Identification of Potential Target Transcription Factor Genes Regulated by Krüppel Homolog 1 in *Chilo suppressalis* (Lepidoptera: Crambidae)[1]

Suijie Kuang[2], Yan Tang[2], Qiao Gao[3], Hualiang He[3], Wenbing Ding[4], Jin Xue[4], Youzhi Li[5], and Lin Qiu[5,6]

Hunan Provincial Key Laboratory for Biology and Control of Plant Diseases and Insect Pests, College of Plant Protection, Hunan Agricultural University, Changsha, 410128, China

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Abstract Juvenile hormone (JH) is a major endocrine hormone that mediates development, metamorphosis, and reproduction in insects. It binds directly to its methoprene-tolerant receptor and recruits a heterodimer partner to form the JH–receptor complex that then activates a JH-inducible gene known as the *Krüppel homolog 1* (*Kr-h1*). There is evidence that this gene is a downstream factor mediating both physiological and biochemical processes; however, the functional mechanism of *Kr-h1* is largely unknown. Using the economically important rice (*Oryza sativa* L.) pest *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae) as a model, we used a combination of RNA interference (RNAi), high-throughput RNA sequencing, and real-time quantitative polymerase chain reaction (RT-qPCR) to identify candidate transcription factor (TF) genes that are regulated by *Kr-h1*. RNAi knockdown of *Kr-h1* identified the Zinc finger proteins, ZBTB, THAP, PAX, MYB, HSF, Homeobox, HMG, CSD, basic helix-loop-helix, STAT, RHD, and MBD families as regulated by *Kr-h1*. RT-qPCR confirmed the transcription levels of these putative TFs and indicated that knockdown of *Kr-h1* can induce or suppress the expression of these proteins in *C. suppressalis*. These results provide the basic information required for in-depth research on the TFs regulated by *Kr-h1* in *C. suppressalis* and other insects.

Key Words Krüppel homolog 1, *Chilo suppressalis*, transcription factor, RNA interference

Juvenile hormone (JH) is a sesquiterpenoid hormone synthesized and secreted by the corpora allata that regulates the development, metamorphosis, reproduction, and other physiological processes of insects (Bernardo and Dubrovsky 2012, Bilen et al. 2013, Flatt et al. 2005, Jindra et al. 2013, Riddiford 2012). Its primary role is to maintain the status quo during larval development, in close cooperation with molting

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[2]These authors contributed equally in this work.
[3]National Research Center of Engineering & Technology for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha, 410128, China.
[4]Hunan Provincial Engineering & Technology Research Center for Biopesticide and Formulation Processing, Changsha, 410128, China
[5]Hunan Provincial Key Laboratory for Biology and Control of Plant Diseases and Insect Pests, College of Plant Protection, Hunan Agricultural University, Changsha, 410128, China.
[6]Corresponding author (email: qiulin@hunau.edu.cn).

hormones (ecdysteroids) (Riddiford 2012). Although the mode of action of JH was described many years ago, the molecular mechanisms underlying JH were largely unknown until the candidate JH receptor, methoprene-tolerant (Met) protein, a basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family of transcription factors (TFs), first described in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Ashok et al. 1998, Wilson and Fabian 1986), was identified. Met homologs have since been identified in other taxa (Kayukawa et al. 2012, Konopova et al. 2011, Lin et al. 2015, Zhu et al. 2010). JH first binds to Met and then recruits the heterodimer partner steroid receptor coactivator (also called Taiman or FISC) to form an active JH–receptor complex that regulates the expression of JH-responsive genes (Furness et al. 2007; Jindra et al. 2013, 2015a, 2015b; Li et al. 2011; Partch and Gardner 2010; Zhang et al. 2011).

