

«Research Note»

Photoperiod-Specific Expression of Eyes Absent 3 Splice Variant in the Pars Tuberalis of the Japanese Quail

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The molecular mechanism underlying photoperiodic response in seasonal breeding animals such as the Japanese quail, red jungle fowl, sheep, mouse, and hamster involves thyroid-stimulating hormone beta subunit (*TSHβ*) mRNA expression in the pars tuberalis stimulated by the extension in day length. Furthermore, this mechanism is regulated by eyes absent 3 (*Eya3*) in mammals. Even in birds, the expression of both *TSHβ* and *EYA3* is induced in the pars tuberalis by the extension in day length; however, the relationship between the two genes is unknown. To clarify the function of *EYA3* in quail photoperiodism, in the present study, we performed mRNA structure analysis of the Japanese quail *EYA3* mRNA using reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot analysis. The results revealed that there are four types of splice variants within regions of exons 7, 8, and 9 of quail *EYA3* mRNA. Among the four splice variants of quail *EYA3*, the splice variant containing exon 7 was expressed in the pars tuberalis on the first long day, when quails were transferred from the short-day condition to the long-day condition. The results indicate that *EYA3* splice variant containing exon 7 is involved in the photoperiodic response of the pars tuberalis in the Japanese quail.

Key words: *EYA3*, Japanese quail, photoperiodism, splice variant

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Introduction

The seasonal change in day length results in various physiological responses in animals and plants, and this phenomenon is called photoperiodism. Photoperiodism in animals involves seasonal breeding, molting, hair replacement, hibernation, and migration. The molecular mechanism underlying photoperiodic response in seasonal breeding organisms, such as the Japanese quail, red jungle fowl, sheep, mouse, and hamster, involves expression of the thyroid-stimulating hormone (*TSH*) β in the pars tuberalis (PT) stimulated by the extension in day length (Nakao *et al.*, 2008; Ono *et al.*, 2008; Dardente *et al.*, 2010; Hanon *et al.*, 2010; Masumoto *et al.*, 2010). TSH secreted by the PT induces the expression of type 2 deiodinase, which is an enzyme con-

verting thyroxine into triiodothyronine (Nakao *et al.*, 2008). Triiodothyronine is synthesized locally in the mediobasal hypothalamus by type 2 deiodinase, which increases the secretion of gonadotropin releasing hormone that then induces the secretion of both follicle-stimulating hormone and luteinizing hormone, which are involved in the development of gonads (Yoshimura *et al.*, 2003; Watanabe *et al.*, 2004). Furthermore, it is clear that the expression of *TSHβ* mRNA encoding TSH in the PT of mouse and sheep is regulated by eyes absent 3 (*Eya3*) genes, which are transcriptional co-activators preceding *TSHβ* mRNA expression (Dardente *et al.*, 2010; Masumoto *et al.*, 2010). In quail, both *TSHβ* and *EYA3* expression is induced, like in mammals, by long-day stimulation (Nakao *et al.*, 2008); however, the mechanism of *EYA3* protein triggering *TSHβ* mRNA expression in the quail PT is unclear.

Eya is reported to be necessary for the formation of the eyes in *Drosophila melanogaster*; the *Eya* gene family includes *Eya1-Eya4* in mammals (Jenc and Rebay, 2007). *Eya3* includes two domains, namely, the *Eya* domain (ED) and *Eya* domain 2 (ED2); in the C terminal, ED is highly conserved among the *Eya* family members (Xu *et al.*, 1997b; Zimmerman *et al.*, 1997). The ED interacts with sine oculis homeobox (SIX) and dachshund (DACH) proteins (Ohto *et*

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al., 1999; Ikeda *et al.*, 2002; Li *et al.*, 2003) and has tyrosine phosphatase activity (Li *et al.*, 2003; Rayapureddi *et al.*, 2003; Tootle *et al.*, 2003). In the N-terminal region, Eyas have threonine phosphatase activity (Okabe *et al.*, 2009; Sano and Nagata, 2011). Furthermore, Eya1, Eya2, and Eya4 that contain proline, serine, and threonine-rich (P/S/T-rich) regions include the ED2 domain and two MAPK phosphorylation consensus sites, which are important for the transactivation function that the activation of the RAS/MAPK pathway potentiates in the transcriptional output of EYA (Xu *et al.*, 1997a; Silver *et al.*, 2003). Thus, the Eya family members possess both transcriptional coactivator function and tyrosine/threonine phosphatase function, and they are involved in embryogenesis and organogenesis. However, the function of quail EYA3 in the photoperiodic response is not well understood. In the present study, to clarify a function of *Eya3* in quail photoperiodism, we performed molecular structure analysis of *Eya3* mRNA.

