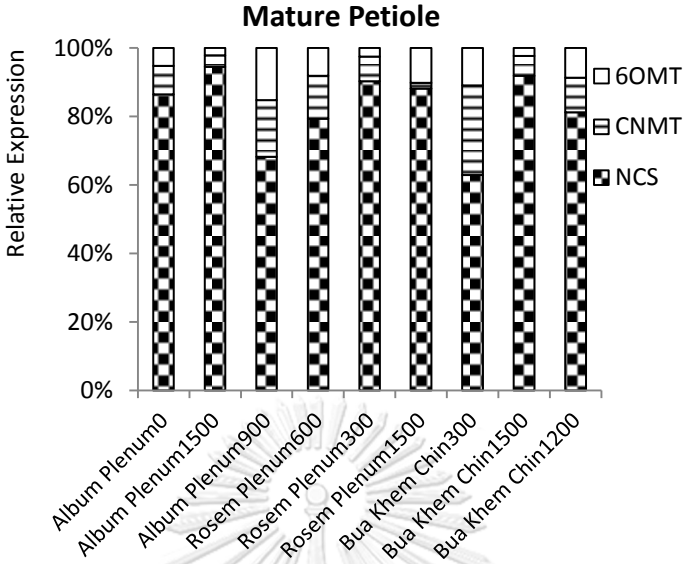


Figure 30 The expression profiles (percent proportion) of putative structural genes in mature petiole of the 9 wild lotus varieties.

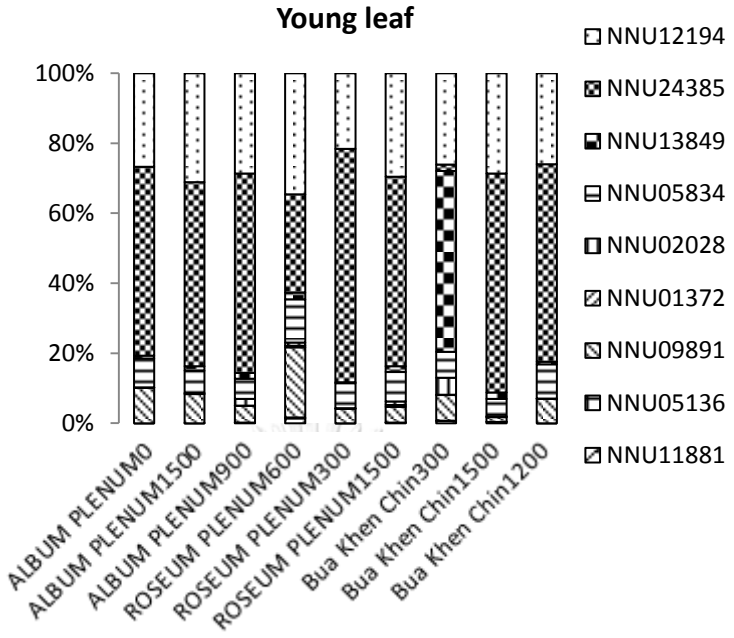


Figure 31 The expression profiles (percent proportion) of 10 putative *WRKY TFs* in young leaf of the 9 wild lotus varieties.

