**TmIKKε Is Required to Confer Protection Against Gram-Negative Bacteria, *E. coli* by the Regulation of Antimicrobial Peptide Production in the *Tenebrio molitor* Fat Body**

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The inhibitor of nuclear factor-kappa B (NF-κB) kinase (IKK) is the core regulator of the NF-κB pathway against pathogenic invasion in vertebrates or invertebrates. IKKβ, -ε and -γ have pivotal roles in the Toll and immune deficiency (IMD) pathways. In this study, a homolog of IKKε (*TmIKKε*) was identified from *Tenebrio molitor* RNA sequence database and functionally characterized for its role in regulating immune signaling pathways in insects. The *TmIKKε* gene is characterized by two exons and one intron comprising an open reading frame (ORF) of 2,196 bp that putatively encodes a polypeptide of 731 amino acid residues. *TmIKKε* contains a serine/threonine protein kinases catalytic domain. Phylogenetic analysis established the close homology of *TmIKKε* to *Tribolium castaneum* IKKε (*TcIKKε*) and its proximity with other IKK-related kinases. The expression of *TmIKKε* mRNA was elevated in the gut, integument, and hemocytes of the last-instar larva and the fat body, Malpighian tubules, and testis of 5-day-old adults. *TmIKKε* expression was significantly induced by *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* challenge in whole larvae and tissues, such as hemocytes, gut, and fat body. The knockdown of the *TmIKKε* messenger RNA (mRNA) expression significantly reduced the survival of the larvae against microbial challenges. Further, we investigated the induction patterns of 14 *T. molitor* antimicrobial peptides (AMPs) genes in *TmIKKε*-silencing model after microbial challenge. While in hemocytes, the transcriptional regulation of most AMPs was negatively regulated in the gut and fat body tissue of *T. molitor*, AMPs, such as *TmTenecin 1*, *TmTenecin 4*, *TmDefensin*, *TmColeoptericin A*, *TmColeoptericin B*, *TmAttacin 1a*, and *TmAttacin 2*, were positively regulated in *TmIKKε*-silenced individuals after microbial challenge. Collectively, the results implicate *TmIKKε* as an important factor in antimicrobial innate immune responses in *T. molitor*.

**Keywords:** *Tenebrio molitor*, IMD pathway, IKKε, antimicrobial peptides, RNAi
INTRODUCTION

The innate immune response represents the first line of defense in vertebrates and it is the only defense arsenal in invertebrates against microbial infections (Hoffmann et al., 1999). This is because of a lack of adaptive immune strategy in invertebrates that necessitates the over-reliance on the innate immune cascades for defense against microbial infection (Hoffmann et al., 1999; Li and Xiang, 2013). Antimicrobial peptides (AMPs) production represents one of the crucial effector mechanisms of innate immunity in insects. AMPs attribute insects most resistant to bacterial infections and shed applications as novel microbicides (Wu et al., 2018). Since the discovery of the first insect AMP called cecropin from the giant silk moth Hyalophora cecropia (Hultmark et al., 1980), over 150 AMPs have been identified, purified, and characterized from insects. Drosomycin, an antifungal peptide, and dipteracin, an antibacterial peptide, have been identified and characterized in Drosophila melanogaster (Lemaitre et al., 1996; Nicolas et al., 1996). Another attacin gene identified from Spodoptera exigua has antimicrobial activity against Escherichia coli DH5α strain, Pseudomonas cichorii, Bacillus subtilis, and Listeria monocytogenes (Bang et al., 2012). In the Tenebrio model, in silico analysis and induction patterns of AMP genes that include tenecin-1 (defensin family), -2 (coleoptericin family), -3 (thaumatin-like protein family), and -4 (attacin family), thaumatin-like protein (TLP)-1, and -2, Defensin and Defensin-like, Coleoptericin-A, -B, and -C, and Cecropin-2 have been studied (Kim et al., 1998, 2017; Roh et al., 2009; Chae et al., 2012; Johnston et al., 2013; Zhu et al., 2014; Noh and Jo, 2016; Jo et al., 2018; Maistrou et al., 2018; Jang et al., 2020a,b; Ali Mohammadie Kojour et al., 2021).