Materials and Methods

Animals and Tissue Collection

Japanese quails (4-weeks-old) were obtained from Motoki Corporation, Saitama, Japan. The quails were maintained under the short-day (SD) condition (6 h light and 18 h dark) for 4 weeks, and then divided into two groups: one group was maintained under SD conditions and the other group was transferred to long-day (LD) conditions (20 h light and 4 h dark). Samples of the mediobasal hypothalamus including the PT were collected at 16 h after dawn of SD or on the first long day (1LD). From the 1LD group, the retina, heart, spleen, lungs, liver, kidneys, testes, gall bladder, skeletal muscle, ovaries, and skin were collected after decapitation and stored at -80°C until further analysis. Food and water were provided *ad libitum*. All procedures were conducted in accordance with the provision for animal welfare of the Nippon Veterinary and Life Science University (approval number: 30S-50).

RNA Isolation and cDNA Synthesis

The total RNA from each tissue was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed at 50°C for 60 min with Super ScriptIII RT (Thermo Fisher Scientific). The polymerase chain reaction (PCR) for cDNA cloning of the coding region of *EYA3*, and reverse transcription-PCR (RT-PCR) and Southern blot-

ting were performed with Ex *Taq* (TAKARA, Tokyo, Japan). The PCR was performed for 30 cycles at 96°C for 10 s, 64°C for 20 s, 72°C for 2 min 30 s (cDNA cloning), 94°C for 10 s, 52.9°C for 20 s, and 72°C for 1 min (RT-PCR and Southern blotting). The sequence of primers is shown in Table 1. The amplified cDNA fragments were ligated to the pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli*. The plasmid DNA was extracted from the bacteria and purified using an alkaline lysis method. The sequence of the cDNA was analyzed using the ABI PRISM 310 Genetic Analyzer (Thermo Fisher Scientific, Tokyo, Japan) with the BigDye Terminator v3.1 sequence kit (Thermo Fisher Scientific).

RT-PCR

PCR was performed to amplify regions from exons 6 to 10 of *EYA3* mRNA under the 1LD condition. The PCR products from the retina, heart, spleen, lungs, liver, kidneys, testes, gall bladder, skeletal muscle, ovaries, and skin were separated using 1.5% agarose electrophoresis. The digital images of electrophoresis gels were acquired using Molecular Imager FX (Bio-Rad, Hercules, CA, USA).

Southern Blotting

The PCR products were separated using 1.5% agarose electrophoresis, and then blotted onto the Hybond-N⁺ membrane (GE Healthcare, Chicago, IL, USA) after alkaline denaturation for 16 h. The blotted membranes were fixed by UV crosslinking. Labeling of DNA probes with alkaline phosphatase and hybridization were performed using the AlkPhos Direct Labelling and Detection System (GE Healthcare) according to the manufacturer's protocol. The membranes were hybridized with alkaline phosphatase-labeled DNA fragments derived from the exon 7 region (92 bp) of *EYA3* cDNA at 55°C for 16 h. After washing twice with primary wash buffer (2 M urea, 0.1% SDS, 150 mM NaCl, 1 mM MgCl₂, 0.2% blocking reagent, and 50 mM phosphate buffer; pH 7.0) at 55°C for 10 min, the membranes were washed twice with secondary wash buffer (2 M NaCl, 2 mM MgCl₂, and 1 M Tris-HCl buffer; pH 9.5) at 25°C for 5 min. After treating the membranes with CDP-Star Detection Reagent (GE Healthcare), an *Eya3* mRNA splice variant containing exon 7 was detected using the LumiCube chemiluminescence analyzer (Liponics, Inc., Tokyo Japan) and quantified using JustTLC image analysis software (Sweday, Södra Sandby, Sweden).

