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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 acts as a red transmittant spectral filter. While the absorption spectrum of the visual pigment peaks at 575 nm, the filter shifts the spectral sensitivity such that it peaks at 600 nm (Arikawa et al., 1999b).

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.

Table 1. *Summary of the characteristics of the three ommatidial types and the distribution of spectral receptors in* Pieris *eyes*

	Pigment			Photoreceptor			
Type	Cluster shape	Colour	Fluorescence	R1	R2	R3/4	R5–8
I	Trapezoidal	Pale-red	–	UV / B	B / UV	L560	L620
II	Square	Deep-red	+	dB		L560-II	L640
III	Rectangular	Pale-red	–	UV		L560	L620

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.

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 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 glaucus* (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.

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 hybridize 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, λ, peaking in the red and deep-red, respectively (Qiu 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_c) 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 $S(\lambda)=(I_{abs}/I_c)_n$, 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:

$$V = (\pi D_r/\lambda)(n_1^2 - n_2^2)^{1/2} , \quad (1)$$

where D_r is the rhabdom diameter and n_1 and n_2 are the refractive indices of rhabdom interior and surrounding medium, respectively. In a *Pieris* rhabdom with D_r=2.0 μm, n_1=1.363 and n_2=1.340, two modes are allowed in the visible wavelength range up to λ=651 nm, because the cut-off *V*-number of the second mode is *V*=2.405. In the wavelength range of the red receptors, ~10% of the light flux in the first mode propagates outside the rhabdom, while for the second mode this is <50% (see, for example, Stavenga, 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 λ, and the transmittance of the dioptrics and distal retinal tier is $T_d(\lambda)$, the light flux entering the proximal rhabdom tier equals $I_c(\lambda)T_d(\lambda)$. 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:

$$T_p = \exp(-\eta\kappa_{max}\alpha L_p) , \quad (2)$$

where $\eta=\eta(\lambda)$ is the light fraction propagating within the rhabdom boundary, κ_{max} is the peak absorption coefficient of the rhabdom medium, and L_p is the length of the proximal rhabdom. The light flux $I_c(\lambda)T_d(\lambda)T_p(\lambda)$ hence reaches the tapetum. When the tapetal 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:

$$I_{abs} = I_c T_d f_j(1 - T_p) + I_c T_d T_p M f_j(1 - T_p) = I_c T_d f_j(1 - T_p)(1 + T_p M) . \quad (3)$$

Hence:

$$\log S = \log(1 - T_p) + \log(1 + T_p M) - A_d + C , \quad (4)$$

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 decadic logarithm here). The proximal transmittance, T_p,

```
                                                                GGAGTAGTCTCCGCAACACAACCTCAG   –91
CACCCGCCGGAGATTGGTTAAACTAAAGACTGGAGTAGTTCGTCAAGCGATTTTCTTCAGTATTTTTGTTTTTAGCTTAGAAATTTAAAA    –1
ATGGCAATCACCAACCTGGACCCAGCACCAGGCGTAGCGGCCATGCAGTCCTTCGGCATCCACGCCGAGGCATTCGGGTCAAACCAGACC    90
M  A  I  T  N  L  D  P  A  P  G  V  A  A  M  Q  S  F  G  I  H  A  E  A  F  G  S  N  Q  T
GTCATAGACAAGGTTCTGCCTGAAATGATGCACCTGATTGACCCACACTGGTACCAGTTTCCACCATTGAACCCACTATGGCACGCTCTT   180
V  I  D  K  V  L  P  E  M  M  H  L  I  D  P  H  W  Y  Q  F  P  P  L  N  P  L  W  H  A  L
CTTGGATTCACCATCAGTGTCCTGGCTTTCATCTCCATAACTGGCAACGGTATGGTGGTTTACATTTTCACGACGACAAAGTCACTCAAG   270
L  G  F  T  I  S  V  L  A  F  I  S  I  T  G  N  G  M  V  V  Y  I  F  T  T  T  K  S  L  K
ACGCCTTCAAACCTGCTAGTTGTTAATTTGGCTTTCTCCGACTTCCTGATGATGGCTATGATGGCTCCGCCTCTGGTGGTCAACAGTTAC   360
T  P  S  N  L  L  V  V  N  L  A  F  S  D  F  L  M  M  A  M  M  A  P  P  L  V  V  N  S  Y
AATGAAACATGGGTTTTTGGTCCGACCGCATGCCAATTCTATGCCTGCTTCGGGTCACTATTTGGATGCGTCTCGATCTGGACTATGACA   450
N  E  T  W  V  F  G  P  T  A  C  Q  F  Y  A  C  F  G  S  L  F  G  C  V  S  I  W  T  M  T
GCCATTGCCTTCGACCGTTACAATGTGATCGTCAAGGGTATCGCTGCAAAACCGATGACCATTAACAGTGCTTTACTGAGAATCCTTGGT   540
A  I  A  F  D  R  Y  N  V  I  V  K  G  I  A  A  K  P  M  T  I  N  S  A  L  L  R  I  L  G
GTATGGCTATTCTCTCTTGCATGGACTCTTGCGCCCATCTTTGGCTGGAGCCGATACGTCCCCGAGGGCAACATGACCGCTTGTGGAACT   630
V  W  L  F  S  L  A  W  T  L  A  P  I  F  G  W  S  R  Y  V  P  E  G  N  M  T  A  C  G  T
GACTACCTTAGCAAGGACTGGGCCAGCCGAAGCTACATCATTCTGTATGCTATCGCCTGCTACTTCCTGCCACTTTTCCTCATCGTCTAC   720
D  Y  L  S  K  D  W  A  S  R  S  Y  I  I  L  Y  A  I  A  C  Y  F  L  P  L  F  L  I  V  Y
TCGTACTGGTTTATTGTACAGGCTGTTGCAGCACACGAGAGGGCCATGAGGGAACAGGCTAAGAAAATGAACGTTGCCTCTCTTAGATCT   810
S  Y  W  F  I  V  Q  A  V  A  A  H  E  R  A  M  R  E  Q  A  K  K  M  N  V  A  S  L  R  S
TCGGAACAGGCTAACACGAGCGCTGAGTGCAAATTGGCTAAGGTTGCACTGATGACCATCTCTCTTTGGTTCATGGCATGGACCCCTTAC   900
S  E  Q  A  N  T  S  A  E  C  K  L  A  K  V  A  L  M  T  I  S  L  W  F  M  A  W  T  P  Y
CTGGTAATCAACTTCGCGGGAGTTTTCGAGACCTCACCAATCAGCCCACTCTCAACCATCTGGGGCTCCGTCTTTGCTAAAGCTAATGCC   990
L  V  I  N  F  A  G  V  F  E  T  S  P  I  S  P  L  S  T  I  W  G  S  V  F  A  K  A  N  A