The TF Krüppel homolog 1 (Kr-h1), first discovered in *D. melanogaster* and homologous with the segmentation gene *Krüppel* (Beck et al. 2004, Pecasse et al. 2000), is one such gene that acts as a key regulator suppressing metamorphosis in insects. Kr-h1 contains eight C2-H2-type Zinc fingers (DNA binding motif) and has been shown to be the direct target of Met and to regulate insect development, metamorphosis, and vitellogenesis. In the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), functional analysis of Kr-h1 has revealed that knockdown of both *TcMet* and *TcKr-h1* causes precocious metamorphosis (Konopova and Jindra 2007, Minakuchi et al. 2009, Parthasarathy et al. 2008). In addition, RNA interference (RNAi) suppression of *Kr-h1* in *Pyrrhocoris apterus* (L.) (Hemiptera: Pyrrhocoridae) causes precocious metamorphosis at the early fourth-instar nymphs (Konopova et al. 2011), whereas overexpression of *Kr-h1* in *Drosophila* and *Bombyx mori* L. (Lepidoptera: Bombycidae) prevented pupation (Kayukawa et al. 2014, Minakuchi et al. 2008).

In addition to repressing metamorphosis, *Kr-h1* has been found to play a role in female reproduction by regulating vitellogenin and oogenesis in the mosquito *Aedes aegypti* (L.) (Diptera: Culicidae) (Ojani et al. 2018, Shin et al. 2012, Zou et al. 2013) and migratory locust *Locusta migratoria* L. (Orthoptera: Acrididae) (Song et al. 2014). Although RNAi suppression of Kr-h1 did not reduce fecundity in bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), it reduced hatchability (Gujar and Palli 2016).

It is now evident that Kr-h1 plays a crucial role in both metamorphosis and reproduction. Recent studies have revealed that Kr-h1 prevents the activation of metamorphosis by suppressing the expression of the *Broad-complex* (*BR-C*) and *Ecdysone induced protein 93F* (*E93*) genes (Belles and Santos 2014, Jindra et al. 2013). Mechanistically, Kr-h1 binds directly to the consensus Kr-h1 binding site (KBS) in the promoter regions of target genes to suppress their transcription (Kayukawa et al. 2016, 2017). It has recently been discovered that Kr-h1 suppresses steroidogenic enzyme by binding to the KBS regions of steroidogenic enzyme gene promotors in *D. melanogaster* and *B. mori*, thereby suppressing 20-hydroxyecdysone (20E) synthesis (Liu et al. 2018, Zhang et al. 2018). Although the role of Kr-h1 in insect development and reproduction is relatively well understood, the potential TF genes that are regulated by Kr-h1 remain largely unknown.

In this study, we used the rice (*Oryza sativa* L.) pest *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae) as a model species and used RNAi and high-throughput RNA sequencing to identify potential TFs that regulated by Kr-h1. The results

indicate that several TFs may be regulated by Kr-h1 to mediate development and reproduction in *C. suppressalis*.

Materials and Methods

Insect rearing. *Chilo suppressalis* larvae were collected from the campus greenhouse of Hunan Agricultural University in April 2017 and reared on water bamboo Zizania latifolia (Griseb.) Turcz. Stapf. at 28 ± 1°C and 80 ± 10% relative humidity under a 16-h photoperiod. Adults were fed on 10% sucrose.

RNA isolation and synthesis of first-strand cDNA. Total RNA was isolated from samples by using RNAiso Reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. Its purity and concentration were quantified with a spectrophotometer (2.1 > OD 260/280 > 1.8; NanoDrop™ 1000, Thermo Fisher Scientific, Waltham, MA). Complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit with gDNA eraser (TaKaRa) according to the manufacturer's recommendations.

RNAi. The target gene fragment was amplified from *C. suppressalis* pupal cDNA by using a PrimeSTAR HS DNA polymerase kit (TaKaRa) with a flanking T7 promoter and T7 terminator site. A segment of enhanced green fluorescent protein (EGFP; accession no. U55762) was the negative control for nonspecific effects in RNAi experiments. For double-stranded (ds)RNA synthesis, a T7 RiboMAX™ Express RNAi System (Promega, Madison, WI) was used to produce dsRNA against Kr-h1 and EGFP from approximately 1 mg of the DNA template. The integrity and concentration of the dsRNA obtained were verified by electrophoresis in 1% native agarose gel.

