Polymorphism of red receptors: sensitivity spectra of proximal photoreceptors in the small white butterfly *Pieris rapae crucivora*

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Summary

The compound eye of the small white butterfly *Pieris rapae crucivora* contains three anatomically distinct types of ommatidia. They differ in pigmentation around the rhabdom, colour of tapetal reflection and violet light-induced autofluorescence, indicating physiological differences between them. We recently reported that the ommatidia are in fact spectrally heterogeneous: in the distal part of the tiered retina they contain different sets of the spectral receptors R1–4. This study examines whether the ommatidia in the proximal retinal tier also show the spectral heterogeneity for the receptors R5–8. We recorded the sensitivity spectra of the proximal photoreceptors, and subsequently injected the dye Alexafluor 568 into proximal photoreceptors, to localize the cell and identify the ommatidial type to which it belonged. We analysed 13 successfully labeled proximal photoreceptors, and found that the sensitivity spectrum of the proximal photoreceptors in types I and III ommatidia peaks at 620 nm, whereas that of type II ommatidia peaks at 640 nm. The difference in the sensitivity spectra can be explained by the anatomical characteristics of each ommatidial type. This is the first demonstration of red receptor polymorphism in insects. The polymorphic red receptor system most probably enhances contrast sensitivity and/or color discrimination in the long wavelength spectral region.

Key words: compound eye, retina, photoreceptor, rhabdom, color vision, butterfly, *Pieris rapae crucivora*.

Introduction

The compound eyes of insects consist of a number of units called ommatidia. The ommatidial lattice is strikingly regular, and the ommatidia are identical in terms of the basic structure. However, recent studies have demonstrated that in many insect species, including flies (Franceschini et al., 1981; Hardie, 1986), moth (Meinecke and Langer, 1984), backswimmer (Schwind and Langer, 1984) and butterflies (Bernard and Miller, 1970; Ribi, 1978a; Arikawa and Stavenga, 1997; Arikawa et al., 1999a,b; Stavenga et al., 2001; Stavenga, 2002a,b), the ommatidia are in fact not identical to each other, but heterogeneous.

The white butterfly, *Pieris*, is a cosmopolitan genus that has long been used for vision research. Early anatomical work revealed that the ommatidia contain nine photoreceptor cells, numbered R1–9, and bear red pigmentation in the ventral half of the compound eye (Kolb, 1978; Ribi, 1978b). The photoreceptors were also shown to be strongly diverse in terms of their sensitivity spectra, with peak sensitivities ranging from the ultraviolet to the red wavelengths (Shimohigashi and Tominaga, 1991). The different spectral receptor types participate in various behavioral tasks such as egg laying and feeding (Kolb and Scherer, 1982). Behavioral aspects related to color vision of *Pieris* have also been studied (Goulson and Cory, 1993; Kandori and Ohsaki, 1996, 1998), and some

behavioral experiments (Scherer and Kolb, 1987) and associated model calculations (Kelber, 2001) have together demonstrated that *Pieris rapae* has true color vision, as in Papilionid species (Kinoshita et al., 1999; Kelber and Pfaff, 1999).

Our research on the yellow swallowtail *Papilio xuthus* has revealed that the eyes contain three types of ommatidia, each with a distinct set of photoreceptors, with sensitivity spectra shaped by the rhodopsin absorption spectra of both visual and filtering pigments (Arikawa and Stavenga, 1997; Arikawa et al., 1999a,b). The accumulated information about the retinal anatomy and the photoreceptor spectra of the *Pieris* compound eye inspired us to investigate whether this butterfly has a similar ommatidial heterogeneity. As expected, we identified three types of anatomically distinct ommatidia in the compound eye of *Pieris rapae crucivora* (Qiu et al., 2002). All ommatidia of a *Pieris* eye are tiered, containing four distal (R1–4) and four proximal (R5–8) photoreceptor cells, the rhabdomeres, of which together form the fused rhabdom, a cylindrical structure along the central axis of the ommatidium. The R9 cell contributes to the rhabdom at the very base (i.e. is most proximal) (Fig. 1). Except for the dorsal part of the eye, all ommatidia are prominently pigmented (Ribi, 1978b). The proximal photoreceptors, R5–8, bear a dense pigmentation
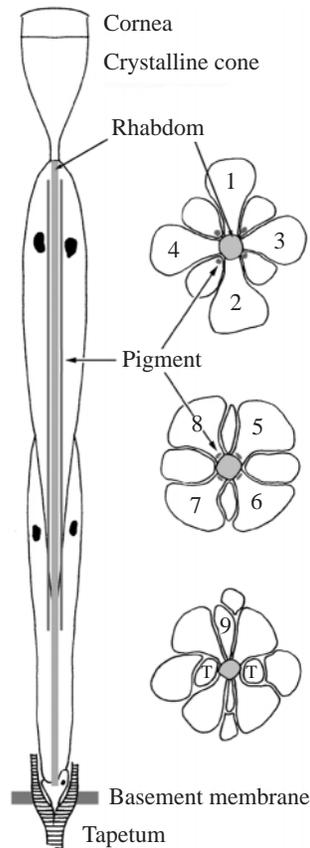
Fig. 1. Diagram of the *Pieris rapae crucivora* ommatidium. Distal photoreceptors (R1–4) bear photoreceptive microvilli in the distal two-thirds of the rhabdom. Proximal photoreceptors (R5–8) contribute their microvilli to the proximal one-third of the rhabdom. R9 adds a few microvilli at the base of the rhabdom. Pigment granules in R5–8 are seen as four spots in transverse sections, and their arrangement is a reliable marker for identifying the ommatidial type (see, for example, Fig. 2C). T, tapetum.

