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BIOLOGY

Genome-wide identification and expression analysis of JmjC domain-containing gene family related to abiotic stress and photoperiodic treatments in Mung bean (*Vigna radiata* L.)

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ABSTRACT: Although the JmjC domain-containing histone demethylases displayed a crucial role in maintaining the homeostasis of histone methylation, while the systematic identification and functional researches of JmjC domain-containing gene family have not been conducted in Mung bean (*VrJMJ* genes). According to the structural characteristics and phylogenetic relationship with their orthologs from *Glycine max*, *Lotus japonicus*, *Medicago truncatula*, *Arabidopsis thaliana*, and *Oryza sativa*, a total of 18 *VrJMJ* genes were identified and divided into four clades (KDM3, KDM5. PKDM8, and PKDM9). Interspecies co-collinearity analysis showed the significant *JmjC* gene duplication events which have occurred during the Papilionoideae evolution. The exon/intron and domain organization of *VrJMJ* genes from the same clade (or subclade) were similar. All VrJMJ proteins contained a conserved JmjC domain, meanwhile other essential domains also have been found in some specific VrJMJ proteins which responsible for their functions. Numerous abiotic stress and light response related cis-elements associating with transcriptional regulation that were demonstrated in the promoter regions of *VrJMJ* genes (Pro_{VrJMJs}). Expression profiles of *VrJMJ* genes in different tissues showed that most genes displayed a tissue-specific expression in roots or leaves. The acronym RT-qPCR results showed that all *VrJMJ* genes displayed different degrees of abiotic stress (drought, salinity, and cold) and photoperiodic responses. Furthermore, *VrJMJ3* and *VrJMJ9* were significantly up-regulated after all three abiotic stress treatments, and *VrJMJ13* exhibited a potential function in the photoperiodic regulation of Mung bean flowering. These results provided a clear understanding of *VrJMJ* genes, and laid a theoretical basis for further verification of their potential biological functions of *VrJMJ* genes.
Key words: Mung bean, JmjC domain-containing gene family, abiotic stress response, light response, gene expression.

Identificação de todo o genoma e análise da expressão da família de genes contendo o domínio JmjC relacionada ao estresse abiótico e tratamentos fotoperiódicos em feijão mungo (*Vigna radiata* L.)

RESUMO: Embora as desmetilases de histonas contendo o domínio JmjC exibam um papel crucial na manutenção da homeostase das metilações de histonas, enquanto a identificação sistemática e a pesquisa funcional da família de genes contendo o domínio JmjC não foram conduzidas em feijão mungo (genes *VrJMJ*). De acordo com suas características de estrutura e relações filogenéticas com os ortólogos de *Glycine max*, *Lotus japonicus*, *Medicago truncatula*, *Arabidopsis thaliana* e *Oryza sativa*, se identificaram um total de 18 genes *VrJMJ* se divididos em quatro clados (KDM3, KDM5, PKDM8 e PKDM9). A análise de colinearidade exibiu eventos significativos de duplicação do gene JmjC ocorridos durante a evolução de Papilionoideae. A organização exon/intron e domínio de genes *VrJMJ* do mesmo clade (ou subclade) foram semelhantes. Todas as proteínas VrJMJ continham um domínio JmjC conservado, enquanto outros domínios essenciais foram encontrados em algumas proteínas VrJMJ específicas que são responsáveis por suas funções. Numerosos elementos cis relacionados ao estresse abiótico e à resposta à luz associados à regulação da transcrição foram encontrados nas regiões promotoras dos genes *VrJMJ* (Pro_{VrJMJs}). A análise do padrão de expressão dos genes *VrJMJ* em diferentes tecidos mostrou que a maioria dos genes exibe uma expressão preferencial em raízes ou folhas. Além disso, os resultados de acronym RT-qPCR mostraram que todos os genes *VrJMJ* apresentam diferentes graus de resposta ao estresse abiótico (seca, salinidade e frio) e tratamentos fotoperiódicos. Além disso, *VrJMJ3* y *VrJMJ9* foi notavelmente expresso na resposta a todos os estresses abióticos mencionados acima, e *VrJMJ13* exibiu funções potenciais na regulação fotoperiódica da floração em feijão-mungo. Estes resultados proporcionam una compreensão clara dos genes *VrJMJ* e estabeleceu uma base teórica para uma maior verificação das possíveis funções biológicas dos genes *VrJMJ*.
Palavras-chave: feijão mungo, família genética que apresenta domínio JmjC, resposta ao estresse abiótico, resposta de luz, expressão gênica.

INTRODUCTION

In the genome of eukaryotes, histones (H2A, H2B, H3, and H4) and genomic DNA are packaged into nucleosomes (LUGER & RICHMOND, 1998; HOLLIDAY, 1987). The N-terminal tails of histones are widely extend out of the nucleosome, which are subject to a wide variety of post-translational modifications including methylation, acetylation, phosphorylation, ADP-ribosylation and ubiquitination (BOWMAN & POIRIER, 2015). As the main histone modifications, methylation and demethylation have played critical roles in regulating gene expression, genome integrity, and epigenetic inheritance (GELATO & FISCHLE,

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2008; LIU et al., 2010). Histone methylation occurs primarily on arginine (R) and lysine (K) residues of histones H3 (K4, K9, K27, K36, and K79) and H4 (K20) (ALLIS et al., 2007; HAN et al., 2016). At the Lysine residues, histone methylation occurs mainly in the forms of monomethylated (Kme1), dimethylated (Kme2), and trimethylated (Kme3). However, histone arginine residues can undergo monomethylation (Rme1), symmetric demethylation (Rme2s), and asymmetric dimethylation (Rme2a) (LIU et al., 2010). Histone methylation can contribute to transcriptional activation or inactivation, H3K9 (H3K9me2/3) and H3K27 (H3K27me3) methylation play roles in transcriptional inhibition, while H3K4 (H3K4me2/3) and H3K36 (H3K36me3) methylation displayed the opposite roles (BINDA et al., 2013; PONTVIANNE et al., 2010). In eukaryotic genomes, Lysine Specific Demethylase 1 (LSD1) and JmjC domain-containing histone demethylases (JHDMs) are known to be the mainly existing histone lysine demethylases (SHI et al., 2004; TSUKADA et al., 2006). As a Flavin adenine dinucleotide (FAD) dependent enzyme, LSD1 catalyzes the removal of single/double lysine residue methylation. However, JHDMs removes the mono/di/tri-lysine residue methylation with the help of ferrous ion (Fe (II)) and a-ketoglutarate (a-KG) (TREWICK et al., 2005; LU et al., 2008).

Many members of JmjC domain-containing gene family have been comprehensively identified and have been known to be involved in the regulation of plant growth and epigenetic processes (KLOSE et al., 2006; KOUZARIDES, 2007; MA et al., 2022). For example, the 21 JmjC domain-containing proteins from *A. thaliana* displayed their functions in regulating leaf growth, floral transition, flowering time, and abiotic stress (LU et al., 2008). In *A. thaliana*, *AtJMJ11*/*ELF6* (*EARLY FLOWERING 6*) and *AtJMJ12*/*REF6* (*RELATIVE OF EARLY FLOWERING 6*) display contrary roles in the regulation of flowering time (YU et al., 2008). In the photoperiodic flowering pathway, *AtJMJ11*/*ELF6* promote early flowering by inhibiting the expression of *FLC* (*FLOWERING LOCUS C*), which is known as a flowering repressor (NOH et al., 2004; LU et al., 2011). As an active histone H3K4 demethylase, *AtJMJ14* suppresses the expression of *FT* (*FLOWERING LOCUS T*) by demethylating H3K4me1/2/3, hence delaying the flowering time of *A. thaliana* (LU et al., 2010; YANG et al., 2010; NING et al., 2015). According to previous experimental and genomic researches, the salt-stress tolerance of plants exhibit a close relationship with histone methylation (SUN et al., 2019). During the adjusting process of dehydration stress response, *AtJMJ17* directly regulate the mRNA abundance of OST1 (OPEN STOMATA 1) via demethylating H3K4me3 (HUANG et al., 2019). When compared with wild-type of *A. thaliana,* gain-of-function mutants of *AtJMJ15* show stronger tolerance to salt stress, while the functionally deficient mutant display more salt sensitiveness (SHEN et al., 2014). As an H3K4me code reader in *G. max*, *GmPHD6* increases the expression of salt-stress response gene via recognizing the H3K4 methylation (WEI et al., 2017). In *M. truncatula*, the cold-dependent alternative splicing of *MtJMJC5* play a role in epigenetic regulation of the link between surrounding temperature fluctuation and circadian clock (SHEN et al., 2016). In *Gossypium hirsutum*, seven *GhJMJ* genes were significantly up-regulated under cold and osmotic stress treatments, which revealing that these genes were closely related to the cold or osmotic stress responses (ZHANG et al., 2020). Under unfavorable environment treatments, varied photoperiod and abiotic stresses modulated the expression of *JmjC* genes to regulate the growth and development of plant.