Five hundred nanoliters of ds*Kr-h1* solution (5 μg/μl) was injected into the abdomen of female pupae 24 and 48 h after pupation by using a nanoinjector (Drummond Scientific Co., Broomall, PA). The control group was injected with dsEGFP according to the same protocol. Four individuals were randomly selected for detection of RNAi efficiency and high-throughput RNA sequencing.

cDNA library construction and transcriptome analysis. Total RNA was extracted from individuals in the control and treatment groups with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA integrity was verified with gel electrophoresis, and its concentration was measured with a Qubit® RNA Assay Kit in a Qubit® 2.0 fluorometer (Life Technologies, Rockville, MD). We used 1.5 μg of total RNA to construct the cDNA library, which was sequenced using a HiSeq platform (Illumina, San Diego, CA).

A method adapted from Qiu et al. (2018) was used to produce clean reads, and a Trinity assembly was created using Trinity software based on the left.fq and right.fq (Grabherr et al. 2011). The putative function of unigenes was deduced by aligning them against Pfam (Protein family), KOG/COG (Clusters of Orthologous Groups of proteins), Nr (National Center for Biotechnology Information [NCBI] nonredundant protein sequences), and Nt (NCBI nonredundant nucleotide sequences).

Differential gene expression. The fragments per kilobase of transcripts per million mapped reads method was used to measure the differential expression of genes in the treatment and control groups (Mortazavi et al. 2008). DESeq R package (Version 1.10.1) was used to analyze the differential expression of genes

in each group. Benjamini–Hochberg approach was used to adjust *P* values to control the false discovery rate. Genes with an adjusted *P* value of <0.05 were considered differentially expressed.

Identification of TFs. TFs were verified by manually using BLASTX with an E-value cutoff of $\leq 10^{-5}$ to check their amino acid sequences against the NCBI nonredundant protein database.

RT-qPCR. Real-time quantitative polymerase chain reaction (RT-qPCR) was used to verify the expression of candidate TFs that were regulated by Kr-h1. Total RNA was isolated from the control and treatment (iEGFP and iKr-h1) groups, and cDNA was synthesized using a PrimeScript RT reagent kit with gDNA eraser (TaKaRa) as described above. Gene-specific primers (Table 2) were designed using the NCBI profile Server (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The *C. suppressalis elongation factor-1* was used as the internal reference gene for RT-qPCR (Hui et al. 2011, Zhu et al. 2016). Quantitative real-time PCR was conducted with a SYBR Premix ExTaq (Perfect Real Time) kit (TaKaRa) according to the manufacturer's instructions and a CFX 96 Touch System (Bio-Rad Laboratories, Hercules, CA). The efficiency of the RT-qPCR primers was assessed from a standard curve (cDNA concentration versus cycle threshold [Ct]) based on a fivefold dilution series of pupal cDNA corresponding to 1 mg of total RNA. The qPCR protocol used was that of Qiu et al. (2018).

Data analysis. Quantitative expression data were analyzed using the $2^{-\triangle\triangle Ct}$ method (Pfaffl 2001), and the statistical significance of differences in treatment group means were assessed with a one-way analysis of variance (ANOVA) in the Statistical Package for the Social Sciences program for Windows (SPSS, Chicago, IL).

Results

RNAi depletion of *Kr-h1* in C. *suppressalis* pupae. High-throughput sequencing combined with Kr-h1 RNAi was used to identify potential TFs that were regulated by Kr-h1. Injection of *Kr-h1* dsRNA significantly reduced transcription of *Kr-h1* by 77% compared with EGFP dsRNA (Fig. 1A). We identified 7,845 differentially expressed transcripts controlled by *Kr-h1* in female pupae (Fig. 1B, C).

***Kr-h1* RNAi depletion revealed differential expression of TFs.** Total RNA from dsRNA-treated pupae was subjected to high-throughput sequencing and screening to identify differentially expressed TFs. The TFs identified included Zinc finger-C2H2, Zinc finger-GATA, Zinc finger-LITAF (lipopolysaccharide-induced tumor necrosis factor–activating factor-like), Zinc finger-BED, ZBTB, THAP, PAX, MYB, HSF, Homeobox, HMG, CSD, bHLH, STAT, RHD, and MBD families (Table 1). Functional annotation identified that iKr-h1 downregulated 29 and upregulated 12 of the above-mentioned genes, suggesting that these 41 genes interact with *Kr-h1* to regulate development and reproduction.

