

Screening and Identification of NsylCBL Family Members Interacting with Protein Kinase NsylCIPK24a in *Nicotiana glauca*

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Abstract The CIPK (CBL-interacting protein kinase) kinase family in plants is a type of serine/threonine protein kinase family. The members of this family interact with the upstream CBL protein (Calcineurin B-like protein) to form the CBL-CIPK signal system, involved in regulating plant growth and development as well as stress response processes. Early research found that AtCIPK24 interacts with AtCBL4 and AtCBL10, respectively, to activate downstream target proteins to respond to high salt stress. Earlier in this study, *NsylCIPK24a* homologous to *AtCIPK24* from *Arabidopsis thaliana* was obtained from *Nicotiana glauca*, but CBL family members interacting with NsylCIPK24a and its specific function are unclear. Therefore, a genome-wide prediction of CBL family members of *N. glauca* was carried out; Predicted *NsylCBL* genes were cloned by RT-PCR, and the analyses of gene structure, protein conserved domain and expression pattern were then conducted. The NsylCBL members interacting with NsylCIPK24a were screened by the yeast two-hybrid system. Results showed that there are 12 potential *NsylCBL* genes in *N. glauca*, and all of them were cloned successfully. Four members including NsylCBL4, NsylCBL5, NsylCBL9 and NsylCBL10 could interact with NsylCIPK24a in yeast. There might be some similar CBL-CIPK pathways in tobacco plants, compared with *Arabidopsis*. The study provides experimental data for the functional identification of *NsylCIPK24a*, and increases understanding of CBL-CIPK network in *N. glauca*.

Keywords *Nicotiana glauca*; Calcium signal; CBL; CIPK24; Yeast two-hybrid

Plants have developed a complex signal network system during the long-term evolution. Ca²⁺, as an important signal molecule, plays an important role in the process of signal transduction. When plants are stimulated by different external environments, the Ca²⁺ concentration in the cells will have specific spatiotemporal changes, forming Ca²⁺ pulses (Chandra et al., 1997), which can be sensed by intracellular calcium receptors (Dong et al., 2015). Calcineurin B-like protein (CBL) is a kind of calcium receptor protein unique to plants which can bind to Ca²⁺ and interact with CBL-interacting protein kinase (CIPK), thus activating downstream target proteins in response to stimuli perceived by the plant (Yu et al., 2014). The CBL-CIPK signaling system plays an important role in plant growth and development and in responses to biotic/abiotic stresses (Mao et al., 2016; Sanyal et al., 2016).

CIPK is a family of serine/threonine protein kinases, also known as SnRK3 (sucrose non-fermenting1-related kinases group 3) (Coello et al., 2011). Generally, CIPK proteins are structurally conserved, possessing an N-terminal kinase catalytic domain and a C-terminal regulatory domain harboring a NAF/FISL motif and a phosphatase interaction motif. The NAF domain has highly conserved NAF motifs (N, Asn; A, Ala; F, Phe) and is an important site for binding to CBL protein. After CBL binds with intracellular Ca²⁺ through the EF elongation factor hand, the physical structure of CBL changes (Sánchez-Barrena et al., 2013). The originally self-inhibited CIPK protein is therefore activated to exert kinase activity, and phosphorylates downstream proteins to triggers plant responses to external signals (Guo et al., 2001).

CIPK24 is one of the important CIPK members which has been reported to be involved in the responses to salt stress through two pathways. The most classic pathway AtCBL4-AtCIPK24-SOS1 was identified in the model