Mung bean is a fast-growing warm-season legume species, which has been mainly grown in Asia by small holder farmers for its edible seeds and sprouts (KANG et al., 2014). The seeds of Mung bean are a good source of dietary proteins, which also contain higher content of folate and iron than most of the other legume crops (KEATINGE et al., 2011). As a legume crop, Mung bean can also fix the atmospheric nitrogen by rhizobial symbiosis, hence to increase the fertility and texture of soil (GRAHAM & VANCE, 2003). So far, there was no systematic identification and function research of histone demethylase gene family in Mung bean. In our study, we conducted a genome-wide identification of *VrJMJ* gene family in Mung bean, and comprehensively analyzed their subfamily classification and architecture, chromosomal location, interspecies co-collinearity, conserved residues, cis-elements in Pro_{VrJMJs}, and expression profiles. Our results will help in better understanding the potential function of Mung bean *VrJMJ* genes in the regulation of abiotic stress and photoperiodic flowering.

MATERIALS AND METHODS

Identification of JmjC domain-containing genes in Mung bean

The genomic sequence and annotation of Mung bean were obtained from the NCBI Genome database (https://www.ncbi.nlm.nih.gov/genome/?term=Vigna+radiata). All the JmjC proteins in Mung bean were identified by two rounds of BLASTP (*P*-value < 1e-10). Firstly, the amino acid sequences of JmjC proteins determined in 20 *O. sativa*, 21 *A. thaliana*, 27 *L. japonicas*, 33

M. truncatula and 48 *G. max* were used to search possible VrJMJ proteins in Mung bean using TBtools (HAN et al., 2016; CHEN et al., 2020; HUANG et al., 2016). Then NCBI Batch CD-Search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) and SMART (http://smart.embl.de/) were used to confirm whether these candidates contained a JmjC domain (PF02373 and SM00558). Consequently, 18 homologous *VrJMJ* genes were finally confirmed in Mung bean after removing all redundant transcripts.

Analysis of the main characteristics of VrJMJ genes in Mung bean

The amino acid number, molecular weights (MW, kDa), theoretical isoelectric point (PI), instability index (II), grand average of hydropathicity (GRAVY), and aliphatic index of *VrJMJ* genes were analyzed using ExPASy software (http://www.expasy.org/tools/) using default parameters. Plant-mPLoc software (http://www.csbio.sjtu.edu.cn/cgibin/PlantmPLoc.cgi) was used to predict the subcellular localization. By aligning the coding sequences with their corresponding genomic sequences, the intronic and exonic positions of 18 *VrJMJ* genes were analyzed using Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/). NCBI-conserved domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to confirm the presence of conserved domains in all *VrJMJ* genes, which have been identified by SMART (http://smart.embl-heidelberg.de/) and Pfam (https://pfam.xfam.org/). Further, the obtained genic (exon-intron) structure and distribution of conserved domains were visualized by TBtools. The tertiary structure of 18 VrJMJ proteins was predicted by SWISS-MODEL server (https://www.swissmodel.expasy.org/interactive).

Phylogenetic analysis of VrJMJ genes in Mung bean

Multiple sequence alignment of all JmjC proteins from *O. sativa*, *A. thaliana*, *L. japonicas*, *M. truncatula*, *G. max*, and Mung bean was performed using Muscle algorithm in MEGA 6.0 (https://www.megasoftware.net) with default parameters. The multi-species phylogenetic tree was constructed using MEGA 6.0 with the Neighbor-Joining (NJ) method. The reliability was assessed with 1000 bootstrap replications and the p-distance model. The obtained phylogenetic tree was visualized and modified using iTOL (LETUNIC & BORK, 2016).

Chromosomal location, synteny analysis, and gene duplication events of VrJMJ genes in Mung bean

Chromosomal location information of 18 *VrJMJ* genes were obtained from the genome annotation of Mung bean, and their distribution in each chromosome was mapped using TBtools. NetNES 1.1 Server (http://www.cbs.dtu.dk/services/NetNES/) and cNLS Mapper (http://nlsmapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) were used to analyze the nuclear export signal (NES) and nuclear localization signal (NLS) of all *VrJMJ* genes. Gene duplication analysis of *VrJMJ* genes was performed using NCBI-BLASTp and MCScanX (http://chibba.pgml.uga.edu/mcscan2/#tm), and synteny analysis of *JmjC* genes among *Pisum sativum,* Mung bean, and *G. max* was performed in TBtools using the default parameters.

Prediction of cis-acting elements in the promoter regions of VrJMJ genes

To identify potential abiotic stress and light responsive cis-acting elements in all Pro_{VrJMJs}, the 2, 000 bp sequence upstream of the initiation codon (ATG) of each *VrJMJ* gene was compared against the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The most frequent abiotic stress and light responsive elements were visualized in all Pro_{VrJMJs} using TBtools.

Expression profile analysis of VrJMJ genes in Mung bean

For determining the tissue-specific expression pattern of *VrJMJ* genes, their expression levels in four different tissues (roots, stems, leaves, and buds) were analyzed using acronym RT-qPCR. To investigate the potential biological functions of *VrJMJ* genes under different abiotic stresses and photoperiod, four-week-old Mung bean seedlings were subjected to these treatments using long day (16 / 8 h), short day (8 / 16 h), cold (4 °C), NaCl (200 mM), and 15% polyethylene glycol (PEG) 6000 mixed with Hoagland solution. Samples were harvested at 0 h, and 12 h after the treatments of cold, NaCl, and PEG 6000. All the samples were immediately snap-frozen in liquid nitrogen after harvesting, then stored at - 80 °C for subsequent RNA extraction. All samples were collected in biological triplicates.

Total RNA was isolated using Spectrum Plant Total RNA Kit (Sigma-Aldrich), RNA purification was done by treating with DNAse I (Sigma-Aldrich) as per manufacturer's protocol. First-strand cDNA was synthetized from 1.0 mg of RNA using the PrimeScript RT reagent kit (Takara Bio). The acronym RT-qPCR analysis was carried out by SYBR-green fluorescence using the Roche LightCycler®480 Real-Time PCR System. Each acronym RT-qPCR reaction mixture contained 10 µL of 2 x *TransStart*® Top Green qPCR SuperMix

(TransGen Biotech), 0.4 μL each of forward and reverse primer (10 μM), 2 μL of cDNA sample, and 7.2 μL of nuclease-free water. At least three biological replicates were performed for each cDNA sample. The Mung bean *Actin* gene (*Vradi03g00210*) was used as the internal control for normalization (LIU et al., 2022; XU et al., 2021). The acronym RT-qPCR run profile was as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s, 60 °C for 1 min. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, and the graphs of gene expression were drawn using GraphPad Prism 5.0.

RESULTS

Identification of VrJMJ genes in Mung bean

According to previous studies, all plant *JmjC* genes both contain a conserved JmjC domain. Then, these criteria were used to identify the putative *JmjC* genes in Mung bean. A total of 18 non-redundant *JmjC* genes were identified in Mung bean, which were designated as *VrJMJ1* ~ *VrJMJ18* based on their phylogenetic relationships with their orthologs from *G. max*, *L. japonicus* and *M. truncatula*. The physiochemical properties of each VrJMJ protein were analyzed, most of VrJMJ proteins had lengths of 601 ~ 1832 amino acids, while the largest VrJMJ11 had 1832 amino acids and the smallest VrJMJ13 had only 601 amino acids (Table 1). The predicted PIs of VrJMJ proteins were ranging from 5.62 to 8.99, and their MWs were in the range of 68.98 ~ 208.99 kDa. Most of the VrJMJ proteins were hydrophilic and unstable, which were supported by the relatively low GRAVY value (< 0) and high Instability index (II) (> 40). The prediction of subcellular location revealed that all VrJMJ proteins were localized in nuclear, which in consistent with their potential functions of histone demethylation. For further correlating the subcellular location and function of VrJMJ proteins, the presence or absence of NLS and NES were also investigated. Except for VrJMJ3 and VrJMJ13 protein, sixteen VrJMJ proteins possessed a NLS signature together with the NES sequence. The lysine/arginine rich sequences of these sixteen VrJMJ proteins might help them to relocate from cytosol to nucleus.

