

Identification of an Interacting Kinase between AmCERK1 and AmLYK3 Mediates CIP-Induced Immune Responses in Atractylodes

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1 **Identification of an Interacting Kinase between *AmCERK1* and *AmLYK3* Mediates**
2 **CIP-Induced Immune Responses in *Atractylodes***

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7
8 **Abstract**

9 The *Sclerotium rolfsii*-induced Southern blight poses a severe threat to the medicinal plant
10 *Atractylodes macrocephala* Koidz (*Atractylodes*), yet effective control strategies for this pathogen
11 remain limited. *Chrysanthemum indicum* polysaccharides (CIP) as an elicitor have been verified to
12 induce the defense response of *Atractylodes* against *Sclerotium rolfsii*. However, the fundamental
13 mechanisms underlying ligand perception remain largely elusive. In this study, a comparative
14 transcriptome analysis was conducted to identify two potential LysM-receptor kinases in
15 *Atractylodes*, namely *AmCERK1* and *AmLYK3*, as pattern recognition receptor (PRR) complexes
16 recognize CIP. Subsequently, the involvement of these genes in CIP-induced defense responses
17 was further confirmed. Notably, *AmCERK1* and *AmLYK3* are orthologs of *Arabidopsis CERK1*
18 and *Medicago truncatula LYK3* respectively, exhibiting up-regulation upon CIP treatment. The
19 BiFC assays demonstrate that the interaction between these two receptors necessitates the
20 presence of CIP. Transient overexpression experiments reveal that CIP treatment enhances the
21 expression of the receptor gene in the plants significantly. Additionally, the CIP treatment
22 effectively mitigated the inhibition of target genes during the silencing of *AmCERK1* and *AmLYK3*
23 in seedlings induced by pTRV-based VIGS application. The CIP is required for the interaction of
24 *AmCERK1* and *AmLYK3* and facilitating the establishment of PTI in *Atractylodes*. This is novel
25 receptor recognition complexes, and is crucial to probe into mechanisms of innate immune
26 recognition in plant.

27 **Keywords** CIP · *Atractylodes* · *AmCERK1* · *AmLYK3* · interacting kinase combination

Introduction

CIP, the polysaccharides from *Chrysanthemum indicum* L., have been identified as elicitors that enhance disease resistance in *Atractylodes* (Du NN et al., 2016). *Atractylodes* as the traditional Chinese medicine is well-known for its anti-tumor, antioxidant, and hepatoprotective properties (Xie F et al., 2012; Han B et al., 2016; Chau CF et al., 2006). However, it is susceptible to *Sclerotium rolfsii* (*S. rolfsii*), which not only causes Southern blight but also reduces products (Zhang XY et al., 2017). Our previous research has confirmed that CIP effectively reduces the disease index of soil-borne diseases in *Atractylodes* infected by *S. rolfsii* and could significantly increase the expression of defense genes, promote the accumulation of phytoalexin in the plants (Zhang PF et al., 2020; Zhou et al., 2021). Studies conducted by Xiong et al. (2021) and Zhou et al. (2021) also have attempted to elucidate the underlying structure responsible for CIP's inducing activity. CIP treatments is expected to be an effective control strategies for soil-borne diseases of *Atractylodes*. Collectively, these findings demonstrate that CIP induces PTI-like responses in the plants. However, it remains unclear which receptor perception induced by CIP triggers enhanced the plant immunity.

Well known that the lack of mobile defender cells and a circulating immune system is a characteristic feature of plants (Couto D et al., 2016). Instead, their defense against pathogens primarily relies on the recognition mediated by immune receptors, which detect non-self or modified-self molecules (Couto D et al., 2016; Cui HT et al., 2015). The frontline of plant immunity is composed of receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Kawai T et al., 2010; Tang DZ et al., 2017), which function as pattern recognition receptors (PRRs) to detect conserved pathogen/damage-associated molecular patterns (PAMPs/DAMPs). This detection leads to the initiation of PAMP-triggered immunity (PTI) (Tang DZ et al., 2016). Previous studies have demonstrated that a variety of endogenous and exogenous molecules derived from plants and pathogens (known as elicitors) are recognized by PRRs to initiate PTI. The chitin, a saccharide elicitor found in fungal cell walls, is primarily composed of a polymer of N-acetylglucosamine (NAG) (Tang J et al., 2015). It can be recognized by lysin-motif (LysM) receptors such as chitin elicitor receptor kinase 1 (*CERK1*), LysM-receptor kinase 4 (*LYK4*), and *LYK5* in *Arabidopsis* (Cao YR et al., 2014; Miya A et al., 2007). Additionally, it is recognized by *OsCERK1* and *LysM-RLP OsCEBiP* in rice (Shimizu T et al., 2010). Interestingly, upon binding to (NAG)₈, *AtCERK1* undergoes homodimerization, suggesting a ligand-induced activation model for *AtCERK1* (Shimizu T et al., 2012). However, subsequent studies have demonstrated that the activation of *AtCERK1* requires the presence of *AtLYK5* as a co-receptor involved in chitin perception (Cao YR et al., 2014). Similarly, chitin binding to *OsCEBiP* induces the interaction between *OsCEBiP* and *OsCERK1*, leading to the activation of *OsCERK1* kinase activity in rice (Shimizu T et al., 2010). Furthermore, *AtCERK1* interacts with LysM-RLPs such as *OsLYP4* and

OsLYP6 (Liu B et al., 2012) as well as *AtLYM1* and *AtLYM3* (Willmann R et al., 2011), which initiate peptidoglycan (PGN) perception and trigger PTI response. Collectively, PRRs are organized in a dynamic manner at the plasma membrane, involving interactions with various coreceptors and regulatory receptor kinases. The signaling components utilized to activate these PRRs differ, and the precise molecular mechanisms underlying their activation remain incompletely understood (Daniel et al., 2016). It is more of a mystery which member of PRRs senses CIP and recruits which member of *LysM-RLKs/RLPs* its family to assemble receptor complexes with signal transduction capabilities in *Atractylodes*. Therefore, the identification of molecules involved in the interaction between CIP and *LysM-RLKs/RLPs* has become crucial for analyzing *Atractylodes*' recognition of CIP and initiation of PTI resistance.

The lack of a genome in *Atractylodes* necessitated the utilization of transcriptome sequencing and differential gene screening in this study to identify PRRs responsible for recognizing CIP elicitors. Through a comparative transcriptome analysis combined with bioinformatic tools, we identified two lysin motif (LysM) receptor kinases (*AmCERK1* and *AmLYK3*). Subsequently, we further investigated functions of these genes, including their interaction through bimolecular fluorescence complementation (BiFC) assays. Furthermore, we utilized transient overexpression and virus-induced gene silencing techniques to further validate the involvement of these two genes in the immune response elicited by CIP treatment. The findings of our study revealed a unique combination of PRRs responsible for perceiving polysaccharide elicitors.

Plant materials and growth conditions

The cultural process of *Atractylodes* plants was conducted according to previous reports (Zhang XY et al., 2017). Briefly, the seeds were soaked in warm water overnight and then cultured in plastic pots containing a mixture of perlite and peat (1:3, v:v) in a greenhouse maintained at 28°C under a 14-hour light:10-hour dark photoperiod. No herbicides were used for daily management. After 90 days (seven-leaf stage), the seedlings were utilized for VIGS and transient overexpression experiments. The *Nicotiana benthamiana* (*N. benthamiana*) seeds were sown in humus-rich coconut bran and subjected to the same growth conditions for *Atractylodes*. After reaching the mature five-leaf stage within 4-5 weeks, the tobacco plants were employed for bimolecular fluorescence complementation (BiFC) assays.