In insects, AMPs are induced through the activation of two key signaling cascade mechanisms – the Toll and immune deficiency (IMD) pathways. In Drosophila, the IκB kinase (DmIKK) complex, which is the major component of the IMD pathway, stimulates the activation of the nuclear factor-kappa B (NF-κB) protein Relish by phosphorylation (Georgel et al., 2001; Vidal et al., 2001; Chen et al., 2002; Choe et al., 2002, 2005; Gottar et al., 2002; Ramet et al., 2002; Cha et al., 2003; Leulier et al., 2003; Silverman et al., 2003; Kaneko et al., 2004). In addition, lipopolysaccharide (LPS) and peptidoglycan (PGN) from gram-negative bacteria stimulate the IMD pathway in Drosophila (Leulier et al., 2003; Kaneko et al., 2004). Further, IKKs in D. melanogaster not only plays a principal role in IMD regulation but also phosphorylates DIAP1 and controls janus kinase (JNK) activation and apoptosis (downstream of IMD) (Gan et al., 2021). This study demonstrated that the activation of the IMD pathway against sindbis virus (SINV) infection is highly dependent on the microbiota present in the gut (GT) of Aedes aegypti (Barletta et al., 2017). In another insect model, Bombyx mori, the expression of CecropinA1 is regulated by Relish in response to gram-negative bacteria (Hua et al., 2016). In addition, the direct functions of B. mori peptidoglycan recognition protein L1 (BmPGRP-L1) and BmIMD in the IMD pathway are suspected but not clearly identified (Zhan et al., 2018). In Plutella xylostella, IMD RNA interference (RNAi) affected the expression of the downstream genes of the IMD pathway (Lin et al., 2018). In fact, several studies have characterized the Toll and IMD pathway responses of diverse insects including Plautia stali stink bugs (Nishide et al., 2019), aphids (International Aphid Genomics Consortium, 2010), kissing bugs (Mesquita et al., 2015; Salcedo-Porras and Lowenberger, 2019), or other arthropods such as Tetranynchus mites (Palmer and Jiggins, 2015; Santos-Matos et al., 2017) and shrimp (Li et al., 2019). Additionally, the crosstalk between Toll and IMD pathways in stink bugs by the RNAi experiments of Toll and IMD pathways-related genes, including PsImd, PsMyD88, PsDorsal, PsPGRP-L1a, PsPGRP-L1b, PsPGRP-L2, PsLysM, PsGNNBP1 have been suggested (Nishide et al., 2019).

In the beetle Tribolium castaneum, the PGRP-LA may be a pivotal sensor of the IMD pathway for both gram-negative and gram-positive bacteria, and both PGRP-LC and -LE acts as IMD pathway-associated sensors, mainly for gram-negative bacteria (Koyama et al., 2015). Based on comparative genomic analysis, around 300 candidate defense proteins were identified and clustered depending on the immune pathway such as Toll, IMD, and JAK-STAT pathways (Zou et al., 2007). Inducible immune-related genes, including Toll, PGRP, and AMP genes such as ferritin, defensin, and others against crude LPS were identified using the suppression subtractive hybridization (SSH) method, and antifungal activity of recombinant TLP was assayed in T. castaneum (Altincicek et al., 2008).

In contrast, a comprehensive study of the IMD pathway in innate immune responses against infections by various pathogens has been partially performed in T. molitor with the functional characterization of TmPGRP-LE, TmIKKα, and TmRelish (Tindwa et al., 2013; Jo et al., 2019; Ali Mohammadie Kojour et al., 2020; Keshavarz et al., 2020a,b,c,d; Ko et al., 2020). In this study, we identified the IκB kinase ε (IKKe) gene, one of the important components for the IMD pathway, from T. molitor RNA and DNA sequence database. We investigated the mRNA expression patterns of TmIKKs depending on different developmental stages, tissues, and microbial challenges to the host. Moreover, we investigated the effects of TmIKKs-specific knockdown on larval mortality, AMP production, and expression of NF-κB genes against various pathogens. The findings that IKKε knockdown beetles are especially susceptible to E. coli but not a gram-positive bacterium or fungus parallels a recent study wherein fruit flies lacking their major AMP genes were specifically susceptible to gram-negative bacteria, but not so much gram-positive bacteria or fungi (Hanson et al., 2019). Collectively, our data provide a better understanding of the IMD pathway in the Tenebrio innate immune response.