Cornea
Crystalline cone
Rhabdom
Pigment
Basement membrane
Tapetum

Japanese yellow swallowtail *Papilio xuthus*, red receptors are found exclusively in the proximal tier of the retina, so we conjectured that the red receptors must be located in the proximal tier in the eye of *Pieris rapae* as well.

Here we have investigated the sensitivity spectra of the proximal receptors, R5–8, in the *Pieris* ommatidia. Using a combination of electrophysiology and histology, we found that the proximal receptors in all three types of ommatidia are specifically sensitive in the long wavelength region, i.e. red. Interestingly, the sensitivity spectra of these receptors differ between the ommatidial types, probably reflecting their differences in pigmentation, tapeta and/or autofluorescence.

Materials and methods
Animals

We used spring-form males of *Pieris rapae crucivora* Boisduval. The butterflies were taken from a laboratory stock culture derived from eggs laid by females caught in the field. 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, prior to experimentation. The adults were used within 4 days after emergence.

Electrophysiology

Electrophysiological methods were as described before (Qiu et al., 2002). Briefly, a butterfly was mounted on a plastic stage set in a Faraday cage. A silver wire inserted in the stump of an antenna served as the indifferent electrode. To insert a glass micropipette into the eye, a hole covering about 10–20 facets was made in the dorsal region of the cornea with a razor blade. The eye was then positioned at the center of a Cardan arm perimeter device. The dorso–ventral axis of the compound eye was adjusted to a vertical orientation.

Monochromatic stimuli were provided by a 500 W Xenon arc lamp through a series of narrow-band interference filters. The light was focused on the tip of an optical fiber that was attached to the perimeter device, where it provided a point source of light (1° in diameter). The quantum flux of each monochromatic stimulus was measured by a radiometer (Model-470D, Sanso, Tokyo, Japan), and the maximum quantum flux of each monochromatic stimulus at the corneal surface was adjusted to 5.0×10^{11} photons cm^{-2} s^{-1}.

A glass microelectrode filled with a fluorescing dye, Alexafluor 568 (excitation at 576 nm, emission at 599 nm), 1% in 50 mmol l^{-1} potassium phosphate, pH 7, resistance approximately 100 MΩ, was inserted into the eye through the hole made in the cornea. After impalement of the electrode into a photoreceptor, the optical axis of the photoreceptor was located by moving the tip of the optical fiber to maximize the response.

Light flashes were of 30 ms duration. First, the sensitivity spectrum of the photoreceptor was determined by stimulating the cell with a series of monochromatic flashes. The stimulus intensity–response function was measured at the peak

around the rhabdom, which appears as four reddish spots in transverse sections. From the arrangement of the pigment clusters three ommatidial types can be clearly distinguished: in type I, the pigment clusters are arranged trapezoidally, in type II, in a square, and in type III, in a rectangle (Qiu et al., 2002; see also, for example, Fig. 2C). The red pigmentation around the rhabdom functions as a red-transmittant spectral filter, with the rhabdom acting as a waveguide, because its diameter in *Pieris* is less than 2 μm (Qiu et al., 2002) and the refractive indices of the rhabdom and surrounding medium only differ slightly. When light propagates along the slender rhabdom, a significant proportion of the light flux travels outside its boundary, and therefore the red pigmentation lining the rhabdom can absorb light in the boundary wave.