Validation of high-throughput sequencing data with RT-qPCR. To verify the differential expression of candidate TFs, total RNA was isolated for analysis via RT-qPCR. Specific primers were designed based on the sequences of 14 randomly selected *Kr-h1*-regulated genes identifed by RNA high-throughput sequencing. For

Table 1. Differential expression of candidate transcription factor genes after RNA interference knockdown of Kr-h1 in *Chilo suppressalis*.

	Transcription Factor Family	Gene No.	Annotation (NR)	iEGFP FPKM	iKr-h1 FPKM	log2 Fold Change
Downregulation	Zinc finger-GATA	CL1640.Contig2_All	Transcription factor BCFI	12.48	0.24	−5.66
	Zinc finger-C2H2	CL569.Contig2_All	PREDICTED: ER lumen protein-retaining receptor	1.15	0.00	−8.15
		Unigene1796_All	PREDICTED: mediator of RNA polymerase II transcription subunit 15-like	175.30	3.68	−5.87
	Zinc finger-LITAF-like	Unigene18554_All	Collagen alpha-1(III) chain-like	1.00	0.00	−4.80
	Zinc finger-BED	Unigene18466_All	PREDICTED: zinc finger BED domain-containing protein 5-like	0.36	0.00	−6.44
	ZBTB	CL1287.Contig1_All	Cuticle protein 19.8-like precursor	13.89	0.045	−8.46
	THAP	Unigene3678_All	PREDICTED: calphotin-like	86.59	3.02	−4.92
		CL2994.Contig5_All	THAP domain-containing protein 5 isoform X2	5.46	0.29	−4.24
	PAX	CL1949.Contig8_All	Putative DNA-mediated transposase	0.91	0	−5.90
		CL2342.Contig1_All	Unnamed protein product	2.81	0.05	−5.59
	MYB	CL22.Contig2_All	PREDICTED: uncharacterized protein LOC105381158 isoform X7	3.97	0.07	−5.55

Table 1. Continued.

Transcription Factor Family	Gene No.	Annotation (NR)	iEGFP FPKM	iKr-h1 FPKM	log2 Fold Change
HSF	CL511.Contig2_All	PREDICTED: protein anoxia upregulated-like isoform X4	4.23	0.15	−4.73
Homeobox	CL3415.Contig1_All	Hypothetical protein KGM_205692	4.09	0	−9.96
	CL564.Contig3_All	P17/29C-like protein DDB_G0287399	1.38	0	−8.46
	CL1022.Contig7_All	PREDICTED: cuticle protein-like	12.92	0.10	−7.03
	CL1045.Contig7_All	PREDICTED: Pre-mRNA-processing-splicing factor 8-like	0.29	0	−5.22
	Unigene16614_All	PREDICTED: DEAD-box ATP-dependent RNA helicase 9-like	2.17	0	−4.90
	Unigene20653_All	PREDICTED: LOW QUALITY PROTEIN: fibril-forming collagen alpha chain-like	2.02	0.07	−4.90
	Unigene14824_All	Collagen alpha-1(I) chain-like	3.44	0.20	−4.02
	Unigene16459_All	Actin-binding LIM protein 3 isoform X2	4.06	0.25	−3.90

Table 1. Continued.

	Transcription Factor Family	Gene No.	Annotation (NR)	iEGFP FPKM	iKr-h1 FPKM	log2 Fold Change
	HMG	Unigene16623_All	Hypothetical protein FL83_24492, partial	36.98	0.39	−6.64
		CL442.Contig4_All	Glycine-rich cell wall structural protein	0.75	0	−5.38
		CL2860.Contig1_All	Larval cuticle protein A2B-like	4.87	0.29	−4.14
	CSD	CL3045.Contig1_All	PREDICTED: serine-arginine protein 55 isoform X3	7.72	0.08	−6.40
	bHLH	CL125.Contig4_All	Protein lingerer-like isoform X1	0.34	0	−5.69
		CL1313.Contig3_All	BAG domain-containing protein Samui-like isoform X1	7.19	0.34	−4.39
		CL275.Contig27_All	Twitchin	24.61	24.61	−9.04
		CL2502.Contig1_All	Hypothetical protein (*Paramecium tetraurelia* strain d4-2)	7.45	0.11	−6.04
		Unigene17244_All	Cross-beta structure silk protein 1	2.83	0.10	−4.75
Upregulation	THAP	Unigene9932_All	Zinc finger MYM-type protein 1-like	0.095	12.08	7.05
		CL2586.Contig1_All	PREDICTED: uncharacterized protein LOC106114734 isoform X2	0.09	4.77	5.70