plant *Arabidopsis thaliana*, also known as the SOS pathway. AtCBL4-AtCIPK24-SOS1 plays a key role in maintaining sodium ion homeostasis and enhancing salt tolerance. AtCIPK24 (SOS2) interacts with AtCBL4 (SOS3) to form a protein complex, phosphorylates and activates the Na⁺/H⁺ transporter SOS1 located on root epidermal cells, and promotes Na⁺ efflux from the root (Qiu et al., 2002; Nunez-Ramirez et al., 2012). The SOS pathway has also been found in other species such as rice (*Oryza sativa*) (Martinez-Atienza et al., 2007), poplar (*Populus trichocarpa*) (Tang et al., 2010), *Populus euphratica* (Lv et al., 2014), *Brassica napus* (Chakraborty et al., 2012) and apple (*Malus domestica*) (Hu et al., 2012). The other pathway is the AtAtCBL10-AtCIPK24-AtNHX pathway (Quan et al., 2007). It was found that AtCBL10-AtCIPK24 complex phosphorylated the Arabidopsis NHX Na⁺/H⁺ antiporter (AtNHX) located on the vacuole membrane, so that excess Na⁺ in the cytoplasm was isolated into the vacuole, thereby reducing Na⁺ concentration in cytoplasm and toxic effect (Quan et al., 2007). The CBL10-CIPK24 pathway has been identified in poplar and *Populus euphratica* (Li et al., 2012; Tang et al., 2014). It was also reported that AtCIPK24 can activate the Ca²⁺/H⁺ antiporters (CAX1) located on the vacuole membrane, and this activation does not depend on AtCBL4, indicating that other AtCBL protein members may cooperate with AtCIPK24 to jointly regulate the Na⁺/Ca²⁺ balance in plants (Cheng et al., 2004).

Tobacco is an economic crop with strong resistance. Tobacco is often used as a model plant to analyze the functions of salt tolerance genes of other species, and the research and utilization of tobacco's own salt tolerance genes and the molecular mechanism of salt stress response are very limited (Jin et al., 2018). Although the functions of CIPK24 have been resolved in many plant species, the specific biological functions of CIPK24 in tobacco are lacking. In the early stage of our group, the homologous gene NsylCIPK24a of AtCIPK24 was cloned from *N. sylvestris* (Xu et al., 2018). On this basis, this study conducted a genome-wide prediction and related analysis of CBL families in *N. sylvestris*; cloned CBL genes in *N. sylvestris*, screened and obtained CBL protein interacting with NsylCIPK24a. This study provides experimental data for further analysis of the function of NsylCIPK24a and CBL-CIPK signaling pathway in tobacco.

1 Results and Analysis

1.1 Genome analysis of NsylCBL proteins

1.1.1 Predictions of NsylCBL gene families

Through homology comparison and bioinformatics prediction, 12 CBL members were predicted in *Nicotiana sylvestris* (Table 1). Predicted CBLs are named according to their evolutionary relationship with the model plant Arabidopsis CBLs in phylogenetic trees (Table 1). The open reading frame length of this family is 636~891 nt, and the encoded protein is composed of 211~296 amino acids. Analysis of protein physical properties of the products encoded by NsylCBL gene families showed that the molecular weight of CBL ranged from 221 40 to 335 60 Da; the theoretical pI was less than 7, ranging from 4.58 to 5.31, which was an acidic protein; the hydrophilicity of the protein was -0.157~-0.373, indicating that the proteins are all hydrophobic proteins.

Table 1 Information of predicted NsylCBL family members

Gene Name	GenBank No.	Coding sequence (bp)	Number of amino acids	Theoretical pI	Molecular weight (Da)	Grand average of hydropathicity (GRAVY)	Aliphatic index (%)
<i>NsylCBL1a</i>	KM396650.1	642	213	4.81	24311.67	-0.157	88.31
<i>NsylCBL1b</i>	KM396652.1	642	213	5.03	24461.01	-0.184	88.73
<i>NsylCBL2</i>	KM396653.1	675	224	4.76	25640.15	-0.228	91.83
<i>NsylCBL3</i>	KM396654.1	669	222	4.97	25347.87	-0.204	93.11
<i>NsylCBL4a</i>	KM658158.1	642	213	4.70	24464.87	-0.191	93.38
<i>NsylCBL4b</i>	XM_009800076.1	660	219	5.14	25293.11	-0.195	88.58
<i>NsylCBL5</i>	XM_009758979.1	642	193	4.66	22140.34	-0.113	102.12
<i>NsylCBL6</i>	XM_009774506.1	636	211	5.31	24819.47	-0.261	96.11
<i>NsylCBL7</i>	XM_009786808.1	891	296	4.95	33488.92	-0.373	86.59
<i>NsylCBL8</i>	XM_009781455.1	891	296	4.94	33560.26	-0.335	94.59
<i>NsylCBL9</i>	KM396651.1	642	213	4.58	24593.84	-0.238	84.65
<i>NsylCBL10</i>	KF667488.1	783	260	4.70	30004.21	-0.184	96.46

