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A unique visual pigment expressed in green, red and deep-red receptors in the eye of the small white butterfly, *Pieris rapae crucivora*

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Summary

The full primary structure of a long-wavelength absorbing visual pigment of the small white butterfly, *Pieris rapae crucivora*, was determined by molecular cloning. *In situ* hybridization of the opsin mRNA of the novel visual pigment (PrL) demonstrated that it is expressed in the two distal photoreceptor cells (R3 and R4) as well as in the proximal photoreceptors (R5–8) in all three types of ommatidia of the *Pieris* eye. The main, long-wavelength band of the spectral sensitivities of the R3 and R4 photoreceptors is well described by the absorption spectrum of a visual pigment with absorption maximum at 563 nm; i.e. PrL is a visual pigment R563. The spectral sensitivities of R5–8 photoreceptors in ommatidial type I and III peak at 620 nm and those in type II ommatidia peak at 640 nm. The large shifts of the spectral sensitivities of the R5–8 photoreceptors with respect to the absorption spectrum of their visual pigment can be explained with the spectral filtering by pale-red (PR) and deep-red (DR) screening pigments that are concentrated in clusters of granules near the rhabdom boundary. The peak absorbance of the two spectral filters appears to be approximately 1 (PR) and 2 (DR).

Key words: compound eye, colour vision, spectral filter, rhodopsin, spectral sensitivity.

Introduction

Animal eyes need to have different classes of spectral photoreceptors for colour vision. The honeybee (*Apis mellifera*), the classical example of an insect species with colour vision, has compound eyes with short (UV)-, middle (blue)- and long (green)-wavelength photoreceptors, which provide the physiological basis of a trichromatic colour vision system. Many butterflies employ so-called red receptors, with spectral sensitivities peaking near 600 nm or even at longer wavelengths (Arikawa et al., 1987; Bernard, 1979; Matic, 1983). These long-wavelength photoreceptors are most likely used for discriminating flower colours upon searching for food (Kelber and Pfaff, 1999; Kinoshita et al., 1999).

The sensitivity wavelength range of a photoreceptor cell is principally determined by the absorption spectrum of its visual pigment. A visual pigment molecule consists of an opsin protein with an 11-cis retinal chromophore. Absorption of light by the visual pigment molecule converts the 11-cis retinal to all-trans retinal, which then triggers the signal transduction cascade, resulting in a change of the membrane potential of the photoreceptor cell. The wavelength range where light effectively isomerizes the chromophore depends on the interaction of certain amino acids of the opsin with the chromophore; i.e. the opsin’s amino acids together with the chromophore determine the spectral sensitivity of a photoreceptor. A distinct spectral sensitivity correlates with a unique amino acid sequence, as has been established for honeybees (Mardulyn and Cameron, 1999; Townson et al., 1998). This also holds for the Japanese yellow swallowtail butterfly, *Papilio xuthus*, where green and red receptors express different mRNAs encoding different visual pigment opsins (Kitamoto et al., 1998).

Optical factors often play a modulatory role in the photoreceptor spectral sensitivity. For example, the spectral sensitivity of the red receptors of *Papilio xuthus* peaks at 600 nm, but the spectrum is considerably narrower than predicted for a visual pigment with peak absorbance at 600 nm. The red receptors of *Papilio* are located in the proximal tier of those ommatidia where the rhabdom is surrounded by red pigmentation, which provide the physiological basis of a trichromatic colour vision system. Many butterflies employ so-called red receptors, with spectral sensitivities peaking near 600 nm or even at longer wavelengths (Arikawa et al., 1987; Bernard, 1979; Matic, 1983). These long-wavelength photoreceptors are most likely used for discriminating flower colours upon searching for food (Kelber and Pfaff, 1999; Kinoshita et al., 1999).

