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Studies of Opsin Genes in a Smelt Fish, Ayu (*Plecoglossus altivelis*)

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To investigate the visual and extra-ocular photoreception, we cloned the opsin genes in ayu (*Plecoglossus altivelis*). Amplified fragments encoding exon-4 (-5) of opsin cDNAs were cloned from the retina and brains of ayu, and sequenced. One clone was identified as rod (AYU-Rh), two as green cone (AYU-G1, -G2), one as red cone (AYU-R), two as ultraviolet cone (AYU-UV1, UV2), one as VA (AYU-VA), and one as extra-ocular rod (AYU-ExoRh) opsins. 335 amino acids sequence deduced from the full-length cDNA of AYU-Rh showed high identity with that of other fish. Southern blotting analysis indicated that ayu possess two 'rhodopsin' genes, one is visual rhodopsin and the other is non-visual extra-ocular rhodopsin. *In situ* hybridization showed that the mRNA of AYU-Rh was localized only in rod cells in the retina. On the other hands, AYU-ExoRh was expressed only in the pineal. We cloned two isoforms (AYU-VAM and -VAL) of VA opsin from ayu. The deduced amino acid sequences of these variants were identical to each other within the first 342 residues, but they showed divergence in the C-terminal sequence. AYU-VAL corresponded to the long isoform found in other fish, and AYU-VAM was identified as a new type of VA opsin variant. Pal-VAM is a new probably functional non-visual photoreceptive molecule in fish.

Key words: *Plecoglossus altivelis*, rhodopsin, cone opsin, vertebrate ancient opsin

INTRODUCTION

Ayu (*Plecoglossus altivelis*) is a smelt fish belonging to the family Osmeridae. A landlocked form of ayu exists in Lake Biwa. Within the landlocked form, there are two main types [1]. One is lake type, which is restricted to the lake throughout its life cycle, feeding on zooplankton and dwarfed in size. The other is the river type, which runs up to the middle reaches of the rivers flowing into the lake at young stage, mainly grazing on adherent algae on the stones on the river bed, and it grow up to the usual size for amphidromous ayu. This fish is expected to possess visual adaptability corresponding to the different. photic environments of its habitats.

Ayu shows photoperiodic responses in testicular development and overt circadian rhythms in which extra-ocular photoreception might be involved [2. 3]. This fish seems to be a suitable experimental model for the study of visual and non-visual photoreception.

In this study, we isolated, sequenced and characterized partial cDNAs of opsin genes expressed in ayu retina and pineal body, and also isolated the full-length cDNA encoding visual rhodopsin (AYU-Rh) and vertebrate ancient (VA) opsins (AYU-VAM, -VAL) using the landlocked form of ayu.

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MATERIALS AND METHODS

Materials. The landlocked lake type of ayu fish were collected at the shore of Lake Biwa (Shiga Pref., Japan) and the river type were collected in the middle reaches of the rivers flowing into the lake during summer.

Exon-4 cDNA cloning. Degenerate primers VVP-F1' and VVP-R2' [4] designed for vertebrate opsin proteins were used to amplify the cDNA fragments encoding ayu visual pigments by PCRs. PCR products of 210 bp were subcloned into plasmid vector and sequenced.

Cloning of complete ORF. 3' and 5' RACE were used to clone AYU-Rh and AYU-VA opsins. After sequencing, cDNAs including complete ORF were amplified, cloned and sequenced.

Southern blotting. Southern blotting analysis was carried out to determine the copy number of opsin genes.

RT-PCR. One step RT-PCR was performed to determine where AYU-Rh, -ExoRh, -VAM and -VAL opsins are expressed.

In situ *hybridization.* To investigate where AYU-Rh, -VAM and -VAL are expressed in the retina, *in situ* hybridization was carried out.

Chromophore analysis by HPLC. The quantitative analysis of retinaloxime and 3-dehydroxyretinaloxime was performed. The amount of oximes was estimated with the absorption coefficients.

RESULTS AND DISCUSSION

Exon-4 cDNA cloning. We cloned 8 opsins from the retina or pineal body. These opsins are belonging to Group-Rh, -Rh, -R, -G, -G, -UV, -UV, -VA and -VA, respectively.

