INTRODUCTION

Mitogen-activated protein kinases (MAPK) are serine/threonine-specific protein kinases that convert extracellular stimuli into a wide range of cellular responses (Garcia-Rodriguez et al., 2012). Mitogen-activated protein kinases are among the most ancient signal transduction pathways and widely used throughout evolution in many physiological processes (Guegan et al., 2013). All eukaryotic cells possess multiple MAPK pathways, which coordinate to regulate mitosis, differentiation, metabolism, motility, apoptosis, and cell survival (Calati et al., 2013; Upadhya et al., 2013). In mammals, 14 MAPK have been characterized into 7 groups. By far the most extensively studied groups of mammalian MAPK are the ERK1/2, JNK, and p38 isoforms, but recent studies have shed some light on the function and regulation of other MAPK groups (Nix et al., 2011; Zhang et al., 2011).

Roman Dziarski had reported that soluble staphylococcal peptidoglycan strongly activates extracellular signal-regulated kinase (ERK) 1 and ERK2 in the mouse macrophage cell line RAW264.7, and moderately activates JNK and weakly activates p38 MAPK. In contrast, lipopolysaccharide (LPS) has significant anti-inflammatory activities involving the activation of ERK 1/2, serine/threonine phosphatases, and PI3 kinase signaling pathways (Zhang et al., 2012). Compared with the extensive research on human and mammal MAPK1, our understanding of bird MAPK1 is lagging behind. There are only several reports on chicken MAPK1 (Xing et al., 2010; Swaggerty et al., 2011; Dupont et al., 2012; Morillo et al., 2012), and none on duck MAPK1. In our studies, the MAPK1 gene was amplified from Cherry Valley duck and cloned into pMD18-T for sequence analysis. Furthermore, a SYBR Green quantitative real-time PCR assay was developed to detect duck MAPK1 expression. Following Riemerella anatipestifer infection by virulent strain Yb2, MAPK1 mRNA level increased more than 200-fold in the duck spleens, suggesting that increased duck MAPK1 expression can be used as an indicator of bacterial infection. Our results provide ground work to warrant further studies of the duck MAPK1 gene in bacterial pathogenesis.

ABSTRACT

Mitogen-activated protein kinase 1 (MAPK1) acts as an integration point for multiple biochemical signals, and is involved in a wide variety of biological processes such as cell proliferation and differentiation, transcription regulation, and development. Mitogen-activated protein kinase 1 plays an important role in inducing cell death in bacterial infections. In this study, the duck MAPK1 gene was cloned for the first time from the Cherry Valley duck. Sequence analysis showed that duck MAPK1 cDNA is 1,557 bp long, with an open reading frame of 1,107 bp. It encodes 368 amino acids, with 85.4, 84.5, and 97.3% homology with the human, mouse, and chicken MAPK1 gene, respectively. Furthermore, a SYBR Green quantitative real-time PCR assay was developed to detect duck MAPK1 expression. Following Riemerella anatipestifer infection by virulent strain Yb2, MAPK1 mRNA level increased more than 200-fold in the duck spleens, suggesting that increased duck MAPK1 expression can be used as an indicator of bacterial infection. Our results provide ground work to warrant further studies of the duck MAPK1 gene in bacterial pathogenesis.

Key words: cloning, duck mitogen-activated protein kinase 1, quantitative real-time PCR
**MATERIALS AND METHODS**

**Birds**

One-day-old Cherry Valley ducklings were obtained from Wuxi Duck Farm (Wuxi, China) and kept under controlled temperature (28-30°C). The ducklings were housed in cages with a 12L:12D cycle and free access to food and water during the study. Care and maintenance of all animals were in accordance with the Institutional Animal Care and Use Committee guidelines set by the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

