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Fluorescence visualization of ultraviolet-sensitive cone photoreceptor development in living zebrafish

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Abstract Cone photoreceptor cells of fish retinae are arranged in a highly organized fashion. However, the molecular mechanisms underlying photoreceptor development and retinal pattern formation are largely unknown. Here we established transgenic lines of zebrafish carrying green fluorescent protein (GFP) cDNA with the 5.5-kb upstream region of the ultraviolet-sensitive cone opsin gene (*SWS1*). In the transgenic fish, *GFP* gene expression proceeded in the same spatiotemporal pattern as *SWS1* in the retinae of embryos. In the adult retina, *GFP* expression was observed throughout the short single cone (SSC) layer where *SWS1* is specifically expressed. Therefore, the transgenic fish provides an excellent genetic background to study retinal pattern formation, photoreceptor determination and differentiation, and factors regulating these processes and SSC-specific expression of *SWS1*.

Key words: Visual pigment; Opsin; Retina; Ultraviolet; Green fluorescent protein; *Danio rerio*

1. Introduction

Cone photoreceptor cells in teleost retinae are typically arranged with a regular mosaic pattern [1] and provide an excellent model to study developmental mechanism of cellular pattern formation of the central nervous system. Among teleosts, the zebrafish (*Danio rerio*) has been a subject of intense scrutiny for genetic and developmental processes of retinal patterning [2]. Recent large-scale mutant screens have identified many zebrafish mutants with defects in retinal and photoreceptor development [2–5].

Detailed observation and characterization of the developmental processes of retinae and photoreceptors necessitate the need for molecular markers to distinguish one photoreceptor cell type from another. Cone photoreceptor cells of zebrafish are classified into four morphologically distinct types: the long (or principal) and short (or accessory) members of double cones (LDC and SDC, respectively), long single cones (LSC), and short single cones (SSC) [6]. It is well established that each type of cone produces a single specific type of opsin, the protein moiety of visual pigments; M/LWS (red) opsin in LDC, RH2 (green) in SDC, SWS2 (blue) in LSC, and SWS1 (ultraviolet) in SSC [1,7,8]. Specific expression of the opsins, together with their early onset of expression before photoreceptor patterning and morphogenesis, has made the opsins ideal markers of photoreceptor cells [9–11].

The introduction of green fluorescent protein (GFP) as a tractable marker in a live organism has significantly facilitated studies of development and gene regulation [12,13]. Combinations of GFP and opsin promoters, therefore, should significantly promote studies of photoreceptor determination and differentiation, retinal patterning, and factors that regulate these processes and cell-specific opsin expression. In fact, GFP visualization has been successfully carried out for the rod photoreceptor cells of *Xenopus* [14] and zebrafish [15–18] using rod opsin promoter regions and the cones of *Xenopus* [19] and mice [20,21] using M/LWS opsin promoters. However, such visualization of other cone types has not been reported for any vertebrates, with the exception of mice where SWS1 (ultraviolet) opsin is co-expressed with M/LWS (green) opsin in a cone subpopulation [22].

We have recently isolated all of the cone opsin genes from the zebrafish genome and described their genomic organizations [23]. This has enabled us to carry out a systematic GFP-reporter assay using the genomic DNA fragments surrounding the opsin genes. In this study, we report the establishment of transgenic zebrafish lines that carry *GFP* with the 5.5-kb upstream region of the zebrafish ultraviolet opsin gene (*SWS1*) and express *GFP* specifically in SSCs. This is the first visualization of a specific class of cone cells in living zebrafish retinae and is the first visualization of the non-M/LWS classes of cones in vivo in all vertebrates.

2. Materials and methods

2.1. Construction of GFP-expression vector

Two overlapping genomic DNA clones, λzf-A34 and λzf-A7, encompassing the zebrafish *SWS1*, were isolated and described in our previous study [23]. The 6-kb *Eco*RI–*Sal*I fragment from λzf-A34 (Fig. 1A) was subcloned into the pBluescript II (SK-) plasmid vector (Stratagene). The 5.5-kb DNA region between the *Eco*RI site and the initiation codon of *SWS1* was amplified from the subclone using a plasmid primer adjacent to the *Eco*RI site and a reverse primer immediately upstream of *SWS1* initiation codon (5′-ccacgcgtcgacggttgtgccgttggaggcctt-3′). The nucleotide sequence of the reverse primer was designed based on our previous data (GenBank accession no. AB087810) and contains a *Sal*I linker (underlined in the sequence). The amplified fragment was recloned into the *Eco*RI/*Sal*I site of the pEGFP-1 vector (Clontech) and the clone was linearized with *Eco*RI.