Table 1. Primers used for PCR

cDNA		Sequence (5' -3')
qEYA3*	Forward	GAATCATGGAAGAGCCACAAGATTACCC
	Reverse	GTCGACGTTACAGGAAGTCTAACTCGAGGG
qEYA3**	Forward	ATCGGAAACAATGAGCCCTTAAC
	Reverse	ACAGAGGGAAAAGGAAAAG
Exon 7 [†]	Forward	GGTGCACGTGTGCCAGGTAT
	Reverse	AACCCTGTAGTGCAGGTAAGAACAC

* Applied for cDNA cloning. ** Applied for RT-PCR Southern blot.

[†] Applied for labelled DNA fragment for Southern blot.

Statistical Analysis

All data were analyzed using the Mann–Whitney U test with the statistics software GraphPad Prism version 4 (GraphPad Software, USA). Statistical significance was set at a *P*-value of less than 0.05.

Results

Identification of *EYA3* Splice Variants in Quail

There were four splice variants (exons 6, 7, 8, 9, and 10; exons 6, 8, 9, and 10; exons 6, 7, and 10; and exons 6 and 10) in the domain from exons 6 to 10 of *EYA3*. Multiple sequence alignments showed the exon 7 region of *Eya3* is not

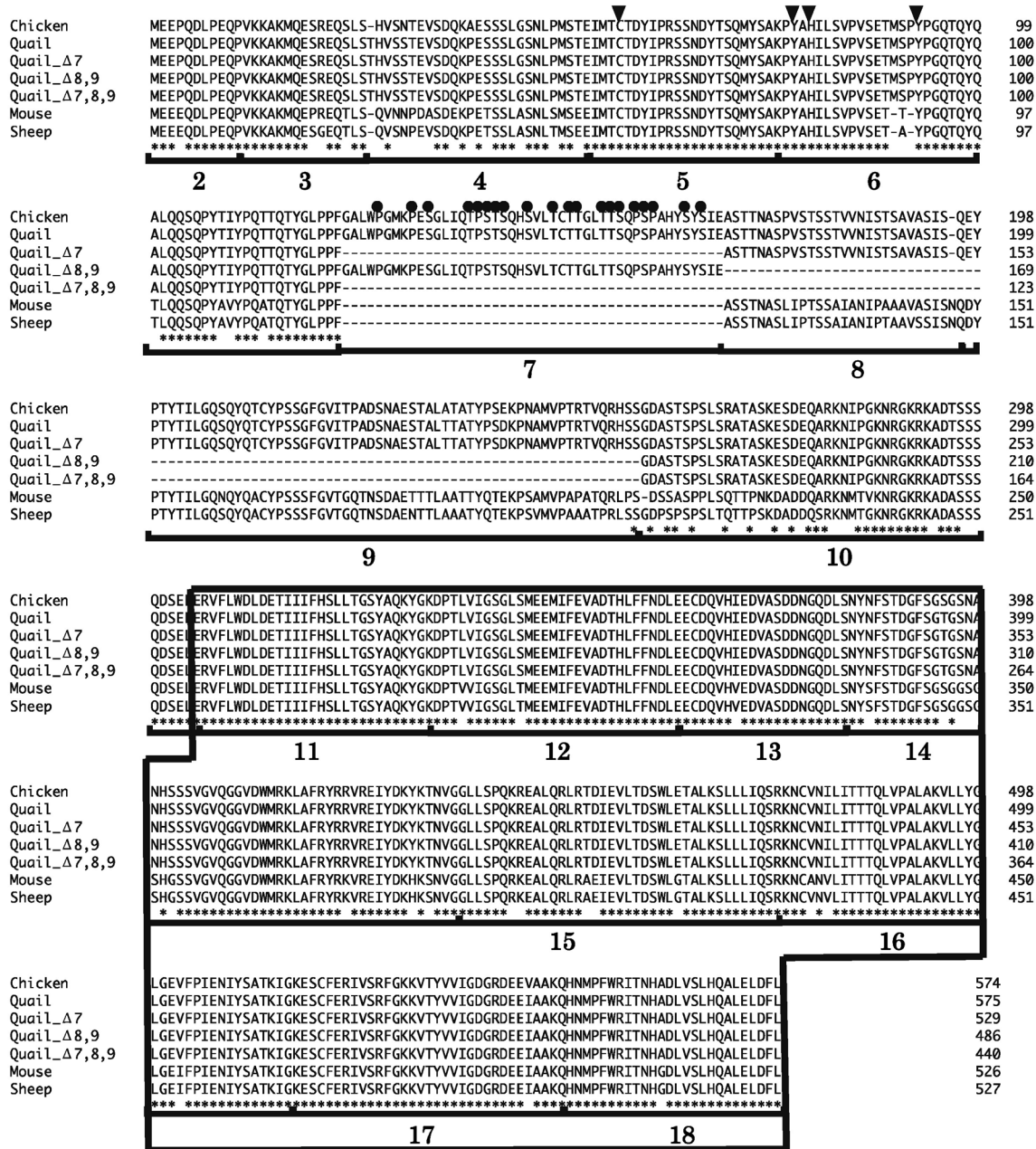