**Escherichia coli** strain DH5α was purchased from ComWin Biotech Co. Ltd. (Beijing, China) and cultured in Luria-Bertani liquid medium or on solid medium containing 1.5% agar at 37°C. *Riemerella anatipestifer* strain Yb2 was isolated in China (Hu et al., 2011) and cultured in tryptic soy agar (Difco, Franklin Lakes, NJ) at 37°C for 24 h in 5% CO₂, or in tryptic soy broth (Difco) shaking at 150 rpm for 8 to 12 h at 37°C. The pMD18-T was obtained from TaKaRa Co. (Dalian, China) and cultured in tryptic soy agar (Difco, Franklin Lakes, NJ) at 37°C for 24 h in 5% CO₂, or in tryptic soy broth (Difco) shaking at 150 rpm for 8 to 12 h at 37°C.

**Bacterial Strains and Plasmids**

**Amplification, Cloning, and Sequence Analysis of the Partial Fragment of Duck MAPK1**

The partial fragment of the duck MAPK1 was amplified from Cherry Valley duck spleen using primer pairs of MAPK1-AF/MAPK1-AR (Table 1), which were designed using Primer Premier 5.0 software according to a conserved region of chicken MAPK1 gene sequence (accession no. NM_204150). The spleen was collected from 10-d-old Cherry Valley duck and homogenized in 10 volumes of ice-cold PBS. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Promega, Fitchburg, WI) according to the manufacturer’s instructions. The cDNA was synthesized using 2 μg of total RNA, oligo d(T)15 primer, and M-MLV reverse transcriptase (Promega). The partial fragment of MAPK1 was amplified from the cDNA template using primers MAPK1-AF/MAPK1-AR with the following PCR cycle: 94°C for 3 min, 25 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s, followed by a 10 min final extension at 72°C. The PCR products were identified by electrophoresis and cloned into pMD18-T vector (TaKaRa Co.) for sequencing using an automatic DNA sequencer (ABI Applied Biosystems Model 377, Grand Island, NY).

**5’- and 3’- Full Rapid Amplification of cDNA Ends and Sequence Analysis**

Both 5’-full/3’-full rapid amplification of cDNA ends (RACE) were performed with the total RNA by using 3’-full/5’-full RACE kits (TaKaRa Co.) as described (Xiao et al., 2011; He et al., 2013). Briefly, 3′ RACE was performed using a 3′ full RACE kit. The outer PCR amplification was carried out using primers MAPK1–5GSP2 and 3’ RACE outer primer (Table 1) and 2 μL of cDNA template. Then the outer PCR product was used as a template for the inner PCR reaction using primers MAPK1–3GSP1 and 3’ RACE inner primer (Table 1). The PCR products were then subjected to gel purification, cloning, and sequence analysis.

For 5’ RACE, the total RNA was first processed using a 5’-full RACE kit through dephosphorylation, 5’ cap structure removal, 5’-RACE adaptor connection, and reverse-transcription reaction steps to provide a template for the outer PCR (Xiao et al., 2011; He et al., 2013). The PCR products were then subjected to gel purification, cloning, and sequence analysis.

