

Temporal and spatial changes in the expression pattern of multiple red and green subtype opsin genes during zebrafish development

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Summary

Zebrafish have two red, *LWS-1* and *LWS-2*, and four green, *RH2-1*, *RH2-2*, *RH2-3* and *RH2-4*, opsin genes encoding photopigments with distinct absorption spectra. Occurrence of opsin subtypes by gene duplication is characteristic of fish but little is known whether the subtypes are expressed differently in the retina, either spatially or temporally. Here we show by *in situ* hybridization the dynamic expression patterns of the opsin subtypes in the zebrafish retina. Expression of red type opsins is initiated with the shorter-wavelength subtype *LWS-2*, followed by the longer-wavelength subtype *LWS-1*. In the adult retina, *LWS-2* was expressed in the central to dorsal area and *LWS-1* in the ventral and peripheral areas. Expression patterns of green type opsins were

similar to those of the red type opsins. The expression started with the shortest wavelength subtype *RH2-1* followed by the longer wavelength ones, and in the adult retina, the shorter wavelength subtypes (*RH2-1* and *RH2-2*) were expressed in the central to dorsal area and longer wavelength subtypes (*RH2-3* and *RH2-4*) in the ventral and peripheral areas. These results provide the framework for subsequent studies of opsin gene regulation and for probing functional rationale of the developmental changes by using the power of zebrafish genetics.

Key words: visual pigment, opsin, zebrafish, retina, differential expression.

Introduction

Vertebrate retinas contain two types of visual photoreceptor cells, rods and cones. Rods are for dim-light vision and cones are for daylight and color vision. Photosensitive molecules, called visual pigments, are located in the outer segments of the visual cells. A visual pigment consists of a protein moiety, opsin and a chromophore, either 11-*cis* retinal or 11-*cis* 3,4-dehydroretinal (vitamin A1 or A2 aldehyde, respectively). Vertebrate visual opsins are classified into five phylogenetic types: RH1 (rod opsin or rhodopsin), RH2 (RH1-like, or green, cone opsin), SWS1 (short wavelength-sensitive type 1, or ultraviolet, cone opsin), SWS2 (short wavelength-sensitive type 2, or blue, cone opsin) and M/LWS (middle to long wavelength-sensitive, or red, cone opsin) (Yokoyama, 2000).

The so-called red and green opsins of humans belong to the M/LWS type and were created by gene duplication of an ancestral single-copy M/LWS opsin gene during primate evolution and, hence, are subtypes of M/LWS opsins. Besides the M/LWS opsins in primates, occurrence of opsin subtypes has only been reported in fish. There are the M/LWS opsins of Mexican cavefish (R007, G101 and G103; Yokoyama and Yokoyama, 1990), RH2 opsins of goldfish (GFgr-1 and GFgr-2; Johnson et al., 1993), SWS2 opsins of cichlid (SWS2-A and SWS2-B; Carleton and Kocher, 2001) and RH1 opsins of eels

(fresh-water and deep-sea types; Archer et al., 1995; Zhang et al., 2000).

We recently screened a zebrafish genomic library for subtype repertoires in all five types of the visual opsins (Hamaoka et al., 2002; Chinen et al., 2003). Zebrafish were found to possess two subtypes of M/LWS opsin genes, *LWS-1* and *LWS-2*, and four RH2 subtype genes, *RH2-1*, *RH2-2*, *RH2-3* and *RH2-4*, in addition to a single-copy of RH1, SWS1 and SWS2 opsin genes (Chinen et al., 2003). The two M/LWS opsin genes are arrayed in tandem with a 1.8 kb interval, 2.5 kb downstream from the SWS2 opsin gene in the chromosomal linkage group (LG) 11 (see also GenBank accession no. AL844847 for a zebrafish genome sequence containing the three genes under symbol names *opn1sw2*, *opn1lw1* and *opn1lw2* for SWS2, *LWS-1* and *LWS-2*, respectively). The four RH2 opsin genes are also arranged in tandem in approximately a 25 kb genomic region in the LG 6 (see GenBank AL732567 for a genome sequence containing the four genes under symbol names *opn1mw1*, *opn1mw2*, *opn1mw3* and *opn1mw4* for *RH2-1*, *RH2-2*, *RH2-3* and *RH2-4*, respectively). Importantly, absorption spectra represented with the wavelength of peak absorbance (λ_{max}), differ markedly between the two M/LWS pigments reconstituted with the A1 chromophore *in vitro* at

558 nm (*LWS-1*) and 548 nm (*LWS-2*). The four A1-reconstituted RH2 pigments are at wavelengths 467 nm (*RH2-1*), 476 nm (*RH2-2*), 488 nm (*RH2-3*) and 505 nm (*RH2-4*) (Chinen et al., 2003). These subtype genes are all expressed in the adult zebrafish retina with *LWS-1* and *RH2-2* having the highest expression level within each type (Chinen et al., 2003).

Occurrence of opsin subtypes in fish may reflect the diversity of aquatic light environments. However, documented variations of spectral sensitivities of visual pigments among fish species are mostly attributed to chromophore type A1 or A2 (Bowmaker, 1995), evolutionary changes of opsin amino acid sequences (Yokoyama, 2000), opsin types expressed in the retina (Carleton and Kocher, 2001), or developmental changes of expressed opsin types in a photoreceptor cell (Cheng and Novales Flamarique, 2004). Only two cases of differential usage of opsin subtypes have been reported; RH1 opsins of eels at different ontogenic stages (fresh-water and deep-sea types; Archer et al., 1995; Zhang et al., 2000) and SWS2 opsins of cichlids where different species inhabiting different niches expressed different subtypes (SWS2-A and SWS2-B; Carleton and Kocher, 2001). Little is known how opsin subtypes are distributed phylogenetically and ecologically among fish species and how differently they are expressed in the retina spatially and temporally in a given species.

In this study we examined expression of subtype opsin genes of zebrafish, the only fish species whose complete repertoires of visual opsin genes have been isolated and spectrally characterized (Chinen et al., 2003). We have shown by *in situ* hybridization (ISH) that the *M/LWS* and *RH2* opsin genes are expressed differently among subtypes in spatial distribution in the retina during development of zebrafish.