CIP preparation and sprayed to leaves of *Atractylodes* and *N. benthamiana* plants

The preparation of CIP was conducted based on previously published studies (Du NN et al., 2016; Xiong QW et al., 2021; Zhou YL et al., 2021). The experimental procedure followed a similar approach as described in our previous reports (Du et al., 2016). In brief, the dried flowers of

Chrysanthemum indicum L. collected from Suichang, Zhejiang province, China were pulverized and subjected to two extractions using purified water (1:15, w/v) at a temperature of 90. Following the filtration process, the filtrate was concentrated by utilizing a rotary evaporator under vacuum conditions. And then followed by addition of a five-fold volume of 95% ethanol to precipitate the crude polysaccharides overnight. This precipitation process was repeated twice. The resulting precipitate was collected through centrifugation and subsequently washed with absolute ethanol and acetone before being dried in vacuo at 40 °C yielding the crude polysaccharide product. This crude polysaccharide was further purified several times by using Sevag reagent (chloroacetic:n-butanol = 5:1,v/v) until the solution underwent scanning from ranging from 400 nm to 200 nm in order to detect protein and nucleic acids present without in it. Subsequently, 30% H₂O₂ was added to the solution to remove the pigment. Then, dialysis (cutoff Mw3500 Da) was performed on the sample, and it was precipitated overnight by adding a five-fold volume of 95% ethanol. The polysaccharide was preliminarily purified and subsequently designated as CIP.

The trial group was subjected to three rounds of spraying leaves of both *Atractylodes* and *N. benthamiana* plants with a CIP aqueous solution (2mg/ml, 50ml/pots) every 3 days. As for the control group, sterile water was used for spraying. Each treatment consisted of a minimum of 30 seedlings, and leaves were sampled from five randomly selected seedlings at specific time points and combined as a material sample. Three technical replicates were performed for each sample, and the data are presented as means ± standard errors (SE; n=3). Three biological replicates were conducted for each of the wild-type (WT), RNA-seq, quantitative real-time polymerase chain reaction (qRT-PCR), Virus-induced gene silencing (VIGS), and overexpressed lines. The leaves from all treatments were collected and promptly flash-frozen in liquid nitrogen, followed by storage at -80°C until further utilization.

Candidate genes screening based on the transcriptome data

In order to identify the candidate pattern recognition receptor involved in the defense response induced by CIP, RNA-seq was performed after 2h CIP-induction in *Atractylodes*. The sequences of *LysM-RLKs/RLPs* from *Arabidopsis* and Rice species were downloaded from Ensembl Plant (<http://plants.ensembl.org>) and used to construct local NR (<ftp://ftp.ncbi.nih.gov/blast/db/>), Swiss-Prot (<http://www.uniprot.org/>), and analysis databases for *Atractylodes*, respectively. The candidate *LysM*-receptor genes were aligned and selected using the published sequences of *Arabidopsis* and *Rice* species as well as our RNA-seq data (No.PRJNA419787) via the BLAST+(E-value = 1e⁻¹⁰) and HMMER software. Sequences containing integrated open reading frames were further filtered using ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). Finally,

the candidate sequences were aligned with the local NR and Swiss-Prot databases for annotation.

Identification of homeolog genes

The subtypes were classified using phylogeny analysis. Separate subphylogenies were generated for the *LysM-RLKs/RLPs* family using neighbor-joining method (P-distance model: bootstrap = 1000 based on the Bayesian criterion) in MEGA 7.0, aiming to provide a clearer understanding of the relationships between *Atractylodes* and other plant species. Homoeologous genes (226), including those from 74 species such as *Oryza sativa*, *Zea mays*, *Arabidopsis thaliana*, *Gossypium spp.*, were obtained from the NCBI database. The resulting sequence tree file was visualized using R packages ggtree and ITOL (<https://itol.embl.de/>). K-means clustering algorithm (K=15; method: complete) was applied to determine the sequence similarity among ten candidate genes and construct a phylogenetic tree in MEGA 7.0.

Physicochemical properties analysis of candidate genes

The properties of *AmLysM-RLKs/RLPs* family proteins were analyzed using the ExPASy translate tool and ExPASy ProParam (<http://web.expasy.org/translate/>). Sub-cellular localization prediction analysis was conducted using the online tool PSORT Prediction (<http://www.genscript.com/psort/>). MEME online tools (<http://meme-suite.org/index.html>) were employed for conserved motif analysis (default motif= 3; amino acid residues in each motif ≤ 200).

Analysis of differentially expressed genes

To identify the differentially expressed genes (DEGs) of *AmLysM-RLKs/RLPs* that play a pivotal role in CIP-induced response, we aligned the reads of *AmLysM-RLKs/RLPs* genes with the unigene datasets of *Atractylodes* RNA-seq data using Bowtie2 ([/bowtie2/index.shtml](http://bowtie2/index.shtml)). Subsequently, the expression level was assessed by integrating RSEM, where the FPKM value signifies the abundance of expression for corresponding unigenes. We utilized the DEseq2 package to analyze the gene expression levels of *AmLysM-RLKs/RLPs* and subsequently employed the complexheatmap package to generate a heatmap for differentially expressed genes (DEGs). Additionally, we created a Volcano Plot using ggplot2 package with filter criteria: $\text{Log}_2(\text{Fold Change}) \geq 1$ and $\text{P-value} \leq 0.05$.

RNA extraction, cDNA synthesis and quantitative real-time (qRT)-PCR

The total RNA was extracted using a plant RNA kit (Bioteke Corporation, China), and its integrity and concentration were assessed. Subsequently, 2 μg of the total RNA was utilized for cDNA synthesis and qRT-PCR employing a Reverse Transcription Kit and universal SYBR qPCR Master

Mix (Vazyme Corporation) on a Quant Studio 3 machine. During the qRT-PCR analysis, the relative expression level of the genes was normalized to the reference gene actin. Additionally, full-length *AmCERK1* and *AmLYK3* were cloned from the cDNA. The primer pairs utilized for qPCR are listed in **Table 1 of the Supporting Material**. Additionally, the trial group underwent three rounds of leaf spraying with a CIP aqueous solution (2mg/ml, 50ml/pots) every 3 days. In contrast, the control group was sprayed with sterile water. Following each round of spraying at intervals of 0, 0.5, 1, 2, 3, 4, 5 and 6 hours respectively, leaf samples weighing approximately 0.5g were collected for qRT-PCR assays.

Gene cloning and vector construction of *AmCERK1* and *AmLYK3*

The *Atractylodes* Chitin elicitor receptor kinase 1 (*AmCERK1*) and *LysM*-containing receptor-like kinase 3 (*AmLYK3*) were cloned from *Atractylodes*. The open reading frames (ORFs) of these two genes were inserted into destination vectors, including the BiFC vector pNC-Enn/Ecn (n/cYFP), and the overexpressed vector pNC-Cam2304-35S via homologous recombination. The 5' -end fragments of *AmCERK1* (300bp) and *AmLYK3* (300bp) were incorporated into the pTRV2 vector, with the GFP sequence serving as a control for VIGS assay.

The BiFC vectors pNC-Enn (nYFP; yellow fluorescent protein) and pNC-Ecn were recombined with *AmCERK1* and *AmLYK3*, respectively, to generate N-terminal YFP (nYFP) fused to *AmCERK1* at its N-terminus (nYFP-*AmCERK1*) and C-terminal YFP (cYFP) fused to *AmLYK3* at its C-terminus (cYFP-*AmLYK3*), or the combination of nYFP-*AmLYK3* and cYFP-*AmCERK1*. The overexpression vector generates recombinants pNC-Cam-*AmLYK3* and pNC-Cam-*AmCERK1*.