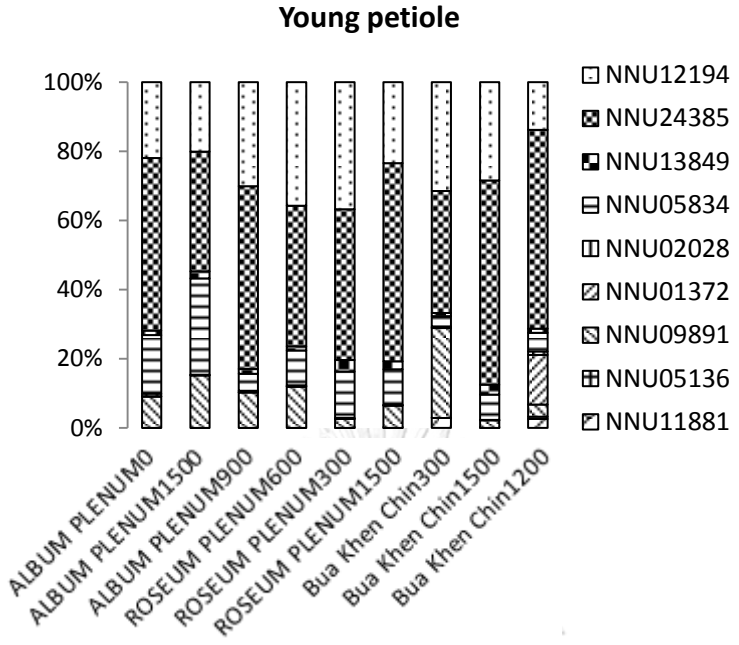


Figure 32 The expression profiles (percent proportion) of 10 putative *WRKY TFs* in young petiole of the 9 wild lotus varieties.

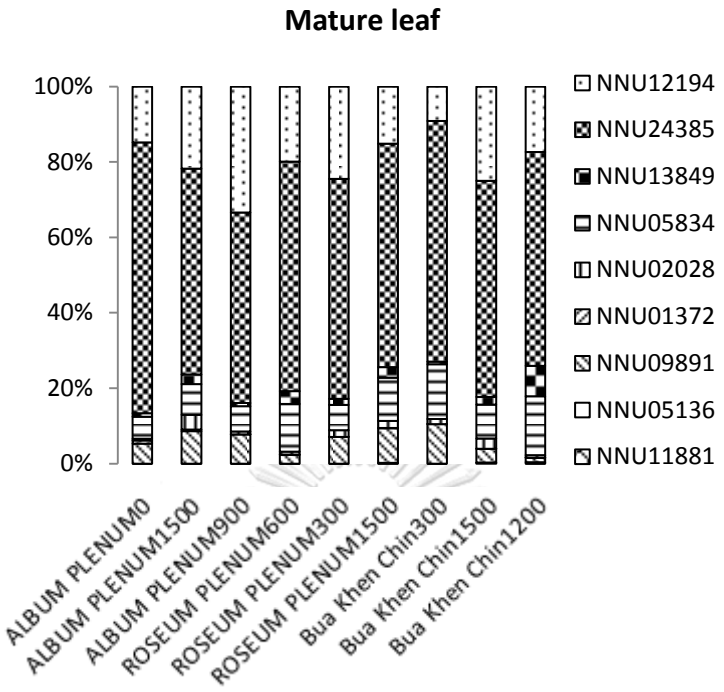


Figure 33 The expression profiles (percent proportion) of 10 putative *WRKY TFs* in mature leaf of the 9 wild lotus varieties.

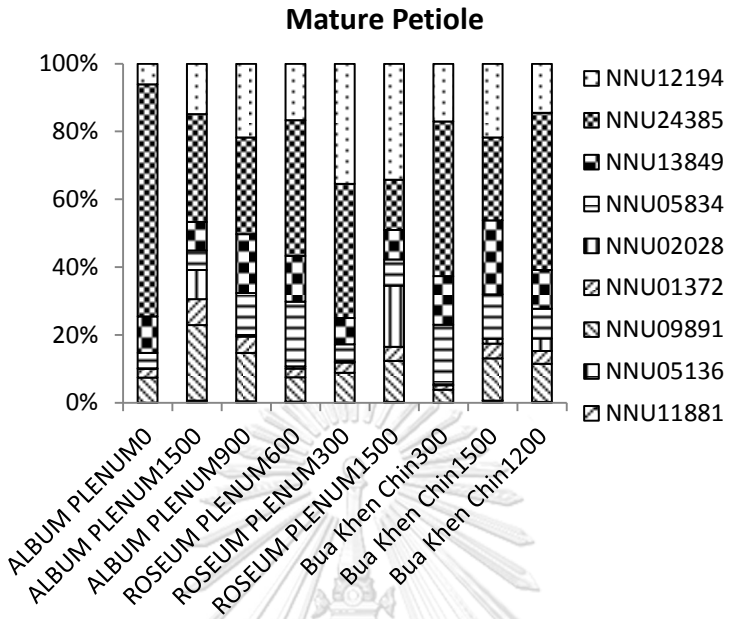


Figure 34 The expression profiles (percent proportion) of 10 putative *WRKY TFs* in mature petiole of the 9 wild lotus varieties.