Phylogenetc analysis of VrJMJ genes in Mung bean

To help the classification and better understanding their evolutionary relationships of *VrJMJ* genes, the JmjC domain sequences of 18 VrJMJ proteins, 20 OsJMJ proteins, 21 AtJMJ proteins, 27 LjJMJ proteins, 33 MtJMJ proteins, and 48 GmJMJ proteins were used to construct an unrooted phylogenetic tree (Figure 1*a*). According to the phylogenetic analysis, these 167 *JmjC* genes

Table 1 - Basic information of *VrJMJ* genes in Mung bean.

Name	CDS (bp)	Protein length (aa)	MW/kDa	pI	Instability index (II)	GRAVY	Aliphatic index	Subcellular localization	NES/NLS
VrJMJ1	2658	885	100.71	6.28	45.3	-0.624	75.21	Cell Nucleus	YES
VrJMJ2	2883	960	109.29	7.17	45.99	-0.704	69.83	Cell Nucleus	YES
VrJMJ3	2196	731	84.32	6.96	49.99	-0.396	82.09	Cell Nucleus	NO
VrJMJ4	2655	884	102.49	8.56	54.83	-0.695	74.99	Cell Nucleus	YES
VrJMJ5	3081	1026	117.70	8.68	60.74	-0.756	70.28	Cell Nucleus	YES
VrJMJ6	2883	960	110.51	8.09	48.11	-0.672	72.18	Cell Nucleus	YES
VrJMJ7	2883	960	108.57	5.62	43.4	-0.599	75.46	Cell Nucleus	YES
VrJMJ8	3138	1045	117.88	5.62	47.45	-0.466	75.49	Cell Nucleus	YES
VrJMJ9	3093	1030	116.88	5.87	48.62	-0.476	75.05	Cell Nucleus	YES
VrJMJ10	3762	1253	140.73	6.45	61.5	-0.546	71.51	Cell Nucleus	YES
VrJMJ11	5499	1832	208.99	6.35	46.46	-0.306	86.2	Cell Nucleus	YES
VrJMJ12	2550	849	95.69	7.95	48.96	-0.519	70.85	Cell membrane, Nucleus	YES
VrJMJ13	1806	601	68.98	8.99	43.09	-0.391	69.3	Cell Nucleus	NO
VrJMJ14	2571	856	97.28	6.58	48.24	-0.469	73.12	Cell membrane, Nucleus	YES
VrJMJ15	2400	799	90.62	8.03	51.61	-0.475	71.43	Cell membrane, Nucleus	YES
VrJMJ16	4560	1519	169.20	6.81	53.7	-0.549	71.88	Cell Nucleus	YES
VrJMJ17	4746	1581	178.02	8.75	55.51	-0.809	61.99	Cell Nucleus	YES
VrJMJ18	4602	1533	174.32	8.88	53.9	-0.821	65.14	Cell Nucleus	YES

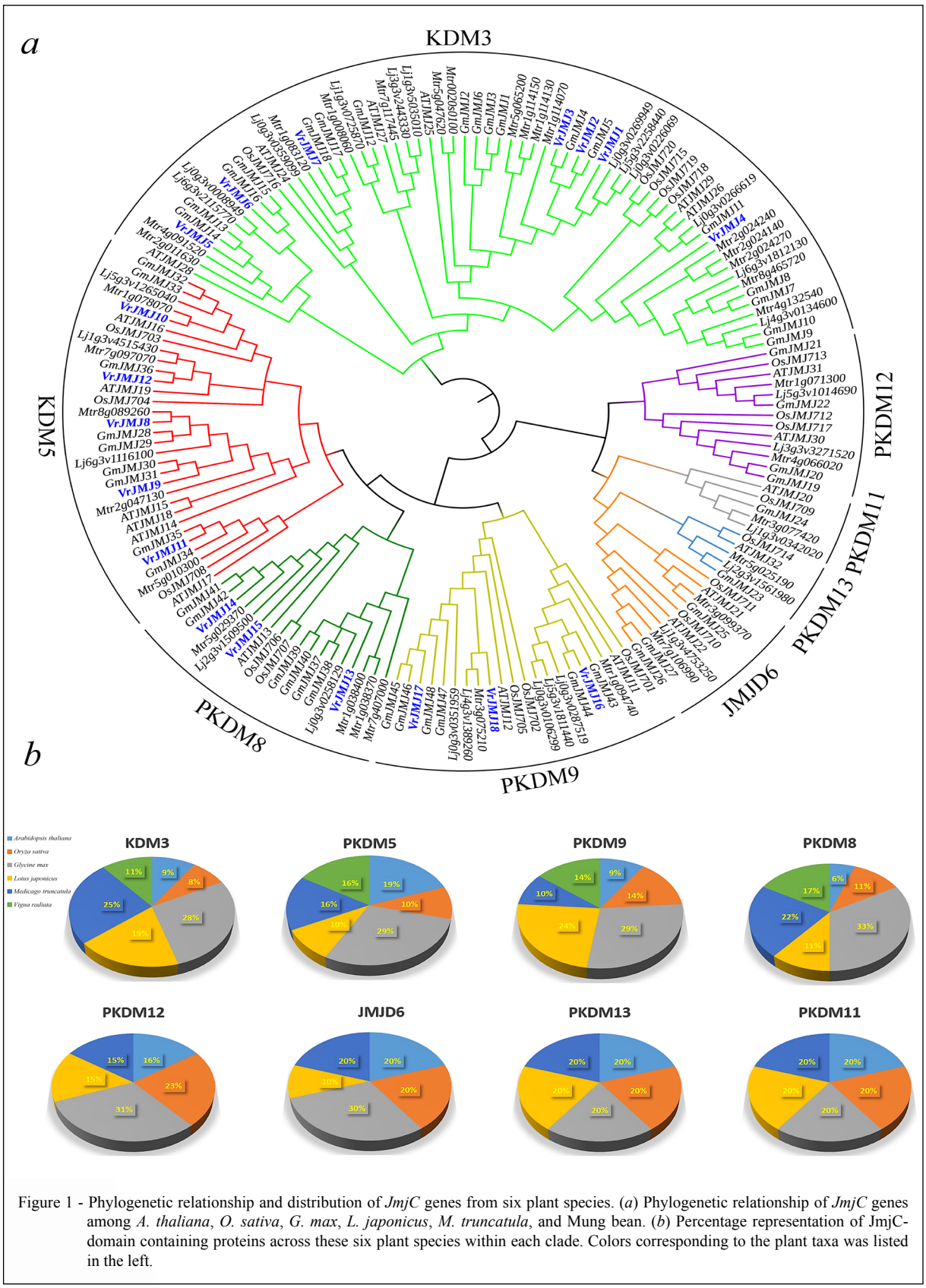

Figure 1 - Phylogenetic relationship and distribution of *JmjC* genes from six plant species. (*a*) Phylogenetic relationship of *JmjC* genes among *A. thaliana*, *O. sativa*, *G. max*, *L. japonicus*, *M. truncatula*, and Mung bean. (*b*) Percentage representation of JmjC-domain containing proteins across these six plant species within each clade. Colors corresponding to the plant taxa was listed in the left.

were divided into eight clades: KDM3, KDM5, PKDM8, PKDM9, JMJD6, PKDM11, PKDM12, and PKDM13, with 18 *VrJMJ* genes were classified into four clades including KDM3, KDM5, PKDM8, and PKDM9. In Mung bean, KDM3 was the largest *JmjC* clade with 7 *VrJMJ* members which accounted for 38.9% of this family, PKDM8 and PKDM9 were the smallest *JmjC* clade that only contained 3 *VrJMJ* genes. Moreover, the proportions of per *JmjC* clade were also not even inconsistent in six species. For instance, there were larger proportions of KDM3 clade genes in *G. max* (28%) and *M. truncatula* (25%) than that in *L. japonicus* (19%), Mung bean (11%), *A. thaliana* (9%), and *O. sativa* (8%) (Figure 1*b*). Different degrees of gene duplication or lose event might have occurred during the evolution of these six species.