MATERIALS AND METHODS

Insect Rearing

Tenebrio molitor larvae were reared in the dark at 27°C ± 1°C and 60% ± 5% relative humidity in an environmental chamber established in the laboratory. The reared larvae were fed an artificial diet (170 g wheat flour, 20 g roasted soy flour, 10 g protein, 100 g wheat bran, 0.5 g sorbic acid, 0.5 mL...
The concentration of microbial cells was adjusted to 1×, was measured using a spectrophotometer (Eppendorf, Germany). PBS and the optical density at 600 nm (OD600) was harvested and washed twice in 1× TmIKK sequences of T. castaneum IKK using EST databases. Local-blastn analysis was performed sequencing (RNA-seq) (unpublished) and expressed sequence DNA (cDNA) translation and predictions of the deduced protein was retrieved using the blastp algorithm, respectively, on the National Center for DNA (cDNA) translation and predictions of the deduced protein were analyzed using BioPHP mini tools software (http://www.biophp.org). FGENESH eukaryotic gene prediction was used to identify and in silico Analysis of TmIKKe The TmIKKe sequence was retrieved from T. molitor RNA sequencing (RNA-seq) (unpublished) and expressed sequence tag (EST) databases. Local-blastn analysis was performed using T. castaneum IKKe amino acid sequence (EEZ99267.2) as a query. The full-length cDNA and deduced amino acid sequences of TmIKKe were determined using the blastx and blastp algorithm, respectively, on the National Center for Biotechnology Information (NCBI) website. Complementary DNA (cDNA) translation and predictions of the deduced protein were analyzed using BioPHP mini tools software (http://www.biophp.org). FGENESH eukaryotic gene prediction was used to predict the TmIKKe open reading frame (ORF) region. The domain architecture of the protein sequences was retrieved using the InterProScan domain analysis program. Representative IKKe protein sequences from other insects were obtained from GenBank and were used for multiple sequence alignments, and percentage identity analysis using Clustal X2.1 (Larkin et al., 2007). A phylogenetic tree was constructed based on the amino acid sequence alignments using the Maximum likelihood method (bootstrap trial set to 1000) with IKKs/TBK1 proteins from representative insects (Supplementary Table 1). The phylogram was analyzed using Tree Explorer view with the Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 program (Kumar et al., 2016) (https://megasoftware.net).

Sample Collection and Microorganism Challenge The TmIKKe mRNA expression was investigated in different developmental stages of T. molitor, eggs (EG), young instar larvae (YL; 10th–12th instar larvae), late-instar larvae (LL; 19th–20th instar larvae), prepupae (PP), 1- to 7-day-old pupae (P1–P7), and 1- to 5-day-old adults (A1–A5). TmIKKe mRNA expression was also measured in the different tissues that included integument (IT), hemocytes (HC), GT, fat body (FB), and Malpighian tubules (MT) dissected under a stereoscopic microscope (SMZ645, Nikon, Japan). The tissues were dissected from both LL and adults. Ovary (OV) and testis (TE) were additionally dissected from 5-day-old adults.

To investigate induction patterns of TmIKKe mRNA against microbial challenge, 1× cells/larva of E. coli and S. aureus, or 5× cells/larva of C. albicans were injected into 12th–15th instar larvae using microinjector with micropipillary. Samples (whole body, HC, GT, and FB) were collected at 3, 6, 9, 12, and 24 h following injection of microorganisms.

Total RNA Extraction and cDNA Synthesis The total RNA was isolated from the developmental stages, tissues, and time-course samples using a Clear-STM Total RNA extraction kit (Invirustech Co., Gwangju, South Korea) according to the manufacturer’s instructions. The total RNA (2µg) was used as the template to synthesize cDNA using the Oligo(dT)12–18 primer on MyGenie96 Thermal Block (Bioneer, South Korea) and AccuPower® RT PreMix (Bioneer) according to the manufacturer’s instructions. The cDNA was stored at −20°C until required.

Expression and Induction Analysis of the TmIKKe mRNA The relative expression level of TmIKKe mRNA was investigated by performing quantitative real-time polymerase chain reaction (qRT-PCR) using an AccuPower® 2× Greenstar™ qPCR Master Mix (Bioneer, Daejeon, Korea) and synthesized cDNAs, and TmIKKe gene-specific primers were designed using the Primer 3 plus program (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Supplementary Table 2). The PCR conditions included an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 30 s. The qRT-PCR assays were performed on an AriaMx Real-Time PCR System (Agilent Technologies, United States). The results were analyzed using AriaMx Real-Time PCR software. The 2−ΔΔCT method (Livak and Schmittgen, 2001) was employed to analyze the TmIKKe mRNA expression levels. The mRNA expression levels were normalized to those of T. molitor ribosomal protein L27a (TmL27a), which acted as an internal control. The results represent mean ± standard error (SE) of three biological replicates (3 pools of 20 T. molitor larvae).

TmIKKe Gene-Silencing For the RNA interference (RNAi) experiments of TmIKKe, a double-strand RNA (dsRNA) fragment of TmIKKe gene was synthesized. Briefly, dsDNA fragment of TmIKKe was amplified using PCR with gene-specific primers conjugated with T7 promoter sequences (Supplementary Table 2). The primers were designed using Snapdragons software (https://www.flyrnai.org) to prevent any cross-silencing effects. The primary PCR for the TmIKKe gene was carried out using an AccuPower Pfu
PCR PreMix (Bioneer) with cDNA and specific primers for the TmIKKε gene (Supplementary Table 2). The second PCR was conducted with primers tailed with T7 promoter sequences and 100× dilution of the second PCR products.