GTTTACAATCCAATTGTATATGGCATCAGCCACCCGAAATACCGCGCTGCTCTATACCAGAGGTTCCCAGCCCTGGCCTGCCAGCCATCA  1080
V  Y  N  P  I  V  Y  G  I  S  H  P  K  Y  R  A  A  L  Y  Q  R  F  P  A  L  A  C  Q  P  S
CCAGCTGAGGAGACGGGATCAGTAGCCTCTGCCGCAACCGCTTGCACCGAGGAGAAACCATCTGCTTAAATTGACGAACCCATTGCGACC  1170
P  A  E  E  T  G  S  V  A  S  A  A  T  A  C  T  E  E  K  P  S  A  *
ACGGCTCGAAAACGACTCTGCCAACACGATTTTATTTTTATACGGCTGTACAAAGTTCTCGACTTGAACACAGAACAGGGGTTCAAAATA  1260
TTCAGGTCATCATTTCATTTGAATGCCTCAAGTAGAATTTTTTTTTGTTTCTCCTCCTAACTTGCATATTTCGGGGTCCACGTTTTGTG  1350
TTTGAAGTGAGACGTTGCACACTTTTGTTTCAACCTTCGCTTTTGGTCGGCGACATTTGAGAAAAATTACAAAAAAATGTTATACGACTT  1440
AAAAAGTGTCGCCAGACCGACCCCTTACCGCGAGACCGCTCCTATTAGACTCTAAAATGTGACGAACTTTCGATGAAAAACGCTTTGTAA  1530
CTTGGACTAATAAATTATTTACATGCAACGTGGTTTATGACCACATT (polyA)                                      1577
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Fig. 1. Nucleotide and deduced amino acid sequence of the PrL opsin of *Pieris rapae crucivora*. Single- or double-headed arrows indicate the regions corresponding to primer attachment: the sequences of degenerate primers are TTGAAGCTTCARTTYCCNCCNATGAAYCC (L01F, forward primer for QFPPLNP), TTGAAGCTTAYGTNCCIGARGGNAAYATGAC (L02F, forward primer for PEGNMTA), CGAATTCGTCATRTTNCCYTCIGGNACRTA (L02R, reverse primer for PEGNMTA) and CGAATTCGGRTTRTANACIGCRTTNGCYTT (L03R, reverse primer for KANAVYNP). Forward and reverse primers have a *Hin*dIII site and *Eco*RI site (underlined), respectively.

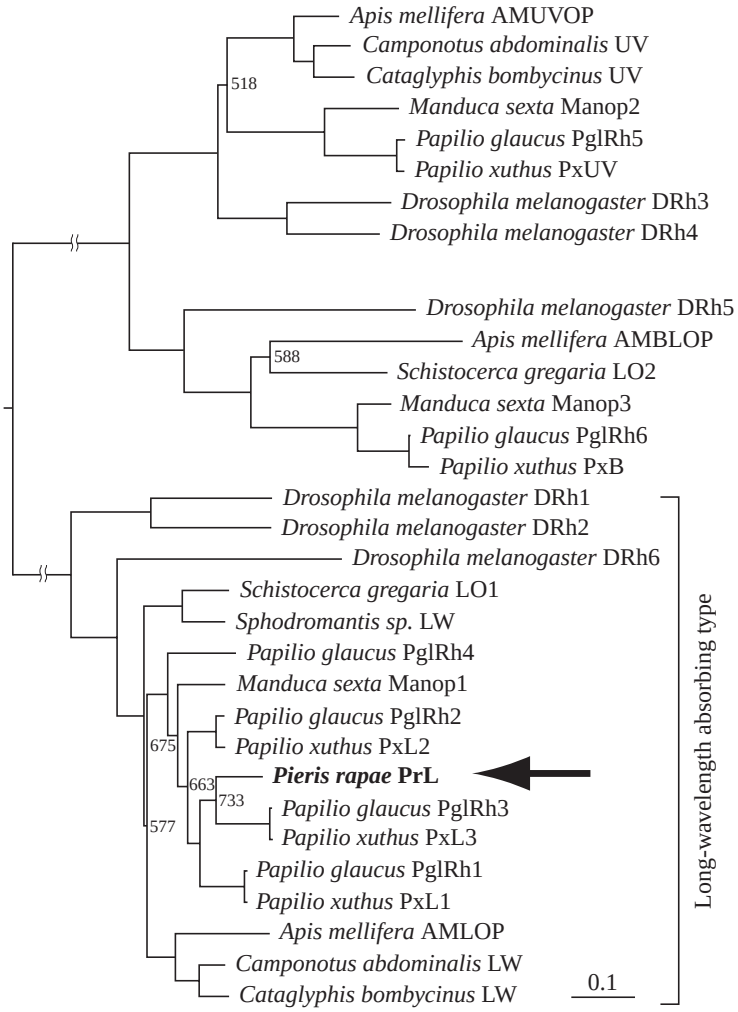

Fig. 2. Phylogenetic relationship of insect visual pigment opsins calculated using the neighbour joining method with octopus opsin as the outgroup. PrL is grouped in the long-wavelength absorbing type (arrow). Data accession numbers: *Apis mellifera* AMBLOP=AF004168; *Apis mellifera* AMLOP=AF091732; *Apis mellifera* AMUVOP=AF004169; *Camponotus abdominalis* LW=U32502; *Camponotus abdominalis* UV=AF042788; *Cataglyphis bombycina* LW=U32501; *Cataglyphis bombycinus* UV=AF042787; *Drosophila melanogaster* Rh1=K02315; *Drosophila melanogaster* Rh2=M12896; *Drosophila melanogaster* Rh3=M17718; *Drosophila melanogaster* Rh4=P08255; *Drosophila melanogaster* Rh5=U67905; *Drosophila melanogaster* Rh6=Y00043; *Manduca sexta* Manop1=L78080; *Manduca sexta* Manop2=L78081; *Manduca sexta* Manop3=AD001674; *Papilio glaucus* PglRh1=AF077189; *Papilio