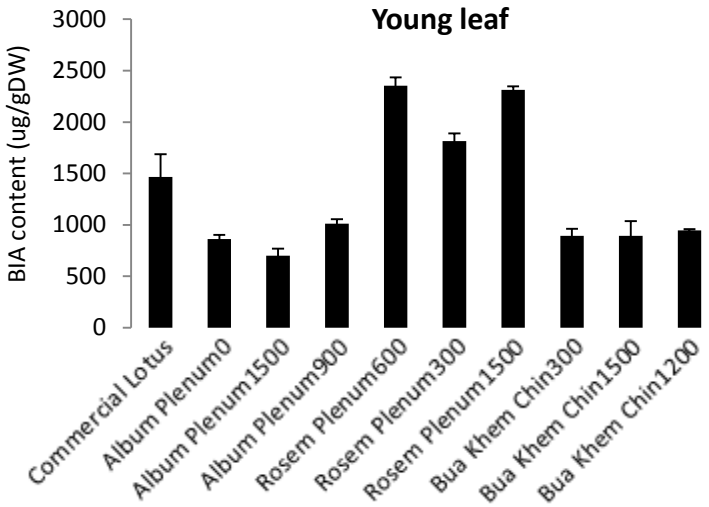


Figure 35 The comparison of the sum nuciferine and *N*-nornuciferine content in young leaf between the 9 wild Thai lotus.

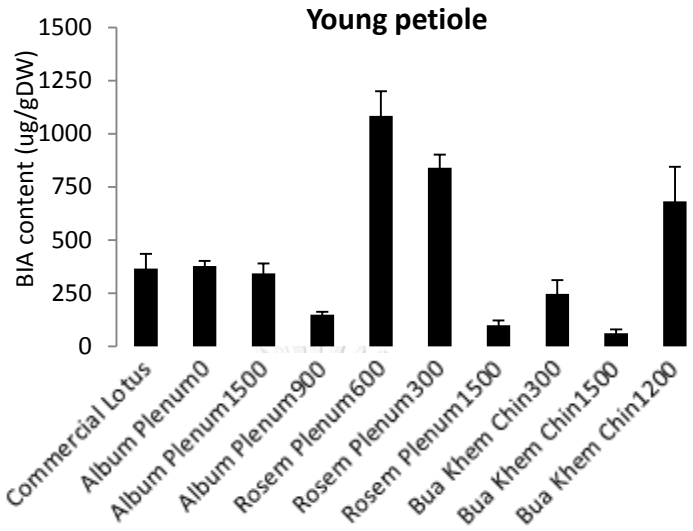


Figure 36 The comparison of the sum nuciferine and *N*-nornuciferine content in young petiole between the 9 wild Thai lotus.