The VIGS vector facilitates the generation of recombinant plasmids pTRV2-*AmCERK1* and pTRV2-*AmLYK3*. Following confirmation through sequencing, these recombinant plasmids, along with their respective empty vectors, were transformed into competent *A. tumefaciens* strain GV3101 (the BiFC strain contained pSoup) using the heat shock method for experimental purposes (Weigel D et al., 2006). All of the aforementioned vectors are kanamycin resistant and were purchased from NC Biotech Corporation in China. The gene-specific primer pairs used for constructing the plasmid vectors are listed in **Supporting material Table 3**.

Bimolecular fluorescence complementation (BiFC) assays

The agrobacterium containing recombinant plasmids were cultured overnight at 28°C/200rpm in Luria-Bertani (LB) medium supplemented with kanamycin (50µg/ml) and rifampicin (35µg/ml).

The culture was harvested by centrifugation at 5000 rpm for 10 minutes, followed by resuspension in infiltration buffer (10mM MES, 10 mM MgCl₂, 150μM acetosyringone, pH 5.6) to achieve a final OD₆₀₀ of 0.8. Subsequently, the suspension was incubated for 3 hours at 25°C. The leaves of *N. benthamiana* were infiltrated with the resuspension containing nYFP-*AmCERK1* along with cYFP-*AmLYK3* or an empty vector using the leaf disc injection method with a needleless syringe (Dong YM et al., 2020). Afterward, the seedlings were further cultivated in a greenhouse for either 48 or 72 hours. Images were captured on a confocal laser-scanning microscope (Olympus, China) at 2h after CIP treatment or without CIP treatment as indicated under excitation wavelength of 515nm and emission wavelength of 528nm.

Transient expression and Virus-induced gene silencing (VIGS) assay

As described previously, an overnight-grown culture of agrobacterium containing the overexpression or VIGS plasmid, was used for the preparation of resuspension, which were injected into *Atractylodes*, following the same method as BiFC assays. The overexpressed and VIGS seedlings were grown in greenhouse after 2d and 25d respectively, the leaf was treated with the CIP or without CIP treatment as indicated , and harvested after 2h and stored at -80°C for further analysis. Quantification of target genes and immunity-related genes was performed using qPCR at the indicated time points. The gene-specific primer pairs used for qPCR in overexpression and VIGS silencing listed separately in **Supporting material Table 2 and Table 4**.

Statistical analysis

Statistical analysis of qRT-PCR was performed by SPSS software (v.26.0, IBM) using one-way ANOVA and pairwise multiple comparisons (or Student's t-tests) to determine the significant differences between the transgenic and control groups, and using the Rv.4.2.0 for data visualization. All treatment data had three biological replicates and three technical duplicates at least.

Results

Genetic relationship of *LysM*-RLKs/RLPs in *Atractylodes*

We employed RNA-seq to investigate the differentially expressed genes (DEGs) in *Atractylodes* after 3 hours of CIP induction. Numerous *LysM*-PRRs (*LysM* receptor-like kinases/receptor-like proteins) were annotated in various databases, including NR and Swiss-Prot, confirming the successful induction of membrane receptor genes by elicitors. Subsequently, we identified 10

candidate sequences using local BLAST+ software, which were annotated as *LysM*-containing receptor-like kinases/proteins in *Lactuca sativa*, *Capsella rubella*, *Fragaria vesca subsp.*, *Medicago truncatula*, *Arabidopsis thaliana*, *Oryza sativa*, *Tribulus alfalfa* and rice from the nucleic acid sequence database (NR) and Non-redundant protein sequence database (Swiss-Prot). Due to incomplete genome sequencing of *Atractylodes* at present, these *LysM*-PRR genes were provisionally named *AmLYK1-LYK10*. The basic information of these *LysM* receptor-like kinases/receptor-like proteins in *Atractylodes* is presented in **Table 1**.

Table 1. Basic information of candidate *LysM*-RLks/RLPs genes

RNA-seq number	Genes name	Sequence length/bp	NR annotation	Swiss-Prot annotation
TD40096	<i>AmLYK1</i>	2470	[<i>Lactuca sativa</i>] <i>LysM</i> -containing receptor-like kinase 3	[<i>Medicago truncatula</i>] <i>LysM</i> -containing receptor-like kinase 3
TD37858	<i>AmLYK2</i>	1846	[<i>Capsella rubella</i>] <i>LysM</i> -containing receptor-like kinase 3	[<i>Arabidopsis thaliana</i>] <i>LysM</i> -containing receptor-like kinase 3
TD36120	<i>AmLYK3</i>	2475	[<i>Lactuca sativa</i>] <i>LysM</i> -containing receptor-like kinase 3	[<i>Medicago truncatula</i>] <i>LysM</i> -containing receptor-like kinase 3
TD36230	<i>AmLYK4</i>	2149	[<i>Capsella rubella</i>] receptor-like kinase	[<i>Arabidopsis thaliana</i>] <i>LysM</i> -containing receptor-like kinase 3
TD28767	<i>AmLYK5</i>	2743	[<i>Capsella rubella</i>] receptor-like kinase	[<i>Arabidopsis thaliana</i>] <i>LysM</i> -containing receptor-like kinase 4
TD31180	<i>AmLYK6</i>	1745	[<i>Lactuca sativa</i>] <i>LysM</i> -containing receptor-like protein 5	[<i>Arabidopsis thaliana</i>] <i>LysM</i> -containing receptor-like kinase 5
TD38603	<i>AmLYK7</i>	2319	[<i>Lactuca sativa</i>] <i>LysM</i> -containing receptor-like protein 5	[<i>Arabidopsis thaliana</i>] <i>LysM</i> -containing receptor-like kinase 5
TD40781	<i>AmLYK8</i>	2092	[<i>Lactuca sativa</i>] chitin elicitor receptor kinase 1	[<i>Arabidopsis thaliana</i>] chitin elicitor receptor kinase 1
TD6162	<i>AmLYK9</i>	1556	[<i>Fragaria vesca subsp. vesca</i>] putative <i>LysM</i> -domain anchored protein	[<i>Oryza sativa</i>] chitin elicitor receptor-like protein
TD57691	<i>AmLYK10</i>	1489	[<i>Capsella rubella</i>] peptidoglycan binding protein	[<i>Oryza sativa</i>] chitin elicitor receptor-like protein

***AmLysM*-RLKs/RLPs genes belong to well-defined subfamilies**

To investigate the relationship between the *AmLysM*-RLKs/RLPs gene family of *Atractylodes* and the *LysM*-RLKs/RLPs gene family of other species, this study collected 226 *LysM*-RLKs/RLPs gene sequences from 74 species including *Arabidopsis thaliana*, rice, maize, and Castor bean from NCBI. These sequences were used to construct a maximum likelihood phylogenetic tree, with the results for *AmLysM*-RLKs/RLPs gene sequences of *Atractylodes* presented in **Fig. 1**.

The results revealed that *Atractylodes* *LysM*-type receptor genes were classified into four major subfamilies: LYKI, LYKII, LYKIII, and LYPI. Within these subclades, a strong phylogenetic association was observed between *AmLYK1-AmLYK10* genes in *Atractylodes* and related plant genes belonging to the LYKI, LYKII, LYKIII, and LYPI subfamilies.

