Evolutionary origin of the NCSI gene subfamily encoding norcoclaurine synthase is associated with the biosynthesis of benzylisoquinoline alkaloids in plants

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Sacred lotus is rich in biologically active compounds, particularly benzylisoquinoline alkaloids (BIAs). Here, we report on isolation of genes encoding (S)-norcoclaurine synthase (NCS) in sacred lotus, which is a key entry-enzyme in BIA biosynthesis. Seven NCS genes, designated NnNCS1 through NnNCS7, were identified in the sacred lotus genome, and five are located next to each other within a 83 kb region on scaffold 8. The NCS genes are divided into two subfamilies, designated NCSI and NCSII. The NCSI genes are universal in plants, while the NCSI genes are only identified in a limited number of dicotyledonous taxa that produce BIAs. In sacred lotus, only NnNCS4 belongs to the NCSII subfamily, whilst the rest NCS genes within the NCSI subfamily. Overall, the NnNCS7 gene was predominantly expressed in all tested tissues, and its expression is significantly correlated with alkaloid content in leaf. In contrast, the NnNCS4 expression shows no significant correlation with alkaloid accumulation in leaf, and its lack of expression cannot inhibit alkaloid accumulation. Taken together, these results suggest that the NCSI subfamily is crucial for BIA biosynthesis, and its origin may represent an important evolutionary event that allows certain plant taxa to produce BIAs.

Sacred lotus (Nelumbo nucifera Gaertn.) is an ancient perennial aquatic plant widely distributed in Eastern Asia. All parts of the lotus plant, including flowers, seeds, rhizomes, and leaves are edible, and some are also used as traditional Chinese herbal medicine1. For example, lotus leaves are used to treat dysentery, diarrhoea, dizziness, and blood vomiting, whereas, lotus flowers are used to treat symptoms such as pain, inflammation, bleeding due to internal and external injury, and skin disorders. Numerous studies have showed that lotus extract and isolated compounds have a variety of biological activities, such as anti-HIV2, anti-obesity3, 4, antimicrobial5, 6, anti-diabetic7, anti-platelet aggregation8, anti-cancer9, 10, anti-acyethylcholinesterase11, 12, and a potential use in Huntington’s disease13.

Alkaloids are recognized as major bioactive constituents in sacred lotus1. The dominant type of alkaloids in sacred lotus is benzylisoquinoline alkaloids (BIAs), which are a structurally diverse group of compounds, including N-nornuciferine, O-nornuciferine, nuciferine, lotusine, nuferine, and roemerine, among others14. BIAs are present in all parts of sacred lotus, but their composition varies among tissue types15. In lotus cotyledons, neferine and lotusine are found, whereas in the leaves, nuciferine is dominant16.

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BIAs are restricted to certain plant families, including Papaveraceae, Ranunculaceae, Berberidaceae, Menispermaceae, and Nelumboaceae. To date, BIAs have been extensively investigated in pharmaceutical plants, such as opium poppy (*Papaver somniferum*), California poppy (*Eschscholzia californica*), meadow rue (*Thalictrum* species), and Japanese goldthread (*Coptis japonica*), and approximately 2,500 BIAs have been reported. BIAs include a range of chemicals used as drug treatments in humans, and the well-known BIA-derived drugs are strong analgesic morphine, antitussive codeine, antispasmodic papaverine, and antibiotic sanguinarine and berberine. Due to their huge diversity in plants, BIAs are divided into different classes, such as morphinan, protoberidine, and aporphine, according to the similarity of their carbon skeleton. In lotus, BIAs mostly are of aporphine type, but their biosynthesis remains largely unknown.

Although BIAs show a wide structural diversity, their biosynthetic pathway is all initiated at the first common committed step, where isoquinoline core structure is generated by norcoclaurine synthase (NCS). NCS catalyzes condensation of dopamine and tyrosine-derived 4-hydroxyphenylacetaldehyde (4-HPAA) to form trihydroxyisoquinoline (s)-norcoclaurine, which is the central precursor of BIAs. NCS was initially isolated and characterized in a meadow rue (*Thalictrum flavum* ssp. glaucum). Later, NCS activity in total soluble protein extracts was observed in various plant species including *N. nucifera*, showing variation between extracts from root, stem, and leaf. However, few studies have been reported on isolation and characterization of genes encoding NCS in plants.