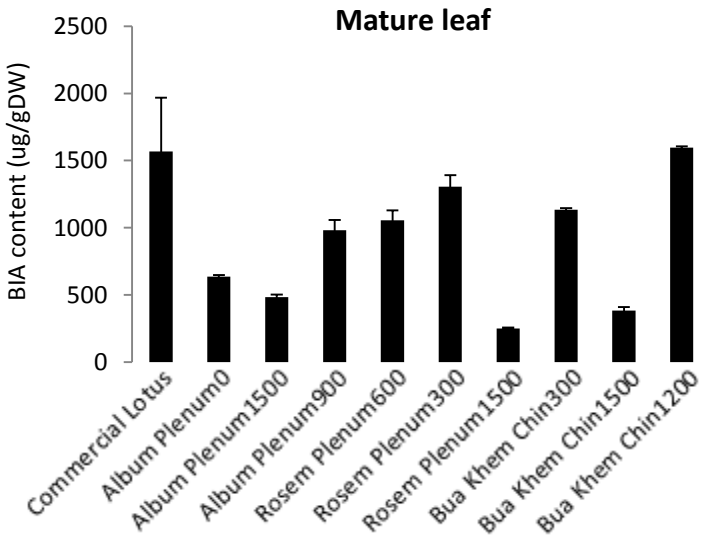


Figure 37 The comparison of the sum nuciferine and *N*-nornuciferine content in mature leaf between the 9 wild Thai lotus.

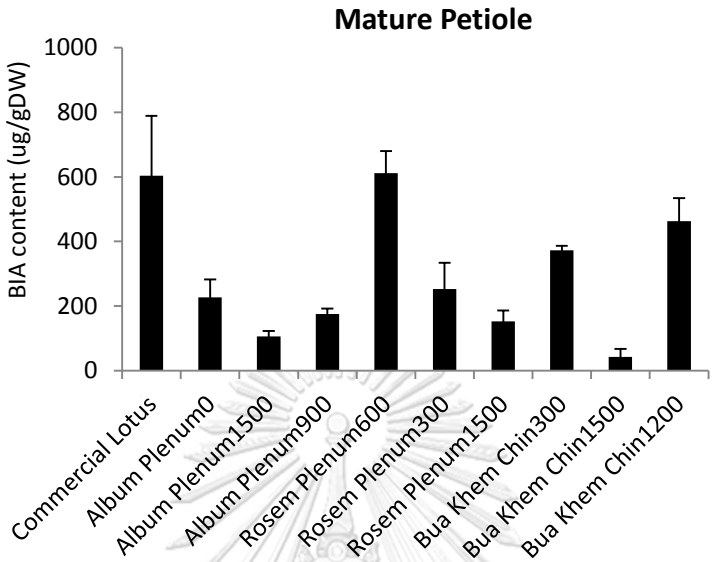


Figure 38 The comparison of the sum nuciferine and *N*-nornuciferine content in mature petiole between the 9 wild Thai lotus.

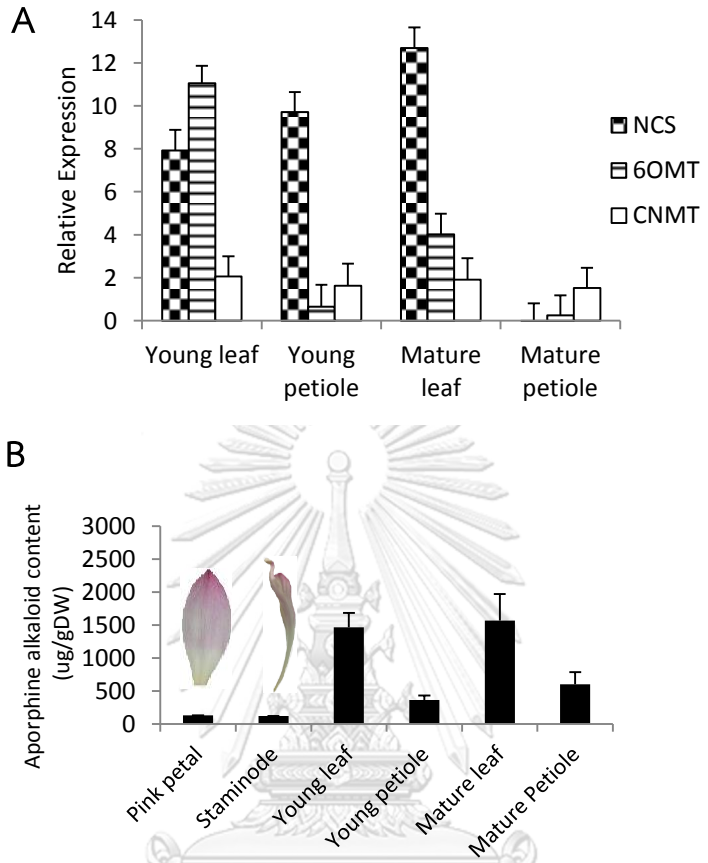


Figure 39 qRT-PCR and HPLC analysis of commercial lotus organs. (A) Bar graph show relative gene expression of structural genes. Leaf and petiole from young and mature stages were subjected to study (B) The comparison of aporphine-type alkaloid content from different organs. Error bars show the SD of the mean.