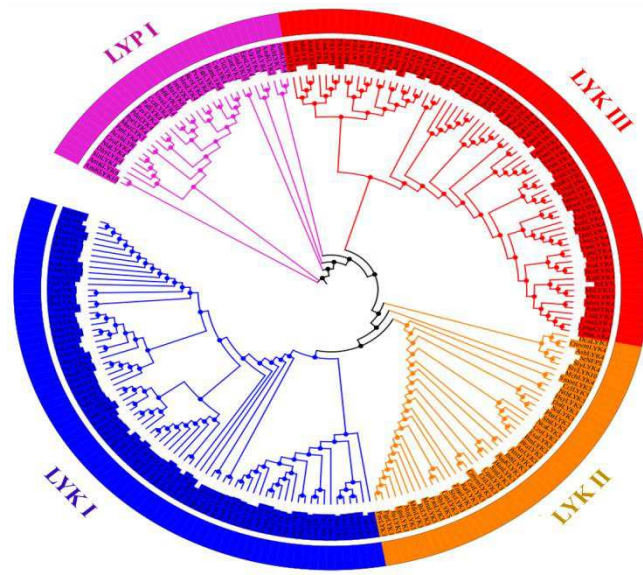


Fig. 1. The Phylogenetic relationships and subgroup designations of the *LYK* proteins in *Atractylodes*. **LYKIII** (red): LYKIII subfamily of LYK4 as main; **LYPI** (purple): LYPI subfamily of LYK4 as main; **LYKII** (yellow): LYKII subfamily of LYK3 as main; **LYKI** (blue): LYKI subfamily of LYK3 as main.

Furthermore, the *AmLYK1-LYK10* genes in the *LysM-RLKs/RLPs* gene family from *Atractylodes* were classified into a phylogenetic tree using MEGA7.0. Their gene types were predicted based on transcriptome annotation results and similar phylogenetic distances. *AmLYK9* and *AmLYK10* clustered together and were predicted to be of the *AmCEBiP*-type genes, while *AmLYK6* and *AmLYK7* formed another cluster and were predicted to be of the *AmLYK5*-type genes. The *AmLYK5* genes were predicted to be of the *AmLYK4*-type, while *AmLYK8* was predicted to be of the *AmCERK1*-type. In addition, there was a clade containing both *AmLYK2* and *AmLYK4* as well as another clade containing both *AmLYK1* and *AmLYK3*; all four genes in these two clades were predicted to be of the same type (*AmLYK3*), but they did not fall within the same branch in **Fig 2**. Based on this observation, we speculate that there has been differentiation among early evolution events for the *AmLYK3*-type genes, and two differentiated *AmLYK3*-type genes have undergone duplication events at recent time points. This hypothesis is supported by phylogenetic analysis conducted on these ten candidate genes.

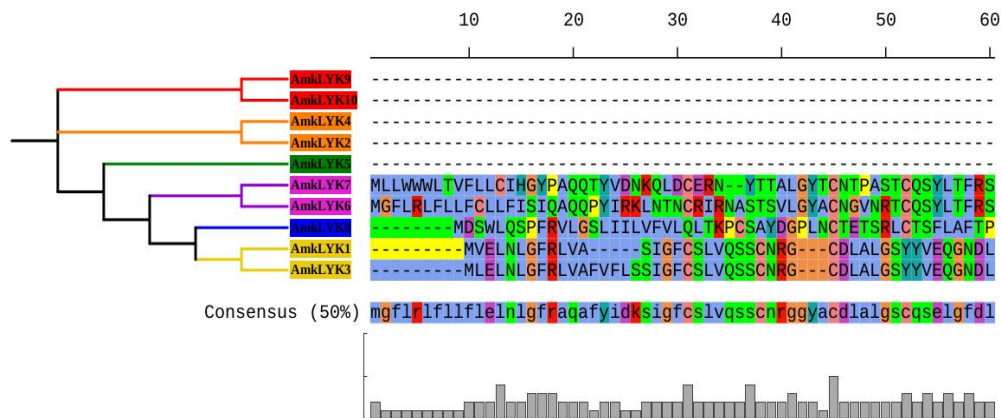


Fig. 2. The phylogenetic tree based on 13 protein-coding genes of the *AmLYK1-LYK10* proteins in *Atractylodes*. Same color branch represents their similar phylogenetic distance respectively.

Physicochemical properties and conserved domain analysis of *AmLysM-RLKs/RLPs*

The protein sequence analysis revealed that the protein encoded by *AmLysM-RLKs/RLPs* family genes consists of amino acids (aa) ranging from 264 to 660 in length (**Table 2**). The *AmLYK6* encoding protein consists of 264aa, making it the shortest in length. On the other hand, the *AmLYK1* protein is composed of 660 aa residues. The molecular weights range from 29757.87 to 73778.68 Da. Proteins *AmLYK1,3,5,6,7,8,9*, and *AmLYK10* have an isoelectric point (PI) below 6.5 and are classified as acidic proteins. Conversely, *AmLYK2* and *AmLYK4* have a PI greater than 7.5 and are considered alkaline proteins. The proteins *AmLYK1,3,7,8,9*, and *AmLYK10* are localized in the plasmalemma, while *AmLYK4, 6* are found in the cytoplasm and *AmLYK2* and *AmLYK5* are located in the mitochondria and endoplasmic reticulum membrane respectively. Positive hydrophilia indicates hydrophobic protein characteristics and vice versa. Therefore, with the exception of *AmLYK1* and *AmLYK9* which exhibit hydrophobic properties, the remaining eight proteins demonstrate hydrophilic characteristics (**Table 2**). The conserved domain analysis revealed that *AmLYK9* and *AmLYK10* exclusively possess N-terminal extracellular lysine motifs (*LysM*) (Couto D et al., 2016), which exhibit structural similarity to *LysM-RLPs*. Conversely, the *AmLYK1-AmLYK8* proteins feature a conserved extracellular *LysM* domain, a single transmembrane domain, and a cytoplasmic kinase domain, resembling *LysM-RLKs*.

Table 2. Basic physicochemical information of *AmLysM-RLKs/RLPs* genes

RNA-seq number	Genes name	Amino acid/aa	Molecular weight/Da	Isoelectric point(PI)	Stability coefficient	Hydrophilia	Fat coefficient	Subcellular localization
TD40096	<i>AmLYK1</i>	660	72942.73	6.04	39.34	0.026	93.91	plasmalemma
TD37858	<i>AmLYK2</i>	466	49582.22	9.55	56.09	-0.448	78.25	mitochondrion
TD36120	<i>AmLYK3</i>	621	67582.43	5.89	33.59	-0.106	91.12	plasmalemma
TD36230	<i>AmLYK4</i>	411	45778.58	9.01	57.67	-0.477	70.00	cytoplasm
TD28767	<i>AmLYK5</i>	639	70788.69	6.04	46.08	-0.086	91.83	Endoplasmic reticulum membrane

TD31180	<i>AmLYK6</i>	264	29757.87	5.15	49.94	-0.434	89.43	cytoplasm
TD38603	<i>AmLYK7</i>	665	73788.69	5.91	42.37	-0.192	87.50	plasmalemma
TD40781	<i>AmLYK8</i>	609	66243.80	5.79	33.02	-0.134	90.61	plasmalemma
TD6162	<i>AmLYK9</i>	448	40535.97	5.02	48.38	0.263	93.95	plasmalemma
TD57691	<i>AmLYK10</i>	372	46300.78	4.74	46.09	-0.020	91.77	plasmalemma

Differential expression analysis of *AmLysM-RLKs/RLPs* genes in *Atractylodes*

Based on the alignment of unigene sequences and assessment of expression levels for DEGs, we obtained an expression matrix in FPKM format to extract datasets representing the expression levels of *AmLysM-RLKs/RLPs* under control check (CK) and CIP treatment conditions. The heatmap analysis of DEGs revealed that the expression patterns of candidate *AmLysM-RLKs/RLPs* could be classified into three categories (**Fig. 3**). In the first category, genes such as *AmLYK3* and *AmLYK8* exhibited high expression levels following CIP treatment.