1.1.2 Evolution of the NsylCBL protein families

In order to gain a deeper understanding of the evolutionary relationship between the CBL protein family in *N. sylvestris* and similar proteins in other species, the CBL protein families of Arabidopsis, rice, poplar, and maize were selected to build a phylogenetic tree. Drawing on the results of others' evolutionary tree analysis, CBL family members can be divided into four groups, A, B, C, and D (Figure 1) (Jiang et al., 2020). *N. sylvestris* has 1 member NsylCBL10 in Group A, 4 members NsylCBL2, NsylCBL3, NsylCBL6 and NsylCBL7 in Group B, 3 members NsylCBL1a, NsylCBL1b and NsylCBL9 in Group C, and NsylCBL4a, NsylCBL4a, NsylCBL5 and NsylCBL8 4 members.

1.2 Cloning and gene structure analysis of NsylCBL families

The primers were designed according to the predicted sequence results, and the CDS sequences of 12 *NsylCBL* genes were amplified by RT-PCR using cDNA of *N. sylvestris* as a template. The genome prediction sequence of each NsylCBL was obtained by searching on GENE using NCBI genbank number. The genetic structures of the 12 CBL family members were analyzed, and it was found that except for NsylCBL6 CDS consisting of one exon, the other members all have multiple intron (Figure 2).