The photoreceptors in the three types of ommatidia appear to be spectrally heterogeneous. Electrophysiological recordings of photoreceptors in the distal tier revealed the existence of UV (U), blue (B), double-peaked blue (dB), green (G) and green with depressed sensitivity at 420 nm (dG) receptors (Qiu and Arikawa, 2003). Using a combination of electrophysiology and histology, we demonstrate that these spectral receptors occur in different combinations in the three ommatidial types (see Table 1). In addition to these spectral receptors, Shimohigashi and Tominaga (1991) reported finding red receptors in the eye of *Pieris rapae*. By contrast, we did not encounter red receptors in the distal tier in our previous study (Qiu and Arikawa, 2003). In fact, in the eye of the
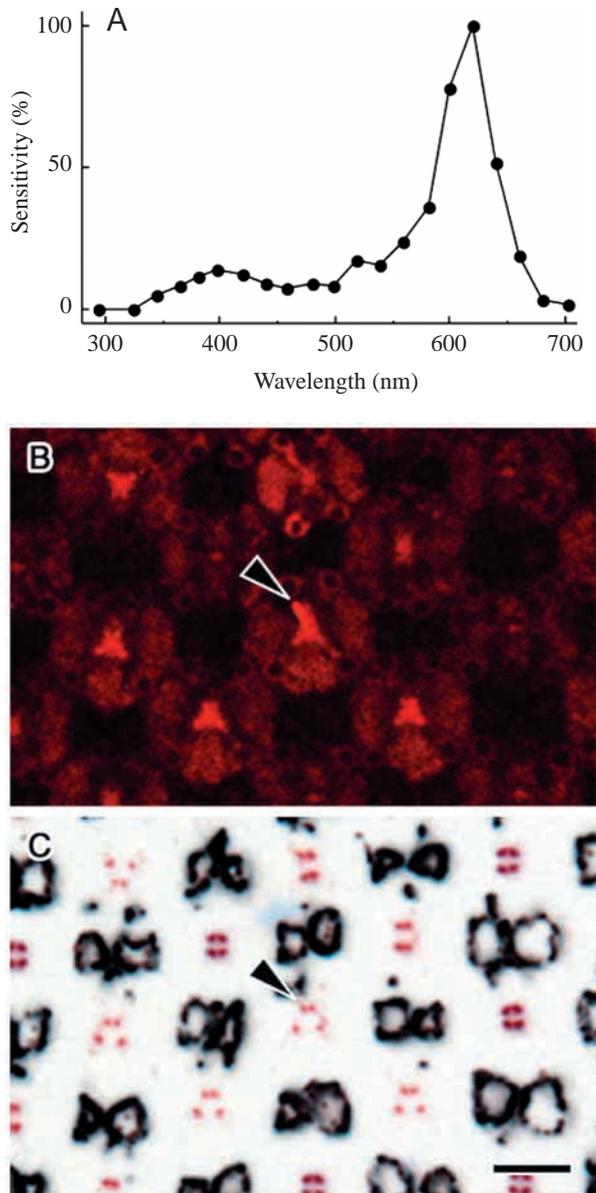
Fig. 2. An example of a LW620 receptor in a type I ommatidium. (A) Sensitivity spectrum. (B) Green-induced fluorescence picture of a transverse section containing the Alexafluor 568-injected receptor shown in A (arrowhead). The receptor is an R8. (C) Same section taken under regular transmission light, indicating that the Alexafluor 568-injected receptor is a member of a type I ommatidium with pigment arranged trapezoidally (arrowhead). Scale bar, 10 μm.

wavelength of the given receptor over an intensity range of 5 log units. The photoreceptor was subjected to further analyses only when the maximal response amplitude exceeded 30 mV.

After recording, photoreceptors were marked by injecting the Alexafluor 568 through the recording electrode into the photoreceptor by applying a 4 nA hyperpolarizing current for approximately 4 min. The eyes were then fixed in 4% paraformaldehyde in 0.1 mol l^{-1} sodium cacodylate buffer, pH 7.4, at room temperature for 30 min, and embedded in

Spurr's resin. Unstained transverse sections of 10 μm thickness were observed and photographed with regular transmission microscopy and with fluorescence microscopy using green excitation light (dichroic cube U-WIG: excitation band-pass filter 550 nm, emission cut-off filter 580 nm), to determine the anatomical identity of the recorded photoreceptor and the type of ommatidium to which it belongs.