**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Description</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK1-AF</td>
<td>5’ AAGTGTTCGACGTGGGG 3’</td>
<td>Amplification of the internal region of MAPK1 cDNA sequences</td>
</tr>
<tr>
<td>MAPK1-AR</td>
<td>5’ TCCTTCGGCAAGTCATC 3’</td>
<td></td>
</tr>
<tr>
<td>RACE-PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK1–3GSP1</td>
<td>5’ TTCTAAGGGTGTTACACAGTC 3’</td>
<td>Amplification of the 3’ and 5’ terminal of MAPK1 cDNA</td>
</tr>
<tr>
<td>3’ RACE outer primer</td>
<td>5’ TACGGTCGTTCCACTAGTTGTTTCAAGGG 3’</td>
<td></td>
</tr>
<tr>
<td>MAPK1–3GSP2</td>
<td>5’ CTATTTGCTTCTTACCCACACC 3’</td>
<td></td>
</tr>
<tr>
<td>3’ RACE inner primer</td>
<td>5’ CGGGATCCTCCACTAGTTGTTTCAAGGG 3’</td>
<td></td>
</tr>
<tr>
<td>MAPK1–5GSP1</td>
<td>5’ GTGGTCTTGGTCTAGGTTGTTGAG 3’</td>
<td></td>
</tr>
<tr>
<td>5’ RACE outer primer</td>
<td>5’ CATGGCTTACTGCTGAGCTGACGTTC 3’</td>
<td></td>
</tr>
<tr>
<td>MAPK1–5GSP2</td>
<td>5’ CTGGCCAGTACGTCTGATGCTCAAA 3’</td>
<td></td>
</tr>
<tr>
<td>5’ RACE inner primer</td>
<td>5’ CGGGATCAGGCTACTGATGATCAGTGCAG 3’</td>
<td></td>
</tr>
<tr>
<td>Real-time PCR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK1-RT-F</td>
<td>5’ CCAGACCATACTGATCACAGG 3’</td>
<td>Real-time quantitative PCR</td>
</tr>
<tr>
<td>MAPK1-RT-R</td>
<td>5’ GGATCCAAGTATGCAAAGGA 3’</td>
<td></td>
</tr>
<tr>
<td>Arbp-RT-F</td>
<td>5’ CGACCTCGAAGTGCCAAACTACT 3’</td>
<td></td>
</tr>
<tr>
<td>Arbp-RT-R</td>
<td>5’ ATCGTCTGATCTGCTGGT 3’</td>
<td></td>
</tr>
<tr>
<td>MAPK1F</td>
<td>5’ CAACGACCACTATTGCTACTTC 3’</td>
<td></td>
</tr>
<tr>
<td>MAPK1R</td>
<td>5’ TCCACGCTACAGGCTACCTACTT 3’</td>
<td></td>
</tr>
</tbody>
</table>

1MAPK1 = mitogen-activated protein kinase 1; RACE = rapid amplification of cDNA ends; Arbp = attachment region binding protein.
al., 2013). The outer and inner PCR amplifications for 5′ RACE were carried out similarly to 3′ RACE, using MAPK1–5GSP1 and 5′ RACE outer primer for the outer PCR reaction and MAPK1–5GSP2 and 5′ RACE inner primer (Table 1) for the inner PCR reaction.

**Sequence Assembly and Homology Analysis of the Duck MAPK1 Gene**

Full-length cDNA of duck MAPK1 gene was assembled using Vector NTI Express Software (Life Technologies, Carlsbad, CA) based on the obtained partial fragment and the 3′-/5′-full RACE products. Amino acid sequence and protein analysis were performed using the ExPASy proteomic tool (http://www.expasy.org/tools/) and the protein domains were predicted using SMART software (http://smart.embl-heidelberg.de/). Homologous sequences were searched using BLAST (http://www.ncbi.nlm.nih.gov/blast/) with default settings on the complete nonredundant GenBank database (Wei et al., 2011; Han et al., 2012). Percent identity of deduced amino acid sequences against human, mouse, and chicken MAPK1 sequences was analyzed using the DNASTar program (DNASTAR Inc. Madison, WI).

**Development of a Real-Time PCR Assay and Analysis of MAPK1 Expression in Duck Tissues**

Based on the duck MAPK1 gene sequence obtained in this study, primers MAPK1-RT-F/MAPK1-RT-R (Table 1) were designed using Primer Express 3.0 to develop a real-time PCR assay for detecting duck MAPK1 expression. The target 213-bp amplicon was selected from a relatively conserved region of the gene (open reading frame 550–762 bp). The expression of attachment region binding protein (Arbp) served as an endogenous control, which was detected using primers Arbp-RT-F/Arbp-RT-R (Table 1). The qPCR was performed using the SYBR Green Quantitative PCR kit (Promega). Total RNA was extracted using Trizol reagent (Invitrogen) and treated with RNase-free DNAse (Promega) according to the manufacturer’s instructions. The cDNA synthesis was performed using oligo (dT)15 primer and PrimeScript RT Master Mix (TaKara Co.). Amplifications were carried out with the following parameters: 2 min at 95°C, followed by 40 cycles consisting of 15 s at 94°C and 60 s at 60°C. A 10-fold dilution series of plasmids containing the target gene (MAPK1) and reference gene (Arbp) fragments was used to compute the PCR efficiency. All experiments were carried out in triplicate. The changes of mRNA expression were calculated using the comparative cycle threshold \((2^{−ΔΔCt})\) method.