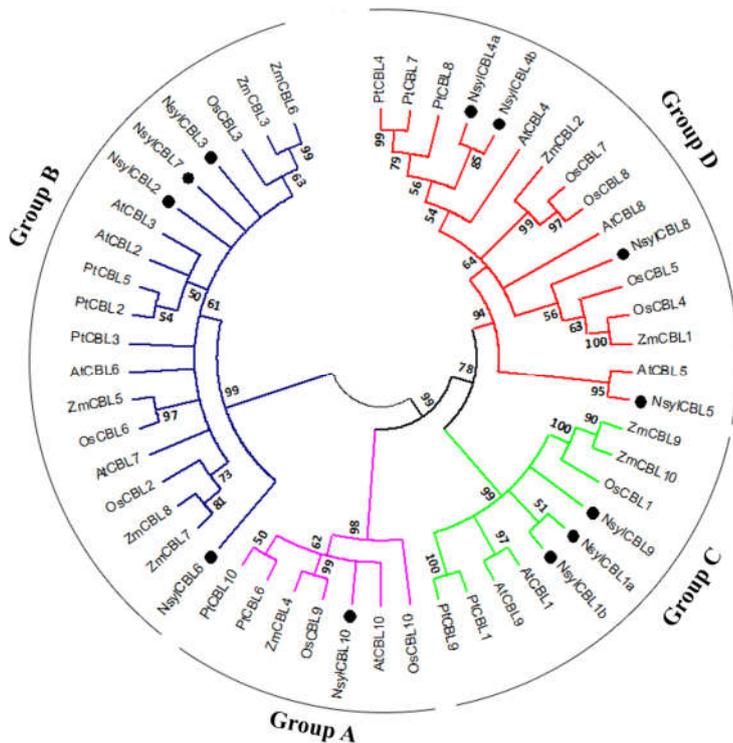


Figure 1 Neighbor-joining phylogenetic tree of NsylCBL family members

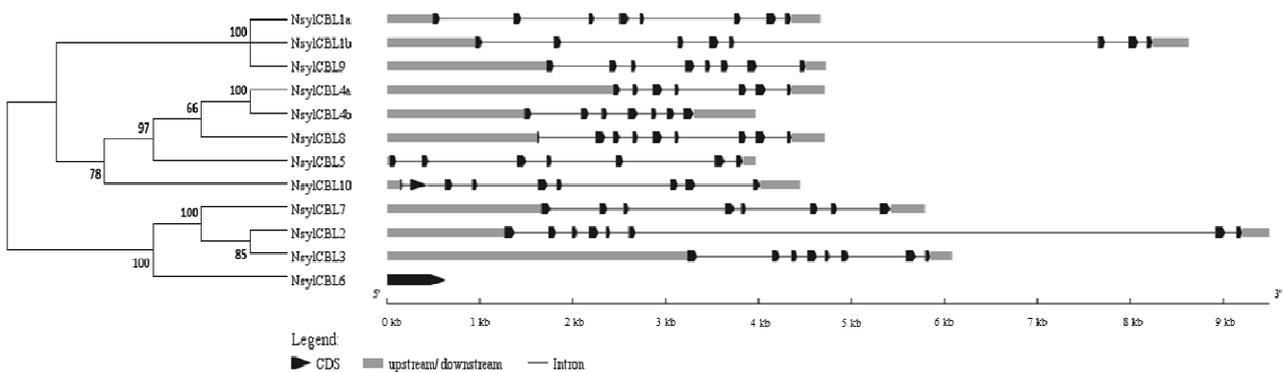


Figure 2 Phylogenetic analysis and gene structures of cloned *NsylCBL* genes

1.3 Protein structure analysis of NsylCBL family members

There are three types of relatively conserved structures in CBL proteins, namely the myristoylation site and palmitoylation site at the N-terminal, the EF hand structure at the middle, and the FPSF motif at the C-terminus. The Ca²⁺ binding function of the EF hand structure gives the CBL protein the ability to perceive changes in intracellular Ca²⁺ (Mao et al., 2016), and the myristoylation site and palmitoylation site at the N-terminus of the protein contribute to the subcellular localization of CBL protein (Dong et al., 2015). Serine residues in the FPSF motif can be phosphorylated by CIPK proteins to strengthen protein interactions, thereby enhancing the ability of the CBL-CIPK complex to activate downstream target proteins (Jiang et al., 2020). Therefore, the above structures of NsylCBL protein family members were predicted and analyzed. The results showed that NsylCBLs all contain four EF-hand motifs, and their conserved sequences are SXXYXDDGLIXKEE, DXKXXGXXXFXE, DLXXXGXIERXE, and DXXXXEXIXDKTF, similar to the results of other known CBL proteins. Most CBL members are conservative at the N-terminus and contain myristoylation and palmitoylation sites, and a few members have amino acid changes at the N-terminus site. The C-terminal FPSF domain sequences of NsylCBLs is relatively conservative (Figure 3).

1.4 Expression profiling of NsylCBL genes in different tissues and developmental stages

Analysis of gene expression patterns can provide basic data for studying the functional differences and similarities of different gene families. We conducted a preliminary analysis of the expression patterns of 12 *NsylCBL* genes in different tissues and different developmental stages (Figure 4). The results showed that *NsylCBL1a*, *NsylCBL1b*, *NsylCBL2*, *NsylCBL3* and *NsylCBL7* were relatively commonly expressed in different tissues and developmental stages of tobacco, and *NsylCBL4a*, *NsylCBL4b* and *NsylCBL9* were highly expressed in tobacco roots. The expression levels of *NsylCBL5*, *NsylCBL6* and *NsylCBL8* are low, and the expressions of *NsylCBL5* and *NsylCBL6* are extremely low.

1.5 Interaction patterns between NsylCIPK24a and NsylCBLs

The previous research of our group has shown that there are two CIPK24 members in *N. sylvestris*, namely NsylCIPK24a and NsylCIPK24b (Xu et al., 2018). Among them, the protein sequence similarity between NsylCIPK24a and AtCIPK24 and OsCIPK24 were 72.76% and 69.76%, respectively.