glaucus* PglRh2=AF077190; *Papilio glaucus* PglRh3=AF067080; *Papilio glaucus* PglRh3=AF098283; *Papilio glaucus* PglRh4=AF077193; *Papilio glaucus* PglRh5=AF077191; *Papilio glaucus* PglRh6=AF077192; *Papilio xuthus* PxB=AB028217; *Papilio xuthus* PxUV=AB028218; *Papilio xuthus* PxL1=AB007423; *Papilio xuthus* PxL2=AB007424; *Papilio xuthus* PxL3=AB007425; *Schistocerca gregaria* Lo1=X80071; *Schistocerca gregaria* Lo2=X80072; *Sphodromantis sp.* LW=X71665.

can be calculated from known parameters: the mode power fraction $\eta(\lambda)$ follows from the diameter and length of the *Pieris* rhabdom, which are approximately D_r=2.0 μm and L_p=150 μm (Qiu et al., 2002); κ_{max} can be taken to be 0.006 μm^{-1} (Warrant and Nilsson, 1998); the spectral absorption coefficient of the visual pigment can be calculated with a template formula (Govardovskii et al., 2000). It follows that $0.5<T_p(\lambda)<1$. The absorbance of the distal layer can then be obtained from:

$$A_d = \log(1 - T_p) - \log S - C' , \quad (5)$$

where $C'=C+\log(1+T_pM)$. The amplitude of the tapetal reflectance of *Pieris* is not known, but certainly $M(\lambda)<1$, and therefore $0<\log(1+T_pM)<0.3$ (it is much smaller when $M \ll 1$). C' is therefore approximately constant throughout the wavelength range of interest compared with the other terms in equation 5. Assuming that the absorbance of the distal retina, A_d, becomes negligible at long wavelengths, C' then equals $\log(1–T_p)$–$\log S$. This is, in practice, determined by shifting the experimental sensitivity spectra along the coordinate axis until the data in the long-wavelength tail of $\log S$ fit with the $\log(1–T_p)$ spectra in the two cases of the pale-red and deep-red pigmented ommatidia.

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 (Stavenga, 2004). The second term is described by A_{dv}=–log(T_{dv}), where the transmittance of the distal visual pigment, T_{dv}, in the green to red wavelength range is given by an expression similar to equation 2: $T_{vd}=\exp(-f_{3,4}\eta\kappa_{max}\alpha L_d)$, where $f_{3,4}=f_3+f_4$ is the sum of the rhabdom volume fractions of photoreceptors R3 and R4, and L_d is the length of the distal rhabdom tier. Anatomical data show that $f_{3,4}$=0.3 and L_d=250 μm (Qiu et al., 2002).

Results

