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BmYki is transcribed into four functional splicing isoforms in the silk glands of the silkworm *Bombyx mori*

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ABSTRACT

Yorkie (Yki), the *Drosophila* homolog of vertebrate yes-associated protein (YAP), is a key effector of the Hippo pathway, which modulates organ size via the transcriptional regulation of downstream targets involved in cell proliferation and survival. YAP has been shown to be expressed as multiple splicing isoforms in mammals, but thus far, no evidence of alternatively spliced Yki isoforms has been reported in insects. Here, we confirmed that the Yki protein of the silkworm *Bombyx mori*, BmYki, is transcribed in the silk gland into at least four splicing isoforms, named BmY1329, BmY1314, BmY1188, and BmY1173. Further analysis revealed that BmY1329 and BmY1314 each contain two WW domains, whereas BmY1188 and BmY1173 each contain only one WW domain. Each BmYki isoform functions in regulating expression of Yki target genes in cultured *B. mori* embryonic cells, and exhibits a few different effects on the expression of Yki targets. Interestingly, the expression of silk fibroin protein genes could also be influenced by each of the BmYki isoforms, suggesting that BmYki is involved in the regulation of silk protein-coding genes. This study provides novel insights into the role of BmYki. The contribution of each BmYki isoform to the modulation of gene expression will be of great interest for further study.

1. Introduction

The Hippo signaling pathway, first defined in *Drosophila* by genetic mosaic screens for tumor suppressor genes, is a key regulator of organ size and is highly conserved between flies and vertebrates (Justice et al., 1995; Xu et al., 1995; Pan, 2007; Zhao et al., 2011). Mutation or dysregulation of this pathway can result in tumor formation, both in *Drosophila* and in mammals (Pan, 2010; Yu et al., 2015). The Hippo pathway functions through a kinase cascade involving two core tumor suppressors, Hippo (Hpo; Mst1/2 in vertebrates) and Warts (Wts; Lats1/2 in vertebrates), that phosphorylates and inhibits a transcriptional coactivator protein known as Yorkie (Yki) in *Drosophila* and yes-associated protein (YAP)/TAZ in vertebrates. This protein is the final destination of Hippo signaling, which regulates the transcription of a number of targets involved in cell proliferation and survival (Reddy and Irvine, 2008; Oh and Irvine, 2010; Staley and Irvine, 2012; Hong and Guan, 2012; Yu and Guan, 2013). Elucidating the regulatory mechanisms of Yki/YAP/TAZ will offer the key to understanding developmental and cancer biology.

As an important downstream effector of the Hippo pathway, Yki/YAP/TAZ proteins have been found in more than one hundred organisms and have been studied intensively in *Drosophila* and in mammals over the past years. YAP was first identified in chickens as an interacting protein of Yes protein tyrosine kinase in 1994, and shortly afterwards, its orthologs were isolated from humans and mice (Sudol, 1994; Sudol et al., 1995; Chen and Sudo, 1995). In mammals, YAP has been shown to exist in multiple protein isoforms generated through alternative splicing. The human YAP gene can be transcribed into nine splicing isoforms based on annotation by NCBI and experimental evidence presented by two groups (Muramatsu et al., 2011; Hong and Guan, 2012; Gaffney et al., 2012). Isoforms 1, 3, 4, 8 and 9 contain two protein-protein interaction domains, i.e., WW domains named after the

Abbreviations: Yki, *Drosophila* Yorkie; BmYki, *Bombyx mori* Yorkie; HaYki, *Helicoverpa armigera* Yorkie; YAP, Yes-associated protein; TAZ, transcriptional co-activator with PDZ-binding motif; BmE, *Bombyx mori* embryonic cells; BmN, *Bombyx mori* ovary-derived cells; *BmA4*, *Bombyx mori actin 4* gene; DAPI, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride; PBS, phosphate buffer saline; siRNA, small interfering RNA; RNAi, RNA interference; RT-PCR, Reverse Transcription PCR; qRT-PCR, quantitative Real Time PCR; *fibH*, fibroin heavy chain gene; *fibL*, fibroin light chain gene; *P25*, fibrohexamerin gene

