Behavioral responses in feeding to green color as visual stimulus with two lepidopteran larvae, *Spodoptera litura* (Fabricius) (Noctuidae) and *Milionia basalis pryeri* Druce (Geometridae)

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Abstract

Larvae of armyworm, *Spodoptera litura*, a polyphagous leaf-eater and the monophagous *Milionia basalis pryeri* feed more on a diet gel containing chlorophyll *b* in the light. Chlorophyll *b* acts as a visual stimulus to feeding rather than as a gustatory or odor stimulus. Furthermore, the larvae are found significantly more frequently on gels placed on silhouettes of gels containing chlorophyll *b* than on that of colorless gels. This reveals that the larval response to the color of chlorophyll *b* is orthokinetic rather than tactic. *S. litura* and *M. b. pryeri* larvae are able to distinguish between colors since consumption varied according to the wavelength of the green silhouettes. Feeding differences between the two species may be due to the color of their host plants. These are novel findings that indicate lepidopteran larvae recognize the green colors of their host plants.

Key words: Chlorophyll; green color; larvae; Lepidoptera; visual stimulus

INTRODUCTION

The larvae of the geometrid moth *Milionia basalis pryeri* Druce feed exclusively on podocarp *Podocarpus macrophyllus* (Podocarpaceae) leaves. During the study of feeding stimulant in *P. macrophyllus* leaves for *M. b. pryeri* larvae, the larvae fed more on a diet gel that contained chlorophyll *b* under a LD16:8h photoperiod condition but did not do so under continuous darkness. This suggests that the visual stimulus from chlorophyll *b* enhances feeding by the larvae. The authors are unaware of any reports of chemical substances role as visual stimuli. There are some reports about interaction between larval behaviors and visual stimuli (e.g., Süffert and Götz, 1936; Wilde and Pet, 1957; Saxena and Khattar, 1977; Raubenheimer and Tucker, 1997). As chlorophyll *b* is a chemical widely distributed in plants, other leaf eaters might have characteristics similar to those of *M. b. pryeri* larvae. Therefore, in addition to *M. b. pryeri* larvae, larvae of the armyworm, *Spodoptera litura* (Fabricius), a polyphagous leaf eater, are also examined to explore how chlorophyll *b* functions as a

visual stimulus. The role of visual stimulus from chlorophyll *b* is discussed in the feeding response of *S. litura* and *M. b. pryeri* larvae.

MATERIALS AND METHODS

Insects. *S. litura* larvae were reared on an artificial diet (Wakamura, 1988) containing soy bean instead of kidney bean. *M. b. pryeri* larvae were collected from *P. macrophyllus*, on Okinawa Island, Japan in July 2002, and successively reared on *P. macrophyllus* leaves in the laboratory. These larvae were reared at 25°C and a LD 14:10 h photoperiod. Third- or 4th-instar larvae within 12 h after ecdysis were used for the bioassays.

Preparation of diet gels for bioassay. Chlorophyll *b* from *Chlorella*, purity: >99.0%, was obtained from Wako Pure Chemical Industries, Ltd. As 150 µg of chlorophyll *b* is contained in 1 g of *P. macrophyllus* fresh leaf, the content of chlorophyll *b* in the diet gel was adjusted accordingly. Chlorophyll *b* (150 µg) dissolved in ethyl acetate (50 µl) was mixed with cellulose powder (0.056 g, Toyo Roshi Kaisha, Ltd.) and the solvent was evaporated

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Fig. 1. Bioassay arena for examining feeding preference and locations of *Spodoptera litura* and *Milionia basalis pryeri* larvae. A: top view, B: side view. A piece of gel containing chlorophyll *b* and that of the control gel were placed directly beneath a clear plastic film (6 cm×6 cm) at 1 cm intervals. On the upper surface of the film, two pieces of chlorophyll-free gel of the same size were pasted in line to the gel silhouettes below. Both sides of the film were covered with a 5 cm Petri dish with a moistened cotton wick. One third-instar *S. litura* larva was released 2 cm from the gels with its head in the center between them. The dishes were lit from below by a 5 W fluorescent lamp, and from above by two 40 W fluorescent lamps.

at room temperature. In a glass Petri dish (3 cm in dia., 1.2 cm depth), sucrose (0.035 g) and agar (0.035 g) were added to distilled water (1 ml). To dissolve the agar, the mixture was warmed in a microwave oven. When the agar was dissolved, the solution was immediately mixed with the cellulose powder and cooled to gel. The gel material was cut into 100 mg pieces for bioassays. Cellulose powder treated with the same amounts of the same solvent was used for control. The gels were prepared just before bioassays.

Measurement of reflectance spectra. Reflectance spectra of the gel diets and the printed silhouettes on the films were measured by using a UV-Vis recording spectrophotometer (UV-2500PC, Shimadzu Co., Kyoto).