Expression of MAPK1 was analyzed in ducks infected with *R. anatipestifer* Yb2. Twenty-four Cherry Valley ducks (15 d old) were divided into 2 groups of 12, injected intramuscularly with *R. anatipestifer* Yb2 culture (0.5 mL, 10⁶ cfu, infection group) or PBS (control group) as described (Hu et al., 2011). Spleens were collected from 3 ducks in each group at 6, 12, 24, and 48 h postinfection and the respective fold expression of MAPK1 mRNA was quantitatively detected as described above. All samples were detected in triplicate. Significance was analyzed by 2-tailed Student’s *t*-test.

**Dot Blot Assay**

The primer pairs MAPK1F/MAPK1R and Arbp-RT-F/Arbp-RT-R were designed using Primer 5.0 software and listed in Table 1. The amplified PCR products were labeled respectively with digoxigenin (DIG) using the DIG Northern Starter Kit (Roche Diagnostics, Mannheim, Germany) and used as probes. Dot blot assay was carried out using the DIG Northern Starter Kit (Roche Diagnostics). Briefly, the total RNA was heated at 100°C for 10 min, and cooled down on ice. The denatured RNA (2 μL) was then manually blotted on a positively charged nylon membrane, which was activated with 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) for 5 min. The blots were hybridized with 10 pM probes at 80°C for 2 h. After hybridization, each blot was washed twice for 5 min in 2 × SSC/0.1% SDS at room temperature with gentle agitation. Blots were then washed in DIG Wash and Block Buffer Set for 2 min and blocked in blocking solution for 30 min at room temperature. The membranes were then incubated with 150 mU/mL alkaline phosphatase conjugated anti-digoxigenin antibody for another 30 min, washed twice in washing solution for 15 min, and equilibrated in 0.1 M Tris-HCl and 0.1 M NaCl (pH 9.5) for 3 min. For colorimetric detection of hybridization, substrate solution (NBT/BCIP) was used as described.
in the manufacturer’s instructions. Color development was performed for 1 h and the reaction was stopped by washing the membrane with TE buffer [10 mM Tris-HCl (pH 8.0), and 1 mM EDTA] for 5 min.

**RESULTS**

**PCR Amplification of the Duck MAPK1 Partial Sequence**

The 973-bp PCR product was identified by 1% agarose gel electrophoresis, which is similar to the theoretical length of the gene (Figure 1A). The PCR product was then cloned into the pMD-18T vector, and transformed into DH5α competent cells. The obtained DNA sequence was analyzed by DNAsstar software, which indicates that the partial sequence is 98.4% homologous to the chicken MAPK1 gene.