2.2. Generation of transgenic zebrafish

A zebrafish strain, WIK, was maintained at 28.5°C in a 14 h light/10 h dark cycle. Embryos were collected and maintained at 28.5°C as

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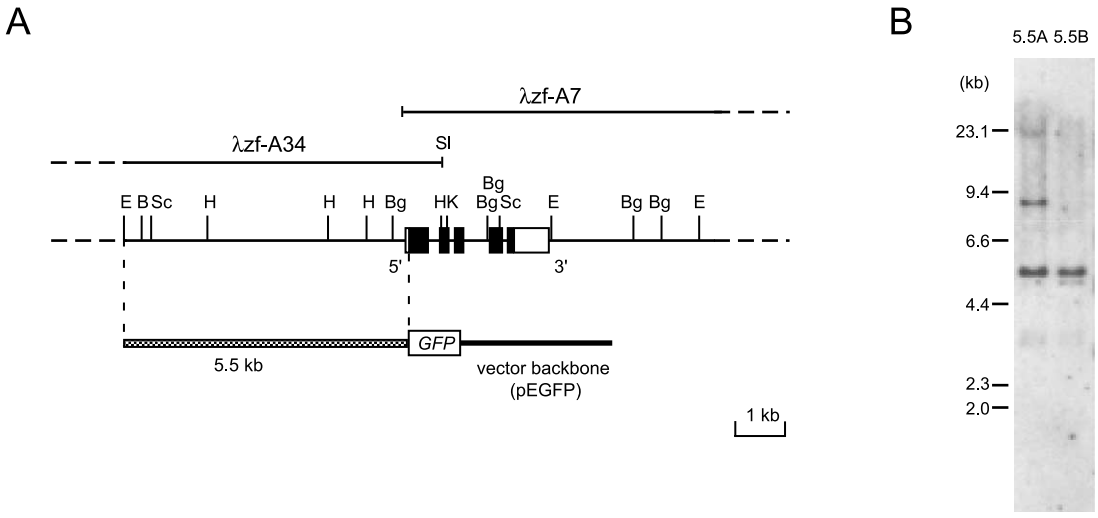

Fig. 1. A: Restriction map of the zebrafish *SWS1* gene and construction of the *GFP*-expression vector. Lines over the map represent phage clones covering the region (λzf-A7 and λzf-A34). Solid and open boxes indicate the coding and untranslated regions of *SWS1*, respectively. Shaded box represents the 5.5-kb upstream region used for construction of the *GFP*-expression vector. A *Sal*I site located in the arm edge of the phage vector is indicated for λzf-A34 clone. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Sc, *Sac*I; Sl, *Sal*I. B: Southern hybridization of the *Bam*HI-digested genomic DNA of transgenic zebrafish, 5.5A and 5.5B, to the *GFP* DNA probe. λ*Hin*dIII size standards are indicated in kb.

described previously [24]. A total of 0.003% 1-phenyl-2-thiourea was added to the water to disrupt pigment formation at 12 h post fertilization (hpf).

The *GFP*-expression constructs were resuspended in 0.1 M KCl at a final concentration of 30–50 ng μl^{-1} with tetramethyl-rhodamine dextran as the tracer. The DNA was microinjected into the cytoplasm of embryos at the 1–4 cell stage [25]. The injected embryos (founder generation; F_0) were raised to sexual maturity and crossed with uninjected fish in pairwise fashion. The resulting embryos were examined for the presence of the transgene by observing GFP fluorescence in the eyes or by applying the polymerase chain reaction (PCR) method to the genomic DNA extracted from a pool of the embryos. The PCR was carried out using a primer set based on the nucleotide sequence of pEGFP-1 vector (5′-tacggcgtgcagtgcttcag-3′ and 5′-tgttgtagttgtactccagc-3′). As a reaction control, the endogenous *Wnt5A* was simultaneously PCR-amplified using the primer set previously described [25]. Fish of the subsequent generation (F_1) were screened for the presence of the transgene by GFP fluorescence in the eyes or by PCR (see above) amplification of the DNA extracted from caudal fins [25].

For Southern blot hybridization, approximately 1 μg per lane of the fin DNA was digested with a restriction enzyme and electrophoresed on a 0.5% agarose gel. A *GFP* DNA probe was constructed by amplifying the full coding sequence of *GFP* from the pEGFP-1 vector, and was labeled with [α-^{32}P]dCTP by the random primer method. The hybridized membranes were washed in 0.1× saline sodium citrate/0.1% sodium dodecyl sulfate at 65°C for 20×4 min.