Table 1. Continued.

Transcription Factor Family	Gene No.	Annotation (NR)	iEGFP FPKM	iKr-h1 FPKM	log2 Fold Change
STAT	Unigene7029_All	PREDICTED: alpha/beta-gliadin A-V-like	0.14	5.11	5.29
RHD	Unigene21569_All	PREDICTED: myotrophin-like	0.45	22.4	5.68
MBD	CL1074.Contig3_All	Protein FAM50 homolog	0	2.71	8.28
	CL2262.Contig1_All	PREDICTED: trichohyalin-like	0	1.9	6.72
HMG	CL1804.Contig1_All	PREDICTED: RNA-binding protein 28	0	0.74	6.63
	CL2348.Contig1_All	Uncharacterized protein LOC110373242 isoform X2	0.04	0.04	5.33
bHLH	CL290.Contig1_All	PREDICTED: uncharacterized protein LOC106129952 isoform X3	0	1.93	8.63
	CL2209.Contig8_All	PREDICTED: cell surface glycoprotein 1-like isoform X4	0	2.01	8.80
	CL2668.Contig2_All	PREDICTED: LOW QUALITY PROTEIN: single-minded homolog 1-like	0	0.57	5.82
	CL238.Contig8_All	Hypothetical protein ALC62_03547	0	0.38	5.57

NR: nonredundant proteins database
FPKM: fragments per kilobase million
EGFP: enhanced green fluorescent protein

Table 2. Specific primers used in real-time quantitative polymerase chain reaction (RT-qPCR) and RNA interference (RNAi).[a]

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
RT-qPCR		
Cs-CL569.Contig2_All	TTGCAATTCTGCCGCAGCTA	GGCCCTATAGGAACCGAGAGC
Cs-Unigene1796_All	TAGTTGTGGGTGCGGGTTGT	AGCAACCAAGTCCCGAGTCC
Cs-CL1640.Contig2_All	CCGCAGTCTTGATGTTCCGC	ACGCGTGCGGACTCTACTAC
Cs-Unigene18554_All	GCTGACCGGGCGTTCCT	TACCCAGGACAACCCGCA
Cs-Unigene18466_All	GCCGAAGCTGATATGAGACTCCAA	ACTTCACCACTTGGTAAGGACAA
Cs-CL1287.Contig1_All	AATCGGTACTGGGGCAAGGG	CAGCGGCTTATGGCAGTGTG
Cs-Unigene3678_All	CACTGCAGCGGTGTATCGTG	GTGGTGCCAGCTCGTATTGC
Cs-CL1804.Contig1_All	GCGCTACTCGCTCGACAAAC	ACGGTCTCACGGTGTTCCTG
Cs-CL2348.Contig1_All	AGTACCCAATGTGTTTGCACGA	TACGCCGGAAATCCTGCACC
Cs-Unigene16623_All	ACGCATCGCGTTTGCGTAAG	AAGTCGGCTGCCTGCTATGT
Cs-CL442.Contig4_All	TGGGGACGCTGTTCTTCAGG	TTCTCGTCCGGTGGCTTCTC
Cs-CL2860.Contig1_All	TGCACCACAGCGTTGAAACC	CCACACTGGCGACTCCAAGA
Cs-CL290.Contig1_All	AGGAACCGGCGTCAGTGAAA	TCGGAGTTGGCCTTGCTGAA
Cs-CL2209.Contig8_All	GGTGTGTTGGCTAAAGGCGG	CTGCGAAAGCAACAGCGAGT
Cs-CL2668.Contig2_All	CGCGAGCAGTTCCTCCTGTA	TCCCGCCAAAATTGCCTTCG
Cs-CL238.Contig8_All	AGCAGTGTTCTCCTCAGCACT	GATGCATGCCCGGTGTTCTG
Cs-CL125.Contig4_All	ACATTACCGCACCGACCACT	ACATTACCGCACCGACCACT
Cs-EF1	TGAACCCCCATACAGCGAATCC	TCTCCGTGCCAACCAGAAATAGG