Gene structure and conserved domains of VrJMJ genes in Mung bean

Intron-exon structure has been proven to play a crucial role in the genic evolution. The number of introns ranged drastically from 6 to 32 in *VrJMJ* genes, with the maximum of 32 introns were found in *VrJMJ11* (Figure 2*a*). In most cases, the neighboring *VrJMJ* genes from the same *JmjC* clade had displayed similar genic structures in terms of numbers and arrangements of intron-exon (DONG et al., 2020). We also reported one exception in the KDM5 clade, *VrJMJ11* contained remarkably 32 introns while other *VrJMJ* genes only had 7 ~ 10 introns, which implying that KDM5 clade could specify into two structural subclades during the evolution process. The location percentages of introns in *VrJMJ* genes at 0, 1, and 2 phase were 62%, 18%, and 20%, which also signifying the conserved structural character of eukaryotic gene evolution (FEDOROV et al., 1992; DONG et al., 2020).

Organization and composition of conserved domains are vital for the fundamental function of proteins. Without any exception, all VrJMJ proteins had only one conserved JmjC domain, meanwhile each VrJMJ protein contained 1 to 9 domains (Figure 2*b*). JmjN domain was the secondly widespread domain, which appearing in all VrJMJ proteins from KDM5, PKDM8, and PKDM9 clades. When interacting with the JmjC catalytic domain, the JmjN domain was shown to be important for Jhd27 (also known as KDM5), a H3K4-specific demethylase in budding yeast (HUANG et al., 2010; QUAN et al., 2011). In PKDM9 clade, the ZnF-C2H2 domain contained two cysteines and histidines, which could create a compact nucleic acid-binding domain by coordinating a zinc atom (CHRISPEELS et al., 2000). Three VrJMJ proteins from KDM5 clade had one FYRN and FYRC domain, which might harbor chromatin binding activity and help the JmjC domain to function by interacting with other proteins (LU et al., 2008). We also found that VrJMJ11 has four uniquely structural domains including the ARID, BRIGHT, PHD and PLU-1 domain. The ARID or BRIGHT domain was associated to sequence-specific DNA binding, the PHD domain might be the important readers of histone codes by recognizing the methylated (modified) histone codes, and the PLU-1 domain could function in chromatin stability and gene regulation (GREGORY et al., 1996; MUSSELMAN & KUTATELADZE, 2009; MADSEN et al., 2003). The structural diversity further suggested the functional differentiation and specification of *VrJMJ* genes.

Cis-acting elements in the promoter regions of VrJMJ genes in Mung bean

When binding with specific cis-acting elements, transcription factors could regulate the expression ability and level of their downstream genes by activating or repressing gene transcription (SUN et al., 2021). To further elucidate the possible regulation mechanism of 18 *VrJMJ* genes under the abiotic stress and light responses, we detected 34 types of cis-acting regulatory elements related to abiotic stress and light responses in Pro_{VrJMJs} (Figure 3). About 2 ~ 6 types of abiotic stress-related elements were identified in each Pro_{VrJMJ}, which including ARE (anaerobic), DRE1 (drought and osmotic stress), TCA (stress-inducible), TC-rich (defense and stress), LTR (cold), WRE3 and WUN-motif (wound responding element), MBS (drought), and STRE (heat shock, osmotic stress, low pH, and nutrient starvation). Among the abiotic stress-related elements, ARE was the most widely distributed one. Twenty-four types of light-responsive elements were identified, such as Box-4, GT1-motif, and TCT-motif. Among the predicted light-responsive elements, the distribution of Box-4 was the most widely, which distributing in all *VrJMJ* genes (Figure 3*b*). At least two types of light-responsive elements were detected in each Pro_{VrJMJ}, which was consistent with their potential regulation of flowering process.

Conserved amino acid residues in active sites of VrJMJ proteins in Mung bean

Fe (II) and α-KG binding sites were crucial cofactors for JmjC demethylase activities. When using the corresponding AtJMJ and GmJMJ proteins

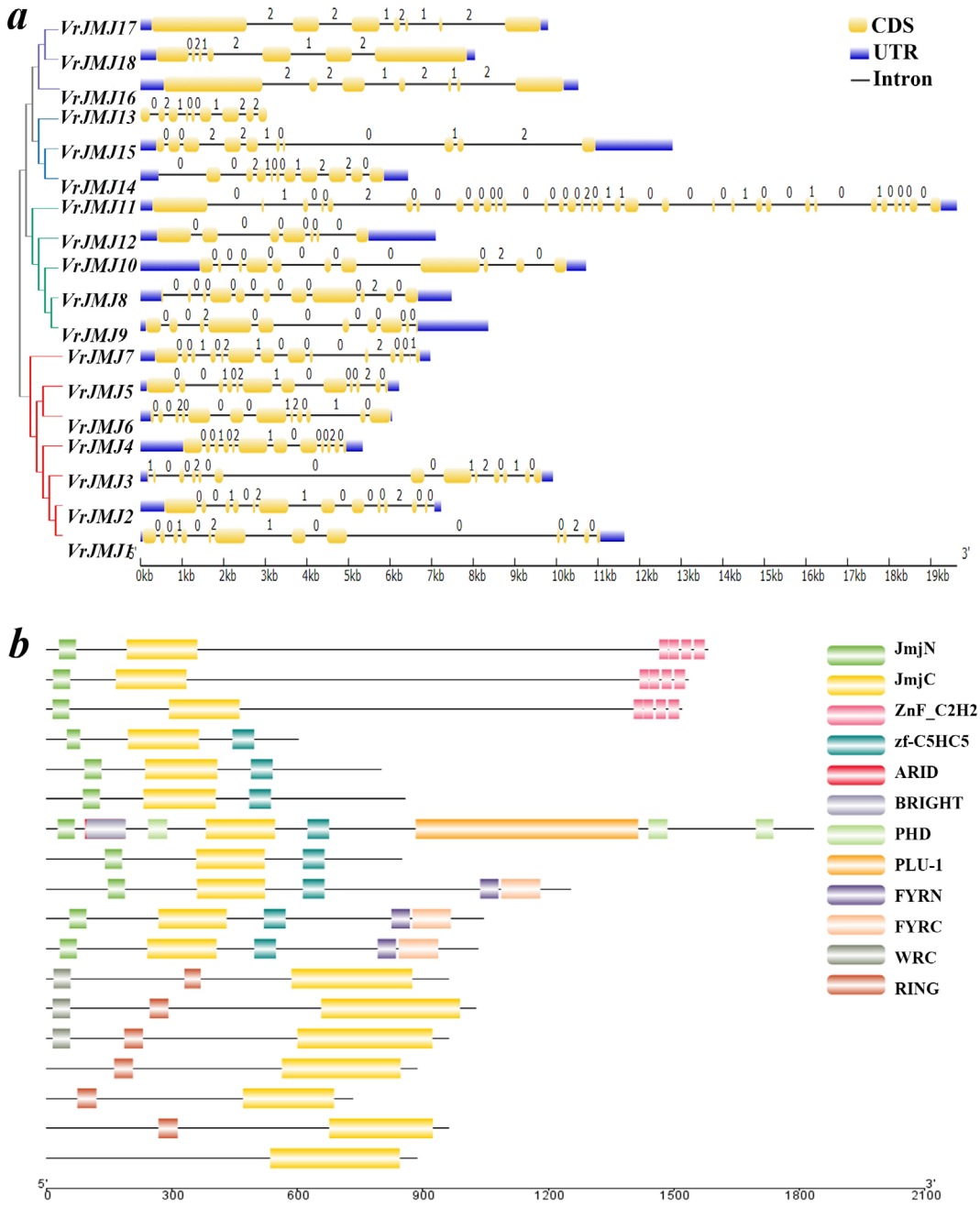

Figure 2 - The genic structure and domain architecture of 18 *VrJMJ* genes. (*a*) Exon/intron structures of *VrJMJ* genes. The yellow box represents exons, and the black line refers introns, and the blue box refers UTR. (*b*) The domain architecture of *VrJMJ* proteins.