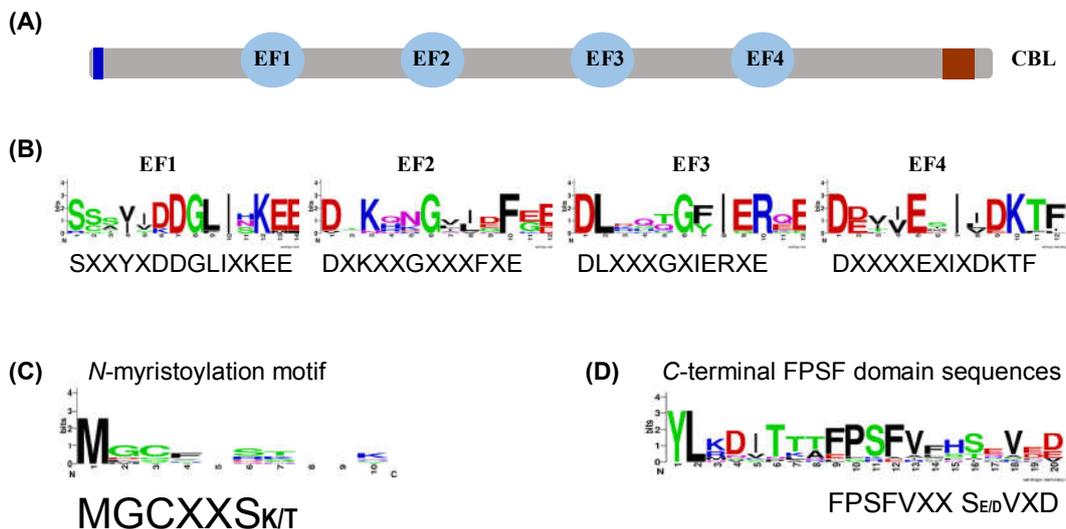


Figure 3 Analyses of motif structures and conserved domains of all CBLs identified in *N. sylvestris*

Note: A: Schematic diagram of CBLs in *N. sylvestris*; There are four EF hands displayed by blue circles; The blue and red box represents the typical N-myristoylation site and C-FPSF motif, respectively; B: Sequence features shown in the form of web logos representing the EF hands of all NsylCBL sequences; C: Detailed comparisons of N-myristoylation motif sequences of NsylCBLs; D: C-terminal FPSF domain sequences of NsylCBLs

To investigate the interaction partners of NsylCIPK24a protein, we performed a yeast two-hybrid system screening. It was found that the combination of pGBKT7-NsylCIPK24a/pGADT7-NsylCBL4a, pGBKT7-NsylCIPK24a/

pGADT7-NsylCBL5, pGBKT7-NsylCIPK24a/pGADT7-NsylCBL9 and pGBKT7-NsylCIPK24a/pGADT7-NsylCBL10 can grow normally on selective SD/-Leu-Trp-Ade-His medium, indicating that NsylCIPK24a interacts with NsylCBL4, NsylCBL5, NsylCBL9, and NsylCBL10 in yeast (Figure 5).

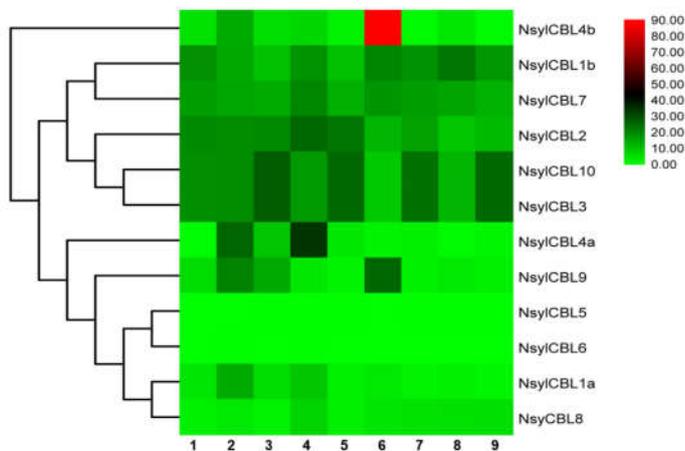


Figure 4 Expression profiles of *NsylCBL* genes in different tissues and developmental stages of tobacco plants

Noted: 1: Seeds of 3 days after sowing; 2: Roots of young seedlings; 3: Leaves of young seedling; 4: Roots of the plants at fast-growing stage; 5: Leaves of the plants at fast-growing stage; 6: Roots of the plants at mature stage; 7: Leaves of the plants at mature stage; 8: Apical buds before budding stage; 9: Detached leaves of 1 day after harvest