Materials and methods

Preparation of digoxigenin-labeled RNA probes

The entire 3'-untranslated region (UTR) was PCR-amplified from either genomic DNA or cDNA analyzed previously (Chinen et al., 2003) for *RH2-1* (252 bp after stop codon; see GenBank AB087805 for sequence), *RH2-2* (549 bp after stop codon; GenBank AB087806), *RH2-3* (277 bp after stop codon; GenBank AB087807) and *RH2-4* (243 bp after stop codon; GenBank AB087808). A portion of 3'-UTR was amplified for *LWS-1* (451 bp after the stop codon; GenBank AB087803) and *LWS-2* (313 bp including the last 10 bp of the coding region [CDR]; GenBank AB087804) from genomic DNA. The amplified DNA fragments were cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and were transcribed using a digoxigenin (DIG) RNA labeling kit (Roche, Basel, Switzerland) after linearization.

Similarly, RNA probes for the full-length CDR were prepared for *LWS-2* (1071 nucleotides [nt]; GenBank AB087804), *RH2-1* (1050 nt; GenBank AB087805), *RH2-4* (1050 nt; GenBank AB087808), *SWS1* (1011 nt; GenBank AB087810), *SWS2* (1065 nt; GenBank AB087809) and *RH1* (1065 nt; GenBank AB087811) from their cDNA clones previously isolated, either in pBluscript II (SK-) (Stratagene,

La Jolla, CA, USA) or pGEM-T easy vector (Chinen et al., 2003).

In situ hybridization (ISH)

Zebrafish (*Danio rerio*) were maintained at 28.5°C in 14 h:10 h light:dark cycle by following a standard procedure (Westerfield, 1995). Embryos [defined here as fish up to 3 days post fertilization (dpf)] of zebrafish WIK strain were raised in 0.003% 1-phenyl-2-thiourea after 12 h pf (hpf) to disrupt pigment formation and were subjected to whole-mount ISH as previously described (Hamaoka et al., 2002). For ISH of larvae (defined as 3 days to 1 month pf) and juveniles (defined as 1–2 months pf), we used an albino strain, B4, to facilitate observation of the photoreceptor layer, which is masked in non-albino strains by the dark retinal pigment epithelium. The albino mutation has been shown not to affect opsin genes and pattern formation of retinal cells (Nawrocki et al., 1985; Ren et al., 2002). Whole bodies of larvae and dissected heads of juveniles were subjected to ISH. For adults (defined as after 2 months pf; WIK strain), light-adapted eyes were enucleated and lenses were removed. The samples were soaked in fixer (4% paraformaldehyde, 0.1 mol l⁻¹ phosphate-buffer) overnight at 4°C. For some samples, the fixer was directly injected into the inside of the eyecup, which is filled with viscid fluid, immediately after enucleation of the eyes and then soaked in the fixer overnight at 4°C to reduce RNA degradation during fixation. Cryosections of retinas were prepared and hybridized to the RNA probes as previously described (Hamaoka et al., 2002). In this study, to compare expression patterns among different subtype opsins, adjacent sections from the same eyes were hybridized with different probes so that entire expression patterns of opsin subtypes in the retina could be reconstructed. After hybridization, samples were washed at 65°C twice in 50% formamide, 2× SSC (1× 0.15 mol l⁻¹ NaCl, 0.015 mol l⁻¹ sodium citrate, pH 7.6) for 30 min each, once in 2× SSC for 10 min and twice in 0.5× SSC for 20 min each. The samples were then rinsed twice in MABT (0.1 mol l⁻¹ maleic acid, 0.15 mol l⁻¹ NaCl, 0.1% Triton-X, pH 7.5) at room temperature, soaked once in 2% blocking reagent (Roche) in MABT for 30 min, incubated with anti-DIG antibody (1:2000 dilution) in the blocking solution for 2 hours, and washed six times in MABT for 10 min each.

Results

RH2 opsin expression in embryonic zebrafish retina

RH2 (green) opsin gene expression has not been examined in embryonic zebrafish retina (Raymond et al., 1995; Robinson et al., 1995; Schmitt et al., 1999). According to sequence similarities in the CDR, the four *RH2* opsins of zebrafish are designated into the *RH2-1/RH2-2* group (85% identity between *RH2-1* and *RH2-2*) and the *RH2-3/RH2-4* group (93% between *RH2-3* and *RH2-4*) (Chinen et al., 2003). Between the two groups, sequence identities are 76–79%. Therefore, to fully view the pattern of *RH2* opsin expression, it is necessary to use both groups of *RH2* probes.

When using the *RH2-1* CDR probe, which detects both *RH2-1* and *RH2-2* transcripts, the first hybridization signal was detected at 45 hpf in a ventral patch, which was in the nasal side of choroid fissure (Fig. 1A). The signal spread to the nasal and central retina (Fig. 1B), then to ventrotemporal retina (Fig. 1C), and finally to the dorsal retina (Fig. 1D), being largely concordant with previously reported progression patterns of the red and blue opsin gene expression (Raymond et al., 1995). We then used *RH2-1* and *RH2-2* 3'-UTR probes (38% identity between the two probes) to distinguish expressions of the two genes. Identical results were obtained using the *RH2-1* 3'-UTR probe, but no reliable signal was detected using the *RH2-2* 3'-UTR probe in embryos examined up to 72 hpf, indicating that the hybridization pattern using the *RH2-1* CDR probe represented the expression pattern of *RH2-1*, and that *RH2-2* is not expressed in the embryonic stage at a detectable level.

When we used the *RH2-4* CDR probe, which detects both *RH2-3* and *RH2-4*, the first signal appeared weakly at 72 hpf in the most marginal side of nasal retina in a broad area (Fig. 1E). This pattern of expression is different to those in other opsins where it is initiated in a ventral spot, as in *RH2-1* (see Fig. 1A). However, no signal was detected using either *RH2-3* or *RH2-4* 3'-UTR probe (70% identity between the two) and we could not distinguish which subtype was expressed between *RH2-3* and *RH2-4*. This is likely due to the shortness of the 3'-UTR probes compared with the CDR probe (see Materials and methods for their lengths).