We here report an extreme case of spectral filtering in the eye of the small white butterfly, *Pieris rapae crucivora*. *Pieris* has three types of long wavelength photoreceptors, peaking at 560 nm (green), 620 nm (red), and 640 nm (deep-red), accordingly called L560, L620 and L640 receptors, respectively (Qiu and Arikawa, 2003a,b). The *Pieris* eye consists of three distinct types of ommatidia, which are characterized by the perirhabdomeral pigmentation: a pale-red pigment in type I and
III ommatidia and a deep-red pigment in type II ommatidia (Qiu et al., 2002). In all ommatidial types, two of the four distal photoreceptors, R3 and R4, are L560 receptors. The proximal photoreceptors of type I and type III ommatidia are L620 receptors, whereas the proximal photoreceptors of type II ommatidia are L640 receptors (see Table 1). These findings pose several questions. First, how are the spectral sensitivities of the long-wavelength photoreceptors of Pieris determined, and do they have different visual pigments? What is the function of the perirhabdomeral pigmentation? At the proximal end of the rhabdom, a tapetal mirror exists that reflects light back into the rhabdom. Do the mirrors affect the spectral sensitivity of photoreceptors? In order to answer such questions, we first performed a molecular cloning study to identify multiple different mRNAs encoding opsins for long-wavelength absorbing visual pigments. What we found was a single opsin of a long-wavelength absorbing visual pigment opsin, which we term PrL (Pieris rapae long-wavelength visual pigment). Furthermore, we carried out histological in situ hybridization to localize the photoreceptors that express PrL mRNA. Quite unexpectedly, the mRNA encoding PrL was detected in all the L560, L620 and L640 receptors in the Pieris eye. The three long-wavelength-sensitive Pieris photoreceptors can be explained with a unique visual pigment when assuming that the two types of perirhabdomeral pigments act as long-wavelength transmittant, spectral filters.

**Materials and methods**

**Animals**

We used spring-form individuals of Pieris rapae crucivora Boisduval. The butterflies were taken from a laboratory stock culture derived from eggs laid by females captured in the field around the campus of Yokohama City University, Yokohama, Japan. The hatched larvae were reared on fresh kale leaves at around the campus of Yokohama City University, Yokohama, Japan. The hatched larvae were reared on fresh kale leaves at 19°C under a light regime of 8 h:16 h light:dark. The pupae were stored at 4°C for at least 3 months and then allowed to emerge at 25°C. The adults were used within 4 days after emergence.

**Molecular cloning**

The method of molecular cloning of Pieris opsins was as described previously (Kitamoto et al., 1998). Briefly, retinal mRNA was extracted using the QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) from eyes rapidly frozen in liquid nitrogen. For amplifying the cDNAs of long-wavelength absorbing visual pigments by RT-PCR, we designed two sets of degenerate primers based on amino acid sequences conserved in long-wavelength absorbing visual pigments of the swallowtail butterflies Papilio xuthus (Kitamoto et al., 1998) and Papilio glauces (Briscoe, 1998) and the hawkmoth Manduca sexta (Chase et al., 1997); for the sequence of the primers, see the legend of Fig. 1. RT-PCR using all of these primers identified a single DNA fragment with an opsin-like sequence. To obtain the full-length cDNA, we carried out 3’- and 5’-RACE.

<table>
<thead>
<tr>
<th>Type Cluster shape Colour Fluorescence</th>
<th>Pigment</th>
<th>Photoreceptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Trapezoidal Pale-red – UV B</td>
<td>R1</td>
<td>L560</td>
</tr>
<tr>
<td>II Square Deep-red + dB</td>
<td>R2</td>
<td>L620-II L640</td>
</tr>
<tr>
<td>III Rectangular Pale-red – UV</td>
<td>R3/4</td>
<td>L560 L620</td>
</tr>
</tbody>
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L560-II, previously termed d-green, is a green receptor with depressed sensitivity at 420 nm, located in type II ommatidia (Qiu and Arikawa, 2003a); for comparison with L560, see Fig. 4A.

To compare the amino acid sequence deduced from the cloned cDNA with opsins of other insects so far identified, the sequences were aligned using an alignment program (CLUSTAL W 1.6), and then a phylogenetic analysis was performed by the neighbour-joining method (PHYLIP 3.572), with octopus opsin as an outgroup.