The second category genes, including *AmLYK1,2,4,6,10*, showed no significant difference in expression between CK and CIP treatments. In contrast, the third category genes (*AmLYK5,7,9*) exhibited repression upon CIP treatment. Therefore, we hypothesize that *AmLYK3* and *AmLYK8* play a crucial role in the CIP-induced immune pathway and are involved in the recognition process of CIP. The lower expression levels of *AmLYK5,7*, and *9* compared to baseline suggest their importance in the developmental growth of *Atractylodes* and their involvement in immune regulation. On the other hand, *AmLYK1,2,4,6*, and *10* were not differentially expressed genes (nDEGs), indicating their potential non-involvement in CIP-induced immune defense mechanisms.

According to the phylogenetic relationship, *AmLYK3* and *AmLYK8* were classified as *LYK3* and *CERK1* types respectively, belonging to the receptor kinase family. The expressions of *AmLYK5*, *AmLYK7*, and *AmLYK9* were assigned to *AmLYK4*, *AmLYK5*, and *AmCEBiP* types respectively. Based on gene subfamily analysis, the expression of genes in the *AmLYK3* and *AmCERK1* types was strongly induced by CIP treatment. On the other hand, genes in the *AmLYK4*, *AmLYK5*, and *AmCEBiP* types were repressed after CIP treatment. This suggests that both *AmLYK3* and *AmCERK1* genes play a crucial role in CIP-induced immune defense while regulating genes in this immune process are also observed for *AmLYK4*, *AmLYK5*, and *AmCEBiP*. Therefore, five candidate genes including renamed as "*AmLYK3*", "*AmCERK1*", "*AmLYK4*", "*AmLYK5*" and "*AmCEBiP*" were further analyzed in subsequent experiments.

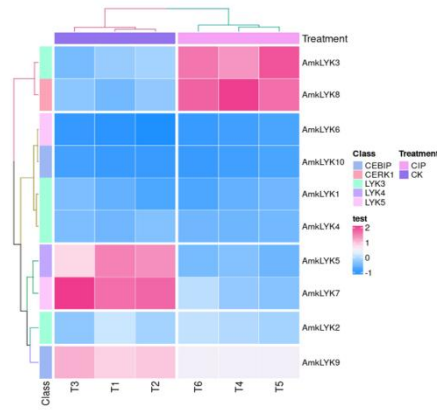


Figure 3. DEGs analysis of *AmLYK1-LYK10*: Heatmap of gene expression of the *AmLYK1-LYK10* in *Atractylodes*, (T₁-T₃: CIP treatment groups; T₄-T₆:Control groups); Red dots represent up-regulated genes, blue represent down-regulated genes.

Analysis of gene expression patterns on five candidate *AmLysM-RLKs/RLPs* genes

In order to further elucidate the involvement of the five candidate *AmLysM-RLKs/RLPs* genes in the response induced by CIP, we assessed the expression levels of these candidate genes at different time points following CIP treatment using Real-time Quantitative PCR (RT-qPCR). The results were visualized using the ggplot 2 package (**Fig. 4**). Five candidate genes exhibited differential expression patterns in response to CIP. The expression levels of *AmCERK1* and *AmLYK3* gradually increased from 0 to 1 hour, followed by a sharp increase between 1 and 2 hours, resulting in a significant fold increase of 3.96- and 3.90-fold ($p < 0.0001$) at the end of the second hour respectively. Subsequently, both genes exhibited a gradual return to basal expression levels from 4 to 6 hours. These similar expression patterns suggest their potential involvement in immune recognition induced by CIP collectively.

The expression levels of *AmLYK4*, *AmLYK5*, and *AmCEBiP* were repressed and remained lower than the basal level during 0-6h, in contrast. Specifically, both *AmLYK4* and *AmLYK5* reached a minimum at 3h with a significant reduction ($p < 0.001$) of 82% and 55%, respectively. Subsequently, they displayed fluctuating patterns from 3-6h, indicating their shared role in immune regulation and involvement in the immune regulatory process induced by CIP treatment. On the other hand, *AmCEBiP* gradually decreased during 0-2h with a significant reduction ($p < 0.0001$) of 42%, reaching its minimum at 2h, then it gradually returned to basal levels at 2-6h, suggesting that this gene is involved in the negative regulation process of CIP-induced immune recognition.

It is obvious that the *LysM-RLKs/RLPs* gene family in *Atractylodes* demonstrates species specificity in the immune recognition process induced by CIP. Particularly, *AmCERK1* and

AmLYK3 assigned to *LYK3* and *CERK1* types respectively(their target gene length was described in supplementary Table 1), are responsible for extracellular ligand recognition. So far, there has not been found these two type genes acts as a co-receptor for ligand recognition in other plant species.This needs to be further validated.

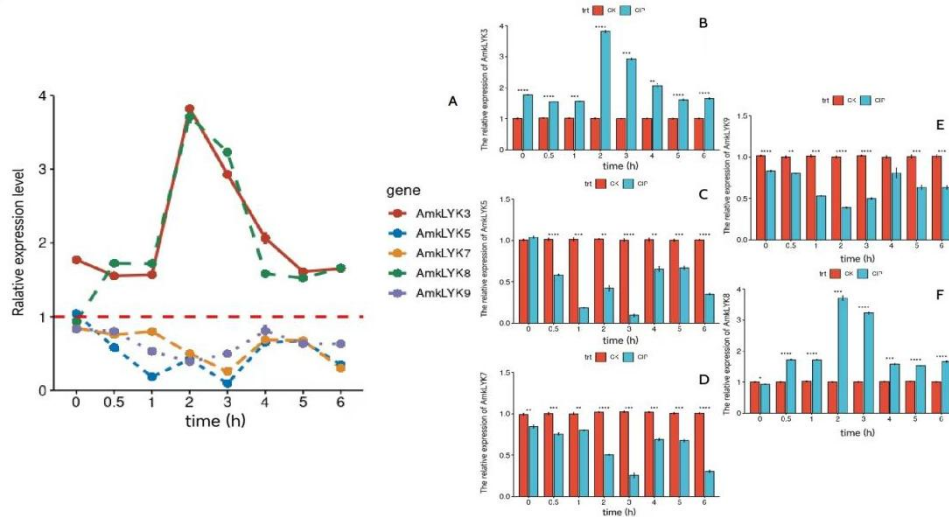


Fig. 4. Expression pattern analysis of *LysM-RLKs/RLPs* genes in *Atractylodes* leaves treated by CIP. **A:** Relative expression level of *LysM-RLKs/RLPs*; **B:** Relative expression level of *AmCERK1*; **C:** Relative expression level of *AmLYK5*; **D:** Relative expression level of *AmCEBiP*; **E:** Relative expression level of *AmLYK4*; **F:** Relative expression level of *AmLYK5*.