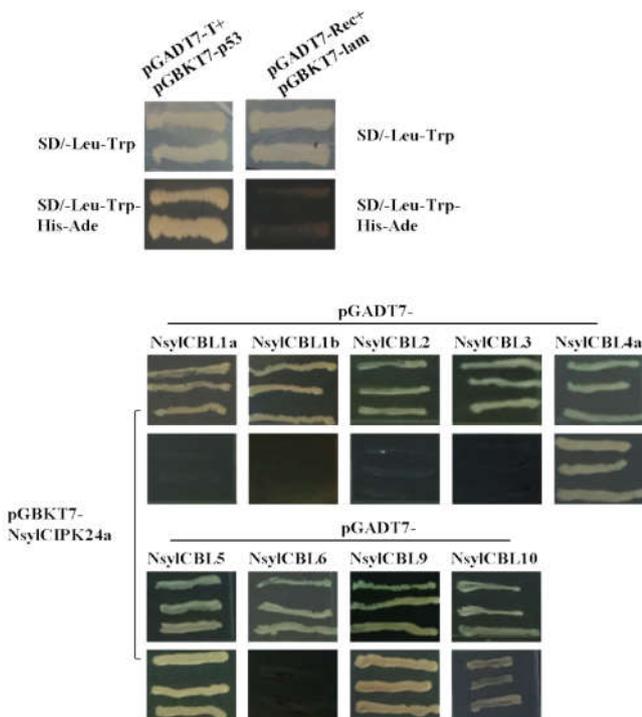


Figure 5 Interaction of NsylCIPK24a and NsylCBLs in yeast

Note: pGBKT7-p53/pGADT7-T combination was used as the positive control, while pGBKT7-lam/pGADT7-Rec as the negative control

2 Discussion

2.1 NsylCIPK24a may be involved in regulating plant responses to salt stress in different ways

Previous studies have shown that AtCIPK24 interacts with AtCBL1, AtCBL2, AtCBL4, AtCBL5, AtCBL9, and AtCBL10 (Hashimoto et al., 2012). Six AtCBL proteins have different subcellular localization patterns: AtCBL1/9 is mainly located on the cell membrane, AtCBL2 is mainly located on the vacuole membrane, AtCBL10 is mainly located on the vacuole membrane and the cell membrane, and AtCBL4/5 is widely distributed in the cell (Dong et

al., 2015). The CBL protein can anchor the CIPK to different positions in the cell, thereby exerting corresponding functions (Batistic et al., 2010). Corresponding to this result, AtCBL4-AtCIPK24 mainly enhances the salt tolerance of plants by regulating the efflux of root Na⁺, while AtCBL10-AtCIPK24 mainly reduces the toxicity of Na⁺ by regulating Na⁺ compartmentalization to the vacuole (Halfter et al., 1999; Kim et al., 2007). This study showed that NsYL CIPK24a also interacts with NsYL CBL4a and NsYL CBL10, so it is speculated that the two pathways NsYL CBL4a-NsYL CIPK24a and NsYL CBL10-NsYL CIPK24a may also participate in the response process of tobacco to salt stress. But further experimental verification is needed.

2.2 NsYL CIPK24a may be functionally redundant with NsYL CIPK3 in response to salt stress

The study found that exogenous expression of ZmCIPK16 and MdCIPK6L in Arabidopsis can functionally complement the salt-sensitive phenotype of *Arabidopsis sos2* mutants, but ZmCIPK16 and MdCIPK6L are not homologous genes of AtCIPK24. This implies that there may be other members of the CIPK family which have functional redundancy with CIPK24 (Zhao et al., 2009; Wang et al., 2012). Similar to this result, in addition to the interaction between NsYL CBL4a and NsYL CBL10 related to salt stress, this study also found that there is a strong interaction between NsYL CIPK24a and NsYL CBL9. NsYL CBL9 is mainly expressed in the tobacco roots. Earlier in this research group, the interaction between NsYL CBL9 and NsYL CIPK3 in yeast was reported, and NsYL CIPK3 was also induced by high salt and its expression was up-regulated (Dong et al., 2015). NsYL CIPK24a is likely to be functionally redundant with NsYL CIPK3, and interacts with NsYL CBL9 to participate in the tobacco response to salt stress.

2.3 Research trend of tobacco response to high salt stress

Tobacco is an economic crop with strong resistance. Most of the work on tobacco salt tolerance uses tobacco as a model crop to study the application of salt tolerance genes from other species in tobacco. The mining and utilization of tobacco's own salt-tolerance genes and the molecular mechanisms of tobacco response to salt stress are very limited (Jin et al., 2018). The salt tolerance of 34 tobacco varieties was evaluated during the germination period. By measuring the different parameters of different varieties during the germination period, the salt tolerance differences of the tested varieties were initially divided (Wang et al., 2020). But the study did not excavate functions at the genetic level. Therefore, the research on tobacco salt tolerance mechanism should be further increased to lay the foundation for effective service production and improvement of tobacco quality.