Table 10 The sum of nuciferine and *N*-nornuciferine content in lotus tissues (mg/g DW). The top three highest amounts were highlighted in bold.

Common name	Collection area	BIA content (mg/gDW)				
		Leaf		Petiole		Total (mg/gDW)
		Young	Mature	Young	Mature	
Album Plenum0	Phichit	0.86	0.63	0.37	0.22	2.10
Album Plenum1500	Phitsanulok	0.69	0.48	0.34	0.10	1.63
Album Plenum900	Prachuap Khiri Khan	1.01	0.98	0.15	0.17	2.31
Bua Khem Chin1200	Krabi	0.94	1.59	0.68	0.46	**3.68**
Bua Khem Chin1500	Satun	0.89	0.38	0.06	0.042	1.37
Bua Khem Chin300	Nakhon Phanom	0.89	1.13	0.24	0.37	2.64
Rosem Plenum1500	Nakhon Pathom	2.31	0.25	0.10	0.15	2.81
Rosem Plenum300	Ubon Ratchathani	1.81	1.31	0.84	0.25	**4.21**
Rosem Plenum600	Amnat Jaroen	2.35	1.05	1.08	0.61	**5.10**

CHAPTER V
Discussion

Sequence analysis of deduced amino acid translated from the BIA-related genes in this study suggested that CNMT and 6OMT can be briefly classified by the presence of the motifs. Motif A, B, and C are presented in 6OMT while CNMT possesses only motif A. According to the phylogenetic analysis, it clearly indicated that Cj6OMT belong to a different branch than CjCNMT (Figure 11). The lotus 6OMT share a close relationship to Cj6OMT and CjSMT while the lotus CNMT share a close relationship with CjCNMT. WRKY transcription factors which control overall BIA biosynthesis pathway were also characterized. Normally, WRKY contains approximately 60 amino acids long with four-stranded β -sheet WRKY DNA binding domain/s (DBD) and zinc-finger motifs. Generally, WRKY TFs are divided into three groups; group I (2 WRKY DBDs), II (single DBD with different C2H2 zinc finger), and III (single DBD with C2HC zinc finger) (Phukan et al., 2016) (Figure 16). All putative WRKY TFs in this study possess a single DBD together with a zinc finger; thus, they are considerably characterized in group III of WRKY family. Such a characteristic has been reported in other alkaloid-producing plant species as follows: AtWRKY70, *Arabidopsis thaliana* (Accession no.AF421157); GmWRKY58, *Glycine max* (Accession no.EU375357); NtWRKY3, *Nicotiana tabacum* (Accession no.AF193770) (Phukan et al., 2016).

Mechanical wounding was reported recently as the method to increase the alkaloid production in lotus (Deng et al., 2016). Herein, the mechanism of the BIA – related genes in the early stage of biosynthesis including *NCS, 6OMT, CN*MT and *WRKY TFs* underlying the production of BIA was determined. It was found that *CNMT* may be a key gene because its expression was responded the most in the defense against mechanical wound. Interestingly, the role of *CNMT* in early day after wounding and high accumulation of nuciferine whose chemical structure required *CNMT* function may suggest that methylation at *N*-position may be able to occur before *O*-position or it would be unnecessary to occur in sequential order. In other word, the biosynthesis of aporphine-type alkaloid may not follow the biosynthetic steps as known in morphinan-type alkaloid. It has been known that *O*-nornuciferine is lack of methyl group at O atom on the 6 position of isoquinoline nucleus implying that 6OMT was not required for its production. Up to date, the order of structural enzymes in the biosynthesis of BIA has been reported where 6OMT occupied on the BIA backbone before CNMT (Facchini, 2001a; Facchini, 2001b; Hashimoto and Yamada, 1994; Stadler et al., 1989). This information also provided the insight into the biosynthetic pathway of nuciferine and *N*-nornuciferine in lotus. There were evidences revealed its low expression level in BIA-producing species (Choi et al., 2002; Staniek et al., 2013). Therefore, It was more likely that *CNMT*, an enzyme responsible for adding methyl group on N position of (*S*)-coclaurine structure via methylation reaction, involved in the bottle-neck step of the BIA production.