The NCS gene is well known to have evolved from a PR10/Bet v 1 ancestor in certain plant taxa, and this evolutionary origin represents a crucial step leading to the biosynthesis of BIAs. In this study, we report for the first time the isolation and characterization of the NCS gene family in sacred lotus. The NCS genes were found to be frequently duplicated in the sacred lotus genome, playing important role in alkaloid accumulation. Phylogenetic analysis showed that the NCS genes can be divided into two subfamilies, NCSI and NCSII. The former was identified in certain plant taxa producing BIAs, while the latter is present in various plant species either or not producing BIAs. This suggests that the origin of the NCSI gene may represent an important evolutionary event that allows certain plant taxa to produce BIAs. Our results will aid our understanding of the mechanisms underlying the accumulation of alkaloids, particularly BIAs in plants.

### Materials and Methods

#### Plant materials

All sacred lotus accessions used in this study are maintained at Wuhan Botanical Garden of the Chinese Academy of Sciences (Wuhan, Hubei province, PRC). A total of 10 lotus accessions, Xiaojinluan (XJL), Simeihuang (SMH), WSL253, Lianxia (LX), Rongjiao (RJ), WSL40, Fenshiba (FSB), Xuehuou (XH), Yupeng (YP), and Shuimeiren (SMR), were selected for real time PCR analysis and quantification of alkaloid content. Leaf and petiole samples were collected at young and mature stages for all cultivars, whereas, petal samples were collected only at full bloom stage. All samples were immediately frozen in liquid nitrogen and stored in −75°C freezer until use.

#### Isolation of the NCS genes in sacred lotus

Seven pairs of primers (Table S1) were designed to amplify NCS genes using cDNA as a template. The amplified cDNA fragments were inserted into pEASY-T1 vector (TransGen Biotech, Beijing, China), and subsequently sequenced. Protein sequence alignment was performed using web-based MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/) and prediction of signal peptide using web-based SignalP4.1 (http://www.cbs.dtu.dk/services/), WoLF PSORT24, and Protcomp 9.0 (http://linux1.softberry.com/berry.phtml).

#### Phylogenetic analysis

Amino acid sequences of the NCS genes were used for phylogenetic analysis. Sequence alignment was performed using MUSCLE in MEGA6 program, and adjusted manually, as necessary. 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 and the branches with less than 50% bootstrap replicates were collapsed.

#### Gene expression profiling using quantitative real-time PCR (qRT-PCR)

Approximately 100 μg of each sample was finely ground in liquid nitrogen and then subjected to total RNA isolation using Universal Plant Total RNA Extraction Kit (BioTeke, Beijing, China) according to the manufacturer’s instructions. Removal of contaminated genomic DNA and cDNA synthesis were conducted in one step using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Real-time PCR assay was performed in Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems). The qRT-PCR assay was carried out in a total volume of 10 μL reaction mixture containing 5 μL of 2× SYBR Green Master Mix (Takara, Dalian, China), 0.2 μM of each primer, and 100 ng of template cDNA. The amplification program consisted of 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec, and 60°C for 30 sec. The accumulation of PCR products was continuously monitored. After the final qPCR cycle, melting curve analysis of the qPCR products was carried out by heating from 60°C to 90°C at a rate of 0.5°C/sec. An actin gene, *GADPH* (GenBank accession no. XM_010244040), was used as a constitutive control. The primer sequences are listed in Table S2. All analyses were performed in triplicates.