Both *AmCERK1* and *AmLYK3* are specific to recognition of CIP

For validating the interactional relationship between *AmLYK3* and *AmCERK1* under CIP treatment, bimolecular fluorescence complementation (BiFC) assays were performed via transient expression recombinant plasmid in *N.benthamiana* leaves (**Fig. 5**). The pNC plant bimolecular fluorescent hybridization (BiFC) vector was utilized to construct nYFP/cYFP-*AmCERK1*/*AmLYK3* positive BiFC vectors in this study. *Agrobacterium tumefaciens* containing the positive plasmids was re-suspended and injected into tobacco leaves using the leaf disc injection method for a 2- days incubation. The leaves were sprayed with CIP, after 30min sampled for observation. The experimental groups were sampled a minimum of three times. The results demonstrated the presence of yellow fluorescence on the cytomembrane of leaf cells upon transient co-expression of nYFP-*AmLYK3* and cYFP-*AmCERK1* or co-expression of cYFP-*AmLYK3* and nYFP-*AmCERK1* after CIP treatment (**Fig. 5A**). However, absence of yellow fluorescence on the cytomembrane of leaf cells when nYFP-*AmLYK3* and cYFP-*AmCERK1*, or cYFP-*AmLYK3* and nYFP-*AmCERK1* were transiently co-expressed without CIP induction (**Fig. 6A**). Similarly, the solitary infiltration group exhibited no fluorescence signal in both the groups treated with CIP (**Fig. 5B** and **5C**) and those not treated (**Fig. 6B** and **6C**). The results indicated that neither the empty vector nor the single linked vector exhibited any impact on fluorescence appearance, while the primary cause of

fluorescence appearance was predominantly attributed to the interaction between two target gene fragments facilitated by CIP-mediated structural changes.

The obtained results provide evidence that these receptor genes encode proteins localized on the cell membrane. Specifically, two receptor proteins function as co-receptors to perceive the CIP ligand and initiate a defense response for immune enhancement (Fig. 5A). Furthermore, it is evident that *AmCERK1* and *AmLYK3*-mediated PTI responses are specifically triggered by recognition of CIP. These receptors form heterologous complexes, which subsequently activate the immune system.

In addition, the homodimer formation test revealed fluorescence signal exclusively in the nYFP-*AmLYK3*+cYFP-*AmLYK3* group induced by CIP(Fig. 5A), while its control group (Fig. 6A) did not exhibit any fluorescence signal, thus ruling out CIP as a source of fluorescence. *AmCERK1* and *AmLYK3*-mediated PTI responses are specific to recognition of CIP.

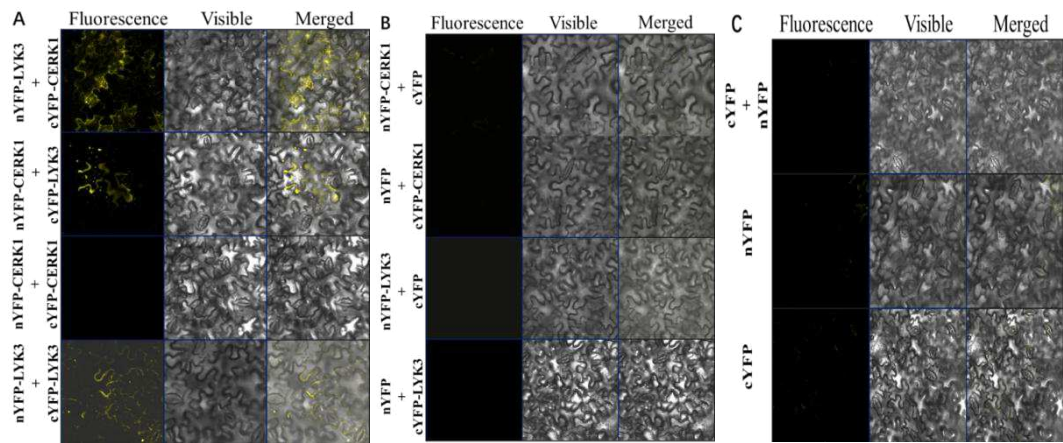


Fig. 5. BiFC analysis of the interaction between *AmCERK1* and *AmLYK3* in *N. benthamiana* leaves after CIP treatment, Yellow fluorescence images were taken 48h post-agroinfiltration. A: nYFP/cYFP-CERK1/LYK3 were co-agroinfiltrated respectively into *N. benthamiana* leaves as treatment groups; B:nYFP/cYFP-CERK1/LYK3 were co-agroinfiltrated separately into *N. benthamiana* leaves as control groups; C: nYFP/cYFP or nYFP+cYFP empty vector were co-agroinfiltrated into *N. benthamiana* leaves as control groups.

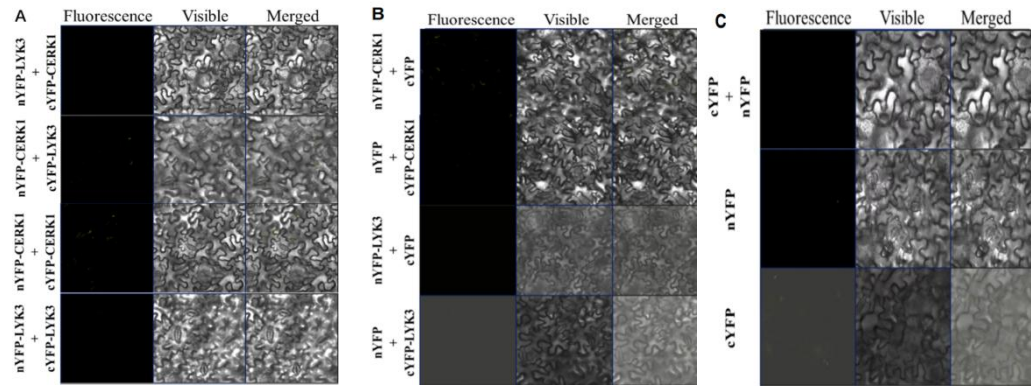


Fig. 6. BiFC analysis of the interaction between *AmCERK1* and *AmLYK3* in *N. benthamiana* leaves without CIP treatment, fluorescence images were taken 48h post-agroinfiltration. A: nYFP/cYFP-CERK1/LYK3 were co-agroinfiltrated respectively into *N. benthamiana* leaves as treatment groups; B: nYFP/cYFP-CERK1/LYK3 were co-agroinfiltrated separately into *N. benthamiana* leaves as control groups; C: nYFP/cYFP or nYFP+cYFP empty vector were co-agroinfiltrated into *N. benthamiana* leaves as control groups.

Transient overexpression of *AmLYK3* and *AmCERK1* in *Atractylodes*

To further functionally characterize the relationship between *AmCERK1* and *AmLYK3* in CIP-induced defense in *Atractylodes*, The vector for overexpressing the target gene was constructed by the pNC-Cam2304-35s vector connected to *AmCERK1* and *AmLYK3*. The leaf disc method will be employed for injecting the agrobacterium suspension containing the expression vector into leaves of *Atractylodes*, followed by incubation for 2 days. Subsequently, the leaves were subsequently treated with separate applications of CIP and water, followed by quantitative detection of the target gene after a 2-hour incubation period. Leaf samples were collected for qPCR analysis, with at least three replicates taken from each experimental group(Fig. 7A-B) .

The results demonstrated that the empty vector did not exert a significant impact on target genes, but it's 35S- promoter can guide the overexpression of the target gene, under the CIP treatment, the *AmCERK1* and *AmLYK3* transcript levels increased 3-fold and 3.13-fold respectively compared with the mock group infiltrated by empty vector. The expression levels of *AmCERK1* and *AmLYK3* in the groups injected with *AmCERK1* or *AmLYK3* expression vectors were significantly upregulated following CIP treatment compared to the water treatment group, exhibiting 12.87-fold and 3.25-fold increases, respectively (Fig. 7A-B). These findings indicate a substantial induction of both *AmCERK1* and *AmLYK3* by CIP treatment.

In addition, the transcript levels of *AmCERK1* were found to be sensitive to CIP treatment in the *AmCERK1*-overexpressed seedling, and the seedlings with *AmCERK1* overexpression exhibited elevated transcript levels of *AmLYK3* compared to those treated with CIP alone, suggesting that *AmCERK1* primarily functions as the receptor for recognizing CIP, while *AmLYK3* may serve as a co-receptor.