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presence of two conserved tryptophans (W) within the amino acid sequences (Sudol et al., 1995; Ilsley et al., 2002), while isoforms 2, 5, 6 and 7 contain only one WW domain. The mouse YAP gene has eight splicing isoforms (based on current annotations from NCBI), each containing two WW domains. TAZ is a paralog of YAP that contains one WW domain and possesses similar but not identical function to that of YAP (Kanai et al., 2000; Zhu et al., 2015). Although three variants of TAZ have been annotated in NCBI, all variants have the same coding region for a protein of 400 amino acids (Hong and Guan, 2012). Regardless, the multiple splicing isoforms of YAP increase the diversity and complexity of the Hippo pathway in the regulation of organ size.

The insect Yki gene was first isolated from *Drosophila* in 2005. This gene has a coding sequence of 1257 bp, encoding a protein of 418 amino acids containing two WW domains and sharing 31% identity with the human YAP (Huang et al., 2005). Interestingly, the transcription activation domain of YAP is missing in Yki, suggesting that the mechanisms of transcription regulation by Yki and YAP are different (Zhu et al., 2015). Since the cloning of *Drosophila* Yki, orthologs of Yki have been identified in more than twenty insect species (based on current annotations from NCBI), but only two orthologs, in *Bombyx mori* Yki (BmYki) and *Helicoverpa armigera* Yki (HaYki), were publicly reported until recently (Liu et al., 2016; Zeng et al., 2017; Wang et al., 2016). Moreover, although NCBI annotation has indicated that the Yki in several insects such as *Drosophila*, *H. armigera*, and *Tribolium castaneum* might be expressed in different types of transcripts, experimental evidence for alternatively spliced Yki in insects remained lacking.

In a recent study that isolated the BmYki gene from *B. mori*, we noticed that two fragments of different sizes are always amplified under stringent Reverse Transcription PCR(RT-PCR) conditions (Zeng et al., 2017). We thus speculated that BmYki might be transcribed into multiple splicing isoforms in *B. mori*. Here, we addressed this question by performing a sequencing analysis of more than 100 cDNA clones and revealed the existence of four BmYki isoforms that differ from each other in coding sequence. We further investigated the expression and subcellular localization of these BmYki isoforms and performed a functional analysis in cultured *B. mori* cells, and we demonstrated that all four BmYki isoforms have similar but distinct roles in the regulation of Yki targets as well as of silk protein-coding genes.

2. Materials and methods

2.1. Animals and cultured cells

Two silkworm strains, *Dazao* (diapause strain) and *Nistari* (non-diapause strain), which have very similar genetic backgrounds, were used to isolate BmYki transcripts from the silk gland. Eggs were maintained at 27–28 °C with 85–95% humidity until hatching, and the larvae were reared on fresh mulberry leaves at 25–26 °C. Cultured *B. mori* embryonic cells (BmE) and ovary-derived cells (BmN) were used to investigate the subcellular distribution and function of BmYki. Cells were maintained at 27 °C in Grace's insect medium containing 10% fetal bovine serum (HyClone, China).

2.2. Sequence analysis

PCR products containing BmYki transcripts were amplified from the silk gland of day-5 fifth instar larvae of *Dazao* and *Nistari* and gel purified separately, followed by subcloning into the pEASY-T5 vector (TransGen, China) and DNA sequencing (BGI-Shenzhen, China). Exon/intron, conserved protein domain and phosphorylation site searches were performed using the Local BLAST Program, GENSCAN (http://www.genes.mit.edu/GENSCAN.html), SMART (http://smart.embl-heidelberg.de/), and Scansite (http://scansite.mit.edu/motifscan_seq.phtml). The BmYki isoforms isolated from *Nistari*, named BmY1329, BmY1314, BmY1188, and BmY1173, were used for subsequent experiments.