RH2 opsin expression in larval and juvenile zebrafish retina

Following *RH2-1*, expression of *RH2-2* was observed throughout the retina by 7 dpf. However, at 16 dpf, expression of *RH2-1* disappeared in the marginal area of retina (Fig. 2A, double arrows), in contrast, *RH2-2* expression disappeared in the central retina in many photoreceptor cells (Fig. 2B, double arrow). This tendency was more obvious at 1 month pf, where expression of *RH2-1* was confined to the central area (Fig. 2D) and that of *RH2-2* in the area surrounding it (Fig. 2E). In addition, *RH2-2* expression at this stage disappeared in the ventral margin of the retina (Fig. 2E, area below the triple stars), and the expression of *RH2-3/RH2-4* filled the space (Fig. 2F), which was detected by the *RH2-4* CDR probe (but not by either the *RH2-3* or *RH2-4* 3'-UTR probe as in Fig. 1E). At 16 days pf, the *RH2-4* CDR probe detected a few signals in this area (Fig. 2C, arrows), which were again not detectable by the 3'-UTR probes for *RH2-3* and *RH2-4*. It was noted that at 1 month pf, expression areas of *RH2-1* and *RH2-2* overlapped at the boundaries (single and double stars in Fig. 2D and E) and so were those of *RH2-2* and *RH2-3/RH2-4* (triple stars in Fig. 2E and F).

RH2 opsin expression in adult zebrafish retina

In adults (2 years old), expression pattern of *RH2-1* was consistent with that at 1 month pf, where it covered the central to dorsal area of the retina (Fig. 2G). By contrast, *RH2-2* expression changed dramatically; the central area of expression

in the retina, which disappeared in the larval to juvenile stages (Fig. 2B,E), was restored (Fig. 2H) so that the *RH2-2* expression covered a larger central to dorsal area than the *RH2-1* area. The restoration of the central expression of *RH2-2* occurred between 1–2 months pf. The hybridization signal of *RH2-2* was much stronger than that of *RH2-1*, which was consistent with our previous observation in real-time RT-PCR, in that the expression level of *RH2-2* in adult retina is

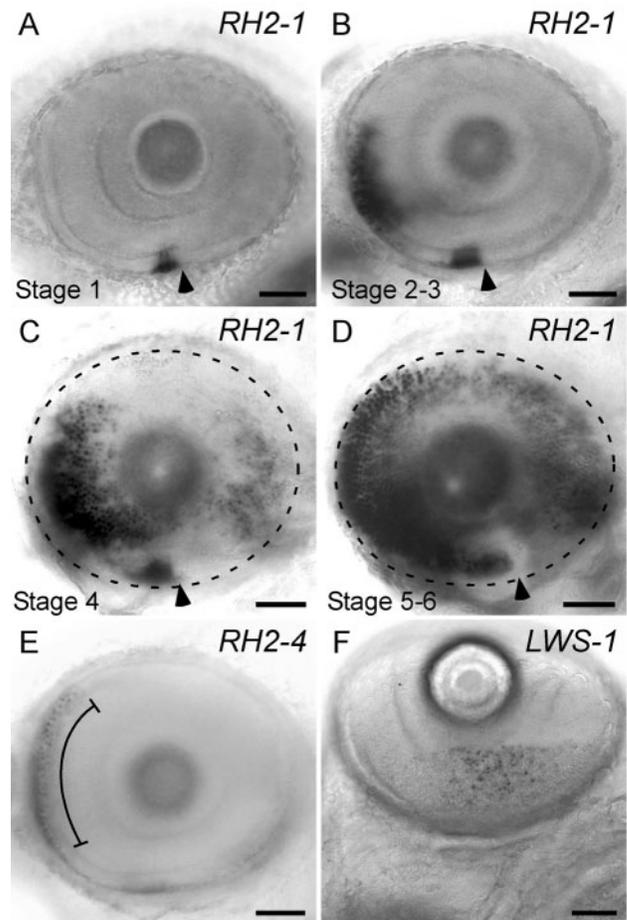


Fig. 1. Whole-mount ISH of *RH2* and *M/LWS* opsin probes to embryonic or larval eyes of zebrafish. (A–D) Lateral views of embryonic eyes (nasal side to the left and ventral to the bottom) hybridized with the *RH2-1* CDR probe, where progress of the gene expression is staged as follows: stage 1, expression is detected in a patch of ventronasal retina; stage 2–3, another patch appears in nasal retina and the two fuse to fill the ventronasal retina; stage 4, expression extends from the ventronasal region to the central region and, less extensively, in dorsal and ventrotemporal directions; stage 5–6, expression is detected in more than three-fourths of the retina (Raymond et al., 1995; Takechi et al., 2003). Dotted line indicates rim of the eye. Arrowhead indicates location of the choroids fissure. (E) A lateral view of an embryonic eye (nasal side to the left and ventral to the bottom) hybridized with the *RH2-4* CDR probe at 72 hpf. Hybridized area is indicated with a bracket. (F) A ventral view of a larval eye (nasal side to the left) hybridized with *LWS-1* 3'-UTR probe at 5.5 dpf. All scale bars, 50 μ m.

significantly higher than the other RH2 subtype genes (Chinen et al., 2003).

In adults we were able to detect expression of *RH2-3* and *RH2-4* separately using the 3'-UTR probes. *RH2-4* was expressed in the ventral side of retina (Fig. 2I, area below the arrow head) and at the ciliary marginal zone (CMZ) (Fig. 2I, arrow). *RH2-3* expression was confined to a narrow zone in the centro-ventral area and dorsal CMZ (Fig. 3A, double arrows).

When expression areas of *RH2-3* and *RH2-4* were compared in adjacent sections prepared from the same eyes, the *RH2-3* and *RH2-4* zones did not appear to overlap (Fig. 3B and C). In addition, in the centro-ventral area the edge of the *RH2-2* zone (Fig. 2H, arrowhead) and that of *RH2-4* zone (Fig. 2I, arrowhead) also appeared not to overlap.