Moreover, *NCS* might have both major and minor roles in the accumulation of target compounds because it is located in the first step of BIA pathway which provides simple BIA skeleton for various BIA structure via complex array (Staniek et al., 2013). It was clearly explained on day 2 that *NCS* diligently performed its role in the initial days after wounding and then the downstream enzymes subsequently took the role for the following modification step. However, the co-expression of *NCS* and *CNMT* in plant or bacterial system would clarify their collaboration. Unlike *NCS* and *CNMT*, the important role of *6OMT* was observed in the later day. Its expression level slightly increased during an observation period when compared with control leaf. These findings demonstrated that *CNMT* was significantly related to the high accumulation of target compound under the stress condition. However, when compared the accumulation content between *N*-nornuciferine and nuciferine during an observation days, we observed massively rapid increase of nuciferine over *N*-nornuciferine. The increase of nuciferine content correlated well with the expression level of *CNMT* on day 2 of the wounded leaf. This may underline the potent function of CNMT toward the production of nuciferine in the response to mechanical wounding. The late-significant increase of *6OMT* also triggered the accumulation of *N*-nornuciferine which. Though they played a role in different point of time, these results suggested that they could be served as a key gene for high demand on nuciferine and *N*-nornuciferine production. In addition, the similar expression patterns of all target genes in the control group and the wounded group on day 7 may suggest that

metabolic event in lotus tissue would be able to recover itself within one week after responding to mechanical wounding.

Mechanical wounding not only affected gene expressions and BIA accumulation in wounded tissues but also in neighboring tissues. *CNMT* greatly expressed in the non-wounded sites on day 3 where the highest BIA content was detected. These results supported that *CNMT* is a key gene for BIA production in both wounded and non-wounded sites. Beside the biosynthetic genes, TFs were known to play an important role in the regulation of BIA production. WRKY transcription factors (WRKY TFs) are responsible for various stress responses and physiological processes in plant. Molecular study exhibited the regulation of WRKY TFs on secondary metabolite production such as phenolic compounds and alkaloids (Phukan et al., 2016; Schluttenhofer and Yuan, 2015). Study on WRKY TF along with other biosynthetic genes would reveal the factor that leads to the regulation of the whole biosynthetic pathway. Putative *WRKY TFs* were found to be related to the biosynthesis of BIA. WRKY TF (*NNU_24385*) in this study showed the high sensitivity in response to the mechanical wound. As shown in the phylogenetic analysis result, *NNU_24385* shares the closest amino acid sequence with *CjWRKY1* which was reported to regulate the biosynthesis of BIA in *C. Japonica* (Suttipanta et al., 2011). It was documented that *WRKY1* from alkaloid producing species; *C. roseus, C. japonica*, and *A. thaliana* has potential function in the biosynthesis of BIAs (Phukan et al., 2016) and wounding could induce the *WRKY TF* expression which subsequently

regulated the BIA pathway in *P. Somniferum* (Mishra et al., 2013). In addition, *WRKY1* from *Arabidopsis* increased the level of cytochrome P450-dependent oxidase berbamunine synthase *(CYP80A1)* leading to the increase of alkaloid production in California poppy callus culture (Ikezawa et al., 2008). This suggested that *NNU_24385* may be responsible for wound-related BIA accumulation in lotus.