**In situ hybridization**

The compound eyes of Pieris were fixed in 4% paraformaldehyde in 0.1 mol l⁻¹ sodium phosphate buffer (pH 7.2) for 0.5–2 h at 25°C. After dehydration with an ethanol series, we embedded the eyes in paraplast. The paraplast-embedded eyes were sectioned at ~8 μm thickness with a rotary microtome.

Probes for in situ hybridization were designed to hydridize to ~400 bases of the mRNA at the non-coding region downstream of the C-terminal. The corresponding cDNA region was first subcloned into pGem-3zf(+) vector, and then digoxigenin (DIG)-labelled cRNA was generated using the DIG-RNA labelling kit (Roche, Mannheim, Germany).

For labelling, sections were first de-paraffinized and treated with hybridization solution [300 mmol l⁻¹ NaCl, 2.5 mmol l⁻¹ EDTA, 200 mmol l⁻¹ Tris-HCl (pH 8.0), 50% formamide, 10% dextran sulphate, 1 mg ml⁻¹ yeast tRNA, 1× Denhardt’s medium], containing 0.5 μg ml⁻¹ of the cRNA probe, at 45°C overnight. After a brief rinse, the sections were incubated in 50% formamide in 2× SSC (saline sodium citrate buffer) at 55°C for 2 h and then treated with RNase (10 μg ml⁻¹) at 37°C for 1 h. The probes were further visualized by anti-DIG immunocytochemistry.

**Calculation of the absorbance spectra of the photoreceptor screening pigment**

Four clusters of pigment surround the rhabdoms distally in all ommatidia of the fronto-ventral eye of Pieris. The pigment clusters thus act as absorption filters for the R5–8 proximal photoreceptors. The pigment is pale-red in type I and III ommatidia and deep-red in type II ommatidia. The R5–8 photoreceptors in the different ommatidial types have spectral
sensitivities depending on the wavelength, $\lambda$, peaking in the red and deep-red, respectively (Qi and Arikawa, 2003b). The central hypothesis of the present paper is that the two types of distal screening pigment create the two types of R5–8 spectral sensitivities by selective spectral filtering.

The spectral sensitivity of a photoreceptor, $S(\lambda)$, is experimentally determined by measuring at several wavelengths the number of photons ($I_0$) necessary to elicit a chosen criterion voltage response, assuming that this response is always the result of the same number of photons absorbed by the visual pigment ($I_{abs}$). The spectral sensitivity is then given by $S(\lambda) = I_{abs}/I_0$, where the index n indicates normalization. The stimulus light first passes the dioptric apparatus and then enters the rhabdom. There, the light propagates in distinct light patterns, the waveguide modes. The number of allowed modes depends on the waveguide number: 

$$V = \left(\pi D_f/\lambda\right)(n_1^2 - n_2^2)^{1/2},$$

where $D_f$ is the rhabdom diameter and $n_1$ and $n_2$ are the refractive indices of rhabdom interior and surrounding medium, respectively. In a Pieris rhomb with $D_f=2.0 \mu m$, $n_1=1.363$ and $n_2=1.340$, two modes are allowed in the visible wavelength range up to $\lambda=651 nm$, because the cut-off $V$-number of the second mode is $V=2.405$. In the wavelength range of the red receptors, about 10% of the light flux in the first mode propagates outside the rhabdom, while for the second mode this is <50% (see, for example, Stavena, 2003a). The distal screening pigment will therefore absorb the second mode much more strongly than the first mode, and hence with dense screening pigment only the first mode will arrive in the proximal tier of the rhabdom. When the light flux entering the ommatidium equals $I_c(\lambda)$ at wavelength $\lambda$, and the transmittance of the dioptrics and distal retinal tier is $T_R(\lambda)$, the light flux entering the proximal rhabdom tier equals $I_c(\lambda)T_R(\lambda)$. When all photoreceptors $R_j$ ($j=5–8$) contain the same visual pigment, with (normalized) absorption coefficient $\alpha(\lambda)$, the transmittance of the proximal rhabdom tier is given by:

$$T_p = \exp(-\eta \kappa_{max} \alpha L_p),$$

where $\eta=\eta(\lambda)$ is the light fraction propagating within the rhabdom boundary, $\kappa_{max}$ is the peak absorption coefficient of the rhabdom medium, and $L_p$ is the length of the proximal rhabdom. The light flux $I_c(\lambda)T_R(\lambda)T_p(\lambda)$ hence reaches the tapetum. When the tapetum reflectance is $M(\lambda)$, and the rhabdom volume fraction of a photoreceptor is $f_j$, the light absorbed by its visual pigment is the sum of the absorbed light that travelled to the tapetum and that was reflected by the tapetum:

$$I_{abs} = I_cT_Rf_j(1 - T_p) + I_cT_Rf_pM_j(1 - T_p) = I_cT_Rf_j(1 - T_p)/(1 + T_p).$$

Hence:

$$\log S = \log(1 - T_p) + \log(1 + T_p)/A_d + C,$$

where $A_d=-\log(T_d)$ is the absorbance of the distal retina including the dioptrics, and $C$ is an unknown constant (log is the decimal logarithm here). The proximal transmittance, $T_p$, $\eta(\lambda)$ is the light fraction propagating within the rhabdom boundary, $\kappa_{max}$ is the peak absorption coefficient of the rhabdom medium, and $L_p$ is the length of the proximal rhabdom. The light flux $I_c(\lambda)T_R(\lambda)T_p(\lambda)$ hence reaches the tapetum. When the tapetum reflectance is $M(\lambda)$, and the rhabdom volume fraction of a photoreceptor is $f_j$, the light absorbed by its visual pigment is the sum of the absorbed light that travelled to the tapetum and that was reflected by the tapetum:

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$$I_{abs} = I_cT_Rf_j(1 - T_p) + I_cT_Rf_pM_j(1 - T_p) = I_cT_Rf_j(1 - T_p)/(1 + T_p).$$

Hence:
where experimental sensitivity spectra along the coordinate axis until fraction can be calculated from known parameters: the mode power

\[ \log(1 - \text{experimental sensitivity spectra}) \]

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and Nilsson, 1998); the spectral absorption coefficient of the visual pigment can be calculated with a template formula\(^\text{(Qiu et al., 2002);}\)

\[ T_p = \log(1 - T_p) - \log S - C' \]

\[ A_d = \log(1 - T_p) - \log S - C' \]

The distal absorbance, \( A_d \), as given by equation 5, consists of three terms: (1) the absorbance of the dioptrics; (2) the absorbance of the various visual (and possibly other absorbing) pigments within the rhabdom and (3) the absorbance of the red screening pigments near the rhabdom boundary. The first term is probably, in very good approximation, a constant, i.e. independent of wavelength\(^\text{(Stavenga, 2004).}\) The second term is described by \( A_d = -\log(T_{d\lambda}) \), where the transmittance of the distal visual pigment, \( T_{d\lambda} \), in the green to red wavelength range is given by an expression similar to equation 2: \( T_{d\lambda} = \exp(-f_3.4k_{\text{max}}d\omega) \), where \( f_3.4 = f_3 + f_4 \) is the sum of the rhabdom volume fractions of photoreceptors R3 and R4, and \( L_\omega \) is the length of the distal rhabdom tier. Anatomical data show that \( f_3.4 = 0.3 \) and \( L_\omega = 250 \mu m \)\(^\text{(Qiu et al., 2002).}\)

\[ \text{Primary structure of a newly identified opsin from the eye of Pieris rapae} \]

With four degenerate primers (Fig. 1), we performed RT-PCR with the mRNA extracted from the \textit{Pieris} eye. In any combination of primers we could amplify cDNA fragments, which eventually appeared to originate from a single mRNA encoding a visual pigment opsin. After performing 3’- and 5’-RACE, we found that the cDNA has a single open reading frame of 1146 bp, encoding 382 amino acids (Fig. 1). The phylogenetic relationship of the novel opsin with other insect opsins indicates that it falls into the group of long-wavelength absorbing visual pigments (Fig. 2). We therefore termed the opsin \textit{PrL} (\textit{Pieris rapae} long-wavelength absorbing visual pigment).
A unique opsin of *Pieris* R3–8 photoreceptors of *Pieris rapae* all contain the same PrL mRNA