as the reference, we analyzed the composition of three amino acid residues (His, Glu/Asp, and His) for Fe (II) cofactor binding and two amino acid residues (Thr/Phe and Lys) for a-KG binding in all VrJMJ proteins (CHEN et al., 2006; JIA et al., 2017). According to their conserved amino acids for Fe (II) and α-KG binding, four phylogenetic clades of VrJMJ proteins were divided into two groups. The first group contained the KDM3 clade which having the conserved amino acids His (H), Asp (D), and

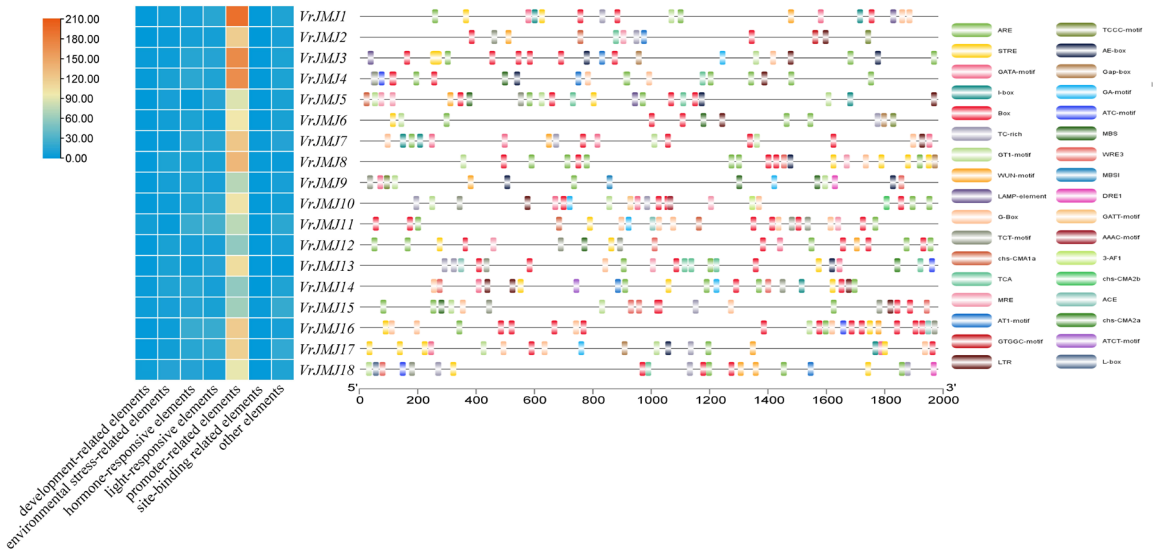

Figure 3 - Cis-elements in the promoter sequences of 18 *VrJMJ* genes. (*a*) Heatmap representing the numbers of cis-acting elements related to development, environmental stress, hormone, light, and so on. Color scale represents the number where blue indicates low number and red indicates high number. (*b*) Distribution of major abiotic stress and light response-related cis-elements in the 18 Pro_{VrJMJs}. The 2,000 bp sequences upstream of the initiation codon (ATG) of the *VrJMJ* genes can be estimated using the scale per 200 bp above.

His (H) for Fe (II) binding, Thr (T) and Lys (K) for a-KG binding (Figure 4*a*). While the second group included the PKDM8, PKDM9, and KDM5 clade, which having the conserved residues H, E (Glu), and H for Fe (II) binding, F (Phe) and K for a-KG binding (Figure 4*b*). Most VrJMJ proteins carried the conserved residues for interacting with Fe (II) and α-KG, though there have some substitutions in KDM3 and KDM5 clade. For instance in the KDM3 clade, substitutions can be seen in the second sites with His (H) changing into Cys (C) in VrJMJ5 and VrJMJ6 protein. But their binding ability with Fe (II) and α-KG might have not been affected because of these substitutions had similar physical and chemical properties. Overall, these highly conservative interaction sites also indicated their significant role in the demethylase activity of plant *JmjC* genes.

Tertiary structures of VrJMJ proteins in Mung bean

The tertiary structures of 18 VrJMJ proteins were shown in figure 4, they were all mainly composed of α-helices, β-folds and random coils. There had VrJMJ1, VrJMJ3, VrJMJ5, VrJMJ6, and VrJMJ7 protein displayed the identical structures, which indicating that they might have similar functions. In addition, VrJMJ8, VrJMJ9, VrJMJ10, and VrJMJ12 protein were also structurally similar, as well as VrJMJ13, VrJMJ14 and VrJMJ15, VrJMJ17, and VrJMJ18 protein (Figure 5). Their different tertiary structures also determined the functional diversity of 18 VrJMJ proteins.

Chromosomal localization and interspecies co-collinearity of VrJMJ genes in Mung bean

Eighteen *VrJMJ* genes were unevenly anchored on 7 of the 11 Mung bean chromosomes, with the distribution of *VrJMJ* genes were as follows, five members on VrChr7, four members on VrChr8 and VrChr11, two members on VrChr5, one member on VrChr3, VrChr6, and VrChr10 (Figure 6*a*). The uneven distribution of *VrJMJ* genes might be attributed to the chromosomal shuffling and gene duplication event during the course of Mung bean evolution.

To investigate the potentially evolutionary process of *JmjC* genes in Papilionoideae, interspecies co-collinearity analysis were conducted to identify these directly homologous *JmjC* genes among *P. sativum*, Mung bean, and *G. max*. From the gray blocks of background, we found that all chromosomes undergo clearly exchange of fragments during the evolution of three Papilionoideae species. In Figure 6*b*, the locations of *VrJMJ* genes and their homologous gene pairs were uncovered. There have 17 directly homologous gene

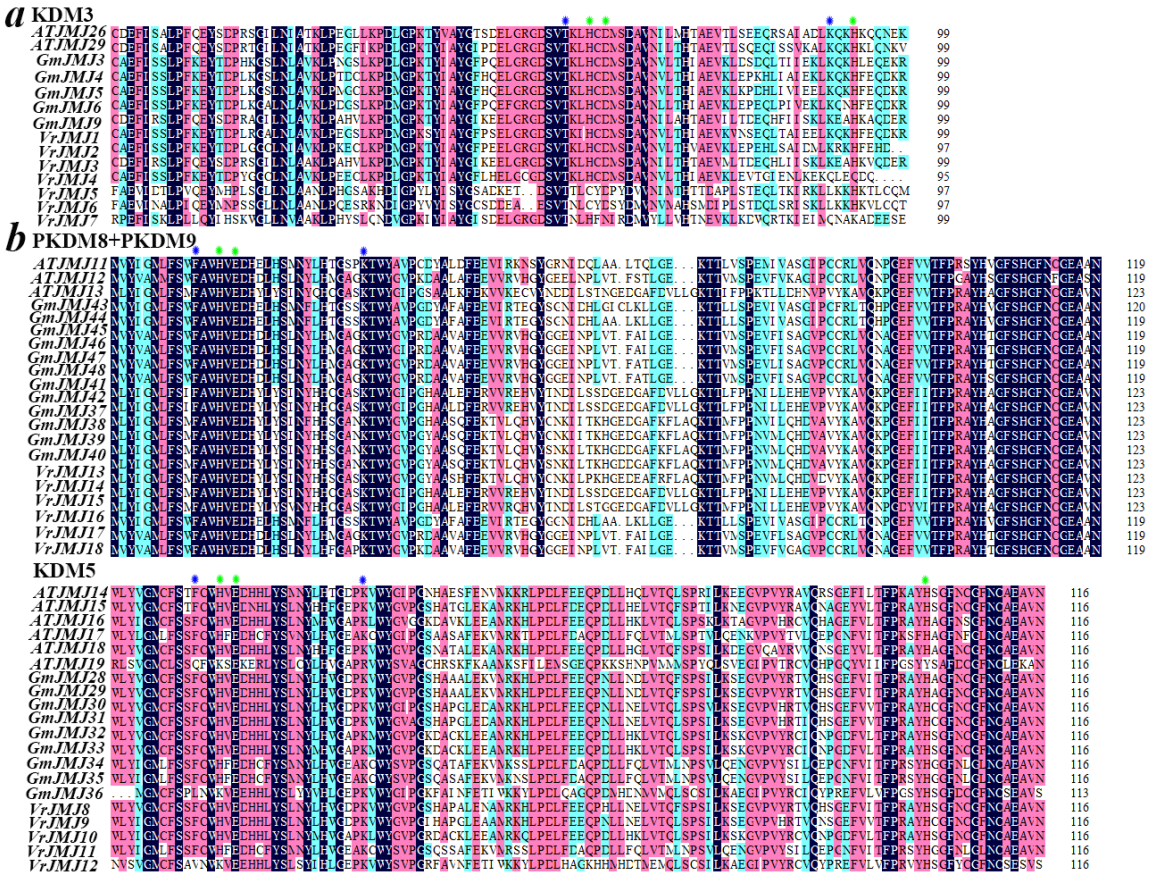

Figure 4 - Conserved residues analysis that were compatible with the demethylation activity within the Fe (II) binding site (blue) α-KG binding site (green) in VrJMJ proteins.