A previous immunohistochemical study using an antibody against a zebrafish green opsin (*zfgr1*, corresponding to

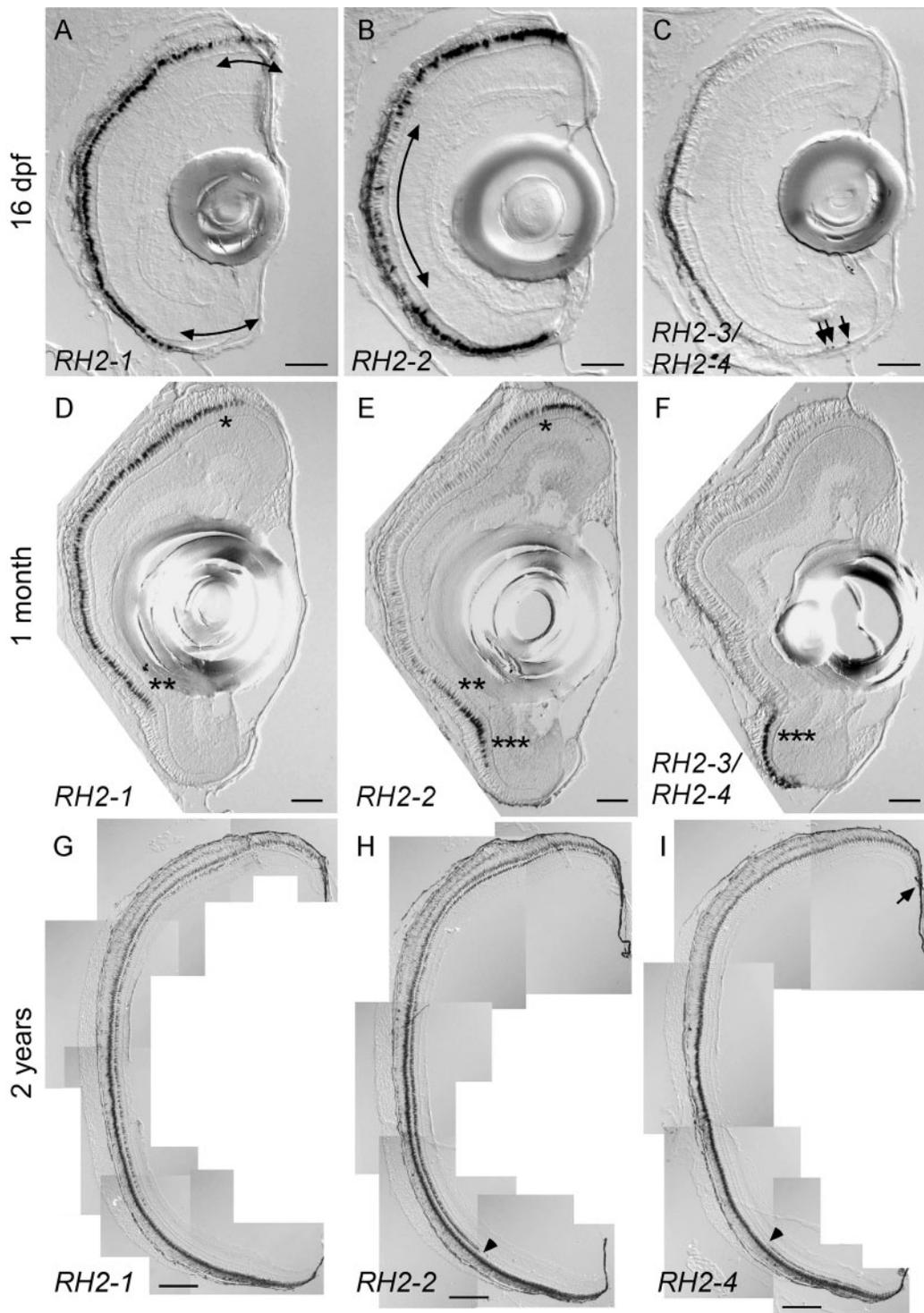


Fig. 2. ISH of RH2 opsin probes to transverse sections of zebrafish eyes. (A–C) Serial eye sections at 16 dpf hybridized with the *RH2-1* 3'-UTR (A), *RH2-2* 3'-UTR (B) and *RH2-4* CDR (C) probes. Double arrows in (A) and (B) indicate the areas where the expression of *RH2-1* and *RH2-2* are disappearing, respectively. Arrows in (C) indicate the scarce hybridization signals detected for *RH2-3/RH2-4*. (D–F) Serial eye sections at 1 month pf hybridized with *RH2-1* 3'-UTR (D), *RH2-2* 3'-UTR (E) and *RH2-4* CDR (F) probes. The single stars, double stars, and triple stars are position markers showing their corresponding locations between sections. (G–H) Serial sections at 2 years pf hybridized with the *RH2-1* 3'-UTR (G), *RH2-2* 3'-UTR (H) and *RH2-4* 3'-UTR (I) probes. Arrowheads in (H) and (I) point to the same location between the sections. An arrow in (I) indicates the hybridization signal of *RH2-4* at ciliary marginal zone. In all panels, the dorsal side is to the top and ventral is to the bottom. Scale bars, 50 μ m (A–F) or 200 μ m (G–I).

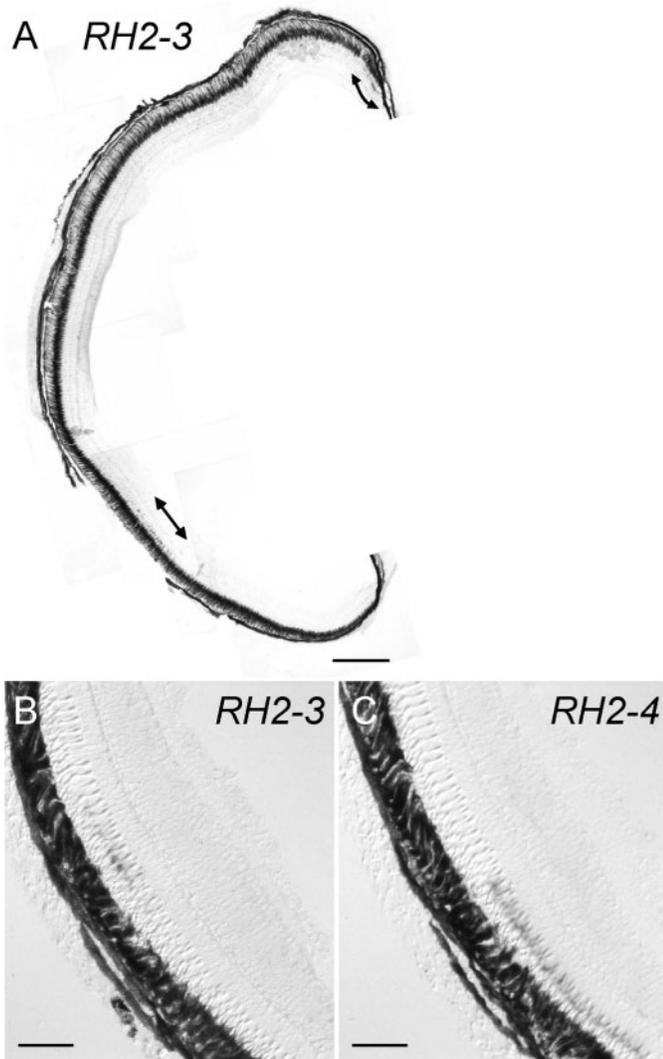


Fig. 3. Expression of *RH2-3* and *RH2-4* in an adult zebrafish retina. (A) A transverse section of an eye at 2 years pf hybridized with the *RH2-3* 3'-UTR probe. Double arrows indicate the areas of *RH2-3* expression. (B,C) Neighboring transverse sections at higher magnification hybridized with the *RH2-3* 3'-UTR (B) and *RH2-4* 3'-UTR (C) probes. In all panels, the dorsal side is to the top and ventral is to the bottom. Scale bars, 200 µm (A) or 50 µm (B and C).