Polymerase chain reaction was conducted using an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 1 min, and extension at 72°C for 30 s on a MyGenie96 Thermal Block (Bioneer). The PCR products purified using the AccuPrep® PCR Purification Kit (Bioneer) were used to synthesize the dsRNA using the EZ1™ T7 High Yield in vitro transcription kit (Enzynomics, South Korea). The dsRNA for enhanced green fluorescent protein (dsEGFP) synthesized from pEGFP-C1 plasmid DNA as described above acted as a negative control. The dsRNA products were purified using the phenol:chloroform:isoamyl alcohol (PCI) method, precipitated from pEGFP-C1 plasmid DNA as described above acted as a negative control. The dsRNA products were purified using the phenol:chloroform:isoamyl alcohol (PCI) method, precipitated from pEGFP-C1 plasmid DNA as described above acted as a negative control. The dsRNA products were purified using the phenol:chloroform:isoamyl alcohol (PCI) method, precipitated from pEGFP-C1 plasmid DNA as described above acted as a negative control. The dsRNA products were purified using the phenol:chloroform:isoamyl alcohol (PCI) method, precipitated from pEGFP-C1 plasmid DNA as described above acted as a negative control. The dsRNA products were purified using the phenol:chloroform:isoamyl alcohol (PCI) method, precipitated from pEGFP-C1 plasmid DNA as described above acted as a negative control. The dsRNA products were purified using the phenol:chloroform:isoamyl alcohol (PCI) method, precipitated from pEGFP-C1 plasmid DNA as described above acted as a negative control. The dsRNA products were purified using the phenol:chloroform:isoamyl alcohol (PCI) method, precipitated from pEGFP-C1 plasmid DNA as described above acted as a negative control. The dsRNA products were purified using the phenol:chloroform:isoamyl alcohol (PCI) method, precipitated from pEGFP-C1 plasmid DNA as described above acted as a negative control. The dsRNA products were purified using the phenol:chloroform:isoamyl alcohol (PCI) method, precipitated from pEGFP-C1 plasmid DNA as described above acted as a negative control. The dsRNA products were purified using the phenol:chloroform:isoamyl alcohol (PCI) method, precipitated from pEGFP-C1 plasmid DNA as described above acted as a negative control.

To measure mortality, microorganisms (1 × 10^6 cells/larva) were injected into TmIKKε gene-silenced T. molitor larvae. Dead larvae were counted each day for up to 10 days following the injection of the microorganisms. Ten insect larvae were used for each group in the mortality assay. Each assay was performed in triplicate. Kaplan–Meier method was used to plot cumulative survival curves of larvae after inoculation, the log-rank chi-squared test was performed as mentioned above using the AMP and NF-κB gene specific primers.

**Mortality Assay**

To measure mortality, microorganisms (1 × 10^6 cells/µl of E. coli or S. aureus, and 5 × 10^4 cells/µl of C. albicans) were injected into TmIKKε gene-silenced T. molitor larvae. Dead larvae were counted each day for up to 10 days following the injection of the microorganisms. Ten insect larvae were used for each group in the mortality assay. Each assay was performed in triplicate. Kaplan–Meier method was used to plot cumulative survival curves of larvae after inoculation, the log-rank chi-squared test was used to assess differences in survival between treatments (Goel et al., 2010).

**Effects of TmIKKε RNAi on the Expression of Antimicrobial Peptide and NF-κB Genes**

To further characterize the function of the TmIKKε gene in the humoral innate immune response, the TmIKKε silenced individuals were challenged with microorganisms and the expression levels of 14 AMP genes as well as three NF-κB genes were investigated. After treatment of TmIKKε dsRNA into T. molitor 10th to 12th instar larvae, E. coli (1 × 10^6 cells/larva), S. aureus (1 × 10^6 cells/larva), or C. albicans (5 × 10^4 cells/larva) were injected into T. molitor larvae. Twenty-four hours post-injection, over 20 larvae (as a group) were dissected and the samples (HC, GT, and FB) were collected. 1 × PBS was used as an injection control. The expression levels of the following 14 AMP genes were measured by qRT-PCR with 14 AMP gene-specific primers (Supplementary Table 2): TmTene1, 2, 3, and 4 (TmTene1, 2, 3, and 4), TmDefensin and TmDefensin-like (TmDef and TmDef-like), TmCecropin A and B (TmCec A and B), TmAnotacin 1a, 1b, and 2 (TmAnot 1a, 1b, and 2), TmCecropin 2 (TmCec2), and TmThaumatin-like protein 1 and 2 (TmLP1 and 2). In addition, the expression profiles of NF-κB genes such as TmDorsal isoform X1 and X2 (TmDorX1 and X2), and TmRelish, were investigated by qRT-PCR. A relative quantitative PCR was performed as mentioned above using the AMP and NF-κB gene specific primers.

**Statistical Analyses**

All experiments were carried out in triplicate and the data were subjected to one-way ANOVA. Tukey’s multiple range tests were used to evaluate the difference between groups (p < 0.05).