Fig. 1. Comparison of amino acid sequences of *Eya3* in chicken, quail, mouse, and sheep. Asterisks indicate identical amino acids. The arrowheads indicate potential residues, Cys-56, Tyr-77, His-79, and Tyr-90, involved in the threonine–phosphatase activity of mouse *Eya3*. The black circles indicate proline, serine, and threonine in exon 7. Boxed region indicates the *Eya* domain (ED). Bold numbers indicate the number of exons, referenced from the chicken ensemble database.

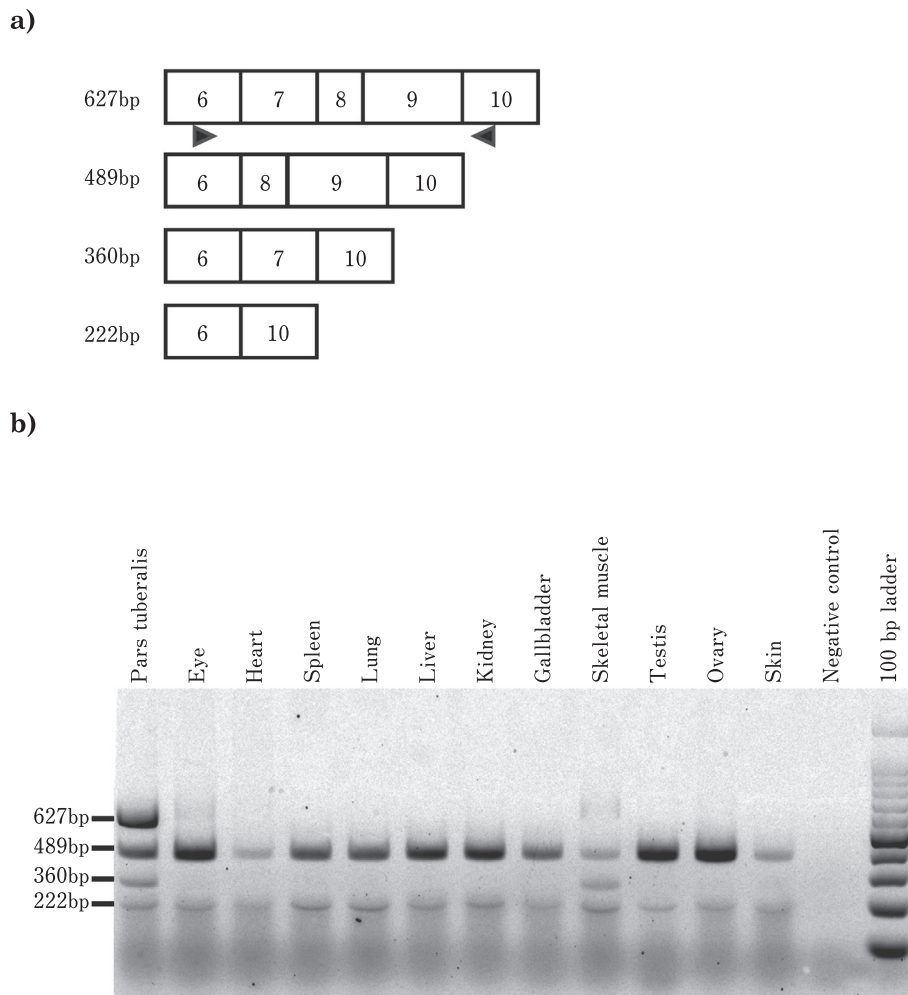


Fig. 2. Expression profiles of *EYA3* mRNA splice variants in quail tissues analyzed by RT-PCR. PCR was performed to amplify regions from exons 6 to 10 of *EYA3* mRNA under the 1LD condition. a: Schematic representation of the cDNA structure of *EYA3* mRNA splice variants. Boxed region indicates the exon. The numerals in the box indicate the number of exons. The 627 bp, 489 bp, 360 bp, and 222 bp indicate amplified cDNA size. The arrowheads show the position of primers used for PCR. b: Tissue distribution of splice variants determined using 1.5% gel electrophoresis. bp indicates base pair.