RH2-1) showed that green opsin is produced only in short double cones (SDC) in adult zebrafish retina (Robinson et al., 1993; Vihtelic et al., 1999). An ISH study using goldfish green opsin cRNA probe, which is more closely related to *RH2-3/RH2-4* than to *RH2-1/RH2-2* (Chinen et al., 2003), also localized the hybridization signal to SDC in adult zebrafish retina (Raymond et al., 1993; Robinson et al., 1993). Our ISH to tangential sections of adult retina confirmed that *RH2-1*, *RH2-2* and *RH2-4* were expressed only in SDC in the square mosaic arrangement of cones (Robinson et al., 1993) using *RH2-1* CDR and *RH2-4* 3'-UTR probes (data not shown). The *RH2-3*-expressing zone was too narrow for us to determine reliably its cell type in the cone

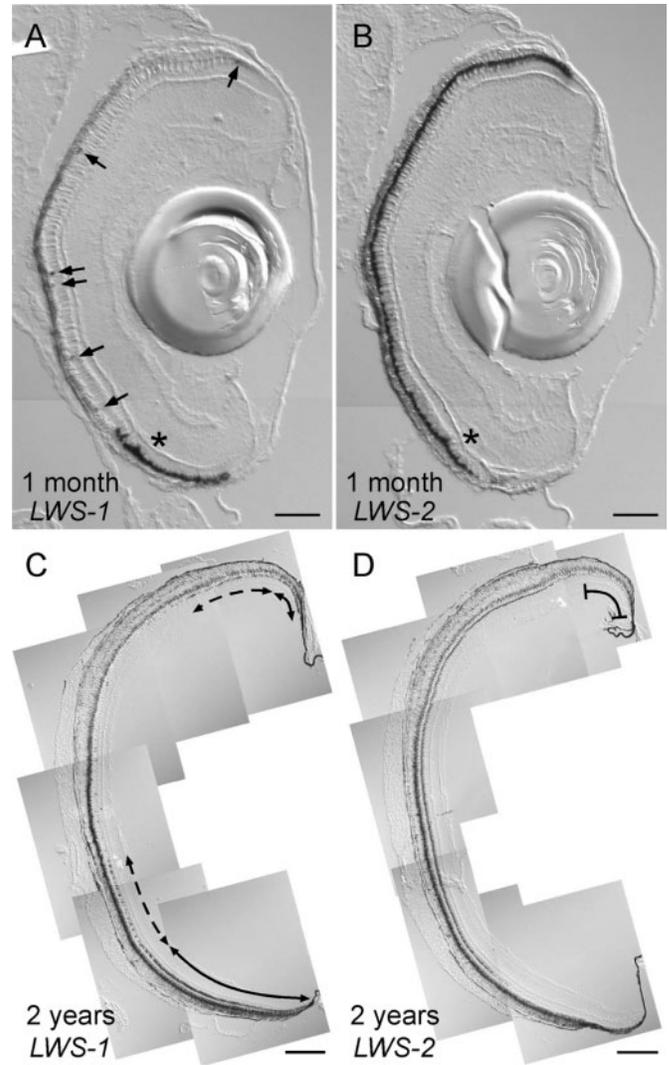


Fig. 4. ISH of M/LWS opsin probes to transverse sections of zebrafish eyes. (A,B) Serial eye sections at 1 month pf hybridized with the *LWS-1* 3'-UTR (A) and *LWS-2* 3'-UTR (B) probes. Arrows in (A) indicate the sparse hybridization signals of *LWS-1*. Corresponding locations between the two sections are indicated with a star. (C,D) Serial sections at 2 years pf hybridized with the *LWS-1* 3'-UTR (C) and *LWS-2* 3'-UTR (D) probes. The solid and dotted double arrows in (C) indicate the areas where *LWS-1* is expressed robustly and sparsely, respectively. The bracket in (D) indicates the area where *LWS-2* expression disappeared on the dorsal side of the retina. In all panels, the dorsal side is to the top and ventral is to the bottom. Scale bars, 50 µm (A,B) or 200 µm (C,D).

mosaic. However, it is most likely that all RH2 subtype genes are expressed in SDC.

LWS opsin expression in zebrafish retina

Transcripts of the two M/LWS (red) opsin genes, highly homologous in CDR (93% identity), were distinguished using the 3'-UTR probes (48% identity between the two genes). Expression of *LWS-2* was first observed at 40 hpf as a ventral patch in the retina. It then spread throughout the retina in the

same spatial pattern as shown for *RH2-1* (Fig. 1A–D). In contrast, initial expression of *LWS-1* was observed in

3.5–5.5 dpf in a broad area in the marginal side of the ventral retina (Fig. 1F). These results indicate that the red opsin