Results

We identified the sensitivity spectra of the proximal photoreceptors, R5–8, of the three types of ommatidia in the eye of male *Pieris rapae crucivora*. We recorded 37 receptors in total, 13 of which were successfully labeled and thus analysed in the present study. Figs 2–4 show typical examples.

The type of receptors encountered most frequently has a peak sensitivity at 620 nm (Fig. 2). Epi-fluorescence microscopy revealed that the particular receptor which yielded the spectrum of Fig. 2A was a proximal photoreceptor R8 (Fig. 2B). Regular transmission microscopy showed that the photoreceptor was located in an ommatidium whose pigment clusters are arranged trapezoidally, meaning that the ommatidium was of type I. We thus identified six cells peaking at 620 nm, which belonged to the R5–8 set of photoreceptors in type I ommatidia. Photoreceptors peaking at 620 nm were also found in type III ommatidia. The particular example of Fig. 3 was identified as an R6 in a type III ommatidium. We identified three such receptors (Fig. 3B,C).

A less frequent class of photoreceptors peaks at 640 nm. Fig. 4 shows an example of a recording from such a photoreceptor in a type II ommatidium. We labeled four cells peaking at 640 nm, all of which were proximal photoreceptors of the R5–8 set in type II ommatidia (Fig. 4B,C).

Fig. 5 shows the mean sensitivity spectra of the two classes, peaking at 620 nm and 640 nm, respectively. Both sensitivity profiles are rather narrow, with half-bandwidth of approximately 40 nm. This value is much lower than that of a normal rhodopsin absorption spectrum (Stavenga et al., 1993). Clear differences can be seen in a few points. For example, in the 640 nm peaking receptors, the sensitivity at 620 nm is less than 50%, and a sensitivity depression exists in the short wavelength region, always with the lowest sensitivity at 420 nm.