2.3. Immunohistochemistry of whole-mounted embryos

Immunostaining was performed as previously described [16] with slight modifications. Embryos were incubated at 4°C overnight with the rabbit polyclonal anti-ultraviolet opsin antibody [7] diluted 1:1000 in 1% goat serum in TBST (250 mM Tris (pH 7.4)/150 mM NaCl/2.7 mM KCl/0.1% Tween 20). After being blocked for 1 h, embryos were incubated with Cy3-conjugated anti-rabbit IgG in 1% goat serum in TBST at room temperature for 1 h and washed in TBST for 15×4 min. Images of GFP and Cy3 fluorescence were captured using Zeiss 510 laser-scanning confocal microscope (Carl Zeiss).

2.4. In situ hybridization

The full-coding region of zebrafish *SWS1* was transcribed using a digoxigenin (DIG) RNA labeling kit (Roche) to generate a DIG-labeled RNA probe. Whole-mount in situ hybridization to embryos was performed as previously described [16]. For hybridization to the adult retina, retinal cryosections were prepared and hybridized in situ to the RNA probe as described previously [16].

3. Results and discussion

3.1. Generation of transgenic lines

The 5.5-kb DNA fragment just upstream from the initiation codon of zebrafish *SWS1* was used to make a *GFP*-expression construct (Fig. 1A). Two injected fish (5.5A and 5.5B) were identified as able to transmit the transgene to the subsequent generation. Southern hybridization using the *GFP* DNA probe showed distinct banding patterns between the two lines (Fig. 1B). The banding patterns were invariant among descendants from the same founders, suggesting that the transgene was concatenated and inserted into one chromosomal location in each transgenic line.

3.2. Spatiotemporal expression pattern of GFP transgene in embryo's retina

The spatiotemporal expression of red and blue opsins in embryonic zebrafish retina has been described in great detail [9,26]. The two opsins appear at around 51 hpf and follow a similar pattern except that red opsin expression precedes blue opsin and spreads more rapidly throughout the retina. The expression pattern of ultraviolet opsin is reported to be comparable with those of red and blue opsins except that its initiation is delayed 5–8 h [26]. In brief, the expression of these cone opsins is first observed in a cluster of cells near the nasal side of the choroid fissure, it then progresses as a continuous wave into the nasal retina, and subsequently proceeds evenly throughout the retina.

The spatiotemporal pattern of the red and blue opsin expression has been divided into stages 0 to 6 [9]. These stages were in large part confirmed in our immunostaining for ultraviolet opsin in the retina of zebrafish embryos (Fig. 2A). For convenience's sake, the seven stages were merged into five for this study as follows: stage 0: no expression is detected; 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