#### Measurement of alkaloid content

Alkaloid extraction was conducted using an ultrasonic protocol as previously described. Briefly, samples of different lotus tissues were ground into fine powder in liquid nitrogen with a mortar and pestle. Approximately 600 mg powder was macerated in 7 mL of extraction buffer (0.3 M HCl-methanol, 1:1 in volume), and sonicated for 30 min at room temperature. After centrifugation (11,000 g, 10 min, room temperature), the supernatant was transferred to a new 15 mL eppendorf tube, and the pellet was...
extracted once again with 5 mL of extraction buffer. The supernatants were combined, and the final volume was adjusted to 15 mL with the extraction buffer.

Alkaloid extracts were filtered through 0.22 μm membranes (Shanghai New Asia purification device factory, Shanghai, China) and then analyzed using high-performance liquid chromatography (HPLC) with the same facility and method as we previously reported. Total alkaloid concentration was determined with a standard curve (Fig. S1) designed with a commercial Nuciferine standard (Yuanye Biotechnology, Shanghai, China). For all tested tissues, three biological samples were measured in this study.

Results

Genes encoding NCS in the sacred lotus genome. We checked the annotation of the sacred lotus genome, and four putative NCS genes, designated NnNCS1 through NnNCS4, were identified, with accession numbers of NNU_21731-RA, NNU_21732-RA, NNU_14334-RA, and NNU_21730-RA, respectively, in the public domain (lotus-db, wbucas.cn). To validate their putative coding sequences, primer pairs for each NCS gene were designed to amplify cDNAs prepared from young leaf of cv. WSL253 and the PCR products were subsequently cloned and sequenced. As a result, full coding sequences of three NCS genes, NnNCS1, NnNCS3, and NnNCS4, were successfully isolated and deposited in GenBank with accession numbers KT963033, KT963034, and KT963035, respectively. Meanwhile, several attempts were made to isolate cDNA fragments of NnNCS2 from three different tissues (leaf, petiole, and petal) of four cultivars, Lianxia, WSL253, Fenshiba, and Shuimeiren. However, no PCR products were amplified. We checked the genomic DNA sequence of the putative NnNCS2 gene, and found that it showed a high level (95%) of similarity with NnNCS1. However, NnNCS2 had a single nucleotide deletion adjacent to the GC dinucleotide at the 5′ border of the intron (Fig. S2). This single nucleotide deletion caused frameshift, resulting in a truncated and nonfunctional protein due to a premature stop codon. Thus, NnNCS2 is deemed to be a pseudogene.

To determine the copy gene number of NCS in lotus, the coding sequences of NnNCS1 were compared against the draft genome of sacred lotus ‘China Antique’. As a result, three additional NCS paralogs, termed NnNCS5, NnNCS6, and NnNCS7, were identified. The full coding sequences of NnNCS5 were confirmed by whole cDNA amplification and sequencing. NnNCS7 was initially identified to be located on scaffold_3159, but this scaffold is very short and only covers the first exon. The second exon was subsequently recovered by PCR-based genome walking. The cDNA sequences of NnNCS5 and NnNCS7 were deposited in GenBank with accession numbers KU234431 and KU234432, respectively. However, the putative NnNCS6 gene contained only partial coding sequences (Fig. S3), and its transcripts were not detected in three different tissues (leaf, petiole, and petal) of four cultivars, Lianxia, WSL253, Fenshiba, and Shuimeiren. Thus, NnNCS6 is deemed to be a pseudogene. In addition, NnNCS3 was located on scaffold 17, while NnNCS1, NnNCS2, NnNCS4, NnNCS5, and NnNCS6 were located next to each other within an 83 kb region on scaffold 8 (Fig. 1A).