Table 2. Continued.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
RNAi		
CsKr-h1	GGATCCTAATACGACTCACTATAGG GCGATTGGTTCGCCACTACAGGA	GGATCCTAATACGACTCACTATAGG GTGAGCTTCCATTTGCTTCTTTGT
EGFP	GGATCCTAATACGACTCACTATAGG GAGGACGACGGCAACTACAAG	GGATCCTAATACGACTCACTATAGG GGTCCATGCCGAGAGTGATCC

[a] T7 promoter sequence is underlined.

the bHLH families, two genes, CL2668.Contig2_All and CL238.Contig8_All, were more highly expressed in the treatment group than in the control group. This result is consistent with that of the transcriptome analysis and confirms that the mRNA levels of these two genes increased in pupae injected with *Kr-h1* dsRNA. Expression levels of the other tested genes, CL290.Contig1_All and CL2209.Contig8_All, did not significantly differ between the treatment and control groups (Fig. 2A). With respect to the Zinc finger-C2H2 families, expression of CL569.Contig2_All was slightly higher in the treatment group but that of Unigene1796_All was not significantly different from that in the control group. These results are inconsistent with those from the high-throughput sequencing data (Fig. 2B). With respect to the HMG TFs, expression of CL1804.Contig1_All in the treatment group was not significantly different from that of the control group, whereas expression of CL2860.Contig1_All was lower in the treatment group than in the control and that of Unigene16623_All was higher (Fig. 2C). We also analyzed the expression levels of CL1287.Contig1_All, Unigene3678_All, and Unigene18554_All, which belong to the ZBTB, THAP, and Zinc finger-LITAF-like families, respectively. Expression of all three genes was lower in the treatment group than in the control (Fig. 2D, F, G). Contrary to the results of the transcriptome analysis, RT-qPCR indicated that expression of CL1640.Contig2_All (Zinc finger-GATA) and Unigene18466_All (Zinc finger-BED) differed between the treatment and control groups (Fig. 2E, H).

Discussion

Kr-h1 has been shown to be induced by JH hormone and to be involved in preventing metamorphosis and promoting reproduction in both holometabolous and hemimetabolous insects (Konopova et al. 2011, Lozano and Belles 2011, Minakuchi et al. 2011, Parthasarathy et al. 2010, Zhu et al. 2010). Consequently, Kr-h1 continues to be one of the most important target molecules for understanding the JH signaling pathway mechanism (Lozano and Belles 2011).

Kr-h1 is not only induced by JH to repress metamorphosis but also is induced by ecdysone signaling (Beckstead et al. 2005, Pecasse et al. 2000). There is evidence to suggest that JH is not necessary to induce *Kr-h1* expression in *B. mori* and, like *BmKr*-h1, *D. melanogaster Kr-h1* can also be upregulated by 20E (Beckstead et al. 2005, Pecasse et al. 2000). *Kr-h1* plays a key role in reproduction; however, previous studies have found that *Kr-h1* does not directly induce *Vg* expression, implying that Kr-h1 activates other regulators of *Vg* expression via other TFs in the fat body (Song et al. 2014). We used RNAi and RNA high-throughput sequencing to identify the candidate TFs that regulated by *Kr-h1* (Table 1) and RT-qPCR to confirm the expression of TF genes that were induced and suppressed by iKr-h1 (Fig. 2).