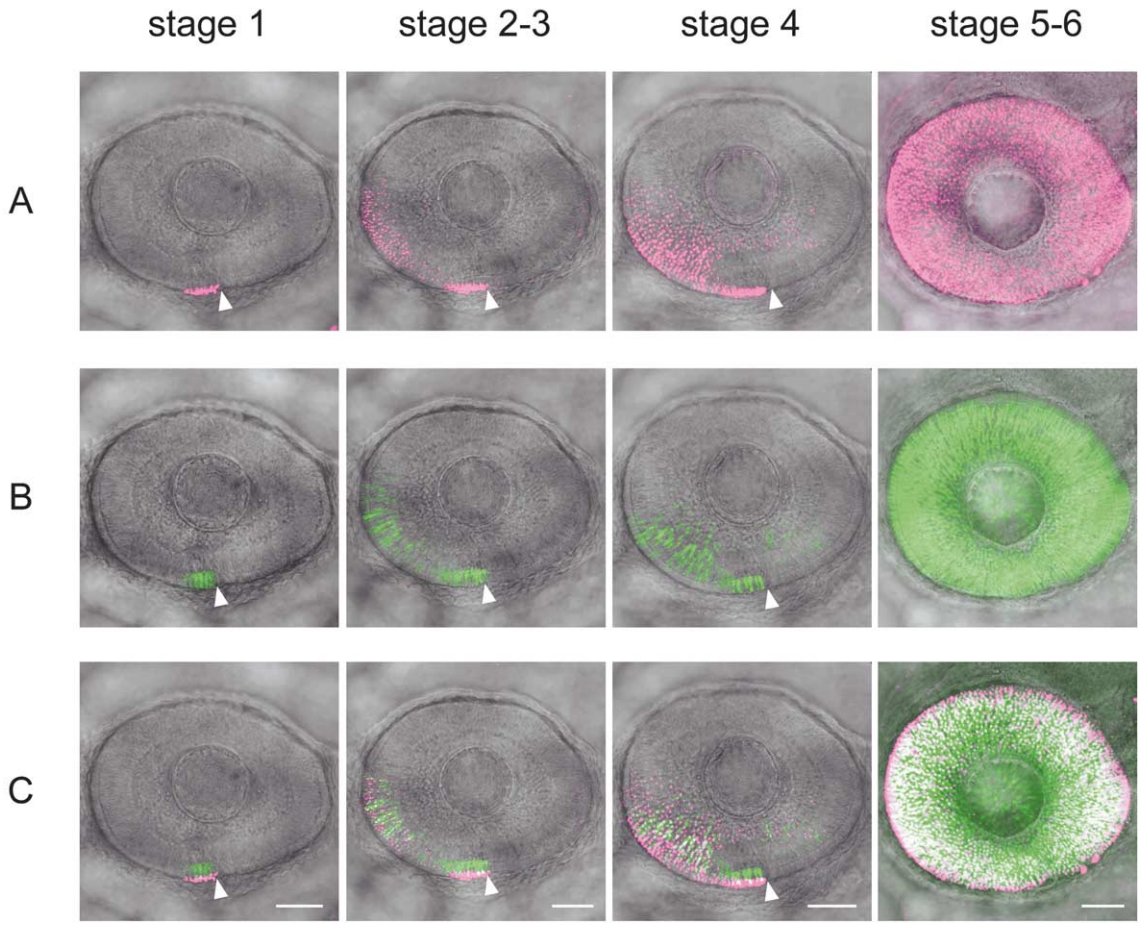

Fig. 2. Spatial distribution of *SWS1* and *GFP* expression in embryonic zebrafish retinae at stages 1, 2–3, 4, and 5–6. In all pictures, nasal is to the left and ventral is to bottom and scale bars represent 50 μm. A: Cy3-immunofluorescent staining of ultraviolet opsin in the eyes of 5.5A transgenic fish. Fluorescent signal is shown with magenta color and is overlaid on light-field images. B: GFP-fluorescent images in the same eyes as shown in A overlaid on the light-field images. C: Overlay of A and B. For fluorescent imaging, a series of 24, 18, 20, and 30 laser confocal images are projected along the *z*-axis for stages 1, 2–3, 4 and 5–6, respectively. The arrowheads indicate the location of the choroid fissure.

and, less extensively, in dorsal and ventrotemporal directions; stage 5–6: expression is detected in more than three-fourths of the retina. Only in stage 4 did our observation differ from the earlier report for the red and blue opsins, where the expression was previously observed to extend uniformly from the ventronasal region to dorsal, central and ventrotemporal directions [9]. The expression patterns observed in this study were also confirmed in whole-mount in situ hybridization using *SWS1* RNA probe (data not shown).

GFP fluorescent signal was examined in the same retinal specimens as used for the opsin immunostaining (Fig. 2B), and the two patterns were shown to be closely overlapped (Fig. 2C). While immunostaining signals showed up as discrete spots, individual GFP signals appeared more diffuse. This is probably due to the immunostaining signals being confined to the outer segment in the cone cell while GFP spreads throughout the entire cell. The slight discrepancies between the signals could also result from incomplete or uneven immunostaining in the whole-mounted samples, degradation of GFP during immunostaining process, or both [16].

It is well known that the extent of opsin expression can be notably different among embryos of the same age and even between the two eyes of a single embryo [9,16,26,27]. To compare the time course of expression between *SWS1* of wild-type and *GFP* of transgenic fish, we examined the eyes of wild-type and transgenic embryos, and assigned them to the appropriate stage at 56, 62, 77, and 86 hpf by whole-mount in situ hybridization using *SWS1* (Fig. 3A) and *GFP* (Fig. 3B) RNA probes, respectively. Because the fish heterozygous for the transgene were crossed with wild-type fish, approximately half of the offsprings were expected to possess the transgene. As expected, *GFP* expression remained undetectable in about half of the offsprings even after 86 hpf (Fig. 3B). In the transgenic population, variance of stages in each age group was similar between *SWS1* and *GFP* RNA expression. The time course of GFP protein production was examined by evaluating the distribution of GFP fluorescence (Fig. 3C). The temporal pattern of protein production was comparable to that of RNA expression in both the 5.5A and 5.5B lines, showing, as expected, a slight delay relative to the RNA expression.