present in mouse and sheep (Fig. 1). In the four *Eya3* splice variants, the ED in the C-terminal domain, and Cys-57, Tyr-78, His-80, and Tyr-93 were well conserved in *Eya3* (Fig. 1). ***Tissue Distribution of EYA3 Splice Variant mRNA in Quail*** Because the exon 7, 8, and 9 regions of *EYA3* mRNA were spliced out, the level of *EYA3* mRNA splice variant containing exons 7, 8, and 9 in the PT, eye, heart, spleen, lungs, liver, kidneys, gallbladder, skeletal muscle, testes, ovary, and skin was determined using RT-PCR analysis. The splice variants containing exons 6, 8, 9, and 10 (489 bp), lacking exon 7, were observed in all the tissues. Furthermore, a low expression of spliced variants of exons 7, 8, and 9 was also observed in all the tissues. In contrast, a splice variant con-

taining exon 7 was expressed only in the PT and skeletal muscle. Notably, the expression of a splice variant containing exons 6, 7, 8, 9, and 10 was higher in the PT than in the other tissues under the 1LD condition (Fig. 2).

Expression of EYA3 Splice Variants was Day Length-dependent in the Quail PT

Splice variants of *EYA3* in the PT under SD and 1LD conditions determined by RT-PCR and Southern blotting showed that a 627 bp variant containing exons 6, 7, 8, 9, and 10 and a 360 bp variant containing exons 6, 7, and 10 were significantly elevated under the 1LD condition compared with that under the SD condition ($p < 0.01$, $n = 5$) (Fig. 3).

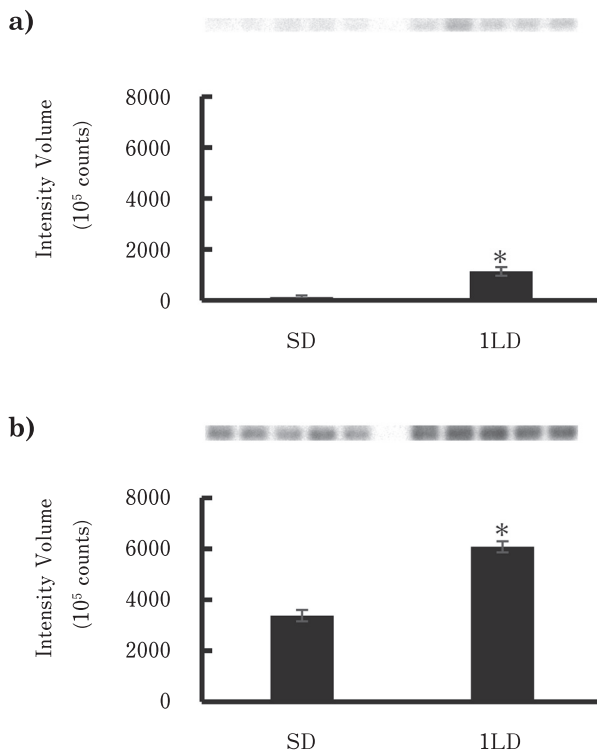


Fig. 3. *EYA3* mRNA containing exon 7 in the quail *parus tuberalis* under the SD and 1LD conditions. PCR was performed to amplify regions from exons 6 to 10 of *EYA3* mRNA. The primer position is shown in Fig. 2 and the probe for Southern blotting was 92 bp of *EYA3* exon 7. Each bar represents the mean \pm SEM ($n=5$). The upper panel of the graph shows the signal containing exon 7 of *EYA3* detected by chemiluminescence. SD and 1LD indicate short day condition and first long day condition, respectively. a: Expression of exons 6, 7, and 10 (360 bp) under the SD and 1LD conditions. b: Expression of exons 6, 7, 8, 9, and 10 (627 bp) under the SD and 1LD conditions. Asterisks indicate significant difference ($P < 0.01$).