Discussion

The compound eye of *Pieris rapae crucivora* contains three anatomically distinct types of ommatidia, which differ in pigmentation around the rhabdom, autofluorescence and color of tapetal reflection (Qiu et al., 2002). Functionally, the different types of ommatidia are spectrally heterogeneous, i.e. each type of ommatidia bears different sets of spectral receptors in the distal tier (Qiu and Arikawa, 2003). The present study demonstrates that the spectral heterogeneity of the ommatidia also occurs in the proximal tier. The sensitivity spectra of the proximal photoreceptors R5–8 peak either at
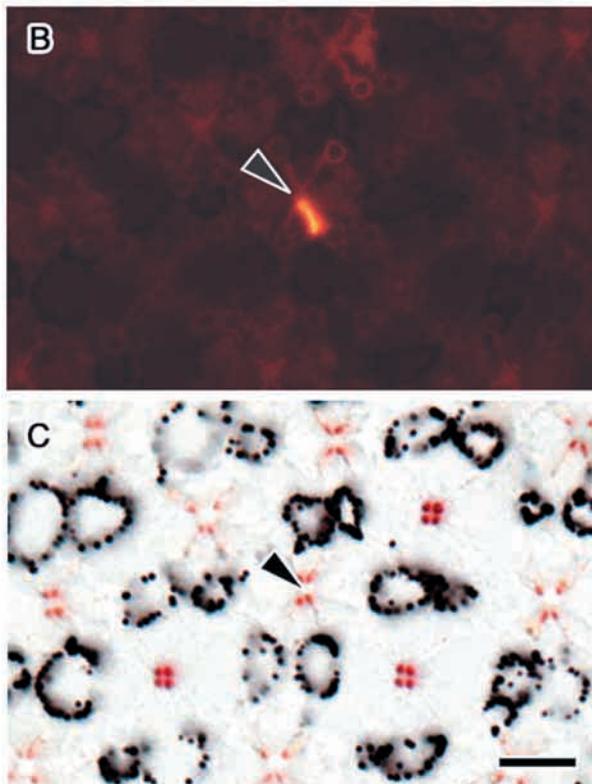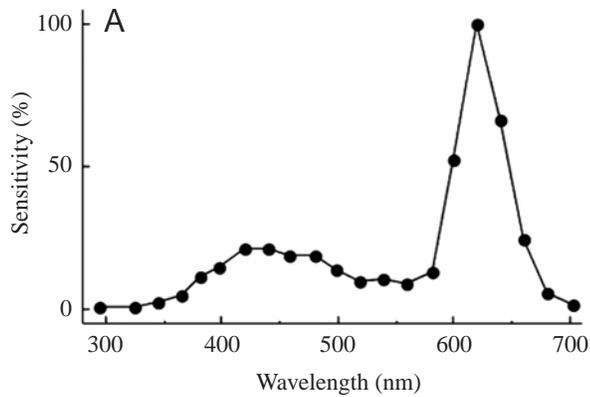
Fig. 3. An example of a LW620 receptor in a type III ommatidium. (A) Sensitivity spectrum. (B) Green-induced fluorescence picture of a transverse section containing the Alexafluor 568-injected receptor shown in A (arrowhead). The receptor is an R6. (C) Same section taken under regular transmission light, indicating that the Alexafluor 568-injected receptor is a member of a type III ommatidium with pigment arranged rectangularly (arrowhead). Scale bar, 10 μm.
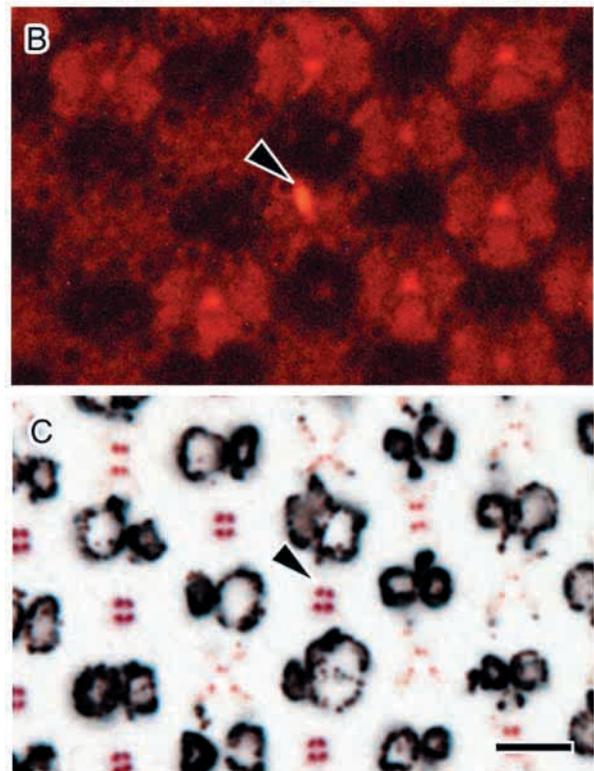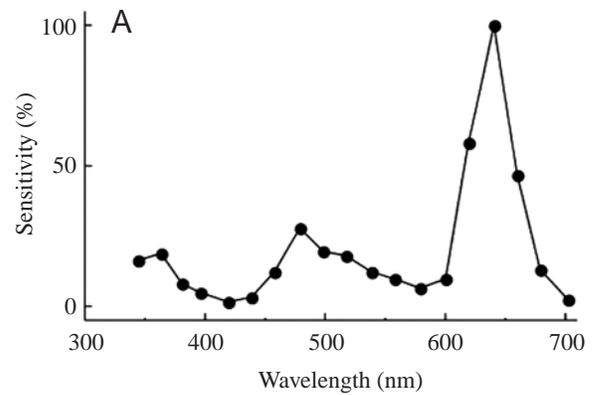

Fig. 4. An example of a LW640 receptor in a type II ommatidium. (A) Sensitivity spectrum. (B) Green-induced fluorescence picture of a transverse section containing the Alexafluor 568-injected receptor shown in A (arrowhead).The receptor is an R8. (C) Same section taken under regular transmission light, indicating that the Alexafluor 568-injected receptor is a member of a type II ommatidium with pigment arranged in a square (arrowhead). Scale bar, 10 μm.

620 nm or at 640 nm, both of which should be categorized as long-wavelength or red receptors. For convenience we hereafter refer to these long-wavelength receptors as LW620 and LW640 receptors, respectively. Types I and III ommatidia have LW620 receptors, whereas type II ommatidia have LW640 receptors. Table 1 summarizes the present results, together with previous results of anatomy and physiology reported for *Pieris* ommatidia.