**RESULTS**

**Gene Organization, Open Reading Frame and in silico Analyses of TmIKKε**

The organization of the TmIKKε gene was deciphered from the T. molitor nucleotide database using the TcIKKε amino acid sequence as a query in a tBLASTn analysis. TmIKKε includes two exons interrupted by a single intron (Figure 1A and Supplementary Figure 1). Exon 1 and exon 2 of TmIKKε were 423 bp and 1,773 bp, respectively. The ORF sequence of 2,196 bp starts with the “ATG” initiation codon and ends with the “TGA” stop codon. The ORF sequence of TmIKKε encoded a protein of 731 amino acid residues (Supplementary Figure 2). Domain analysis predicted a serine/threonine-protein kinase catalytic domain (residues 12–258), a ubiquitin-like domain (residues 310–387), and a TANK-binding kinase 1 coiled-coil domain 1 (residues 412–653) in TmIKKε protein. A 5’-untranslated region (UTR) sequence of 581 bp and 3’-UTR of 930 bp was also predicted for TmIKKε. The 3’-UTR sequence contains a consensus polyadenylation signal (AATAAA) located in 173–178 bp following termination codon (TGA). The TmIKKε cDNA sequence and deduced protein sequence have been submitted to GenBank (GenBank ID: MZ708789).

The alignment of the predicted amino acid sequence (restricted to the serine/threonine protein kinase catalytic domain) of TmIKKε with IKKs and serine/threonine protein kinase TBK1 from other known insect IKKs revealed a high degree of conservation (Figure 1B). A phylogenetic tree was constructed using the Clustal X2 alignment file and MEGA 7.0 program to assess the evolutionary position of TmIKKε among the orthologs (Figure 1C). Homo sapiens IKKs (HsIKKs) was used as the outgroup in the phylogenetic analysis. Two clear clade divisions were observed. TmIKKε was placed with TcIKKs and TBK1 from other beetles (AgTBK1, LdTBK1, and AtTBK1). The same clade also placed the IKKs isoforms from the order Diptera in a separate cluster. The high bootstrap values supported the tree topology in this clade. While the mosquito IKKs/TBK1 formed one sub-cluster, the Drosophila and blowfly IKKs/TBK1 formed the other sub-cluster. In the second clade, the orthologs of other insect IKKs proteins were placed.

**Expression of TmIKKε During Development and in Different Tissues**

In order to examine the TmIKKε mRNA expression during development and in different tissues of T. molitor, we performed
qRT-PCR using SYBR Green dye-binding assay. Developmental expression patterns indicate that TmIKKε mRNA was highly expressed in the pupal stage-2, -3, and -4 followed by variable expression in the adult stages (Figure 2A). Further, the expression of TmIKKε mRNA was measured in tissues of T. molitor late-instar larvae and 5-day old adults using qRT-PCR. In the
late-instar larval tissues, the TmIKKε mRNA was expressed in a tissue-dependent manner (Figure 2B). TmIKKε mRNA was found to be higher (especially in FB, MT, and TE) in the 5-day-old adult T. molitor (Figure 2C).

Temporal Expression of TmIKKε After Microbial Infection

To understand the biological function of TmIKKε in innate immunity of T. molitor, the mRNA expression levels were monitored at different time-points (3, 6, 9, 12, and 24 h) in whole-larvae, HC, GT, and FB after exposure to PBS (as injection controls), and microorganisms (E. coli, S. aureus, and C. albicans) challenge (Figure 3). The expression level of TmIKKε at various time points was analyzed relative to PBS control. In the whole body, TmIKKε mRNA was induced early at 3 h and declined at 6, 9, and 12 h post-injection of microorganisms towards the level of PBS-injected control. At 24 h, the expression of TmIKKε mRNA was found to be lower compared to the expression at 3 h (Figure 3A). In hemocytes, TmIKKε mRNA was induced at 3 and 12 h post-injection by E. coli. S. aureus induced TmIKKε mRNA early at 3 and 9 h post-challenge (Figure 3B). But in the gut tissue, expression of TmIKKε mRNA was drastically high at 6 h-post-injection of S. aureus, E. coli, and C. albicans (Figure 3C). Expression of TmIKKε mRNA in the FB was higher at 9 h post-injections with E. coli and is decreased at 12 hpi relative even to 3 hpi (Figure 3D).

Effects of TmIKKε RNAi on Larval Survivability

To further substantiate the function of TmIKKε in the host immunity against pathogens, we silenced TmIKKε mRNA by synthesizing dsRNA and injected it into the T. molitor larvae. The silencing of TmIKKε mRNA was compared relative to the injection of dsEGFP (as negative control) to a separate set of larvae. The RNAi efficiency of TmIKKε was found to be approximately 75% relative to the dsEGFP control in the whole body of the larvae (Figure 4A). In addition, the tissue-specific knockdown efficiency by injecting TmIKKε dsRNA was investigated, which showed the results that the expression of TmIKKε was significantly down-regulated by
dsTmIKKε-treatment in all tissues including FB (86%), GT (68%), HC (89%), and integuments (84%) (Supplementary Figure 3).