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 *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 PrL (*Pieris rapae* long-wavelength absorbing visual pigment).

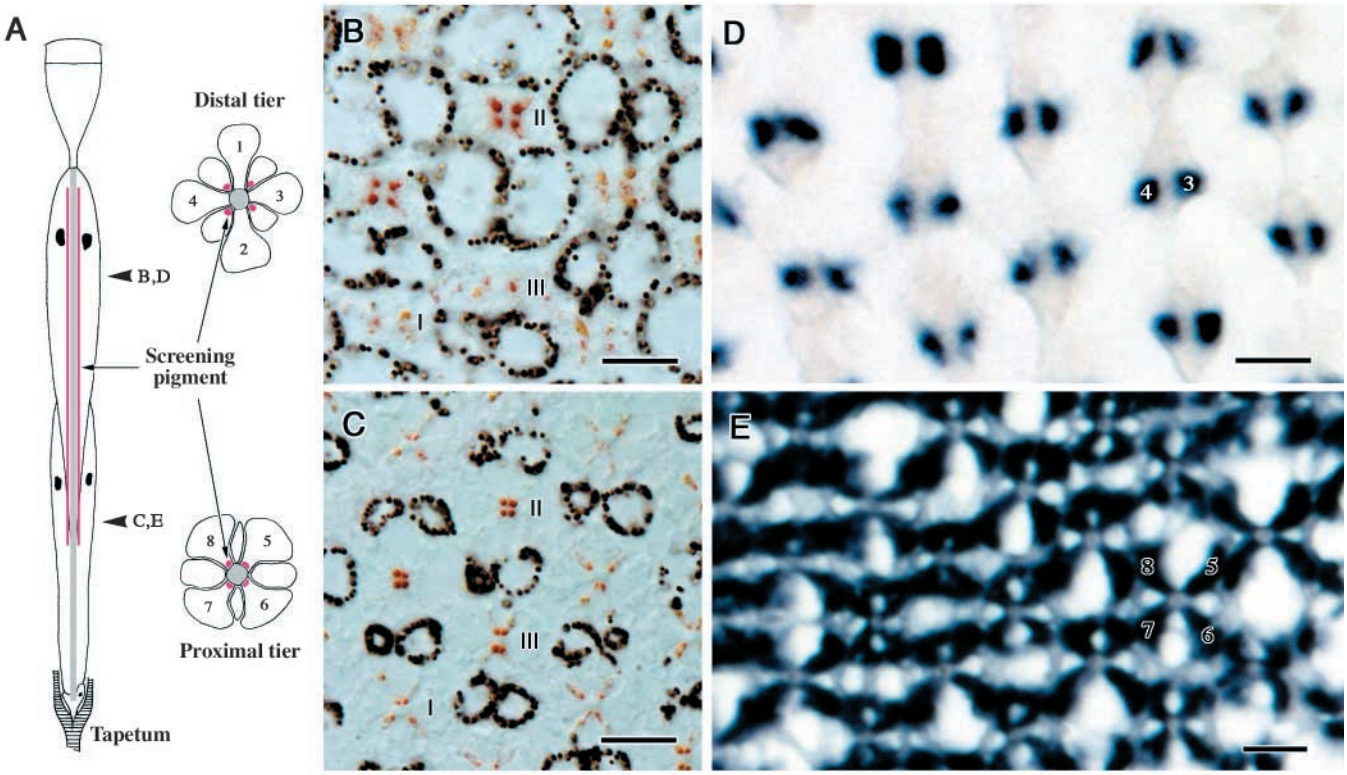

Fig. 3. *In situ* hybrization of the PrL mRNA of *Pieris rapae crucivora*. (A) Diagrammatical sketch of a *Pieris* ommatidium. Arrowheads indicate the planes where the transverse sections B,D and C,E were obtained. (B) Non-stained transverse section through the distal tier. Roman numbers indicate the ommatidial type I, II and III. (C) Unstained transverse section of the identical region of the eye shown in B but through the proximal tier. (D) *In situ* hybridization of PrL mRNA on a transverse section through the distal tier. R3 and R4 photoreceptors in all ommatidia were labelled. (E) PrL mRNA *in situ* hybridization in the proximal tier. R5–8 photoreceptors in all ommatidia were labelled. Scale bars, 10 μm.

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

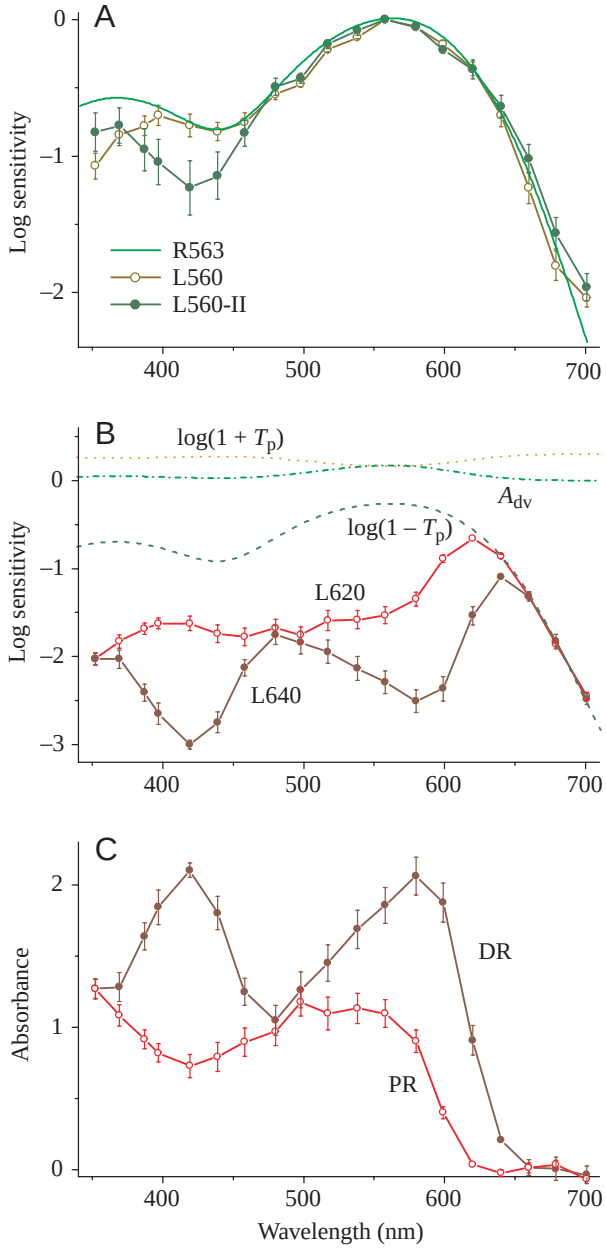

Fig. 4. Spectral sensitivities of *Pieris* photoreceptors and absorbance spectra of screening pigments. (A) Log spectral sensitivity data (error bars are S.E.M.) of green sensitive photoreceptors determined by intracellular recordings of R3,4 photoreceptors of type I and III ommatidia (open symbols; L560) and type II ommatidia (filled symbols; L560-II); from Qiu and Arikawa (2003a). The continuous green line gives the absorption spectrum of a visual pigment with peak absorption at 563 nm. (B) Log spectral sensitivity data (error bars are S.E.M.) determined by intracellular recordings on R5–8 photoreceptors of type I and III ommatidia (open symbols; L620) and type II ommatidia (filled symbols; L640); from Qiu and Arikawa (2003b). The dashed and dotted lines give log(1–T_p) and log(1+T_p), respectively, where T_p is the transmittance of the proximal rhabdom tier, length L_p=150 μm, diameter D_r=2.0 μm, with a visual pigment R563, with maximum absorption coefficient κ_{max}=0.006 μm^{-1}, in all R5–8 photoreceptors. The dot/dash green line gives the absorbance due to the visual pigment in photoreceptors R3 and R4 of the distal rhabdom tier, A_{dv}=–log(T_{dv}), where T_{dv} is the transmittance. The summed volume fraction of R3 and R4 is assumed to be $f_{3,4}$=0.3 (see Materials and methods). The used spectral sensitivity data are normalized to the peak value, meaning that the log sensitivity data have a zero