The monochromatic stimuli used in the present study had 20 nm periodic intervals. Therefore, one might argue that the difference between the two spectra could be attributed to some experimental error. It is true that the precise profiles of the sensitivity spectra may be slightly distorted by the low sampling rate, and therefore the peak values, 620 and 640 nm, are only approximate. Nevertheless, clear differences exist between them, which strongly indicate that the physiological properties of LW620 and LW640 receptors are in fact
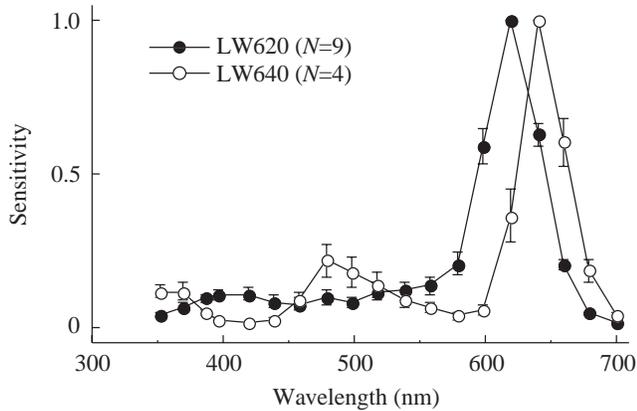
Fig. 5. Sensitivity spectra of the two different classes of red receptor, LW620 and LW640, identified in the proximal tier of the ommatidia of *Pieris rapae crucivora*. Values are means ± S.E.M., *N*=number of cells analyzed in the present study.

different. Most importantly, the two receptor classes exist in different types of ommatidia. As described by Qiu et al. (2002), the pigmentation around the rhabdom of types I and III ommatidia has the same, pale-red color. The pigmentation of type II ommatidia is deep-red. Pigments accumulated near the rhabdom act as spectral filters, which thus can considerably alter the sensitivity spectrum of the photoreceptors (Arikawa et al., 1999b; Stavenga, 1989, 2002a,b). Deep-red pigment will shift the sensitivity spectrum of the proximal photoreceptors to longer wavelengths more strongly than pale-red pigment (Qiu et al., 2002; Stavenga 2002a), in agreement with the finding that the ommatidia with pale-red pigment and deep-red pigment have LW620 and LW640 receptors, respectively.

This view is further supported by the difference in the properties of the tapetum. The tapetum is a tracheole folded into a stack of layers, alternately consisting of air and cytoplasm, thus creating an interference reflection filter. Incident light that has propagated along the rhabdom is reflected at the tapetum, travels back through the rhabdom and leaves the eye again, visible as the so-called eyeshine. The eyeshine reflectance spectra of all ommatidia have a more-or-less gaussian shape, with a half-bandwidth of approximately

50 nm, but the spectra nevertheless distinctly differ between the types of ommatidia: the reflectance spectra of types I and III ommatidia are approximately the same and peak at 635 nm, whereas the spectrum of type II peaks at 675 nm (Table 1). The eyeshine is due to light reflected by the tapetum and filtered by the clusters of screening pigment located adjacent to the rhabdom (Qiu et al., 2002; Stavenga, 2002a). The slope of the screening pigment's absorption spectrum at the long-wavelength side determines the left-hand (short-wavelength) slope of the eyeshine reflectance spectrum, and the long-wavelength tail of the tapetal reflectance spectrum determines the right-hand (long-wavelength) slope of the eyeshine reflectance spectrum. The types I and III ommatidia thus have a pale-red screening pigment coupled with a tapetum reflecting up to approximately 670 nm, and the type II ommatidia have a deep-red screening pigment coupled with a tapetum reflecting up to approximately 710 nm (Qiu et al., 2002). The wider spectral range of the latter tapetum will be beneficial for an extended red sensitivity.

A point of specific interest is the depressed sensitivity of the LW640 receptors in the short wavelength region. LW640 receptors exist in type II ommatidia, which fluoresce under epi-illumination of 420 nm light (Qiu et al., 2002). In the distal tier of type II ommatidia, we encountered dB receptors and dG receptors, both having a depressed sensitivity at 420 nm (Qiu and Arikawa, 2003). The depressed sensitivity is presumably due to the filtering effect of the fluorescing material, absorbing strongly at 420 nm, functioning as a blue–violet-absorbing filter, similar to the 3-hydroxyretinol in *Papilio* type II ommatidia, which functions as a UV-absorbing filter (Arikawa et al., 1999a). If this is the case, the blue–violet filter must also act on the proximal photoreceptors. Interestingly, the LW640 receptor has a depressed sensitivity in the short wavelength region, with the minimum sensitivity always at 420 nm.