The survival of the larvae was recorded for 10 days after injection of *E. coli*, *S. aureus*, and *C. albicans* to TmIKKε-silenced larvae. After *E. coli* challenge to TmIKKε-silenced larvae, the mortality observed was 100% at 6-day post-challenge but was not significant compared to dsEGFP-treated larvae (log-rank chi-squared test; *p* = 1.28E-09) (Figure 4B). The mortality of TmIKKε-silenced larvae was 70% at 10-day post-challenge with *S. aureus* (log-rank chi-squared test; *p* = 0.1129) (Figure 4C). After infection with the fungus, *C. albicans*, the mortality was close to 90% in TmIKKε-silenced larvae (log-rank chi-squared test; *p* = 0.0378) (Figure 4D). The results indicate that depletion of TmIKKε caused increased larval mortality against *E. coli*, not *S. aureus* and *C. albicans*.

**Antimicrobial Peptide Expression Levels in TmIKKε Knockdown *T. molitor* Larvae**

In order to investigate the requirement of TmIKKε gene in the regulation of AMP production in immune organs (hemocytes, gut, and fat body) of *T. molitor* larvae, we injected gram-negative bacteria *E. coli*, gram-positive bacteria *S. aureus* or fungus *C. albicans* into TmIKKε knockdown *T. molitor* larvae. The transcriptional expression levels of fourteen *T. molitor* AMP genes were measured in TmIKKε knockdown individuals in comparison with dsEGFP-treated group. In the hemocytes of *T. molitor* larvae, the expression of TmTene2 was significantly upregulated in TmIKKε knockdown individuals post-inoculation with *E. coli*, *S. aureus*, and *C. albicans* (Figure 5B). TmTene1 (Figure 5A) and TmTene3 (Figure 5C) expression were also upregulated post-inoculation of *E. coli* and *C. albicans*, respectively. Further, the expression of TmAtta1a was upregulated post-inoculation of *E. coli* (Figure 5J) and TmAtta1b upregulated post-inoculation of *E. coli* and *S. aureus* (Figure 5K) in TmIKKε knockdown individuals. The upregulation of AMPs in response to TmIKKε gene knockdown suggests negative regulation during the pathogenic challenge.

Alternatively, in the gut, the expression of eight AMP genes including TmTene1 (Figure 6A), TmTene4 (Figure 6D), TmDef (Figure 6E), TmColeA (Figure 6G), TmColeB (Figure 6H), TmAtta1a (Figure 6J), TmAtta1b (Figure 6K), and TmAtta2 (Figure 6L) out of the fourteen AMP genes were significantly decreased in TmIKKε knockdown individuals. On the other hand, the expression of only two AMP genes including TmTene2 (Figure 6B) and TmDef-like (Figure 6F) was significantly upregulated by TmIKKε RNAi. Overall, in the gut of *T. molitor* larvae, TmIKKε RNAi leads to decreased transcriptional regulation of most AMPs and might be putatively involved in the survival of the larvae against pathogenic stress. We also noticed the downregulation of TmIKKε transcripts in the gut tissue following systemic injection of dsTmIKKε. Moreover, in the
silenced individuals, eight AMPs were downregulated suggesting putative role in gut immunity.

In *T. molitor* FB, the expression of twelve AMP genes including *TmTene1* (Figure 7A), -2 (Figure 7B) and -4 (Figure 7D), *TmDef* (Figure 7E) and -2 (Figure 7F), *TmColeA* (Figure 7G) and -2 (Figure 7H), *TmAttata* (Figure 7J), -1b (Figure 7K), and -2 (Figure 7L), *TmTLP1* (Figure 7M), and -2 (Figure 7N), out of fourteen AMP genes were significantly decreased by *TmIKKε* RNAi. Downregulation of AMPs in *TmIKKε* knockdown individuals after challenge with microorganisms ascertains the role of *TmIKKε* in the innate immunity of the insect.

**Effects of *TmIKKε* RNAi on the Expression of *Tenebrio* NF-κB Genes**

Furthermore, to understand the effect of *TmIKKε* RNAi on expression of *Tenebrio* NF-κB genes, *TmIKKε*-silenced *T. molitor* larvae were challenged with microorganisms and the expression patterns of *Tenebrio* NF-κB genes such as *TmRelish*, *TmDorX1*, and *TmDorX2* were investigated at 24 h by qPCR analysis. The results showed that the mRNA level of three NF-κB genes was dramatically decreased by *TmIKKε* RNAi in the FB of *T. molitor* larva. In addition, the expression of *TmDorX1* and *TmDorX2* transcripts were significantly decreased by *TmIKKε* RNAi in the GT (Figure 8).