All the NnNCS genes were composed of two exons and one intron. The coding-sequence lengths of NnNCS1, NnNCS3, NnNCS4, NnNCS5, and NnNCS7 were 489, 486, 468, 513, and 492 bp, respectively. NnNCS1 and NnNCS7 shared the highest level (83.8%) of identity in coding DNA sequences, and they showed approximately 65% identity in coding DNA sequences with NnNCS3 and NnNCS5. NnNCS4 had approximately 55% identity in coding DNA sequences with other NCS genes. The deduced amino acid sequences of NnNCS1, NnNCS3, NnNCS4, NnNCS5, and NnNCS7 genes were 162, 161, 155, 170, and 164 in length, respectively. NnNCS1, NnNCS3, NnNCS5, and NnNCS7 genes shared over 50% identity in amino acid sequences with each other, while they showed approximately 40% identity in amino acid sequences with NnNCS4. In addition, all the deduced amino acid sequences of the NnNCS genes contained a glycine-rich loop (Fig. 1B), the ligand binding domain of Bet v1 protein family. Subcellular localization of the NnNCS amino acid sequences was predicted using several programs, including SignalP4.1, WoLF PSORT, and Protcomp 9.0. However, the result indicated that none of the lotus NnNCS proteins contained any signal peptides. This suggests that the lotus NnNCS proteins are probably localized in cytoplasm.

Phylogenetic analysis of NCS genes and their homologs. The NCS enzyme belongs to Bet v 1 protein family and its activity was biochemically characterized in a limited number of species, including opium poppy (Papaver somniferum)33, yellow meadow-rue (Thalictrum flavum)31,32, Japanese goldthread (Coptis japonica)34, and Sinopodophyllum hexandrum35. Besides NCS, Bet v 1 family also includes plant intracellular pathogenesis-related class 10 (PR-10) proteins, cytokinin binding proteins (CSBPs), major latex proteins (MLPs), and ripening-related proteins. However, the PR-10/Bet v1 allergen proteins do not possess NCS activity although they share similarity to NCS proteins34. Thus, phylogenetic analysis was conducted to investigate the relationship between the lotus NnNCS genes and members of the Bet v1 family.

All the NCS genes were tested divided into two subfamilies, designated NCSI and NCSII clades, but separated from the PR-10/Bet v1 allergen family (Fig. 2). The NCSI subfamily consisted of four NnNCS genes, NnNCS1, NnNCS3, NnNCS5, and NnNCS7, and functionally characterized NCS genes from Ranunculus such as California poppy, opium poppy, Japanese goldthread, and yellow meadow-rue. The NCSII subfamily was composed of the lotus NnNCS4 gene and NCS genes from both eudicots, such as tea, cotton, and cacao, and monocots, such as rice, wheat, and maize.

Gene expression profiles of the NnNCS genes in sacred lotus. Spatial and temporal expression patterns of the five lotus NCS genes, NnNCS1, NnNCS3, NnNCS4, NnNCS5, and NnNCS7, were investigated in 10 cultivars (Fig. 3). Overall, NnNCS7 was the predominant NnNCS transcript in leaf, petiole, and petal of most tested cultivars (Fig. 4). In contrast, the transcript levels of NnNCS5 were extremely low or undetectable in leaf, petiole, and petal of most tested cultivars, but NnNCS5 was relatively highly expressed in leaf and petiole of cv. Xuehouou. Similarly, NnNCS4 showed extremely low or undetectable expression levels in leaf, petiole, and petal of most tested cultivars, but predominantly expressed in young and mature leaves of two cultivars, Rongjiao and...
WSL40. The transcripts of the remaining two NnNCS genes, NnNCS1 and NnNCS3, were detected in all tested tissues. Expression of NnNCS3 was high in petioles of three cultivars, Rongjiao, Fenshiba, and Yupeng, and in petals of most tested cultivars, such as Simeihuang, WSL253, Rongjiao, WSL40, Fenshiba, Yupeng and Shuimeiren. However, the expression levels of NnNCS1 were very low compared with NnNCS7 and/or NnNCS3 transcripts. In addition, the tested cultivars were composed of white-flower and pink-flower genotypes. However, no relationship was observed between the expression pattern of NnNCS genes and the flower color.