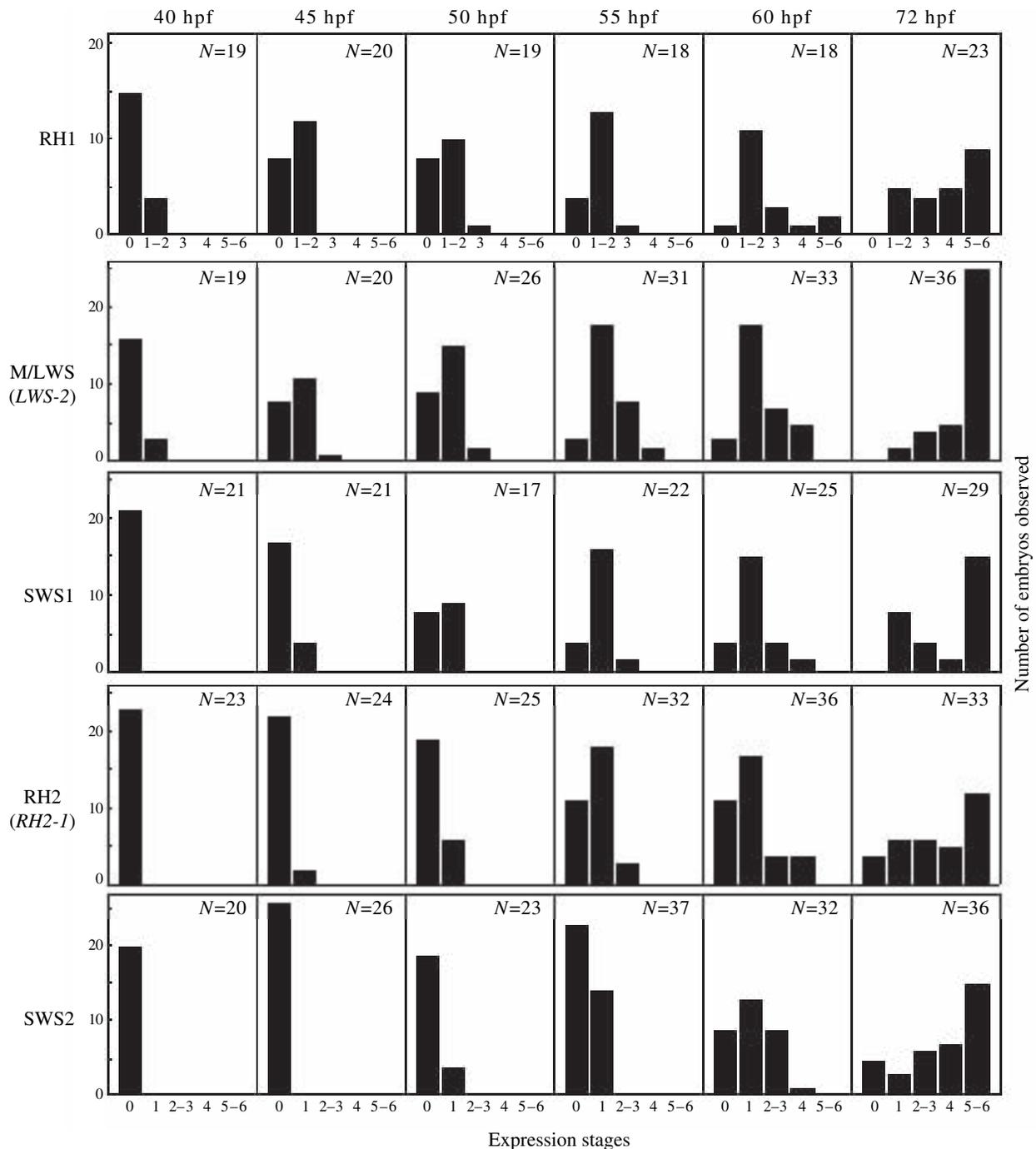


Fig. 5. Progression of gene expression of the five opsin types (RH1, M/LWS, SWS1, RH2 and SWS2) in embryonic zebrafish retina sampled at 40, 45, 50, 55, 60 and 72 hpf. Embryos sampled at each hpf were examined for staging opsin expression by whole-mount ISH with CDR probes of the five opsin genes (total number of embryos examined, N , are indicated in each panel). The number of embryos (vertical axis) showing certain expression stages (horizontal axis) was counted at each hpf for each gene. One eye was examined per embryo. Stage 0 indicates that no expression was detected in an observed retina. For RH1, subsequent stages are defined as follows (Raymond et al., 1995; Hamaoka et al., 2002): stage 1–2, expression is detected in a patch of ventronasal retina; stage 3, expression is detected across the choroid fissure from the primary ventronasal patch; stage 4, hybridization signal increases in the ventrotemporal patch and scatters outside the ventral region; stage 5–6, ventrotemporal and ventronasal patches are at equivalent density and fuse across the fissure to form a dumbbell shape, and density of the signal outside the ventral region further increases. For cone opsins (M/LWS, SWS1, RH2 and SWS2), refer to Fig. 1 legend for definitions of stages.

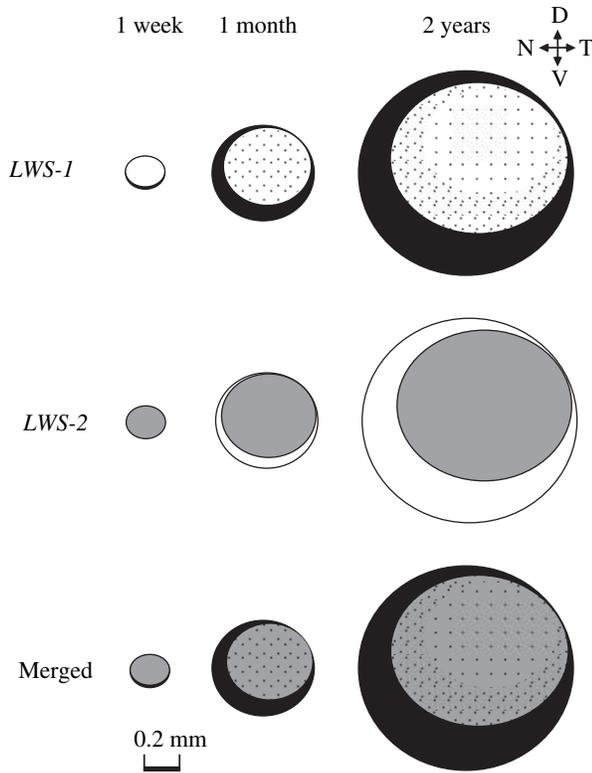


Fig. 6. Schematic representation of the spatiotemporal expression patterns of M/LWS opsin subtypes in zebrafish retina (lateral views) reconstructed from ISH to serial sections. Expression areas of *LWS-1* and *LWS-2* are indicated with black and gray, respectively. Black dots indicate the sparse expression of *LWS-1* in the central area of the retina. D, dorsal; V, ventral; N, nasal; T, temporal.

expression reported in previous studies (Raymond et al., 1995; Schmitt et al., 1999) is that of *LWS-2* but not of *LWS-1* (*LWS-1* was originally known as a sole cDNA species of zebrafish red opsin, Schmitt et al., 1999; Vihtelic et al., 1999; Chinen et al., 2003).