**3’ and 5’ Full RACE of Duck MAPK1 Gene**

There were 3 potential inner PCR products resulting from the 3’ RACE (Figure 1B). We subsequently identified the middle band as the real 3’ inner PCR product.
Figure 3. The nucleotide sequence alignment of duck, *Gallus gallus* (NM_204150.1), *Homo sapiens* (NM_002745.4), and *Mus musculus* (NM_011949.3) mitogen-activated protein kinase 1 (*MAPK1*) genes. Real-time PCR primers MAPK1-RT-F and MAPK1-RT-R were selected from a conserved region and are marked by arrows.
<table>
<thead>
<tr>
<th>Residue Position</th>
<th>Amino Acid</th>
<th>Species</th>
<th>Nature of Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>2163</td>
<td>CLONING OF DUCK MITOGEN-ACTIVATED PROTEIN KINASE 1</td>
<td>Duck MAPK1</td>
<td>seq</td>
</tr>
<tr>
<td>2163</td>
<td>CLONING OF DUCK MITOGEN-ACTIVATED PROTEIN KINASE 1</td>
<td>Gallus gallus</td>
<td>MAPK1</td>
</tr>
<tr>
<td>2163</td>
<td>CLONING OF DUCK MITOGEN-ACTIVATED PROTEIN KINASE 1</td>
<td>Homo sapiens</td>
<td>MAPK1</td>
</tr>
<tr>
<td>2163</td>
<td>CLONING OF DUCK MITOGEN-ACTIVATED PROTEIN KINASE 1</td>
<td>Mus musculus</td>
<td>MAPK1</td>
</tr>
</tbody>
</table>

Figure 4. The amino acid sequence alignment of duck, *Gallus gallus*, *Homo sapiens*, and *Mus musculus* mitogen-activated protein kinase 1 (MAPK1) proteins. The numbers on the left indicate the residue positions. Gaps are marked by dashes, and the residues that differ from the duck MAPK1 gene are shaded with solid black.
product and others as nonspecific products following cloning and sequencing of all 3 bands. A single 5' inner PCR product was obtained (Figure 1C). The PCR products were cloned into pMD18-T vector and transformed into DH5α competent cells. The DNA sequences were analyzed by DNAstar software, indicating the partial sequences at the 3' and 5' ends, which were 97.7 and 97.3% homologous to chicken MAPK1, respectively.

**Sequence Assembly and Homology Analysis of the Duck MAPK1 Gene**

Based on the obtained partial fragment and 3'-/5'-full RACE product sequences, the full-length duck MAPK1 cDNA was assembled using the DNASTar program and submitted to GenBank (accession no: DUCK MAPK1 Duck KF482375). The duck MAPK1 full cDNA sequence is 1,557 bp long, including a 55-bp 5'-untrans-
lated region, a 395-bp 3′-untranslated region, and a 1,107-bp open reading frame encoding 368 amino acids (Figure 2). The nucleotide sequences of duck, *Gallus gallus* (NM_204150.1), *Homo sapiens* (NM_002745.4), and *Mus musculus* (NM_011949.3) MAPK1 genes were aligned and compared using the Vector NTI Express and DNAStar programs. The alignment was performed using Clustal W v2.0. The residues that differ from duck MAPK1 were shaded with solid black (Figure 3). Duck MAPK1 gene is 85.4, 84.5, and 97.3% homologous to human, rat, and chicken MAPK1, respectively. The amino acid sequence of duck MAPK1 protein was shown in Figure 4. Gaps are marked by dashes and the residues that differ from duck MAPK1 gene were shaded with solid black. The theoretical molecular weight of duck MAPK1 is 41.9 kDa.

**Figure 6.** A) Quantitative real-time PCR analysis. Mitogen-activated protein kinase 1 (*MAPK1*) mRNA level in duck spleen at 6, 12, 24, and 48 h following infection by *Riemerella anatipestifer* strain Yh2. The duck attachment region binding protein (*Arbp*) gene was used as an internal control. The increased *MAPK1* mRNA was detected and indicated as fold expression. Data were presented as means from 3 independent experiments. Error bars correspond to the SD of the means (±SD; n = 3). Significance was analyzed by 2-tailed Student’s *t*-test (**P < 0.01). B) Dot blot assay. Section a: Expression of *MAPK1*. Section b: Expression of *Arbp*. Samples in lanes 1, 3, 5, and 7 were from normal, healthy ducks. Samples in lanes 2, 4, 6, and 8 were from *R. anatipestifer* infected ducks. The expression of *MAPK1* in *R. anatipestifer* infected duck spleens was confirmed to be upregulated.