pairs been identified between *P. sativum* and Mung bean, 31 directly homologous gene pairs were identified between Mung bean and *G. max*. Except for *VrJMJ1*, *VrJMJ3*, *VrJMJ8*, *VrJMJ13*, and *VrJMJ15*, 10 *VrJMJ* genes had one-to-one and 3 *VrJMJ* genes had one-to-two relationships with their *JmjC* homologs from *P. sativum*. Besides *VrJMJ1*, *VrJMJ3*, *VrJMJ13*, and *VrJMJ15,* 2 *VrJMJs* had one-to-one, 9 *VrJMJs* had one-to-two, 1 *VrJMJs* had one-to-three, and 2 *VrJMJs* had one-to-four relationships with their *JmjC* homologs from *G. max*.

Expression analysis of VrJMJ genes in different tissues of Mung bean

The tissue-specific expression profiles were the first step to explore the gene functions, then the transcriptional expression of 18 *VrJMJ* genes in roots, stems, leaves, and buds were evaluated (Figure 7). From the expression profiles, *VrJMJ* genes showed distinct expression patterns in different tissues, meanwhile most genes were expressed at higher levels in roots and leaves. Moreover, eight *VrJMJ* genes (*VrJMJ3*, *VrJMJ5*, *VrJMJ10*, *VrJMJ11*, *VrJMJ14*, *VrJMJ15*, *VrJMJ17*, and *VrJMJ18*) showed relatively high expression levels in roots, and three *VrJMJ* genes (*VrJMJ8*, *VrJMJ9*, and *VrJMJ13*) were highly expressed in leaves.

Expression profiles of VrJMJ genes under different abiotic stress and photoperiodic treatments

The investigation of cis-acting elements had proven that all Pro_{VrJMJs} contain abiotic stress and light response elements. To further gain insight into the responses of *VrJMJ* genes to various abiotic stresses and photoperiods, a comparative acronym RT-qPCR analysis was conducted on the Mung bean seedlings subjected to NaCl, cold, PEG 6000, long-day and short-day treatments. According to the expression profiles, all *VrJMJ* genes were differentially expressed in response to different abiotic stresses (Figure 8). When compared with non-treated controls (0 h), all *VrJMJ* genes were significantly

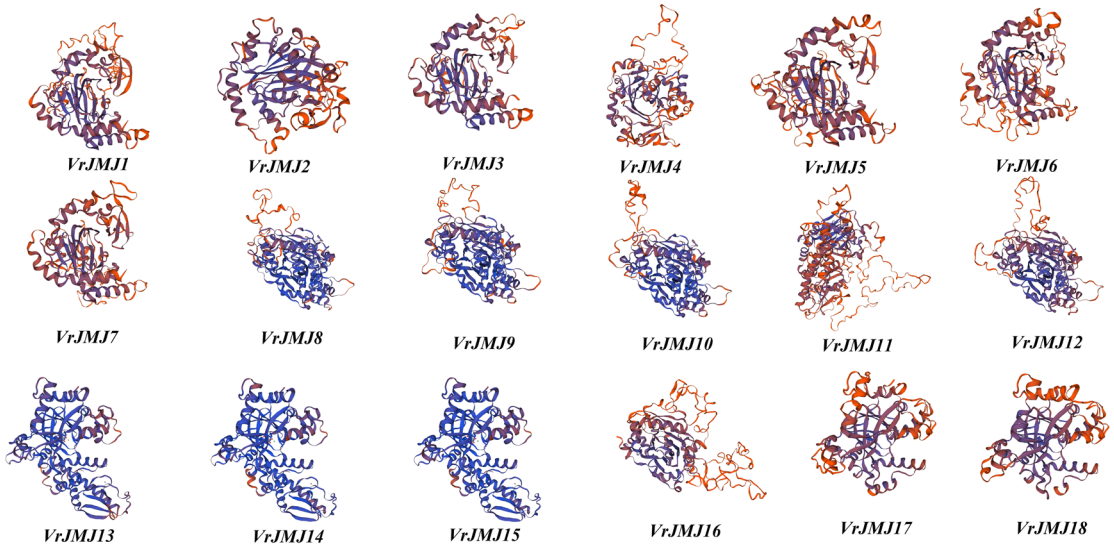

Figure 5 - Predicted three-dimensional domains of VrJMJ proteins. The low-energy structure was indicated in blue, while the high-energy structure was indicated in orange.

up-regulated under both PEG 6000 and NaCl stress treatments. Moreover, the expression levels of *VrJMJ14*, *VrJMJ15*, and *VrJMJ18* were relatively higher under PEG 6000 than that under NaCl stress treatment. Interestingly, the transcriptional degrees of all *VrJMJ* genes were remarkably lower in response to cold stress treatment. Under cold treatment, *VrJMJ4*, *VrJMJ6~7*, *VrJMJ10~13*, and *VrJMJ18* were slightly down-regulated, and *VrJMJ1~2*, *VrJMJ5*, *VrJMJ8*, and *VrJMJ14~17* were slightly up-regulated at 12 h after treatments. Furthermore, *VrJMJ3* and *VrJMJ9* were significantly up-regulated under all three abiotic stress treatments.

When compared with their expression under LD conditions, the expression levels of 12 *VrJMJ* genes were down-regulated in response to SD treatment, while *VrJMJ3~4*, *VrJMJ7~8*, *VrJMJ12*, and *VrJMJ17* displayed up-regulated expressions (Figure 9). Except for *VrJMJ6*, *VrJMJ11*, and *VrJMJ17*, the other *VrJMJ* genes showed significantly expression levels under LD or SD conditions. Interestingly, it was worth noting that *VrJMJ8*, *VrJMJ9*, and *VrJMJ13* also predominantly expressed in leaves. The DNA binding domain (DBD) analyses suggested that there have two Zinc-coordinating DBD profiles (MA0372.1 and MA0306.1) been found in *VrJMJ13*. We also scanned the tranion factor binding sites of MA0372.1 in the promoter regions of *VrFT* genes, which demonstrating the important function of *VrJMJ13* in the photoperiodic regulation of flowering in Mung bean.

DISCUSSION

In the epigenetic regulation of gene expression, histone methylation has played an important role in plant growth and development (CHEN et al., 2011). The JmjC domain-containing proteins represented a large family of histone demethylases in plants, which comprised a significant part of epigenetics and displayed essential roles in maintaining homeostasis of histone methylation (KLOSE et al., 2006). Until now, a few plant *JmjC* gene families have been successfully analyzed to reveal their evolutionary history and biological functions at the whole-genome level (CHENG et al., 2022; HAN et al., 2016). However, none systematic research has been performed on the *JmjC* gene family of Mung bean. In present study, a comprehensive identification and functional analysis of *VrJMJ* genes were performed using the latest version of the Mung bean genome database, including their phylogenetic relationships, gene structure, domain composition, chromosomal location, interspecies co-collinearity, cis-acting elements, and expression profiles.

Initially, our phylogenetic analysis provided novel insights into the evolution of gene multiplicity and family members in Mung bean.