In conclusion, we propose here that there exist at least two subclasses of red sensitive photoreceptor cells in the eye of *Pieris rapae crucivora*. To the best of our knowledge, this is the first example of polymorphism of red receptors in insect compound eyes. In crustaceans, the amazingly complex eye of the mantis shrimp contains at least two types of long wavelength receptors in the wavelength region between 600 and 700 nm (Marshall et al., 1991). The possible benefit of

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

Type	Pigment clusters	Reflectance peak (nm)	Fluorescence	Photoreceptor sensitivity spectrum				
				R1	R2	R3/4	R5–8	R9
I	Trapezoid	635	–	UV, Blue	Blue, UV	Green	**LW620 (Red)**	Red*
II	Square	675	+	dBlue	dBlue	dGreen	**LW640 (Red)**	Red*
III	Rectangle	635	–	UV	UV	Green	**LW620 (Red)**	Red*

UV, ultraviolet; the prefix d indicates depressed sensitivity (see text for an explanation).

R5–8 characteristics were obtained in the present study (bold). Others are based on data reported by Qiu et al. (2002) and Qiu and Arikawa (2003).

*Data for R9 was reported by Shimohigashi and Tominaga (1991).

having polymorphic red receptors is to increase the color discrimination ability in this region of the spectrum. This needs to be tested by appropriate behavioral experiments, however.

In the present study we focused on the sensitivity spectra of four proximal photoreceptors R5–8, and did not try to record from the small basal photoreceptor R9. Shimohigashi and Tominaga (1991) successfully identified one R9 as a red receptor peaking at 620 nm, which is similar to the LW620 receptor described in this study. At that time, however, ommatidial heterogeneity of the *Pieris* retina had not yet been discovered, so the ommatidial type of the recorded R9 was not characterized. The R9 photoreceptors occupy the most proximal location of the ommatidium (Arikawa et al., 1999b) and therefore should be even more severely affected by the spectral filtering. It is therefore likely that the red-sensitive R9 recorded by Shimohigashi and Tominaga (1991) was a member of either a type I or type III ommatidium. If the R9 receptors of type II are also red sensitive, their sensitivity spectrum would probably be similar to that of the LW640.

As stated above, the precise profiles of the sensitivity spectra of LW620 and LW640 receptors could be slightly distorted due to our low wavelength sampling rate. Electrophysiological measurements using a higher sampling rate will be necessary for a more detailed characterization of the sensitivity spectra. Interestingly, our preliminary studies on the molecular biology of visual pigment opsins expressed in the proximal receptors indicated that all proximal receptors contain identical mRNA, encoding a long wavelength-absorbing visual pigment, so the LW620 and LW640 receptors may be identical at the molecular biological level. However, we have also found in the *Papilio* retina that some photoreceptors coexpress multiple types of visual pigments (Kitamoto et al., 1998, 2000), resulting in distinct sensitivity spectra (Arikawa et al., in press). Therefore, more complete knowledge of the visual pigment opsins expressed in the proximal photoreceptors in the *Pieris* eye is needed to substantiate our view that *Pieris* has two different classes of red photoreceptors. Such molecular biological information would also provide insight into how the polymorphism of red receptors is established.

The existence of three different types of ommatidia in a single compound eye has also been demonstrated in *Papilio xuthus* (Kitamoto et al., 2000), *Vanessa cardui* (Briscoe et al., 2003) and *Manduca sexta* (R. H. White, H. Xu, T. A. Munch, R. R. Bennett and E. A. Grable, manuscript submitted), and seems to be a basic design for the eyes of lepidopteran insects. One type of ommatidia commonly contains two different short-wavelength receptors, namely UV and blue receptors. This is probably important for discriminating colors of very small targets in the short wavelength region of the spectrum. The difference is obvious in the proximal tier. The sensitivity spectra of the proximal photoreceptors are either the green, red or broad-band types in *Papilio xuthus* (Arikawa et al., 1999b, in press), whereas there are two types of red-sensitive receptors in *Pieris*. In *M. sexta* and *V. cardui*, these photoreceptors are probably all green-sensitive (Briscoe et al., 2003; R. H. White, H. Xu, T. A. Munch, R. R. Bennett and E. A. Grable,

manuscript submitted). The difference may reflect the difference in the color vision properties, which should be demonstrated by careful behavioral analyses.

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