**Real-Time Quantitative PCR and Dot Blot Analyses of MAPK1 Expression in Ducks Infected with R. anatipestifer**

We successfully developed a qRT-PCR assay to measure duck *MAPK1* gene expression. Amplifications generated a single expected amplicon with single, sharp fusion curves (Figure 5A). The PCR efficiency values of all genes were above 1.92. The detection limit for the assay is 10 copy numbers (Figure 5B). Levels of *MAPK1* expression in the spleens of *R. anatipestifer*-infected ducks were analyzed. Levels of *MAPK1* mRNA were significantly increased by 224-, 604-, 480-, and 449-fold at 6, 12, 24, and 48 h postinfection (*P < 0.01), respectively (Figure 6A).
The expression of MAPK1 in *R. anatipestifer* infected duck spleens was also confirmed to be upregulated by dot blot assay (Figure 6B).

**DISCUSSION**

Mitogen-activated protein kinases are a series of intracellular Ser/Thr protein kinase that are highly conserved. Six identical positions of cysteine residues (C1, C3, C4, C5, C6, and C8) were found in both vertebrate and invertebrate MAPK1 (except for the *Apis mellifera* homolog, where C1 is replaced by S; Ponza et al., 2011). Mitogen-activated protein kinases are involved in directing cellular responses to a diverse array of stimuli such as mitogens, osmotic stress, heat shock, and pro-inflammatory cytokines. They regulate gene expression, mitosis, proliferation, differentiation, cell survival, and apoptosis through intracellular phosphorylation of 3 consecutive enzymatic reaction cascade. The cascade consists of MAPKKK (MAPK-kinase kinase), MAPKK (MAPK kinase), and MAPK, of which MAPKKK is a both Ser/Thr and tyrosine bifunctional kinase. The MAPK family has multiple subgroups, of which the ERK, JNK, p38 lightning, and ERK5 groups are most characterized at the present. The first identified MAPK family member was ERK (Jiang et al., 1996; Irving et al., 2000; Hii et al., 2004).

The orthologs of mammalian MAPK1 were identified previously in chicken cells (Duchene et al., 2008; Tummlala et al., 2011). In this paper, we describe the cloning of duck MAPK1. The duck MAPK1 gene was successfully cloned from Cherry Valley ducks and shares 97.3% sequence homology with chicken MAPK1 gene. A qRT-PCR assay was also developed in this study to measure duck MAPK1 mRNA levels. Amplification generated a single expected amplicon with single, sharp fusion curves, indicating the high specificity of the amplicons. Furthermore, MAPK1 expression was increased more than 200-fold in duck spleens following *R. anatipes-tifer* infection. This suggests that duck MAPK1 expression can be used as an indicator of bacterial infection.

The activation of MAPK and nuclear factor κB by membrane protein in *Listeria monocytogenes*-infected macrophages was confirmed by the induction of ERK and IκB phosphorylation (Zhang et al., 2012). Nuclear factor κB triggers destructive inflammation accompanied by MAPK activation. Inflammation is driven by massive tumor necrosis factor production, which results in expression of p38 and ERK (Guma et al., 2012). Nitrous oxide production induced by bacterial stimulation was most effectively reduced by inhibition of p38 MAPK and least effectively reduced by inhibition of IκB in chicken macrophage cell line HD11 (Crippen, 2006). *Brevibacillus texansporus* (BT peptides) enhance leukocyte functional and pro-inflammatory cytokine and chemokine gene transcription activities through MAPK pathways, and may prove useful as immune modulators in neonatal poultry (Kogut et al., 2012). In this study, the complete sequence of duck MAPK1 gene was obtained and the increased expression of duck MAPK1 was observed after *R. anatipes-tifer* infection. The role of duck MAPK1 in bacterial pathogenesis needs further experimental verification.

**ACKNOWLEDGMENTS**

This study was supported by the Chinese National High-Technology Research and Development Program (863 Program, 2011AA10A209), the Special Fund for Agro-Scientific Research in the Public Interest (grant no. 201003012), and the National Natural Science Foundation of China (31272591).

**REFERENCES**