It was noted that the onset of *SWS1* expression in our study was earlier than that reported previously [26], where the expression initiated between 55 and 60 hpf with about half of the embryos showing expression at 60 hpf but remaining in stage 1. The aforementioned study differed from ours in that only fish that had achieved certain developmental characteristics at certain time points were examined for opsin ex-

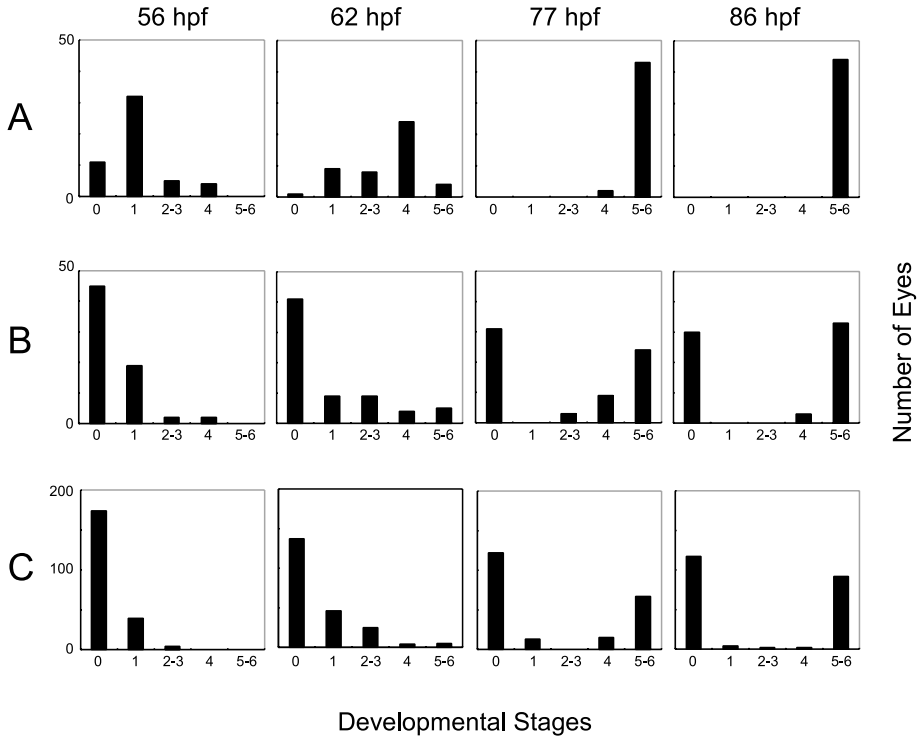

Fig. 3. Histograms showing stages at 56, 62, 77, and 86 hpf of *SWS1* RNA expression in wild-type fish (A) and *GFP* RNA (B) and protein (C) expression in 5.5A transgenic fish. For each individual, one eye was examined. Vertical and horizontal axes indicate the number of eyes and developmental stages, respectively.

pression to minimize the individual variation [26], while we did not perform any such selection. The time discrepancy between the two studies is most likely due to this difference.

3.3. GFP *expression in adult retina*

Retinae of adult transgenic fish from the 5.5A and the 5.5B lines were examined by fluorescent microscopy. The retinal whole-mount from an adult 5.5A fish demonstrated robust expression of GFP across the entire retina (Fig. 4A). In the 5.5B line the fluorescence was found to be much weaker than that in the 5.5A line, although signal was observed throughout the retina (data not shown). In vertical retina sections,