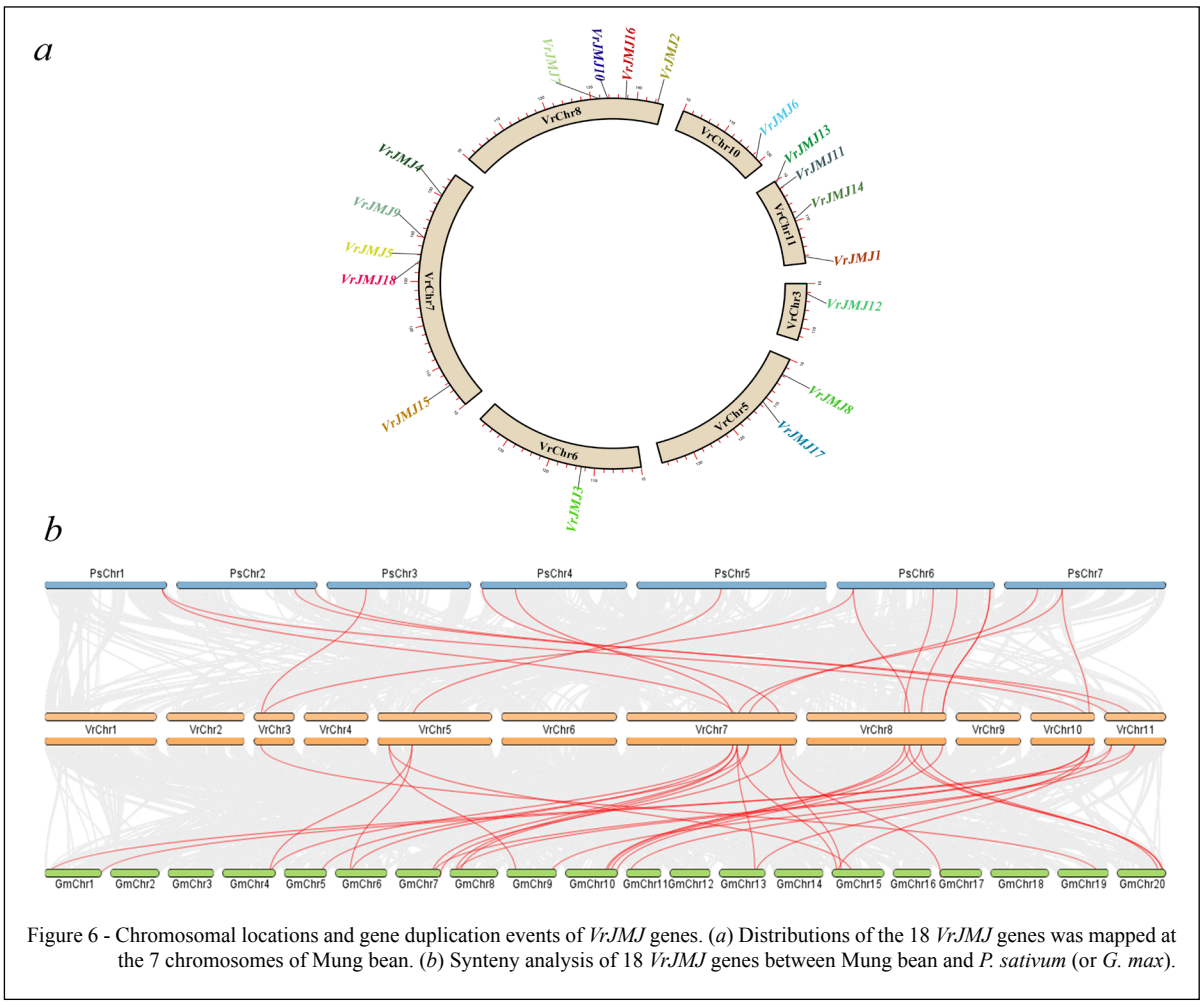

Figure 6 - Chromosomal locations and gene duplication events of *VrJMJ* genes. (*a*) Distributions of the 18 *VrJMJ* genes was mapped at the 7 chromosomes of Mung bean. (*b*) Synteny analysis of 18 *VrJMJ* genes between Mung bean and *P. sativum* (or *G. max*).

According to their phylogenetic relationships, 18 *VrJMJ* genes were mainly categorized into four distinct clades, which was similar with previous studies in *Z. mays* (19), *O. sativa* (20) and *A. thaliana* (21) (LU et al., 2008; QIAN et al., 2019). However, the genome size of Mung bean (579 Mb) was larger than *A. thaliana* (125 Mb) genome and *O. sativa* (389 Mb) genome but much smaller than *Z. mays* (2,300 Mb) genome. This phenomenon might result from a less gene duplication or large gene loss event of *VrJMJ* genes Mung bean evolution, which further demonstrated that the *JmjC* genes was relatively stable in plants, was highly conserved in evolution, and had little to do with genome size. Interspecies co-collinearity analysis of *JmjC* genes among Papilionoideae species exhibited one-to-one, two, three, and four direct homology existed between Mung bean and *P. sativum* (or *G. max*). Although the unusual amplification of JmjC genes existed in *G. max*, but relatively gene duplication events had occurred during the Papilionoideae evolution.

Almost all of our results proved that *VrJMJ* genes were significantly conservative in the same phylogenetic clade, which sharing a similarly genic structure, conserved domain, and conservative residues, meanwhile different *JmjC* clades also displayed a largely diversity. The PKDM8 and PKDM9 clade genes had a conservative composition of genic exon/intron and domain, the KDM5 clade genes showed a conservative exon/intron but diversified domain composition, while the KDM3 clade genes displayed diversified exon/intron and domain compositions. There were nine amino acid substitutions for a-KG or Fe (II) binding in KDM3 clade, two substitutions in KDM5 clade, PKDM8 and PKDM9 clade genes carried the conservative residues for cofactors binding. We speculated that the structural diversity of *VrJMJ* genes accounted for the

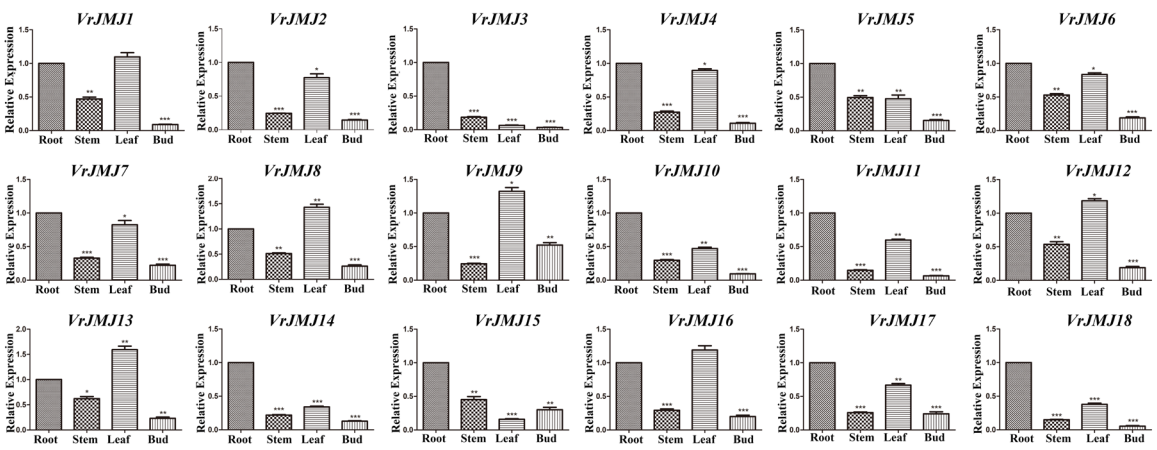

Figure 7 - The acronym RT-qPCR analysis of *VrJMJ* genes in different tissues (roots, stems, leaves, and buds). The expression of *VrJMJ* genes in root was set to "1". Data are the mean ± standard errors of three independent replicates. Significant differences relative to the expression in roots are indicated by asterisks (***P < 0.001; **P < 0.01; and *P < 0.05).

functional differentiation and specification during the evolutionary process, meanwhile these genes might share a variety of demethylation roles responsible for different physiological activities.