The biosynthetic genes and TFs are co-regulating the biosynthesis of BIA. In this study, *CNMT* showed the most response to mechanical wounding with the collaboration of WRKY TF *NNU_24385*. The dramatic increase of *WRKY TFs* was observed immediately after wounding in day 0 of non-wounded leaves. It clearly showed the sensitivity of early response in active tissues which required for late response in both wounded and non-wounded sites resulted in the increasing of nuciferine and *N*-nornuciferine. The result from relative gene expression would answer some parts of this mechanism. The study on the function of these genes should be further conducted for better understanding their roles in the alkaloid production.

The decreasing and increasing of alkaloid content on day 2 and 3 of non-wounded site were observed in the tissue where its cells were still biosynthetically active suggesting that translocation of metabolites may occur in the tissues where the production of compounds can either accumulate in its generative tissues or translocate to other organs (Savatin et al., 2014) because high quantity of secondary metabolite can also be toxic to plant tissues itself (Fürstenberg-Hägg et al., 2013;

Green and Ryan, 1972). It is possible that the active tissue would be able to produce or translocate secondary metabolite to another compartment or wounded site through plasma membrane via active transport system. This phenomenon has been reported in alkaloid-producing species such as *C. roseus, Lupinus polyphyllus, Fumaria capreola*, and *Senecio vulgaris* of which its indole, isoquiniline, quinolizidine, and pyrrolizidine alkaloids were uptaken into vacuoles, respectively (Furuya et al., 1972). Besides, isoquinoline alkaloids could be excreted into the extracellular medium as observed in *Thalictrum minus* cell culture (Brodelius, 1990). In addition, wound-related alkaloid accumulation can be triggered by signal transduction in intra- and inter- cellular around the wounded site where secondary metabolite deposit (Savatin et al., 2014) because these produced alkaloids could play a role as a physical barrier or antimicrobial substances. It has been observed in *Arabidopsis* leaves where wound induced damage-associate molecular pattern at the level of plasma membrane and subsequently triggered wound signaling through MAPK cascades and calcium channel (Rehrig et al., 2014). Then, jasmonic acid, WASPs, and ROS waves generated the alert message in undamaged tissues. This phenomenon was observed in our study on day 0 when the relative expression level of putative *WRKY TFs* in the non-wounded leaf was exaggeratedly increased higher than that of the wounded leaf (Deng et al., 2016).

BIA-related genes displayed different manners in normal condition and abiotic stress condition of lotus. To determine the expressions of BIA-related genes in

normal condition, tissues of various lotus varieties were used. It was found that *6OMT* had high percentage of relative expression in young tissue; while, *NCS* took a major role in mature tissues. The relative expression of *6OMT* correlated well with nuciferine and *N*-nornuciferine accumulations in young leaf as same as of *NCS* in mature leaf (Table 8). Each gene deems to take a different role in different developmental stages of lotus. Normally, BIA biosynthesis begins with the condensation of 2 precursors using *NCS*; *CNMT* and *6OMT* are then subsequently convert the product of *NCS* to form methylated BIA structure (Liscombe et al., 2009). The expression of *6OMT* in young tissue might indicate that *6OMT* is needed for the production of some aporphine-type alkaloids whose chemical structure has methylation at OH group of 6C position such as nuciferine and *N*-nornuciferine. Likewise, *NCS* which synthesizes a central-branch precursor and highly expresses in mature tissue, is required for the production of monobenzylisoquinoline-type or bisbenzylisoquinoline-type alkaloids such as liensinine, isoliensinine, neferine, anonaine, roemerine, and so on (Duan and Jiang, 2008; Kashiwada et al., 2005). These types of BIAs rarely accumulate in leaf and petiole but translocate from leaf tissue to reproductive tissues such as embryo (Deng et al., 2016). It may be common to find high accumulation of aporphine-type alkaloids in young tissue of lotus organ emerged in water because the related genes simultaneously express throughout their life time but they start to decrease during the senescence period (Mei et al., 2017). Moreover, the individual alkaloid acts in the defense against pathogen, and stress

condition in both abiotic and biotic condition (Fürstenberg-Hägg et al., 2013; Green and Ryan, 1972; Rehrig et al., 2014). The findings in this study showed a parallel result with the study on total BIA accumulation in various tissues of 15 Chinese lotus cultivars in which the accumulation of total alkaloid content was higher in young tissues than mature tissues (Vimolmangkang et al., 2016).