Bioassay 1. Two-choice tests were conducted to examine larval preferences to chlorophyll *b*. A piece of gel (100 mg) containing chlorophyll *b* and that of the control gel were placed on a 5 cm plastic Petri dish at 1 cm intervals. One *S. litura* larva (third-instar) or *M. b. pryeri* larva (fourth-instar) was released 2 cm from the gels with the head positioned between the two gels. To prevent desiccation, a moistened cotton wick was placed on the inner side of the lid of the Petri dish. The dishes were placed in a clear plastic box (40×30×8 cm) at a LD photoperiod of 16:8 h or 0:24 h at 25°C. After 3 d of feeding, the larva and feces were removed and the remaining gels were dried at 60°C for 2 h to measure consumption. The test of *S. litura* was replicated six (LD 16:8 h) or seven (LD 0:24 h) times, and that of *M. b. pryeri* was repli-

cated six (LD 16:8 h) or five (LD 0:24 h) times.

Bioassay 2. Visual response to chlorophyll *b* was examined by a two-choice test. A piece of gel containing chlorophyll *b* and that of the control gel were placed directly beneath a clear plastic film (6×6 cm) at 1 cm intervals (Fig. 1A). On the upper surface of the film, two pieces of the same size of chlorophyll-free gel were pasted in line with the gel silhouettes below (Fig. 1B). Both sides of the film were covered with a 5-cm Petri dish with moistened cotton wick. One third-instar *S. litura* larva was released 2 cm from the gels with its head in the center between them. The dishes were lit from 35 cm below by a 5 W fluorescent lamp, and from 2 m above by two 40 W fluorescent lamps. To avoid an increase in temperature from the lighting, a 30 cm of air layer, a 2 cm of water layer and another 2 cm of air layer were placed between the lower lamp and the test dishes. The lighting intensities were 1.94 W/m² (670 lx) from below the test dish, and 2.84 W/m² (980 lx) from above. Reflectance spectra of the gels on the film are shown in Fig. 2A. For the experiments under dark conditions, the test dishes were covered with aluminum foil. After 2 d of feeding at 25°C, the remaining gels were dried at 60°C for 2 h to measure consumption. Each test was replicated six times.

During bioassay 2, the larval locations were recorded: (1) on the gel with a chlorophyll *b* color silhouette, (2) on the control gel, and (3) on other place in the Petri dish. Observations were conducted at 10 min intervals for 180 min (resulting in 19 observations), from 90 min after the initiation of

Fig. 2. Reflectance spectra of color gels and films. A: gels+films, B: gray films, C: green color films; c: control (no printing) film, b: bluish green film, g: green film, and y: yellowish green film.

assay when the larvae were placed in the dishes. This assay had six replicates and resulted in a total of 114 observations. In a separate experiment, using the same bioassay system, continuous observation was conducted to assess the first choice by the larvae. This experiment had 22 replicates.

Bioassay 3. To examine the larval response to light intensity, gray silhouettes with different light-

ness were printed on the clear plastic film (6×6 cm): dark gray, gray, and light gray (reflectance spectra: Fig. 2B). Bioassay apparatus used was the same as Bioassay 2. One control gel was placed in line with the printed silhouette, and a second control gel was placed on the first gel at 1 cm intervals. One fourth-instar *S. litura* larva was released 2 cm from the gels with its head placed in the center between them. A Petri dish with a moistened cotton wick beneath the lid was placed on the gels. After 2 d, the remaining gels were dried and weighed in the same manner as above. Each test was replicated six times.

Bioassay 4. Responses of fourth-instar larvae of *S. litura* or *M. b. pryeri* to different green colors were evaluated in the same manner as Bioassays 2 and 3. In this assay, three different green silhouettes were printed on clear plastic film: bluish green, green, and yellowish green (reflectance spectra: Fig. 2C). Each test was replicated six times.

Statistics. The mean value of the differences in larval consumptions between test and control gels were applied to paired *t*-test.

RESULTS

Two-choice test between gel containing chlorophyll *b* and the control gel (Bioassay 1)

S. litura larvae consumed significantly more gel containing chlorophyll *b* than the control under lighted conditions (Fig. 3A, $p=0.004$). The consumption of the two gels was not significantly different under the continuous dark condition. A similar tendency was also observed in *M. b. pryeri* larvae (Fig. 3B, light condition; $p=0.02$).

Two-choice test between gel with chlorophyll *b* color silhouette and the control gel (Bioassay 2)

To clarify whether the larvae identify the preferred gel containing chlorophyll *b* through visual stimulus, Bioassay 2, in which gustatory or odor effects of chlorophyll *b* were completely eliminated was performed. In this bioassay, the gel containing chlorophyll *b* was inaccessible but visible to the larvae (Fig. 1). *S. litura* larvae consumed significantly more gel placed on the silhouette of gel that contained chlorophyll *b* than that placed on a colorless control under the lighted conditions (Fig. 4, $p=0.002$). This difference was not observed under

Fig. 3. Two-choice test between gel containing chlorophyll *b* and control gel by the larvae under the light and dark conditions (Bioassay 1). A: third-instar *Spodoptera litura*, LD 16:8 h, $N=6$; LD 0:24 h, $N=7$. B: fourth-instar *Milionia basalis pryeri*, LD 16:8 h, $N=6$; LD 0:24 h, $N=5$. Values indicated are means and SE (bar) of gel consumption for 3 d.

the dark conditions. Reflectance spectra indicated that the film does not reflect a shorter wavelength than 310 nm (Fig. 2A).