At 1 week pf, while expression of *LWS-1* was confined to the ventral side of the retina, the expression of *LWS-2* was present in the entire retina. At 1 month pf, ISH in transverse retinal sections showed that expression of *LWS-2* disappeared in the ventral side (Fig. 4B, area below the star) where complementarily *LWS-1* expression was observed (Fig. 4A). It was noted that expression areas of the two genes overlapped at the boundary (Fig. 4A,B, stars). Expression of *LWS-1* was also observed sparsely in photoreceptor cells in the central to dorsal retina (Fig. 4A, arrows).

In sexually mature adults (2 years old), expression of *LWS-1* was observed not only in the ventral area but also in the dorsal periphery (Fig. 4C, solid double arrows). In addition, the number of cone cells expressing *LWS-1* appeared to increase in the central and dorsal areas (Fig. 4C, dotted double arrows). Conversely, *LWS-2* was expressed in the central to dorsal retina but was not in the dorsal peripheral region (Fig. 4D, bracket). The expression profiles of the two genes are complementary in

the retina, although cells at the boundary of the two fields and those expressing *LWS-1* in the central *LWS-2* zone appeared to express both gene subtypes. It was noted that the hybridization signal of *LWS-1* was stronger than that of *LWS-2* using the same amount of probe, which is consistent with our previous observation by real-time RT-PCR, that the *LWS-1* transcript is more abundant than that of *LWS-2* in the adult retina (Chinen et al., 2003). Previous immunohistochemical and ISH studies showed that red opsin is produced only in long double cones (LDC) in adult zebrafish retina (Raymond et al., 1993; Robinson et al., 1993; Vihtelic et al., 1999). Our ISH to tangential sections of retina confirmed that both *LWS-1* and *LWS-2* genes are expressed in LDC (data not shown).

Sequence of expression among opsin types in embryonic zebrafish retina

We also examined the onset times of other opsin types SWS1, SWS2 and RH1, together with M/LWS and RH2 opsins to re-evaluate relative onset timing among the five types of opsins by using their full-length CDR probes. *LWS-2* and *RH2-1* were used to represent M/LWS and RH2 types, respectively, because they are the first subtypes expressed in their opsin types. Whole-mount ISH was performed on embryos sampled at 40, 45, 50, 55, 60 and 72 hpf and expression patterns of the opsin genes were assigned to appropriate stages according to Raymond et al. (1995), Hamaoka et al. (2002) and Takechi et al. (2003) (Fig. 5). RH1 and M/LWS were the first opsins showing expression in the retina (at 40 hpf), consistent with previous studies (Raymond et al., 1995; Schmitt et al., 1999), and were followed by SWS1 and RH2 (at 45 hpf) and then SWS2 (at 50 hpf), which differed from the order suggested in a previous study, i.e. green, blue and ultraviolet (Schmitt et al., 1999). We noticed that the DNA sequence assigned to the zebrafish blue opsin gene (GenBank AF104903) in Schmitt et al. (1999) was in fact that of SWS1 (i.e. ultraviolet) but not of SWS2 opsin genes, which could have introduced an error in the evaluation of the expression order among the opsin genes.

Onset times of the five opsin types in this study (40–50 hpf) were overall earlier than those reported in the previous studies (50–55 hpf) (Raymond et al., 1995; Schmitt et al., 1999). The early onset times may be partly due to the fact that Schmitt et al. (1999) selected only embryos that had achieved certain developmental characteristics at certain time points to minimize individual variation whereas we did not do any selection (see also Takechi et al., 2003).

Discussion

We previously showed that zebrafish has two spectrally differentiated M/LWS (red) opsin genes, *LWS-1* and *LWS-2*, which are arrayed in tandem in the genome, and four RH2 (green) opsin genes, *RH2-1*, *RH2-2*, *RH2-3* and *RH2-4*, also spectrally distinct and arranged in tandem (Chinen et al., 2003). In this study we demonstrated by ISH that these subtype opsin genes are expressed at different times and in different