It is also worth noting that the behavior of BIA-related genes in mature stage of wild Thai lotus and wounded lotus were different. *CNMT* maintain its low transcript level in normal condition as shown in all subjected organs of wild Thai lotus. Interestingly, the *CNMT* exaggeratedly increased its transcript level under abiotic tress condition in the early day after mechanical wounding. This showed its sensitivity and important role in wound defense mechanism. Though *NCS* and *6OMT* showed their high level of relative expression in normal condition, but they played lesser role in this response when compared with the behavior of *CNMT* in wounded lotus leaf. The role of WRKY TF (*NNU_24385)* which controls the transcription of structure genes in this study also highlighted. *NNU_24385* is the most outstanding transcript among all selected *WRKY TFs* and it showed close relationship to *WRKY1* from *C. roseus* which has been reported its role in the production of catharanthine (Schluttenhofer and Yuan, 2015). From the phylogenetic tree analysis, it also located the deduced amino acid sequence of *NNU_24385* next to the branch of *WRKY1* from *C. japonica* which known to control the expression of berberine biosynthetic genes (Phukan et al., 2016). This indicated the possible role of *NNU_24385* as BIA regulator.

Similar to *CNMT*, its transcript level massively increased more than 10 times after wounding in both wounded and non-wounded sites higher than its expression in normal condition. It showed even more sensitivity compared with the transcription level of structural genes.

In addition, the wild and commercial lotuses which provide high BIA content in this study have had common morphology and it was found that they are in the same group which is a group of Rosem Plenum; pink flower-producing variety. The Rosem Plenum group was observed to be a good source for harvesting nuciferine and *N*-nornuciferine. These results suggested that this group could be a good candidate for development of BIA production in lotus. Altogether, these findings would be helpful for the selection of high yielding alkaloids in lotus and it should be noted that screening for alkaloid content in Thai lotus germplasm would provide better understanding toward the new breeding of lotus cultivars.

CHAPTER VI
Conclusion

Altogether, the expression profile of *NCS*, *CNMT*, *6OMT*, and *WRKY TFs* were investigated in mechanically wounded lotus leaf. It was found that the putative *CNMT* and *6OMT* contain the characteristics of methyltransferase enzyme. Similarly, all 10 putative *WRKY TFs* were closely related to the reported BIA-regulated TF of *C. roseus.* The accumulation of BIA was significantly increased in mechanically wounded leaf in accordance with the relative expression of *CNMT* and one WRKY transcription factor (*NNU_24385)*. The transcript level of *CNMT* directly related to the rapid accumulation of nuciferine alone on the early day after wounding. The study on corresponding gene and BIA content in wild Thai lotus also provided some insight into the key gene in BIA biosynthesis. Three groups of wild Thai lotuses (Album Plenum, Rosem Plenum, and Bua Khem Chin) and a commercial lotus were subjected to this study. We found that in normal condition, *CNMT* maintained its relatively low transcript level in both young and mature stage of leaf and petiole while the expression of *NCS* and *6OMT* were the highest in mature and young tissues, respectively. Moreover, the relative expression level of *NCS* and *6OMT* were correlated with the BIA content in their tissues. These results suggested that when the lotus tissue experiences the abiotic stress condition, *CNMT* and the *WRKY TF* may play an important role in BIA production. Furthermore, we found that Rosem Plenum, a pink-flower producing lotus, provide high level of our interesting

compound, especially the young leaf. Thus, this group could be a good candidate for development of BIA production in lotus. To conclude, this study provides the understanding of gene regulating the BIA biosynthesis under the stress which would lead to the improvement on BIA production in the commercial lotus.

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