Deduced amino acid sequences of AYU-Rh. AYU-Rh retained the conserved feature of functional opsin protein. AYU-Rh has aspartate and alanine at position 83 and 292, respectively, where blue sensitive rhodopsins have substitutions. In addition, glutamate at 122 and tryptophan at 265, which are known to affect the spectral sensitivity of green sensitive rhodopsins, were conserved in AYU-Rh [5]. These findings suggest that AYU-Rh is the green sensitive rhodopsin, consistent with the report that the λmax of vitamine A1 based ayu rhodopsin is 503 nm [6].

Southern blotting analysis of two 'rhodopsin' genes. Southern blotting revealed two hybridization bands in each lane, and the two bands showed the same molecular weight when each probe was used. These results indicated that each probe recognizes both the AYU-Rh and -ExoRh gene, and that two 'rhodopsin' genes are present in the ayu genome.

Expression of AYU-Rh and -ExoRh. RT-PCR experiment revealed that AYU-Rh is expressed only in the eye, and that AYU-ExoRh is expressed only in the pineal body. The AYU-Rh cRNA probe recognized only the rod cells in the retina. These results reveal that AYU-Rh is certainly the visual rhodopsin of ayu, and that AYU-ExoRh is one of the non-visual photoreceptive opsins.

Comparative analysis between lake and river type. No amino acid substitution was found between the corresponding opsins of two types. The results of Southern blotting, RT-PCR and *in situ* hybridization experiments on river type ayu were identical to those on lake type. In lake type ayu, the proportion of retinal was 87.2 ± 4.3 % (n=5) In the river type ayu, the proportion was 43.8 ± 6.5 % (n=5), significantly lower ($p<0.001$). These results imply that ayu adapts to photic environments through a change the chromophore composition.

Two isoforms of VA opsin. We isolated two kinds of cDNA variants, AYU-VAM and -VAL [7]. The deduced amino acid sequences of these variants were identical to each other within the first 342 residues. AYU-VAM and -VAL retained the conserved features required for opsin function. Two doublets of amino acids were lacking in the second extra-cellular loop and third intra-cellular loop. The deletion in the second extra-cellar loop were also observed in other non-visual photoreceptive molecules. The deletions in the third-cellar loop were observed only in salmon and ayu. That suggest VA opsin lacked this doublet in the Salmoniformes lineage, and that these opsins use different phototransduction cascades from other known opsins. AYU-VAL clearly corresponded to zebrafish VAL and carp VA opsin. AYU-VAM was a newly identified

variant of the VA opsin group, and has a carboxyl terminus of usual length. Some physiological functions of non-visual photoreceptors are considerable, such as entrainment of circadian rhythms, behavioral orientation, detection of seasonal changes in the photoperiod, regulation of body color, and regulation of serum hormone levels. As each role requires a distinct wavelength and distinct time of response, vertebrates would need to obtain photopigments with different characteristics and / or different phototransduction cascades. AYU-VAM and -VAL might play different roles in some of these important functions.

Southern blotting analysis of VA opsin gene. The result of Southern blotting experiment showed that only a single copy of the VA opsin gene exists in the genome of ayu, and indicated that these two isoforms are derived from this single gene. However, the boundary between the common and isoform-specific region is not the conserved splice site. This suggested that the generation mechanism of the two isoforms in the smelt is different from that in zebrafish [8].

Expression of VA opsins. RT-PCR experiment showed that both AYU-VAM and -VAL are expressed in the retina and brain. *In situ* hybridization revealed that AYU-VAM and / or -VAL are expressed in the amacrine cells in the retina. In contrast to previous studies indicating expression of VA opsins in the horizontal cells [8, 9], the hybridization signals were not detected in the horizontal cells of the smelt. The reasons for this discrepancy remain unknown. The expression pattern might change with the time of the day, the growth stage of the fish or environmental photic conditions reasons for this discrepancy remain unknown. The expression pattern might change with the time of the day, the growth stage of the fish or environmental photic conditions.

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