Fig. 3 shows the results of histological *in situ* hybridization, together with a diagrammatical sketch of the ommatidium (Fig. 3A) and unstained histological sections of the *Pieris* retina through the distal (Fig. 3B) and the proximal (Fig. 3C) tier of the retina. In the distal tier, the probe for PrL mRNA hybridized the R3 and R4 photoreceptors in all ommatidia (Fig. 3D). In the proximal tier, the same probe labelled the R5–8 photoreceptors in all ommatidia (Fig. 3E). We could not identify any obvious labelling in the R9 basal photoreceptors.

**Filtering by photoreceptor screening pigment results in different photoreceptor classes**

The spectral sensitivity of the distal R3,4 photoreceptors, determined by intracellular recordings (Qiu and Arikawa, 2003a), well approximates the absorption spectrum of a visual pigment peaking at 563 nm (Fig. 4A). We therefore may conclude that PrL, expressed in R3,4, is an R563 visual pigment. This conclusion immediately implies that all R5–8 photoreceptors have an R563, although their spectral sensitivities severely deviate from an R563 absorption spectrum (Fig. 4B). The R5–8 photoreceptors form two classes, L620 and L640, that correlate with the colour of the pigment clusters in the ommatidium of the photoreceptor, being pale-red (PR; in type I and III ommatidia) or deep-red (DR; in type II ommatidia), respectively.

The two photoreceptor classes can be explained with a simple model, based on the assumption that the pale-red and deep-red pigments act as spectral filters, which are positioned fully distally, i.e. in front of the proximal rhabdom, formed by the rhabdomeres of photoreceptors R5–8. The absorbance spectra of the screening pigments can be estimated with the procedure outlined in the Materials and methods, as visualized in Fig. 4B,C. The first step is the calculation of the log absorptance of the proximal photoreceptors, \( \log(1 - T_p) \), where \( T_p \) is the transmittance of the proximal tier of the rhabdom. Red pigments are assumed to be transparent at long wavelengths, and hence the log sensitivity curves should match the log absorptance spectrum at long wavelengths. This indeed occurs for both L620 and L640 receptors (Fig. 4B). The difference between the log absorptance and log sensitivity curves then yields the absorbance spectra for the retinal material situated distally (Fig. 4C).

**Discussion**

A unique long-wavelength absorbing opsin in the eye of *Pieris rapae*

We identified a single long-wavelength absorbing opsin (L-opsin) mRNA in the eye of *Pieris rapae crucivora*. This is
without exception, express the unique PrL mRNA strongly.

The complementary observation that all R3–8 photoreceptors, more than the unique long-wavelength opsin, PrL, in

therefore we used various sets of degenerate primers to check

sensitive (Shimohigashi and Tominaga, 1991). Of course, we

If R9 photoreceptors do not have PrL, they must express some

long-wavelength photoreceptors. We note here that our PrL

cannot exclude the possibility that the, as yet unknown, visual
pigment of R9 is expressed in the other red receptors, R5–8,
together with PrL: coexpression of multiple visual pigments
has been found in many photoreceptors in the eye of Papilio xuthus (Arikawa, 2003). Furthermore, it should be mentioned
that the actual absorbance spectrum of PrL in the different
classes of photoreceptors could, in principle, be different due
to post-translational modification or specific interactions with
unique cellular components, but there is no clear evidence for
such phenomena.