Total alkaloid content in various tissues of sacred lotus. Total alkaloid content was measured for the same accessions selected for qRT-PCR analysis (Table 1). On average, the alkaloid contents in young leaf, mature leaf, young petiole, mature petiole, and petal were 2.28, 2.26, 0.34, 0.19, and 0.35 mg/g FW, respectively. There was a wide range of alkaloid content in leaf across the tested cultivars, while a relatively narrow range of alkaloid content was observed for either petiole or petal across the tested cultivars. Overall, alkaloids were most abundant in the leaf, with a 6 to 12-fold higher amount as compared to both petiole and petal. The alkaloid content in the leaf ranged from 0.66 to 4.32 mg/g FW. Three cultivars, Lianxia, Rongjiao, and Simeihuang, had higher levels of alkaloids in young leaf than in mature leaf, whilst three cultivars, Xiaojinluan, Yupeng, and Fenshiba, showed higher levels of alkaloids in mature leaf than in young leaf. The remaining cultivars, WSL253, Xuehuou, Shuimeiren, and WSL40, showed no great difference in total alkaloid content between young and mature leaves.

Alkaloid content in petiole ranged from 0.08 to 0.50 mg/g FW. Five cultivars, Rongjiao, Xiaojinluan, Simeihuang, Yupeng, and Fenshiba, showed 2- to 3-fold decrease in total alkaloid content compared with young petiole, while a slight decrease was observed for the rest five cultivars. On average, mature petiole showed an approximately 2-fold decrease in total alkaloid content compared with young petiole. In petal, the highest level of alkaloid accumulation (0.88 mg/g FW) was observed for cv. Yupeng bearing red-colored flowers, whilst the lowest level of alkaloid accumulation (0.07 mg/g FW) was observed for white flower cultivar Xiaojinluan and red flower cultivar Fenshiba. No relationship was observed between alkaloid accumulation and petal color.

Relationship between the NnNCS gene expression and alkaloid accumulation. To investigate the potential role of NCS genes in alkaloid accumulation, we analyzed the relationship between the NCS gene expression and alkaloid accumulation. NnNCS4 showed almost undetectable expression levels in leaf, petiole, and petal of three cultivars, Xiaojinluan, Simeihuang, and Xuehuou. Similarly, NnNCS5 showed almost undetectable levels of expression in leaf, petiole, and petal of four cultivars, Shuimeiren, WSL40, Yupeng, and Fenshiba.
As mentioned above, all tested cultivars accumulated alkaloids in different tissues, with relative abundance of alkaloids in young and/or mature leaves. These results indicated that both \textit{NnNCS4} and \textit{NnNCS5} play little role in alkaloid accumulation in sacred lotus. The other three \textit{NCSI} genes, \textit{NnNCS1}, \textit{NnNCS3}, and \textit{NnNCS7}, were expressed in various tissues of all tested cultivars. Since bisbenzylisoquinoline-type alkaloids are synthesized mainly in laminae, we further conducted correlation analysis to investigate the degree of linear relationship between the \textit{NCS} gene expression level and alkaloid content in leaf. The expression of \textit{NnNCS7} (\(r = -0.404, P < 0.05\)) was significantly correlated with alkaloid contents in leaf, whereas, no significant correlation was identified for either \textit{NnNCS1} (\(r = 0.140, P > 0.05\)) and \textit{NnNCS3} (\(r = 0.110, P > 0.05\)).

Taken together, the results above suggested that the \textit{NCSI} genes are involved in the biosynthesis of alkaloids in sacred lotus.

Discussions

Secondary metabolites play a crucial role in the adaptation of plants to their environment and represent an important source of pharmaceuticals. Plant secondary metabolites are highly diversified, which is obviously related to recruitment of genes for secondary metabolism. Gene duplication is assumed to be a major driving force for evolution of genes in secondary metabolism, and duplicated genes are often in tandem repeats, forming clusters within the plant genome\(^{35,36}\). For example, tandem duplication has been frequently observed for genes encoding enzymes involved in flavonoid biosynthesis, such as chalcone isomerase in soybean\(^{37}\) and \textit{Lotus japonicus}\(^{38}\) and flavonoid 3-\textit{O}-glycosyltransferase in peach\(^{39}\) and grapevine\(^{40}\). Two clusters of genes \textit{O}-methyltransferases involved in lignin biosynthesis have been identified in the apple genome\(^{41}\). A cluster of three genes encoding terpene synthases involved in volatile biosynthesis have been identified in \textit{Arabidopsis}\(^{42}\) and \textit{tomato}\(^{43}\). More recently, tandem duplication of genes encoding known enzymes of the caffeine biosynthetic pathway has been proven to be crucial for the convergent evolution of caffeine biosynthesis in coffee\(^{44}\).