We screened Zinc finger TF families, including Zinc finger-C2H2, Zinc finger-GATA, Zinc finger-LITAF, and Zinc finger-BED. TFs (GATAs) have been demonstrated to be involved in the proliferation, differentiation, and development of eukaryotes (Patient and McGhee 2002). In some blood-feeding insects, GATA is synthesized after a blood meal and it, in turn, activates the expression of *Vg* (Attardo et al. 2005, Boldbaatar et al. 2010, Park et al. 2006, Sun et al. 2018). Functional analysis has shown that *Bm*GATAβ4 binds directly to the GATA *cis*-

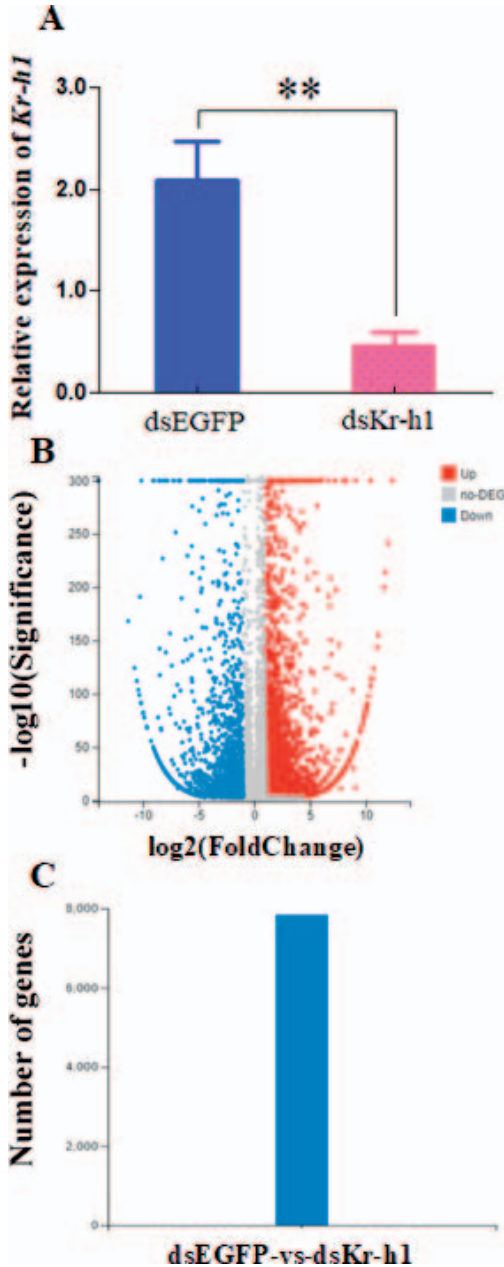

Fig. 1. Transcriptomic analysis of differentially expressed genes after RNA interference knockdown of *Kr-h1* in *Chilo suppressalis*. (A) Transcription level of *CsKr-h1* determined by quantitative real-time polymerase chain reaction in pupae treated with *Cs*Kr-h1 double-stranded RNAs (dsRNAs). Enhanced green fluorescent protein (EGFP) was the