Absorbance spectra of red screening pigments

The absorbance spectra of the screening pigments in the
pale-red (PR) ommatidia (type I and III) and deep-red (DR)
ommatidia (type II) in the distal retina of the Pieris eye were
estimated by subtraction of the experimental log spectral
sensitivities from the calculated log absorbance spectrum of
the proximal rhabdom tier. The resulting spectra show a
substantial absorption of approximately 1 and 2 log units at
wavelengths of <600 nm, but moreover clearly show a
difference in spectral cut-off for the two types of screening
pigments. Whereas the PR absorbance is low above ~620 nm,
the DR absorbance is only minor above 660 nm. This is in full
agreement with measurements of the eye shine, which showed
that eye reflectance sharply drops at wavelengths below 620
and 660 nm for the two ommatidial types that can be
distinguished with optical methods (Qiu et al., 2002). It should
be noted, however, that the derived absorbance spectra of

quite parsimonious compared with Papilio xuthus, which has
three different L-opsins, PxL1–3 (Kitamoto et al., 1998), and
therefore we used various sets of degenerate primers to check
extensively whether other long-wavelength opsin mRNAs exist in the Pieris eye. So far, we have been unable to trace
more than the unique long-wavelength opsin, PrL, in Pieris.
The complementary observation that all R3–8 photoreceptors,
without exception, express the unique PrL mRNA strongly indicates that Pieris relies on a single opsin for the different
long-wavelength photoreceptors. We note here that our PrL
labelling has so far failed to clearly label R9 photoreceptors.
If R9 photoreceptors do not have PrL, they must express some
other long-wavelength absorbing visual pigment, because
electrophysiological recordings indicate that R9s are red
sensitive (Shimohigashi and Tominaga, 1991). Of course, we
The long-wavelength visual pigments characterized photochemically in the nymphalids Vanessa cardui (Briscoe et al., 2003) and Polyogonia c-album (Vanhouthe, 2003) absorb maximally at ~530 nm, and the similarly dominant visual pigment of the moth Manduca sexta peaks at 520 nm (White et al., 2003). This can be compared with the honeybee, where six of the eight main photoreceptors have a 530 nm visual pigment (M. Kurasawa, M. Giurta and K. Arikawa, manuscript in preparation). (Note also that, in flies, six large photoreceptors have the same visual pigment, peaking at 490 nm.) In Apis, Vanessa and Manduca, the two additional photoreceptors are UV and/or blue receptors, which are distributed heterogeneously in the ommatidial lattice. The same long-wavelength visual pigment appears to exist in all long-wavelength receptors, however, indicating that these animals have a homogeneous retina, when considering the long-wavelength range. This agrees with optical observations on common nymphalids, which show a homogeneous eye shine (Stavenga, 2002a).

The heterogeneous eye shine observable in many butterflies, including Pieris, demonstrates that many butterflies have diversified their long-wavelength receptors. As a first example, the dorsal parts of the eyes of the satyrine Bicyclus anynana have ommatidia, which all have the same green–orange eye shine. Ventrally, red-reflecting ommatidia intersperse the yellow–orange shining ones. Spectral measurements strongly suggest that in the red-reflecting ommatidia red spectral filters occur, presumably shifting the spectral sensitivity of sets of long-wavelength receptors (Stavenga, 2002b). Pieris applies one of two types of red spectral filters in the ommatidia in the fronto-ventral eye area. Papilio xuthus also uses two types of screening pigment, i.e. either a red or a yellow pigment, but moreover combines these pigments with different types of long-wavelength-sensitive visual pigments. Papilio xuthus exercises further extravagance by expressing two different long-wavelength visual pigments in certain photoreceptors. Presumably, these differences increase the potential for colour vision. At least, Papilio xuthus can boast extreme colour discrimination capacities (Kinoshita and Arikawa, 2000; Kinoshita et al., 1999).

Eye shine studies suggest that the diversification of spectral properties strongly depends on species and eye region (Stavenga, 2002a). For example, whereas the ommatidia in the main part of the eye of Pieris rapae reflect red or deep-red light, the ommatidia in the dorsal part of the eye reflect in the yellow wavelength range, in agreement with the absence of red screening pigment dorsally (Ribi, 1979). The red spectral filters in the fronto-ventral area create extremely long-wavelength shifted photoreceptors, presumably to improve the capacity to discriminate food plants (Kelber, 1999), which are more often seen with the ventral than the dorsal eye area.

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