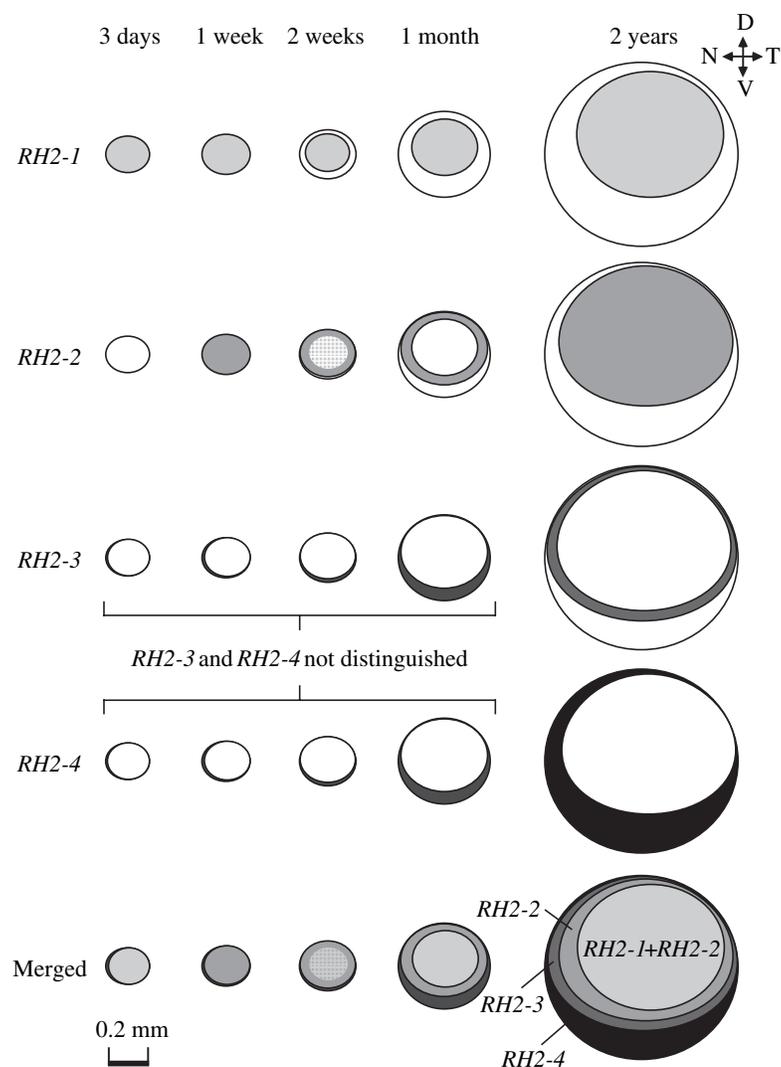


Fig. 7. Schematic representation of the spatiotemporal expression patterns of RH2 opsin subtypes in zebrafish retina (lateral views) reconstructed from ISH to serial sections. Expression areas of *RH2-1*, *RH2-2* and *RH2-3* are indicated with different shades of gray (light, middle and dark, respectively). That of *RH2-4* is indicated with black. Dots in *RH2-2* expression at two weeks pf indicates sparse expression in the central retina. Up to 1 month pf, expression of *RH2-3* and *RH2-4* was not distinguishable. D, dorsal; V, ventral; N, nasal; T, temporal.

areas in the retina. Expression patterns of the subtypes of M/LWS and RH2 opsin genes were similar in two aspects: (1) expression started with the shorter-wavelength subtypes followed by longer-wavelength ones and (2) in the adult retina, shorter-wavelength subtypes were expressed in the central to dorsal area and longer-wavelength subtypes were in the ventral and peripheral areas. Expression patterns of the M/LWS and RH2 opsin genes throughout development are summarized in Figs 6 and 7, respectively.

In early development of zebrafish, expression of *LWS-2*, the shorter-wavelength subtype of M/LWS opsins (λ_{\max} at 548 nm), precedes expression of *LWS-1*, the longer-

wavelength-sensitive subtype (558 nm), and is distributed more widely in the retina than *LWS-1* expression (Fig. 6). Conversely, in the adult retina expression level of *LWS-2* is lower than that of *LWS-1* (Chinen et al., 2003), indicating an overall spectral shift of M/LWS opsin type from short to long wavelength direction throughout development. This is consistent with an earlier observation by microspectrophotometry (MSP) that λ_{\max} of a class of double cone shifts from ~540 nm in early larvae (6–8 dpf) to ~560 nm in late larvae (11–17 dpf) and adults (1–2 years old) (Nawrocki et al., 1985).

Among the four RH2 opsin genes, the shortest-wavelength subtype *RH2-1* (467 nm) is first expressed throughout the retina by ~3 dpf (Fig. 7). Subsequently, expression of *RH2-2* (476 nm) spreads throughout the retina by ~1 week pf and passes through a period of disappearance in the central retina at around two weeks to 1 month pf; its expression level becomes highest among the four subtypes in the adult retina (Chinen et al., 2003). However, unlike M/LWS opsins, longer-wavelength subtypes *RH2-3* (488 nm) and *RH2-4* (505 nm) remain expressed at the periphery and at a low level (Chinen et al., 2003) throughout development. Although there is no MSP data before 6–8 dpf for zebrafish, MSP for middle-wavelength-sensitive cones after this period shows that their mean λ_{\max} values are at around 475–480 nm, with a relatively lower one (475 nm) found at 11–17 dpf (Nawrocki et al., 1985), which appears consistent with our results.

In adult retina, the shorter-wavelength-sensitive subtypes *LWS-2*, *RH2-1* and *RH2-2* are expressed in the central to dorsal area and the longer ones *LWS-1*, *RH2-3* and *RH2-4* are in the periphery, especially in the ventral area (Figs 6 and 7). *RH2-3* is expressed circularly surrounding the *RH2-2* zone and *RH2-4* is expressed outside of the '*RH2-3* ring' with a broader area in the ventral side of the retina.

Ontogenic and regional differentiations of photoreceptors in their spectral sensitivity are classical and well documented phenomena in many vertebrates including fish and mammals (Levine and MacNichol, 1982; Ahnelt and Kolb, 2000). The phenomena have been ascribed to differential usage of opsin types or chromophore types. The loss of UV cones and the change to blue cones in salmonid fish is a well recognized example (Cheng and Novales Flamarique, 2004). However, in zebrafish the single-copy SWS1, SWS2 and RH1 opsins are expressed uniformly in the retina throughout development (Vihtelic et al., 1999; Hamaoka et al., 2002; Takechi et al., 2003) and only A1 chromophore is used throughout development under natural conditions (Nawrocki et al., 1985; Saszik and Bilotta, 1999). Besides primate M/LWS opsin genes, occurrence of opsin subtypes is characteristic of fish, and the resulting variations of opsin repertoire among species should be intricately involved in the evolutionary adaptations of fish to diverse

aquatic photo-environments. To date, however, the only known example of differential usage of opsin subtypes in a given fish species has been the ontogenic shift of rod opsin expression between fresh-water and deep-sea subtypes in eels (Archer et al., 1995; Zhang et al., 2000).

The present study provides the first evidence that subtype differentiation of cone opsins in fish contributes not only to temporal but also to regional differentiation of the retina with distinct spectral sensitivities. Spectral difference between opsin subtypes is generally smaller than that between different cone opsin types. Subtype differentiation of opsins in absorption spectra and in expression pattern might be another way for fish to achieve finer spatial and temporal grading of spectral sensitivity in the retina. It is of great importance to probe the functional rationale and behavioral significance of the revealed changes in expression patterns of the zebrafish M/LWS and RH2 opsin subtypes by using the power of zebrafish genetics in future studies. The results obtained in this study revealed the excellence of the zebrafish M/LWS and RH2 opsin genes for studying functional divergence of duplicated genes in a general sense and will provide the framework for subsequent studies of opsin gene regulation.

Abbreviations

bp	base pair(s)
CDR	coding region
CMZ	ciliary marginal zone
DIG	digoxigenin
dpf	days post fertilization
hpf	hours post fertilization
ISH	<i>in situ</i> hybridization
kb	kilo bp
LDC	long double cones
M/LWS	middle to long wavelength-sensitive or red
MSP	microspectrophotometry
nt	nucleotide(s)
PCR	polymerase chain reaction
pf	post fertilization
RH1	rod-opsin or rhodopsin
RH2	RH1-like or green
SDC	short double cones
SWS1	short wavelength-sensitive type 1 or ultraviolet
SWS2	short wavelength-sensitive type 2 or blue
UTR	untranslated region.

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