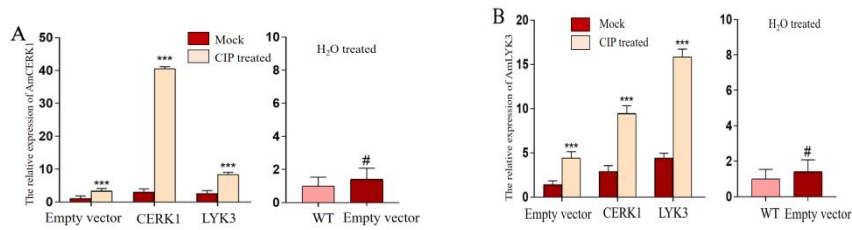


Fig. 7. The results of *AmCERK1* and *AmLYK3* transient overexpression after CIP treated. **A:** *AmCERK1* expression in pNC-Cam2304-35S-*AmCERK1* infiltration groups ; **B:** *AmLYK3* expressed in pNC-Cam2304-35S-*AmLYK3* infiltration groups. [Compared with the mock control, # represent no significant difference ($P>0.05$) , * represent statistical difference ($p<0.05$) , ** represent statistically significant difference $p<0.01$, *** represent very significant difference ($p<0.001$)].

Virus-induced gene silencing of *AmCERK1* and *AmLYK3*

The identification of several PRRs and their co-receptors as key defense players has been extensively studied (Böhm H et al., 2014; Jamieson PA et al., 2018). To investigate the role of *AmCERK1* and *AmLYK3* as PRRs in response to CIP recognition, the VIGS approach was employed in *Atractylodes*, as previously described (Cna'ani A et al., 2015). The application of TRV-based VIGS in functional genomics studies of several medicinal plants has been demonstrated, except for *Atractylodes* (Singh AK et al., 2015; Liscombe DK et al., 2011). Additionally, due to the lack of genomic data, we are unable to identify leaf-albino phenotype genes that can serve as visible silencing markers. Therefore, we conducted experiments using two types of VIGS vectors (pTRV1 and pTRV2) in order to establish an efficient protocol for VIGS. The pTRV-VIGS system was employed to construct VIGS silencing vectors targeting the 450-500bp interference fragment in *AmCERK1* and *AmLYK3* genes. Subsequently, the constructed HGS-*AmCERK1* and HGS-*AmLYK3* silencing vectors were transferred to agrobacterium, then introduced into *Atractylodes* leaves via leaf disc method and incubated for 25 days. Following a 2-hour treatment with CIP, the expression levels of the target genes were assessed in the silenced plants. The results revealed that the interest genes consistently exhibited a silencing frequency of over 60% following infiltration of agrobacterium carrying the pTRV1 and pTRV2-*AmCERK1/AmLYK3* vectors. No significant discrepancy was observed in the transcripts between seedlings infiltrated with empty vector controls (pTRV1 and pTRV2-GFP) and mock controls. These findings highlight the efficacy of pTRV-mediated VIGS for investigating gene function in *Atractylodes*. Lastly, our study demonstrated the effectiveness of disc injection method combined with pTRV.

In the *AmCERK1* and *AmLYK3*-silenced seedlings, the transcript levels of *AmCERK1* and *AmLYK3* in the mock control group and the untreated group with CIP were 0.42-fold and 0.79-fold,

respectively (**Fig. 8**). Similarly, in the same seedlings compared to wild type, indicating an effective silencing efficiency of 69% for *AmCERK1* and 73% for *AmLYK3*. However, when these silenced seedlings were treated with CIP, which alleviated the inhibition of target genes, the transcript levels of *AmCERK1* and *AmLYK3* in the CIP-treated group increased to 3.05-fold (**Fig. 8A**) and 1.67-fold (**Fig. 8B**), respectively, compared to those in the mock control group.

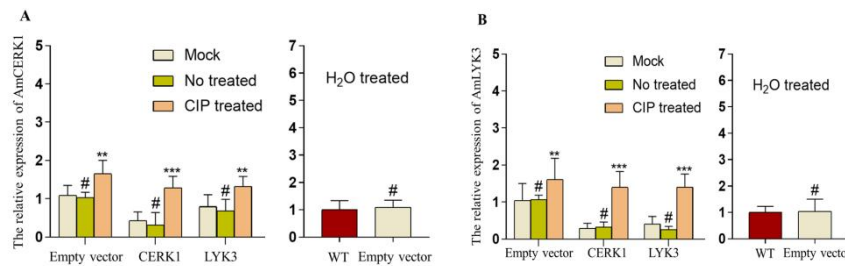


Fig. 8. Comparison of relative *AmCERK1* and *AmLYK3* gene expression in mock, no treated and CIP treated groups of VIGS-plants at 2h. [Compared with the mock control, # represent no significant difference ($P>0.05$), * represent statistical difference ($p<0.05$), ** represent statistically significant difference $p<0.01$, *** represent very significant difference ($p<0.001$)]. A: Silencing of *AmCERK1* in *Atractylodes* leaves by pTRV2-*AmCERK1*, silencing efficiency 69%; B: Silencing of *AmLYK3* in *Atractylodes* leaves by pTRV2-*AmLYK3*, silencing efficiency 73%.

The effect of *AmCERK1* and *AmLYK3* silencing on downstream defense gene expression

Following the aforementioned VIGS protocol, we assessed the transcript levels of two defense enzyme genes in silenced seedlings after a 30-hour treatment with CIP. In both *AmCERK1* and *AmLYK3*-silenced seedlings, the transcript levels of LOX (Lipoxygenase, LOX) were found to be (0.80-fold, 0.88-fold) and (0.71-fold, 0.59-fold) reduced in the mock control and untreated groups respectively (**Fig. 9A**). Compared to the mock control, the expression levels of LOX transcripts were significantly upregulated in the CIP treatment group, showing a 1.93-fold and 2.54-fold increase respectively (**Fig. 9A**). In *AmCERK1* and *AmLYK3*-silenced seedlings, the transcript levels of CHI (Chitinase, CHI) in the mock control and untreated groups were reduced by approximately 0.75-fold and 0.69-fold, as well as 0.71-fold and 0.42-fold respectively (**Fig. 9B**). However, upon CIP treatment, the expression of CHI increased by approximately 2.47-folds and 3.4-folds compared to the mock control (**Fig. 9B**). These findings demonstrate that CIP treatment can alleviate the suppression of *AmCHI* and *AmLOX* in plants with silenced *AmCERK1* or *AmLYK3* genes, highlighting the crucial role of activated *AmCERK1* and *AmLYK3* functions in overall plant immunity. Their target gene length was described in **supplementary Table 4**

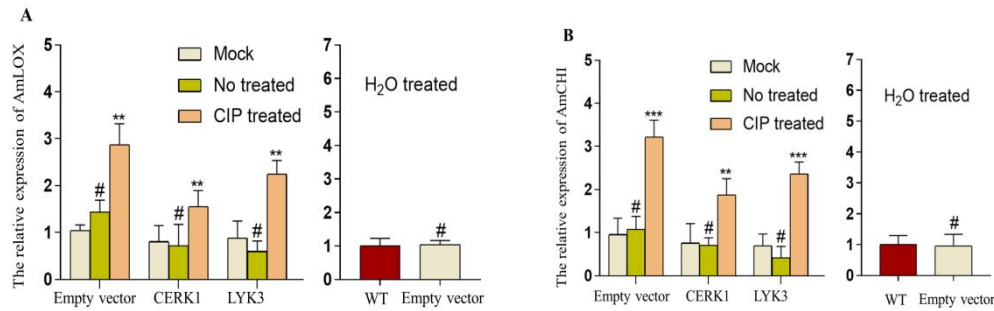


Fig. 9. Transient silencing of *AmCERK1* and *AmLYK3* via VIGS compromised expression of downstream defense genes in *Atractylodes* leaves (empty vector control plants were treated with pTRV1 and pTRV2, whereas *AmCERK1/AmLYK3*-silenced plants were treated with pTRV2-*AmCERK1* and pTRV2-*AmLYK3* respectively). **A:** The transcript levels of *AmLOX* as quantified by quantitative RT-PCR, mean $\pm$ SE, n = 3; **B:** The transcript levels of *AmCHI* as quantified by quantitative RT-PCR, mean $\pm$ SE, n = 3. (Compared with the mock control, # represent no significant difference ($P > 0.05$), * represent statistical difference ($p < 0.05$), ** represent statistically significant difference ($p < 0.01$), *** represent very significant difference ($p < 0.001$)).