3 Materials and Methods

3.1 Screening of NsYL CBL gene family

N. sylvestris genome-wide data are downloaded from the NCBI (<https://www.ncbi.nlm.nih.gov/>) and China Tobacco Genome Database (<http://218.28.140.17/>); the transcriptome data of *N. sylvestris* is downloaded from the China Tobacco Genome Database (<http://218.28.140.17/>); the CIPK protein sequences of Arabidopsis, rice and poplar are obtained from the protein database Uniprot (<http://www.uniprot.org/>) and the Arabidopsis database Tair (<http://www.arabidopsis.org/>).

3.2 Bioinformation analysis of NsYL CBL

The online software ProtParam (<http://web.expasy.org/protparam/>) was used to analyze the physical properties of the obtained NsYL CBL protein; the software MEGA6 was used to build a phylogenetic tree; the online software GSDS2.0 (<http://gsds.cbi.pku.edu.cn>) was used to analyze gene structure; the online software WebLogo (<http://weblogo.berkeley.edu/logo.cgi>) and InterProScan (<http://www.ebi.ac.uk/interpro/search/sequence/>) was used to analyze conservative amino acids and protein conserved domains, respectively.

3.3 Materials

The *Escherichia coli* strain DH5 α , *Saccharomyces cerevisiae* strains AH109 and Y187, enzymes and MiniBEST Agarose Gel DNA Extraction Kit used in the experiment were all purchased from akara Biomedical Technology (Dalian) Co., Ltd.; yeast expression vectors pGADT7 (Amp resistance) and pGBKT7 (Kan resistance) were saved by our laboratory.

3.4 Cloning and plasmid construction

The phenol-based method was used to extract the total RNA of *N. sylvestris* (Shi et al., 2020), and the RNA was reserved after analyzing its integrity and purity. cDNA was synthesized according to the PrimeScript RT-PCR Kit operating instructions. By using Primer Premier 6.0 software, the corresponding specific primers were design based on the predicted *NsylCBLs* CDS (Table 2). Using the synthesized cDNA as a template, PCR amplification was performed to obtain *NsylCBL* gene. The amplified CDS was ligated into yeast vector pGADT7. The bait vector pGBKT7-*NsylCIPK24a* was constructed by our research group (Xu et al., 2018). The primers used in this experiment were synthesized by Ruibotech (Qingdao) Co., Ltd.