In this study, a cluster of five \textit{NnNCS} genes involved in alkaloid biosynthesis was identified on linkage group 8 of sacred lotus. Among these clustered genes, \textit{NnNCS1} showed high levels of nucleotide sequence identity with \textit{NnNCS2} and \textit{NnNCS6}. This suggests that \textit{NnNCS1}, \textit{NnNCS2}, and \textit{NnNCS6} share a common progenitor, but \textit{NnNCS2} and \textit{NnNCS6} become pseudogenes due to the accumulation of deletions and/or point mutations.
Besides the NnNCS cluster, two additional NnNCS genes, NnNCS3 and NnNCS7, were also identified in the sacred lotus genome, but they have not been physically mapped onto any linkage groups. NnNCS3 and NnNCS7 are phylogenetically closely related to NnNCS1. The sacred lotus genome lacks the paleo-triplication observed in other eudicots. Thus, it is reasonable to speculate that the NnNCS1, NnNCS3, and NnNCS7 genes could have arisen by tandem duplication from a common progenitor. In addition, we checked the assembled sequence of the NnNCS cluster, and several unsequenced gaps remain in the gene cluster. Further studies are needed to clarify whether NnNCS3 and NnNCS7 are also located within the NnNCS cluster on linkage group 8.

NCS is an important enzyme catalyzing the first committed step in benzylisoquinoline alkaloid biosynthesis in plants. In this study, we retrieved the NCS gene sequences from the genome sequences of eleven plant species, including dicots, such as sweet orange, strawberry, peach, Medicago truncatula, cucumber, and cacao, and monocots, such as maize, rice, Brachypodium distachyon, sorghum, and millet. These NCS genes along with previously characterized NCS genes were subjected to phylogenetic analysis, and the result indicates that all the NCS genes are divided into two subfamilies, NCSI and NCSI. Overall, the NCSI genes are present in all tested species, including both monocots and dicots, while the NCSI genes are identified in a limited number of dicotyledonous taxa that produce benzylisoquinoline alkaloids. A possible explanation for this finding is that the NCSI genes likely evolved through duplication of the NCSI genes in certain plant taxa. This explanation appears to be true in sacred lotus, in which the NCSI gene NnNCS4 is clustered with the NCSI genes, such as NnNCS1 and NnNCS5.

Figure 3. NCS gene expression profiles in flower-producing cultivars of lotus. YL, young leaf; ML, mature leaf; YP, young petiole; MP, mature petiole; and P, petal.
and it has a closer relationship with the NCSII genes in monocots than those in dicots. Therefore, the NnNCSI genes in sacred lotus are likely derived from duplication of the NnNCSII ancestor.

An ancient recruitment of the NCS gene from a PR10/Bet v 1 ancestor is proposed to be the primary evolutionary event that allowed certain plant taxa to produce BIAs. As mentioned above, the NCSI gene probably evolved from the NCSII ancestor. Thus, it seems that the NCSII gene was initially recruited from a PR10/Bet v 1 ancestor, and its subsequent duplication resulted in the generation of NCSI genes. The NCSII gene is not only present in BIA-producing plant species, but also in plant species devoid of BIAs, such as Poaceae and Rosaceae. In contrast, the NCSI gene is exclusively present in certain plant taxa that produce BIAs. These results suggest that evolutionary origin of NCSI genes from the NCSII ancestor could be associated with BIA biosynthesis in plants. In addition, previous study raised a question whether Arabidopsis contain the NCS gene. We compared the amino acid sequences of the sacred lotus NnNCS genes against the Arabidopsis genome sequences using the BLASTP program, and only two Bet v 1 homologs were identified. This suggests that the Arabidopsis genome lacks any NCS genes, which is consistent with its deficiency in BIAs. Given gene loss has been frequently observed in Arabidopsis, the NCSI gene is thus deemed to be lost during its evolution.