peak value. The log sensitivity data of L620 and L640 were shifted 0.66 and 1.1 log unit, respectively, to fit the log (1–T_p) spectrum at the longest four (L620) or three (L640) wavelength values. (C) Absorbance spectra of screening pigments in the pale-red (PR) ommatidia and the deep-red (DR) ommatidia derived by subtracting the log spectral sensitivities of L620 and L640 from the log (1–T_p) spectrum; the error bars are taken to be identical to those in B.

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 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 absorptance 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

Fig. 4C are not exclusively due to the screening pigments. The visual pigments in the distal rhabdom tier also function as spectral filters. The absorbance, given by spectrum A_{dv} in Fig. 4B (see Materials and methods), is maximally ~0.17, so the effect of the distal visual pigment is minor. The absorbance spectra include the possible spectral effects of the dioptric apparatus. Although the facet lens and crystalline cone will be fully transparent, the channelling of light into the rhabdom will not be spectrally flat. Presumably, the spectral modulations are very minor (see the case of fly eyes: Stavenga, 2003a,b). Furthermore, as explained in the Materials and methods, the light reflected by the tapetal mirror slightly modulates the absorptance spectrum of the proximal photoreceptors. The maximal contribution from the tapetum is given by $\log(1+T_p)$, but it is probably much smaller. At any rate, the absorbance spectra of Fig. 4C will not essentially change when the contributions of distal visual pigment filtering and tapetal mirror are fully accounted for. A detailed analysis cannot be given here because first the absorption characteristics of the short-wavelength visual pigments and then the existing short-wavelength spectral filters have to be analyzed in more detail (Arikawa et al., 1999a).

Variability of long-wavelength photoreceptors

Many lepidopterans appear to have a long-wavelength absorbing visual pigment in six of the eight main photoreceptors. The long-wavelength visual pigments characterized photochemically in the nymphalids *Vanessa cardui* (Briscoe et al., 2003) and *Polygonia c-album* (Vanhoutte, 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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