Discussion

Here, the structural analysis of *EYA3* mRNA elucidated the function of *EYA3* in the photoperiodic response of the Japanese quail. There are four types of *EYA3* mRNA splice variants. Interestingly, exon 7, which is expressed in quail, is not present in mouse and sheep. A splice variant of *EYA3* containing exon 7 might participate in the function of the encoded protein in the photoperiodic response of quail. In the C-terminal of *EYA3*, the ED is conserved among the four splice variants of *EYA3*. Residues Cys-56, Tyr-76, His-79, and Tyr-90 in mouse *Eya3* are known to be essential for the threonine-phosphatase activity (Sano and Nagata, 2011). In quail, residues Cys-57, Tyr-78, His-80, and Tyr-93 were well conserved among the four splice variants of *EYA3*. Taken together, the four splice variants of *EYA3* have conserved sequences that function as transcriptional coactivators and

tyrosine and threonine phosphatases; however, their functions in the quail are unknown.

The tissue distribution of *EYA3* mRNA including exon 7 demonstrated that the expression of mRNA with exon 7 spliced out was widespread and was similar to the expression pattern of mouse *Eya3* (Zimmerman *et al.*, 1997); however, the variant containing exon 7 was only expressed in the PT and skeletal muscle. In particular, the mRNA expression of *EYA3* containing exons 7, 8, and 9 in the PT has been known to play a central role in photoperiodic control, and its expression was higher than that in the skeletal muscle. Moreover, comparative expression analysis of *EYA3* in the PT under the SD and 1LD conditions demonstrated that *EYA3* including exon 7 was the splice variant of the 1LD-specific gene. The amino acid sequence that exon 7 encodes corresponds to none of the domains that are necessary for the threonine phosphatase activity of *Eya3* (Sano and Nagata, 2011), and it involves ED and ED2, which are specific to the *Eya* family members (Zimmerman *et al.*, 1997). In contrast, the amino acid sequence of exon 7 was rich in proline, serine, and threonine, which is similar to the P/S/T-rich domain of the *Eya* family members, and might be involved in the transactivation function (Xu *et al.*, 1997a; Silver *et al.*, 2003).

The photoperiodic control in mammals involves TSH, which is regulated by *Eya3* expression before *TSH β* expression (Dardente *et al.*, 2010; Masumoto *et al.*, 2010). In quail, the peak expression of *EYA3* was almost concomitant with *TSH β* expression under the 1LD; *EYA3* expression decreased after the second long day under the 2-week-long day condition (Nakao *et al.*, 2008). To clarify how the transcriptional activity of *TSH β* mRNA is affected by *EYA3* under different long photoperiod stimuli, it is necessary to perform future studies on the subcellular localization of *EYA3* and *TSH β* based on *in situ* hybridization and the homo- and/or heterotypic dimerization-driven activation of *EYA3* protein.

In conclusion, we identified four types of splice variants of Japanese quail *EYA3* mRNA and showed that the splice variants containing exon 7 were expressed in the PT by the first long day stimulation. The exon 7 of *EYA3* is considered to be an important key element for elucidating the action of *EYA3* in the quail photoperiodic response. Detailed analysis of exon 7 is important to clarify the role of *EYA3* in the photoperiodic response.

Acknowledgments

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Conflicts of Interest

The authors declare no conflict of interest.

References

- Dardente H, Wyse CA, Birnie MJ, Dupré SM, Loudon AS, Lincoln GA and Hazlerigg DG. A molecular switch for photoperiod responsiveness in mammals. *Current Biology*, 20: 2193-2198.