Figure 4. The proportion of different NnNCS transcripts in various tissues of fifteen lotus cultivars.

Table 1. Total alkaloid contents in lotus tissues (mg/g FW). Top three highest amounts in each tissue were highlighted in bold. N/A, not applicable.
NCS plays an important role in the regulation of BIA biosynthesis due to its entry-point location in the pathway. Our study indicates that the leaves of sacred lotus contain abundant BIs, which is consistent with the previous report. The expression level of the NCSI gene NnNCS4 ($r = 0.278$, $P > 0.05$) shows no significant correlation with alkaloid content in leaves, and lack of the NCSI expression does not inhibit alkaloid accumulation. In contrast, NnNCS7 are highly expressed in leaves and shows a significant correlation with alkaloid content. It is worth noting that the correlation is negative, which suggests that high or low levels of alkaloids may inhibit or induce, respectively, the expression of NnNCS7 via feedback mechanism. Moreover, it is also worth noting that BIs occur in Rutaceae. As above mentioned, sweet orange (Citrus sinensis) contains two copies of NCSI genes, but lacks any NCSI genes. These results further demonstrate that the NCSI genes, rather than the NCSI genes, are crucial for BIA biosynthesis in plants. To date, all the functional NCS genes previously characterized belong to the NCSI subfamily, while no members of the NCSI subfamily have been functionally characterized. It is worthy of further study to ascertain that the NCSI gene has no BSI activity. In addition, previous study suggests that the NCS proteins appear to assemble as dimers for their catalytic activities. Besides NnNCS7, other NCSI genes such as NnNCSI and NnNCS3 are also expressed in leaves of sacred lotus. More studies are still needed to clarify whether NnNCS7 can act as a heterodimer with other NCSI genes to induce the biosynthesis of BIs.

In general, our study shows that the sacred lotus leaves have the most abundant NCS transcripts, followed by petiole and petal. This is consistent with the finding that bisbenzylisoquinoline-type alkaloids are synthesized mainly in lamina. Unlike sacred lotus, Thalictrum flavum ssp. glaucum and Papaver somniferum have low level of the NCS transcripts in leaf, but the NCS transcripts are abundant in rhizome/petiole and root/stem, respectively. Overall, these studies show that the NCSI gene expression is correlated with alkaloid accumulation in different tissues. The sacred lotus petiole predominantly accumulates the transcripts of the NCSI gene, NnNCS7, and its transcript level is significantly correlated with alkaloid accumulation. BIs accumulate in the cytoplasm of specialized cells, also known as laticifers. The sacred lotus leaf and petiole contain laticifers, in which latex sap is produced. These results suggest that petiole is likely able to produce BIs. However, a previous study showed that NCSI activity was only observed in leaf, but not in petiole and root of lotus. Here, NnNCSI is also highly expressed in petiole of sacred lotus, however, with no significant correlation ($r = -0.332$, $P > 0.05$) with alkaloid content in petiole. Thus, further studies are still needed to address whether NnNCSI plays similar roles in leaf and petiole.

Currently, lotus leaf has attracted more and more interest because of its use in tea/beverage production. Alkaloids are one of the most active ingredients in lotus leaf. Thus, understanding the mechanism underlying natural variation in alkaloid content will be useful for development of new rhizome-producing lotus cultivars with high level of alkaloid accumulation.

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Author Contributions

Y. H. and S. V. conceived and designed the experiments. S. V. and X. D. performed the experiments. Y. H. and S. V. wrote the paper. A. O., C. O. and T. M. revised the manuscript.

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