2.3. Vector construction

Subcellular localization constructs. The coding sequences of BmY1329, BmY1314, BmY1188, and BmY1173 with the stop codon deleted were digested with *BamH* I and *Not* I, then inserted separately into the pUC57S[hr3BmA4-EGFP-S1PA] (abbreviated as hr3A4-EGFP). The resulting constructs were named Y1329-EGFP, Y1314-EGFP, Y1188-EGFP, and Y1173-EGFP, respectively. The expression of the fusion protein was driven by a regulatory element consisting of the *hr3* enhancer (Wang et al., 2013) and the *B. mori actin 4* (*BmA4*) promoter.

Overexpression constructs. The fragments encoding the BmY1329, BmY1314, BmY1188, and BmY1173 were digested with *BamH* I and *Not* I and inserted separately into the pUC57S[10 × UAS-B/N-Ser1pA] vector. Then, each of the expression cassettes was subcloned into the *Asc* I site of pB[3 × P3EGFPafm] (Horn and Wimmer, 2000). The final constructs were abbreviated as UY1329, UY1314, UY1188, and UY1173, respectively. The Gal4 expression vector pB[BmA4-Gal4,3 × P3DsRed] (abbreviated as A4G4) was constructed previously in which the *BmA4* promoter was used to control Gal4 expression. A4G4 is not known to impact endogenous gene expression on its own (Zeng et al., 2017; Gong et al., 2017).

2.4. Cell transfection

2.4.1. Subcellular localization analysis

A mixture of 2 μL of transfection reagents (Roche, USA) containing 2 μg of Y1329-EGFP, Y1314-EGFP, Y1188-EGFP, or Y1173-EGFP plasmid DNA was transfected into BmN cells. After 24 h of culture at 27 °C, the cells were harvested, washed three times with 1 × PBS, fixed with 4% paraformaldehyde for 15 min, washed three times with 1 × PBS, dyed with DAPI Staining Solution (Beyotime, China) for 20 min at room temperature, and washed three times with 1 × PBS. Then, the fixed cells were mounted on slides with 2 μL of Antifade Mounting Medium (Beyotime, China) and observed using an FV1000 confocal microscope (Olympus, Japan). The hr3A4-EGFP plasmid DNA was used as a positive control.

2.4.2. Overexpression analysis

The plasmid DNA of UY1329, UY1314, UY1188, and UY1173 was mixed separately with A4G4. Two micrograms of each plasmid mixture was incubated with 2 μL of transfection reagents (Roche, USA) and then transfected separately into BmE and BmN cells. After 48 h of culture at 27 °C, the cells were washed three times with 1 × PBS and used to prepare cDNA templates.

2.4.3. RNA interference (RNAi) analysis

Three pairs of siRNAs targeting the first exon of all BmYki isoforms were commercially synthesized (GenScript, China), but only siRNA-BmYki-1 (sense: 5′-GGAUUCAGACUCUGUAUUAdTdT-3′; antisense: 5′-UAAUACAGAGUCUGAAUCCdTdT-3′) was effective in inhibiting the expression of BmYki (data not shown). Therefore, we selected siRNA-BmYki-1 to perform the RNAi experiment. Briefly, a mixture of 150 μL of Grace's insect medium containing 2 μL transfection reagents (Roche, USA) and 50 nM of siRNA-BmYki-1 was transfected into BmE and BmN cells. After 48 h of culture at 27 °C, the cells were washed three times with 1 × PBS and used to prepare cDNA templates. All the above transfections were performed in triplicate in 24-well plates.

2.5. Quantitative Real Time PCR (qRT-PCR) analysis

The total RNA from various developmental stages of *Nistari*, tissues of day-5 fifth instar larvae and cultured *B. mori* cells were extracted using the E.Z.N.A.® Total RNA Kit II (Omega, USA). The cDNA template was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Japan). The mRNA levels of all four BmYki isoforms, six known Yki targets and three silk fibroin genes were analyzed by qRT-