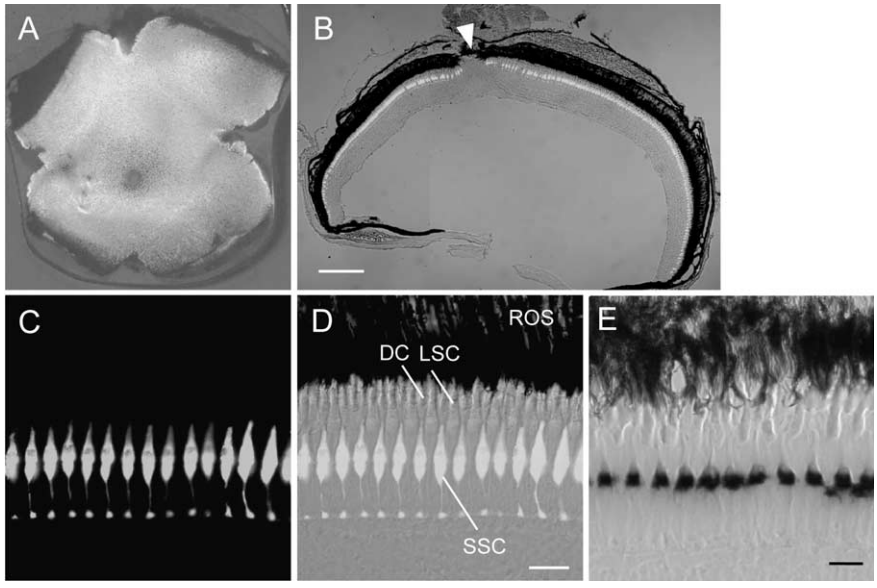

Fig. 4. GFP fluorescence of the eyes in adult 5.5A transgenic fish. A: An entire view of an eye flattened by dissection. Nasal is to the right and ventral is to bottom. B: An overview of an eye cryosection showing GFP fluorescence overlaid on light-field image. Arrowhead indicates optic nerve exit point. Scale bar = 200 μm. C: GFP-fluorescent image of the vertical section of the retina at higher magnification. The image is viewed under dark field with FITC filter. D: Image in C overlaid on light-field image. (DC, double cone; LSC, long single cone; ROS, rod outer segments; SSC, short single cone.) Scale bar = 20 μm. E: In situ hybridization of the *SWS1* RNA probe to an eye cryosection from an adult wild-type zebrafish. Scale bar = 10 μm.

GFP fluorescence was observed as a single layer (Fig. 4B) in both lines. At a higher magnification, the fluorescent layer was shown to correspond to the SSCs (Fig. 4C,D). Their cell type was determined on the basis of their characteristic morphology and location in the layer of the retina [1]. In the region where fluorescence appeared sparsely at low magnification (Fig. 4B), SSC was found to be absent at higher magnification in the section preparation. The cell type was further confirmed by in situ hybridization using *SWS1* RNA probe, which specifically recognizes the cell body of SSCs (Fig. 4E). We also confirmed that GFP fluorescence was confined to the retina by examining the entire body of both embryos and adults.

3.4. Perspective

In this study we established two transgenic zebrafish lines possessing *GFP* with the 5.5-kb upstream region of *SWS1* in the genome and expressing *GFP* in the retina with the spatiotemporal pattern identical to that of endogenous *SWS1* in both embryos and adults. This is the first visualization of a specific class of cone photoreceptor cells in living zebrafish. After collecting data presented in Figs. 2–4 using the F_1 generation (for Fig. 3, embryos from F_1 vs. wild-type cross), we established F_2 generation fish homozygous for 5.5A transgene from crossing heterozygous F_1 fish. The F_3 embryos resulting from the cross between homozygous F_2 fish showed fluorescent signal in the eye with a pattern similar to that shown in Fig. 3A (data not shown). We have seen no apparent defects in these homozygous fish.

The transgenic lines described in this report should be of great use for the study of photoreceptor development and retinal patterning as demonstrated in previous studies using other opsin promoters [18,21]. These fish should also be useful for screening retinal mutants, in particular the ones affected with either SSC development or *SWS1* expression since identification of such mutants would otherwise require tedious behavioral observation and molecular staining. Using these transgenic lines, one could also generate changes in the retinal cells such as inducing apoptosis using C-2 ceramide or light, or changes in the expression of opsin to provide novel information regarding the regulatory mechanisms in retinal and photoreceptor development. The transgenic fish also enable us to isolate SSC cells by flow cytometry, which should facilitate exploration of transcription factors involved in *SWS1* expression and SSC development.

This study indicates that regulatory elements necessary for SSC-specific gene expression reside in the 5.5-kb region upstream of *SWS1*. To identify the elements, however, it is necessary to examine a series of *GFP*-expression constructs with varying lengths of regions upstream of *SWS1*. When regions 1.8-kb and 1.4-kb upstream were used, GFP fluorescence in the resulting transgenic fish (two and one lines, respectively) was faint and delayed in the embryos' retinae and was confined to the central region of the adult retina (data not shown). Alternatively one transgenic line with 0.4-kb region upstream of *SWS1* appeared to recapitulate normal *SWS1* expression in our preliminary observation. Systematic assay using a series of *GFP*-expression constructs together with sequence analysis of the 5.5-kb region is currently in progress to localize regulatory elements.

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