**DISCUSSION**

The Toll and IMD pathways constitute an important defense arsenal to protect insects from non-self-discriminating pathogens. Our research group has been successful in elucidating key genes of the Toll and IMD intracellular pathways, which are relevant in the context of humoral immunity in the coleopteran pest *T. molitor* (Patnaik et al., 2013, 2014; Tindwa et al., 2013). Our focus has been on the transcriptional activation of AMPs elicited by diverse groups of microorganisms and mediated through the Toll/IMD signaling cascade (Jo et al., 2017, 2019; Keshavarz et al., 2019, 2020a,b,d; Park et al., 2019; Ali Mohammadie Kojour et al., 2020; Edosa et al., 2020a,b; Ko et al., 2020). The IKK family of proteins act upstream of the NF-κB factor Relish in the IMD pathway by phosphorylating
FIGURE 5 | The antimicrobial peptides (AMPs) expression levels in TmIKKε-knockdown T. molitor larval hemocytes upon microorganism challenge. E. coli (Ec), S. aureus (Sa), or C. albicans (Ca) were injected into TmIKKε-silenced T. molitor larvae. The transcriptional expression levels of TmTene1 (A), TmTene2 (B), TmTene3 (C), TmTene4 (D), TmDef (E), TmDef-like (F), TmColeA (G), TmColeB (H), TmCec2 (I), TmAtta1a (J), TmAtta1b (K), TmAtta2 (L), TmTLP1 (M), and TmTLP2 (N) were measured using qRT-PCR. EGFP dsRNA was used as a silencing control, and TmL27a was used as an internal control. Data represent the mean ± SE of three independent biological replicates. Asterisks indicate significant differences between dsTmSpz5- and dsEGFP-treated groups when compared using Student’s t-test (p < 0.05).
FIGURE 6 | AMP expression levels in TmIKKε-knockdown T. molitor larval gut upon microorganism challenge. E. coli (Ec), S. aureus (Sa), or C. albicans (Ca) were injected into TmIKKε-silenced T. molitor larvae. The transcriptional expression levels of TmTene1 (A), TmTene2 (B), TmTene3 (C), TmTene4 (D), TmDef (E), TmDef-like (F), TmColeA (G), TmColeB (H), TmCec2 (I), TmAtta1a (J), TmAtta1b (K), TmAtta2 (L), TmTLP1 (M), and TmTLP2 (N) were measured using qRT-PCR. EGFP dsRNA was used as a silencing control, and TmL27a was used as an internal control. Data represent the mean ± SE of three independent biological replicates. Asterisks indicate significant differences between dsTmSpz5- and dsEGFP-treated groups when compared using Student’s t-test (p < 0.05).
FIGURE 7 | AMP expression levels in TmIKK-knockdown T. molitor larval fat body upon microorganism challenge. E. coli (Ec), S. aureus (Sa), or C. albicans (Ca) were injected into TmIKK-silenced T. molitor larvae. The transcriptional expression levels of TmTene1 (A), TmTene2 (B), TmTene3 (C), TmTene4 (D), TmDef (E), TmDef-like (F), TmColeA (G), TmColeB (H), TmCec2 (I), TmAtta1a (J), TmAtta1b (K), TmAtta2 (L), TmTLP1 (M), and TmTLP2 (N) were measured using qRT-PCR. EGFP dsRNA was used as a silencing control, and TmL27a was used as an internal control. Data represent the mean ± SE of three independent biological replicates. Asterisks indicate significant differences between dsTmSpz5- and dsEGFP-treated groups when compared using Student's t-test (p < 0.05).
Relish and regulating the transcriptional activation of AMP genes. The involvement of the IKK isoforms IKKβ (ird5 in Drosophila) and IKKy (Kenny in Drosophila) in phosphorylation of Relish has been described earlier (Erturk-Hasdemir et al., 2009; Kleino and Silverman, 2014). The IKKe isoform of the IKK family of proteins encodes a serine-threonine kinase that has
been implicated in NF-κB activation. The isoform also forms an essential component of the interferon regulatory factor 3 (IRF3) signaling pathway (Fitzgerald et al., 2003; Seccareccia et al., 2014; Dubois et al., 2018). IKKs in the black carp (Mylopharyngodon piceus) was functionally characterized to participate in activating the expression of interferons in zebrafish and epithelioma papulosum cyprini (EPC) cells (Qu et al., 2015). TBK1, which is structurally identical to other IKK proteins, may also have a putative role as an important immunoregulator for IRF3 and IFNγ induction in chickens (Wang et al., 2017). However, the functional characterization of the IKKs homolog in insects is less described. In this study, we characterized the IKKε isoform in the coleopteran pest T. molitor by identifying its sequence, tissue distribution, and possible role in humoral immunity by studying the transcriptional regulation of 14 T. molitor AMP genes after microorganism challenge. We have also examined the expression of downstream NF-κB factors, including TmDorX1 and TmDorX2, which are involved in the Toll signaling pathway, and Relish, which is involved in the IMD signaling pathway, under TmIKKε-silenced conditions. Our results have improved understanding of the putative regulatory pathway involving TmIKKε.