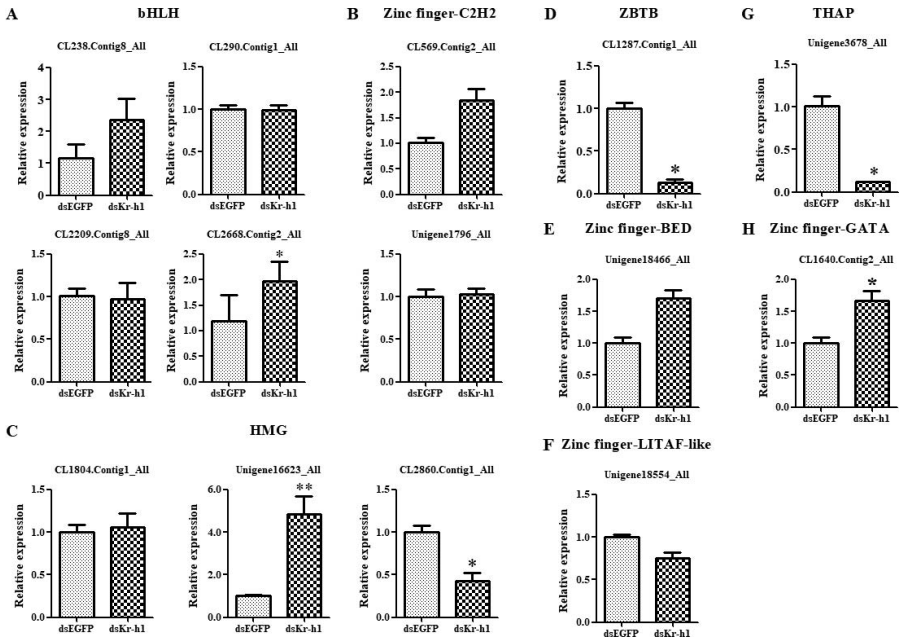

Fig. 2. Depletion of *Kr-h1* changed the expression of transcription factors in female *Chilo suppressalis* pupae. The *EF1* gene was the reference gene for normalization. Values are expressed as the means ± SEM of three independent biological replicates. Asterisks indicate significant differences (analysis of variance followed by Tukey's honestly significant difference post hoc test). *, $P < 0.05$; **, $P < 0.01$. (A) bHLH families. (B) Zinc finger-C2H2 families. (C) HMG families. (D) ZBTB families. (E) Zinc finger-BED. (F) Zinc finger-LITAF-like. (G) THAP family. (H) Zinc finger-GATA.

response element 1 (CRE1) and CRE2 in the promoter of *Bm*Vg to upregulate *Vg* expression (Liu et al. 2019). Although Kr-h1 may induce *Vg* expression via the JH signaling pathway, there is no evidence that it binds directly to the *Vg* promoter. Consequently, we speculate that Kr-h1 regulates the transcription of *Vg* in combination with GATAs or antagonized GATAs.

We postulate that Kr-h1 belongs to Zinc finger families that directly bind to the *Kr-h1* binding site (KBS) to promote the pupal specifier gene *Broad-Complex* (*BR-C*)

←

negative control. Asterisks indicate significant differences (analysis of variance followed by Tukey's honestly significant difference post hoc test). *, $P < 0.05$; **, $P < 0.01$. (B) Volcano plots of differentially expressed genes in pupae injected with Kr-h1 dsRNA. (C) Numbers of differentially expressed genes in the treatment (Kr-h1 dsRNA) and control (EGFP dsRNA) groups.

and the adult specifier gene *Ecdysone induced protein* (*E93*), thereby suppressing larval-to-pupal transition and adult metamorphosis, respectively (Belles and Santos 2014; Jindra et al. 2013; Kayukawa et al. 2016, 2017). Kr-h1 has also recently been found to bind directly to KBS in the promoter region of steroidogenic enzyme genes to downregulate ecdysone biosynthesis in *Drosophila* and *Bombyx* (Zhang et al. 2018). As part of this study, we confirmed the transcription levels of *E93* and *BR-C* in *C. suppressalis* with qPCR and iKr-h1, and the results were consistent with those of previous studies (data not shown). Based on our qPCR data (Fig. 2), the expression of other candidate Zinc finger TF genes were up- or downregulated by RNAi; however, the mechanism regulating the interaction between Kr-h1 and these factors needs further study.

Our results identified bHLHs family genes as likely TFs, and transcripts of CL238.Contig8_All and CL2668.Contig2_All were higher in the treatment than in the control group, suggesting that this was due to the knockdown of *Kr-h1*. Met is a well-established JH receptor that belongs to the bHLH-PAS TF family. Previous studies have demonstrated that the bHLH domain is indispensable for inducing *Kr-h1* transcription (Cui et al. 2014); hence, we suspect that bHLH proteins may mediate Kr-h1 expression either directly or indirectly by interacting with other bHLH factors like Met via JH signaling. Functional analysis suggests that Kr-h1 mediates the bHLH gene to regulate develoment and reproduction, but confirming this requires further gene identification and characterization.

In conclusion, a combination of RNAi, high-throughput sequencing, and qPCR identified candidate TFs that regulated by *Kr-h1* in *C. suppressalis*, including members of the bHLH, Zinc finger, and other protein families. This the first study to comprehensively identify candidate TFs that regulated by Kr-h1 in *C. suppressalis*. Consequently, our results provide a useful foundation for future research on these proteins in both *C. suppressalis* and other insect pests.

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