Discussion

The presented data supports the notion that *AmCERK1* and *AmLYK3* function as interacting kinases in the perception of CIP trigger innate immunity in *Atractylodes*. Specifically, the analysis of differentially expressed genes (DEGs) revealed that two membrane receptor genes (*AmCERK1* and *AmLYK3*), which belong to the LysM family of receptor kinases based on genetic sequence analysis in the transcriptome, exhibited upregulation and a consistent expression pattern in *Atractylodes* treated with CIP. Subsequently, the BiFC analysis demonstrated that in the absence of CIP involvement, these two receptor proteins are spatially separated; however, upon binding of CIP to *AmCERK1* and *AmLYK3*, they form a receptor complex by acting as a 'molecular glue'. Additionally, we utilized VIGS and overexpression techniques to further confirm the functional role of *AmCERK1* and *AmLYK3* in plant interactions with CIP elicitors. In a further step, downstream defense gene expression data reveals that plants silenced for *AmCERK1/AmLYK3* exhibit significantly reduced levels of defense enzyme gene expression, but this immune inhibitory state can be alleviated by CIP. These results indicate that both *AmCERK1* and *AmLYK3* exhibit specificity towards CIP.

It has long been hypothesized that different microbial patterns, recognized by various types of PRRs of plants, activate common intracellular signaling pathways to initiate effective defense responses against invaders (Wan WL et al., 2019). The ligand recognition mediated by PRRs plays a crucial role in triggering plant innate immunity. Based on their ectodomain structure, PRRs can be classified into subfamilies including lysin motif (*LysM*)-containing, leucine-rich repeat (*LRR*)-containing, lectin-containing, and other subfamilies (Couto D et al., 2016). Lectin-containing and *LysM*-containing PRRs preferentially bind carbohydrate-based ligands such as bacterial peptidoglycan or fungal chitin (Böhm H et al., 2014; Zipfel C et al., 2014). Though, Previous studies have confirmed that plant-derived polysaccharides can activate the innate

immunity of plants, but due to the complex structure of polysaccharides, mediated the polysaccharides perception were few reports (Zheng et al., 2020). CIP, a plant-derived polysaccharide inducer, is essential for the interaction between *AmCERK1* and *AmLYK3* in *Atractylodes*. Our investigation conducted on the recognition of CIP by PRRs in *Atractylodes* offers novel insights into the mechanism underlying polysaccharide elicitor recognition in plants.

Plants have been found to possess a wide array of diverse and dynamic receptors on their membranes, which are intricately interconnected and engage in extensive cross-talk within immune networks. This phenomenon plays a crucial role in adjusting phenotypic responses to various environmental conditions (Hayafune M et al., 2014). Thus, further investigation is required to explore the capacity of plant *LysM-RLKs/RLPs* to form heterodimers or multimers and the specific recognition of different glycan inducers based on the number of *LysM* in their extracellular domain (Daniel et al., 2016). Previously, rice *CERK1* (*OsCERK1*) and *Arabidopsis* *CERK1* (*AtCERK1*) have been identified as crucial receptors in both immunity and symbiosis processes (Lee WS et al., 2014; Miyata K et al., 2014; Zhang X et al., 2015). In *Arabidopsis*, chitin binding to *AtLYK5* promotes the heterodimerization of *AtCERK1* and *AtLYK5* in five *LysM-RLKs*, which is crucial for the chitin-triggered response (Han Z et al., 2014). On the other hand, in *rice*, chitin binding to *OsCEBiP* dimer recruits *OsCERK1* and facilitates the homodimerization of *OsCERK1*, leading to subsequent phosphorylation and activation of immunity (Hayafune M et al., 2014). Unlike them, our findings suggest that both *AmCERK1* and *AmLYK3* receptors kinase play specific roles in recognizing CIP, and the receptor-like kinase combination of *AmCERK1* and *AmLYK3* initiates CIP-induced immunity in *Atractylodes*. In the chitin recognition system, *CEBiP* is widely conserved (Kaku et al., 2006), and it interacts with co-receptors of *LysM-RLKs/ RLPs* family members including *CERK1*, *LYK5*, *LYP4* and *LYP6* to activate intracellular kinase activity (Cao et al., 2014). However, the recognition system of CIP, specifically *AmCERK1*, acts as the central receptor for CIP recognition, whereas *AmLYK3* functions as a recruited co-receptor that triggers immune responses. This sets it apart from other glycan inducers like chitin, which may have a potential association with structural differences among these inducers. Our discovery is essential for comprehending plant-based immunity, and it provides new content for deepening the understanding of how different regulatory mechanisms in plants work together to integrate the immune signals of different PRRs to trigger PTI.

Conclusion

To the best of our knowledge, so far, studies on the role of ligand recognition in medicinal plants is less compared to model plant and crops. This is the first time that *Atractylodes* plants have been

used to study the effects of its receptor kinase recognizing CIP on immune activation behaviour. we explicitly excluded the possible effects of such *AtLYK5-AtCERK1* (Cao Y et al., 2014) or *OsCEBiP-OsCERK1* receptors (Cao Y et al., 2014; Hayafune M et al., 2014) combinations identified in other plants to recognized CIP, which is a novel discovery in PRRs combination of plants ligand recognition except for polysaccharide inducer such as chitin, β -glucan etc. To mitigate the inherent susceptibility of BiFC to false positives, we employed transient overexpression and pTRV-based VIGS as corroborative evidence to validate our conclusion that *AmCERK1* and *AmLYK3* constitute a novel interacting kinase involved in CIP-triggered immunity in *Atractylodes*. However, further investigations are warranted to confirm the protein-protein interaction categorically between *AmLYK3* and *AmCERK1* using yeast two-hybrid and co-immunoprecipitation techniques. The current understanding of immune recognition of polysaccharides in plants remains limited (Zheng et al., 2020). Our discovery is significant for comprehending the molecular basis of immune recognition upon ligand binding and subsequent activation of PRRs complexes, as well as for assembling these immune receptors to confer durable broad-spectrum resistance in medicinal or crop plants.

Author contribution statement Lei Chen and Xiangbing Chang designed the study. Kun Wu, Chuntao Wu .Xiangbing Chang and Kun Wu contributed to genomic analysis. Xiangbing Chang and Kun Wu generated the data. Xiangbing Chang and Kun Wu analyzed the result and wrote the manuscripts. All authors reviewed and approved the submitted version. Wei Tian: Supervision, Conceptualization, Funding acquisition, Resources, Project administration, Writing-review & editing.

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Declarations

Conflict of interest: On behalf of all authors, the corresponding author states that there is no

conflict of interest.

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