The induction pattern of TmIKKε against injection of representative gram-negative bacteria, gram-positive bacteria, and fungi indicated that TmIKKε mRNA expression was mainly induced by E. coli challenge in the hemocytes. Furthermore, the knockdown of TmIKKε transcripts resulted in larval death upon E. coli challenge. The findings suggest that TmIKKε may be putatively involved in the defense against gram-negative bacteria. Interestingly, the TmIKKε transcript was significantly induced by S. aureus in hemocytes. This result may indicate the involvement of TmIKKε in the canonical IMD pathway. Further, the role of S. aureus in the activation of the Toll signaling cascade is well established in insects, including T. molitor (Patnaik et al., 2014). However, in the gut defense system of D. melanogaster, IMD pathway is required for clearance of S. aureus, possibly independently from AMP expression and via Duox system that produces reactive oxygen species (ROS) (Hori et al., 2018).

The transcriptional regulation of fourteen AMP genes in T. molitor in TmIKKε-silenced condition was investigated in hemocytes, gut, and fat body tissues post-injection with gram-negative bacteria E. coli, gram-positive bacteria S. aureus, and the fungus C. albicans. Interestingly, most AMP genes were not positively affected by TmIKKε RNAi in hemocytes, except to some extent TmDef. Other critical AMP genes were mostly negatively regulated after microorganisms challenge in TmIKKε-silenced individuals. Further, the inconsequential role of TmIKKε knockdown on the activation of the NF-κB genes including TmRelish, TmDorX1, and TmDorX2 suggest that TmIKKε is not required for AMP production in hemocytes. Contrastingly, in the gut, seven AMP genes were significantly downregulated by TmIKKε dsRNA-treatment post-injection of E. coli. We also find that the TmDorX1 and TmDorX2 mRNA (NF-κB regulator of Toll signaling pathway) were critically downregulated in the gut in TmIKKε-silenced individuals. This possibly suggests that TmIKKε regulates the transcriptional activation of seven AMP genes in the host gut in response to the systemic infection of microorganisms. Studies in mosquitoes highlight the promiscuous intervention of hemocytes in the activation of the anti-plasmodial gut immune system (Ramirez et al., 2014; Castillo et al., 2017). These pieces of evidence suggest the relationship between systemic infections and gut innate immune system.

In the fat body tissue of T. molitor larvae, the expression of ten AMP genes was downregulated and all the three NF-κB genes were significantly affected by TmIKKε RNAi in response to microbial challenge. These results indicate that TmIKKεs is a key regulator for Toll and IMD pathways in the fat body of T. molitor larvae. Cross-talk between Toll and IMD pathways is proposed in D. melanogaster (Tanji et al., 2007), T. castaneum (Yokoi et al., 2012a,b), P. stali (Nishide et al., 2019), and T. molitor (Ko et al., 2020). It was also suggested that several AMP genes were co-regulated by those pathways in D. melanogaster (De Gregorio et al., 2002). Further, DmIKKε phosphorylates DIAPI1 leading to DIAPI1 degradation and apoptosis in development downstream of IMD. In Drosophila, the function of DmIKKε is not especially Toll or IMD related, particularly for its role in IMD activation (Kuranaga et al., 2006; Oshima et al., 2006). In addition, the activation of Toll and IMD pathways is totally dependent on invading microbes. For instance, the gram-positive bacteria including Micrococcus luteus, Bacillus subtilis, Bacillus megaterium, Enterobacter cloacae, and fungi such as Beauveria bassiana, Saccharomyces cerevisiae, Metarhizium anisopliae, and Geotrichum candidum activate the IMD pathway (Hedengren-Olcott et al., 2004). Drosophila Cecropin A was induced by the gram-positive bacteria M. luteus and S. aureus independent of the NF-κB factor Relish. The transcriptional activation of AMPs such as Cecropin A1 and Cecropin A2 in response to M. luteus infection required Relish and Dif, respectively. Even, it is well known that the Tenecin 3 protects T. molitor against infection by the fungus Beauveria bassiana exemplified by increased larval survivability (Maistrou et al., 2018). Contrastingly, our results showed that the TmIKKεs RNAi does not mainly affect the expression of Tenecin 3 gene and larval mortality against fungal infection.

Taken together, TmIKKε plays a critical function in the production of nine AMPs in the fat body by regulating both Toll (TmDorX1 and -X2) and IMD (TmRelish) pathways. Interestingly, seven AMP genes were positively regulated by TmIKKε RNAi in the gut, carefully suggesting that the systemic infection may positively regulate AMP production in the gut through the Toll (TmDorX1 and -X2) pathway.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

YH and YJ: conceptualization, methodology, supervision, and project administration. YH: software, validation, resources,
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.758862/full#supplementary-material
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