The functions of plant *JmjC* genes are definitely diverse, which also involved in the plant response to abiotic stress and photoperiod. In addition, the *JmjC* genes could enforce the demethylase activity to silence the redundant parts of the genome, which reaching the function to regulate the expression of related genes and ensure the structural and functional integrality of the genome (CUI et al., 2013). In *A. thaliana* and *O. sativa*, *AtJMJ30/32* and *OsJMJ705* helped them to resist the adversely environmental conditions by removing the methylation of H3K27me3 (WU et al., 2019). Therefore, we inferred that the orthologous *VrJMJ* genes of *AtJMJ30/32* and *OsJMJ705* might display a similar function in Mung bean. Under both PEG 6000 and NaCl stress treatments, *VrJMJ16*, *VrJMJ17*, and *VrJMJ18* were significantly up-regulated. Meanwhile most of VrJMJ proteins possessed NLSs and located

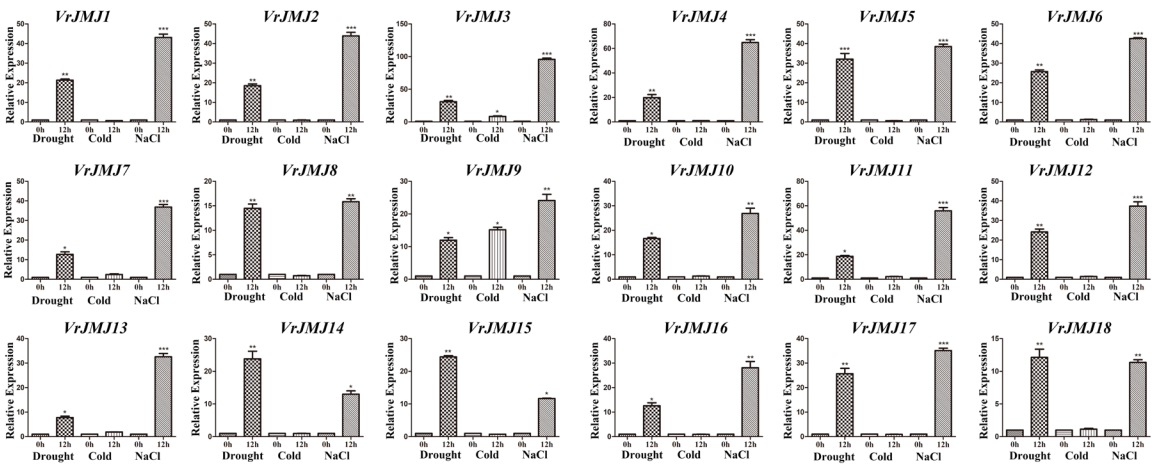

Figure 8 - Relative expression of *VrJMJ* genes under drought, cold, and NaCl treatments. The expression of *VrJMJ* genes at 0 h under each abiotic stress treatment was set to "1". Data are the mean ± standard errors of three independent replicates. Significant differences relative to the expression at 0 h under each abiotic stress treatment are indicated by asterisks (***P < 0.001; **P < 0.01; and *P < 0.05).

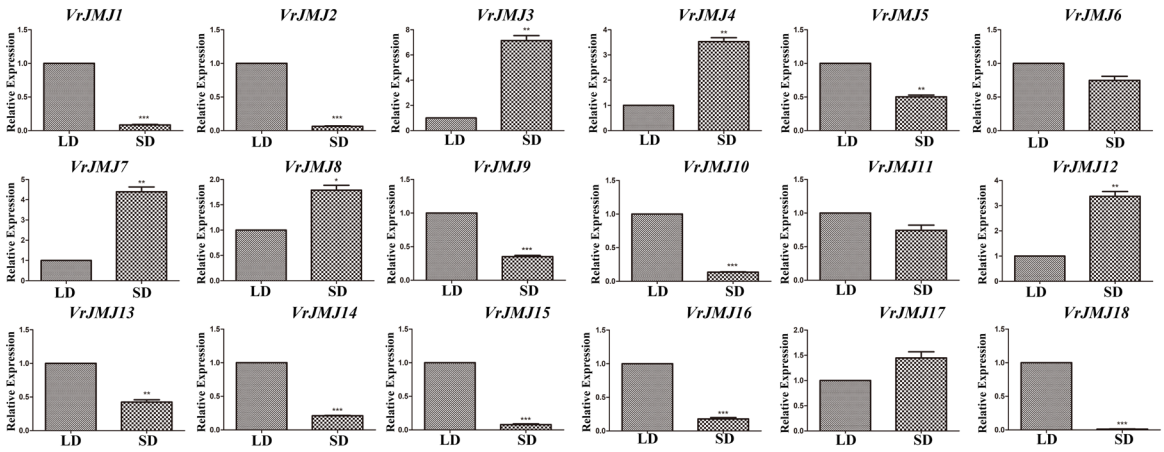

Figure 9 - Relative expression of *VrJMJ* genes under LD and SD treatments. The expression of *VrJMJ* genes under LD treatment was set to "1". Data are the mean ± standard errors of three independent replicates. Significant differences relative to the expression under LD conditions are indicated with asterisks (***P < 0.001; **P < 0.01; and *P < 0.05).

in the nucleus, which also provided the evidence to support the above theory. As we all known, JmjC domain didn't work alone during the demethylation. So far, some studies had revealed that the tandem ZnF_C2H2 domain at the C-terminus of the REF6 protein (*AtJMJ12*) could recognize the CTCTGYTY motif, then recruited the ATPase BRM to remodel chromatin in *A. thaliana* (CUI et al., 2016). As the orthologs of *AtJMJ12*, *VrJMJ16* and *VrJMJ18* were highly expressed under LD while *VrJMJ17* displayed a high expression level under SD treatment. This revealed that these three genes might be the same pathway genes to regulate the flowering time of Mung bean. In *A. thaliana*, *AtJMJ15* is a KDM5 clade gene with FYRN and FYRC domains. The increased expression of *AtJMJ15* preferentially down-regulated these H3K4me2/3-marked stress-related genes and enhanced the salt stress tolerance of mutants (SHEN et al., 2014). As the orthologous genes of *AtJMJ15*, *VrJMJ10* ~ 12 also exhibited a strong response to NaCl stress treatment. In KDM5 clade, *VrJMJ11* and *AtJMJ17* were both the PHD domain-containing genes, meanwhile *AtJMJ17* displayed crucial roles in response to osmotic stresses (HUANG et al., 2019). The *VrJMJ11*, an ortholog of *AtJMJ17*, was highly expressed under osmotic stresses which indicating its potential regulatory role in Mung bean.

In the promoter regions, cis-acting elements have displayed vital roles in the transcriptional initiation and regulation of gene expression (HERNANDEZ-GARCIA & FINER, 2014). We also uncovered some important cis-acting elements related to abiotic stress and light responses in the all Pro_{VrJMJs}, such as DRE1 (drought and osmotic stress), MBS (drought), and GT1-motif (light response). According to the expression profiles obtained in our study, almost all of *VrJMJ* genes were involved in the abiotic stress and light responses. Thus, we speculated that *VrJMJ* genes were functionally related to the abiotic stress or light responses in Mung bean. Meanwhile, our comparative acronym RT-qPCR analysis of these differential expressed *VrJMJ* genes also has provided novel insights into the responses of Mung bean to abiotic stress or light. Interestingly, *VrJMJ3* and *VrJMJ9* were remarkably up-expressed in responding to all three abiotic stresses, and *VrJMJ13* displayed potential role in the light-responsive regulation. However, the molecular mechanisms of how *VrJMJ* genes achieved their functions still needing further investigations.

CONCLUSION

In this study, we have conducted a genome-wide identification and expression analysis of the *JmjC* gene family in Mung bean. Based on their structural characteristics and phylogenetic relationships, a total of 18 *VrJMJ* genes have been identified and further separated into KDM3, KDM5. PKDM8, and PKDM9 clade. The structural profiles of 18 *VrJMJ* genes were considerably conservative among the same clade or subclade, which suggesting that they might have experienced differentiation and specification during the evolution. Interspecies co-collinearity analysis showed significant gene duplication events which have occurred during the Papilionoideae evolution.

According to the cis-acting elements analysis, all Pro_{VrJMJs} contained cis-acting elements responsive to different abiotic stresses or light. Furthermore, all *VrJMJ* genes were predominantly expressed in roots or leaves. Comparative expression profile analysis also revealed differing responses of *VrJMJ* genes to light, cold, and osmotic stresses. Our results provided valuable clues for further precise identification of the genetic diversity and specific functions of *JmjC* gene families in the *Vigna* genus.

DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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