Table 2 Primers used in the experiments

Name	Sequence of primer (5'-3')	Note
NsylCBL1a-2F	<u>CCATGGG</u> GCTGTTTCCAGTCTACAGC	correspond to 1-23 nt of <i>NsylCBL1a</i> CDS sequence, and <i>NcoI</i> restriction site introduced
NsylCBL1a-1R	TTAGGTAGCCATCTCATCAACTTC	antisense to 619-642 nt of <i>NsylCBL1a</i> CDS sequence; and insert <i>NsylCBL1a</i> by <i>BamH I</i> restriction site of pMD19-T to construct the pGADT7 vector.
NsycBL1b-2F	<u>CCATGGG</u> GCTGCTTTCATTCTAAGG	correspond to 1-22 nt of <i>NsycBL1b</i> CDS sequence, and <i>NcoI</i> restriction site introduced
NsycBL1b-1R	TTATGTAGCAACTTCATCAACT	antisense to 621-642 nt of <i>NsycBL1b</i> CDS sequence; and insert <i>NsycBL1b</i> by <i>BamH I</i> restriction site of pMD19-T to construct the pGADT7 vector
NsylCBL2-2F	<u>CCATGGATG</u> TTGCAGTGCCTAGACGG	correspond to 1-20 nt of <i>NsylCBL2</i> CDS sequence, and <i>NcoI</i> restriction site introduced
NsylCBL2-1R	TCAGGTATCCTCAACTCTGGAATG	antisense to 652-675 nt of <i>NsylCBL2</i> CDS sequence; and insert <i>NsylCBL2</i> by <i>BamH I</i> restriction site of pMD19-T to construct the pGADT7 vector
NsylCBL3-2F	<u>CCATGGATG</u> TCGCATTGTTTAGAGGGGAT	correspond to 1-22 nt of <i>NsylCBL3</i> CDS sequence, and <i>NcoI</i> restriction site introduced
NsylCBL3-1R	<u>GGATCCT</u> CAGGTATCCGGGACTCTTG	antisense to 659-678 nt of <i>NsylCBL3</i> CDS sequence, and <i>BamH I</i> restriction site introduced
NsylCBL4a-1F	<u>CCATGGG</u> GCTGCTTTCACTCC	correspond to 1-18 nt of <i>NsylCBL4a</i> CDS sequence, and <i>NcoI</i> restriction site introduced
NsylCBL4a-1R	TTAGACTTCCAAATCTTCAACC	antisense to 621-642 nt of <i>NsylCBL4a</i> CDS sequence; and insert <i>NsylCBL4a</i> by <i>BamH I</i> restriction site of pMD19-T to construct the pGADT7 vector
NsylCBL5-1F	<u>CCATGGG</u> GCTGTGCTTTAAGAAAGCAAGA	correspond to 1-26 nt of <i>NsylCBL5</i> CDS sequence, and <i>NcoI</i> restriction site introduced
NsycBL5-1R	<u>GGATCCT</u> CAGAAATCCTTGTAATCTCATC	antisense to 618-642 nt of <i>NsycBL5</i> CDS sequence, and <i>BamH I</i> restriction site introduced
NsylCBL6-2F	<u>CCATGGAGT</u> GAATTGTTGCGATTCTTGTATATG	correspond to 1-28 nt of <i>NsylCBL6</i> CDS sequence, and <i>NcoI</i> restriction site introduced
NsylCBL6-2R	<u>GGATCCT</u> CACGTATCTTCAACTTGAGAG	antisense to 614-641 nt of <i>NsylCBL6</i> CDS sequence, and <i>BamH I</i> restriction site introduced
NsycBL9-2F	<u>CCATGGG</u> GCTGTTTTAGTTCTACAGC	correspond to 1-23 nt of <i>NsycBL9</i> CDS sequence, and <i>NcoI</i> restriction site introduced
NsycBL9-3R	<u>GGATCCG</u> GTACCTCAAGTAGCTACTTC	antisense to 627-641 nt of <i>NsycBL9</i> CDS sequence, and <i>BamH I</i> restriction site introduced
CBL10-1-2F	AAGCTT <u>CCATGG</u> ATTCAACCCGCG	correspond to 1-14 nt of <i>NsylCBL10</i> CDS sequence, and <i>NcoI</i> restriction site introduced
CBL10-1-3R	<u>GGTACCG</u> GATCCCAACAAATGGCTTTTC	antisense to 664-679 nt of <i>NsylCBL10</i> CDS sequence, and <i>BamH I</i> restriction site introduced

Note: The underlined sequences represent the restriction sites

3.5 Yeast two-hybrid assays

The pGADT7-NsycCBL and pGBKT7-NsycCIP24a were transformed into yeast strains AH109 and Y187 by PEG/LiAc method, respectively. The successfully transformed colony was selected, and the AH109 strains containing different pGADT7-NsycCBL plasmids and the Y187 strain containing pGBKT7-NsycCIP24a were co-cultivated in YPDA liquid medium for 24 h. pGBKT7-P53/pGADT7-T was used as a positive control, pGBKT7-lam/ pGADT7-Rec was used as a negative control. The co-cultivation conditions were 28°C and 50 r/min. The bacterial solution after co-cultivation was spread on SD/-Leu-Trp and SD/-Leu-Trp-Ade-His medium, and then placed in a constant temperature incubator at 30°C for 3 to 5 days. After the growth of colony, it will plate on SD/-Leu-Trp and SD/-Leu-Trp-Ade-His medium, and the photographs were recorded after culturing for 3 to 5 days.

Authors' contributions

An Lulu, Mao Jingjing and Shi Sujuan were the main executives of this research experiment; An Lulu completed the writing of the paper; Che Yuhao, Shi Sujuan, Dong Lianhong and Song Yufeng cloned some of the NsycCBLs; Xu Dizhi cloned the NsycCIPK24a; Xu Fangzheng participated in the data analysis; Liu Haobao participated in guiding the design of this experiment And the execution process; Wang Qian is the conceiver of this experiment, guiding the experiment and revising the paper. All authors read and approved the final manuscript.

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