- 2010.
- Hanon EA, Routledge K, Dardente H, Masson-Pévet M, Morgan PJ and Hazlerigg DG. Effect of photoperiod on the thyroid-stimulating hormone neuroendocrine system in the European hamster (*Cricetus cricetus*). *Journal of Neuroendocrinology*, 22: 51–55. 2010.
- Ikeda K, Watanabe Y, Ohto H and Kawakami K. Molecular interaction and synergistic activation of a promoter by Six, Eya, and Dach proteins mediated through CREB binding protein. *Molecular and Cellular Biology*, 22: 6759–6766. 2002.
- Jemc J and Rebay I. The eyes absent family of phosphotyrosine phosphatases: properties and roles in developmental regulation of transcription. *Annual Review of Biochemistry*, 76: 513–538. 2007.
- Li X, Oghi KA, Zhang J, Kronen A, Bush KT, Glass CK, Nigam SK, Aggarwal AK, Maas R, Rose DW and Rosenfeld MG. Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. *Nature*, 426: 247–254. 2003.
- Masumoto KH, Ukai-Tadenuma M, Kasukawa T, Nagano M, Uno KD, Tsujino K, Horikawa K, Shigeyoshi Y and Ueda HR. Acute induction of Eya3 by late-night light stimulation triggers TSH β expression in photoperiodism. *Current Biology*, 20: 2199–2206. 2010.
- Nakao N, Ono H, Yamamura T, Anraku T, Takagi T, Higashi K, Yasuo S, Katou Y, Kageyama S, Uno Y, Kasukawa T, Iigo M, Sharp PJ, Iwasawa A, Suzuki Y, Sugano S, Niimi T, Mizutani M, Namikawa T, Ebihara S, Ueda HR and Yoshimura T. Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature*, 452: 317–322. 2008.
- Ohto H, Kamada S, Tago K, Tominaga SI, Ozaki H, Sato S and Kawakami K. Cooperation of six and eya in activation of their target genes through nuclear translocation of Eya. *Molecular and cellular biology*, 19: 6815–6824. 1999.
- Okabe Y, Sano T and Nagata S. Regulation of the innate immune response by threonine-phosphatase of Eyes absent. *Nature*, 460: 520–524. 2009.
- Ono H, Hoshino Y, Yasuo S, Watanabe M, Nakane Y, Murai A, Ebihara S, Korf HW and Yoshimura T. Involvement of thyrotropin in photoperiodic signal transduction in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 105: 18238–18242. 2008.
- Rayapureddi JP, Kattamuri C, Steinmetz BD, Frankfort BJ, Ostrin EJ, Mardon G and Hegde RS. Eyes absent represents a class of protein tyrosine phosphatases. *Nature*, 426: 295–298. 2003.
- Sano T and Nagata S. Characterization of the threonine-phosphatase of mouse eyes absent 3. *FEBS Letters*, 585: 2714–2719. 2011.
- Silver SJ, Davies EL, Doyon L and Rebay I. Functional dissection of eyes absent reveals new modes of regulation within the retinal determination gene network. *Molecular and Cellular Biology*, 23: 5989–5999. 2003.
- Tootle TL, Silver SJ, Davies EL, Newman V, Latek RR, Mills IA, Selengut JD, Parlikar BE and Rebay I. The transcription factor Eyes absent is a protein tyrosine phosphatase. *Nature*, 426: 299–302. 2003.
- Watanabe M, Yasuo S, Watanabe T, Yamamura T, Nakao N, Ebihara S and Yoshimura T. Photoperiodic regulation of type 2 deiodinase gene in Djungarian hamster: possible homologies between avian and mammalian photoperiodic regulation of reproduction. *Endocrinology*, 145: 1546–1549. 2004.
- Xu PX, Cheng J, Epstein JA and Maas RL. Mouse Eya genes are expressed during limb tendon development and encode a transcriptional activation function. *Proceedings of the National Academy of Sciences of the United States of America*, 94: 11974–11979. 1997a.
- Xu PX, Woo I, Her H, Beier DR and Maas RL. Mouse Eya homologues of the Drosophila eyes absent gene require Pax6 for expression in lens and nasal placode. *Development*, 124: 219–231. 1997b.
- Yoshimura T, Yasuo S, Watanabe M, Iigo M, Yamamura T, Hirunagi K and Ebihara S. Light-induced hormone conversion of T4 to T3 regulates photoperiodic response of gonads in birds. *Nature*, 426: 178–181. 2003.
- Zimmerman JE, Bui QT, Steingrímsson E, Nagle DL, Fu W, Genin A, Spinner NB, Copeland NG, Jenkins NA, Bucan M and Bonini NM. Cloning and characterization of two vertebrate homologs of the Drosophila eyes absent gene. *Genome Research*, 7: 128–141. 1997.