Choices and locations of gels were examined under the lighted conditions (Fig. 5). The frequency of the first choice by *S. litura* larvae was equal between chlorophyll *b* color (50%) and colorless (50%) gels ($N=22$, Fig. 5A). However, the larvae were observed more frequently on the gels with silhouettes of the diet gel containing chlorophyll *b* (46%) than that of the colorless gel (16%) ($N=6$, Fig. 5B, $p<0.001$).

Two-choice test between gel with gray silhouette and control gel (Bioassay 3)

To examine whether the larvae visually recognize the chlorophyll *b* color by lightness, the bioassay was conducted using the gels on gray silhouettes with three different grades; dark gray, gray, and light gray. Reflectance spectra of the films were measured and the spectra were similar to each other but the intensity was different, which suggests that the difference of the three films arises from the lightness (Fig. 2B). The consumption by *S. litura* larvae was not significantly different between gels on gray silhouettes and the colorless

Fig. 4. Two-choice test between gel with chlorophyll *b* color silhouette and colorless gel by *S. litura* larvae under light and dark conditions (Bioassay 2). $N=6$. Third-instar larvae were used and fed for 2 d. Values indicated are means and SE (bar) of gel consumption for 2 d.

Fig. 5. *Spodoptera litura* larval behavior during Bioassay 2. A: First choice of the two gels ($N=22$). B: Location of the larvae. Third-instar larvae were used ($N=6$), and location of the larvae was checked every 10 min for 3 h, resulting in $6 \times 19 = 114$ observations. The frequencies of first choice and location were submitted to χ^2 -test.

control gel (dark gray: $p=0.99$, gray: $p=0.69$, and light gray: $p=0.074$, Fig. 6).

Two-choice test between gel with green color silhouette and control gel (Bioassay 4)

The consumption of the gels on green and yellowish green silhouettes by *S. litura* larvae was significantly larger than the colorless gel (bluish green: $p=0.28$, green: $p=0.05$, and yellowish green: $p=0.004$, Fig. 7A). *M. b. pryeri* larvae showed a slightly different response: the consumption of the gels on green and bluish green silhouettes was significantly higher than that on the colorless control (bluish green: $p=0.009$, green: $p=0.02$, and yellowish green: $p=0.25$, Fig. 7B).

Fig. 6. Two-choice test between control gel and gels with three different gray color silhouettes by *Spodoptera litura* larvae (Bioassay 3). Fourth-instar larvae were used and fed for 2 d under lighted conditions. $N=6$. Values indicated are means and SE (bar) of gel consumption for 2 d.

DISCUSSION

The importance of visual stimulus has been well studied, especially on butterflies and bees (e.g. Silberglied, 1989; Barth, 1991). Furthermore, certain species of Lepidoptera and Coleoptera have been revealed to use visual cues for mate location (Hidaka, 1972; Silberglied, 1989; Fukaya et al., 2004a, b). There are few studies about visual stimuli on larvae; current information deals primarily with the receptors of larval eyes (Saxena and Khattar, 1977; Ichikawa, 1987; Raubenheimer and Tucker, 1997; Lin et al., 2002). The reports about interaction between visual stimulus and behavior in larval stages were as follows: e.g. Söffert and Götz (1936) showed that the larvae of *Vanessa urticae* and *V. io* in the feeding stage are attracted by the green color of leaves and of paper fragments. Saxena and Khattar (1977) reported the use of glass-screened films of freshly-excised leaves of the larval host plant *Citrus limettoides* as visual stimuli, and that the color of the leaves had been found to attract *Papilio demoleus* (Lepidoptera: Papilionidae) larvae. Fifth-instar African migratory locusts, *Locusta migratoria* (Orthoptera: Acrididae), displayed higher preference for yellow than green in a two-choice test (Raubenheimer and Tucker, 1997). In those reports, colors are considered to function as attractants, but it is unclear if behavioral response to the colors is kinesis.

In this study, since the larvae of both *S. litura* and *M. b. pryeri* consumed more gel containing chlorophyll *b* compared to the control gel, chlorophyll *b* seemed to function as a feeding stimulant under the lighted conditions (Fig. 3). These results

Fig. 7. Two-choice test between control gel and gels with three different green color silhouettes by the larvae (Bioassay 4). A: *Spodoptera litura*. B: *Milionia basalis pryeri*. Fourth-instar larvae were used and fed for 2 d under lighted conditions. $N=6$. Values indicated are means and SE (bar) of gel consumption for 2 d.

suggest that the larvae identified the preferred gel containing chlorophyll *b* through visual stimulus. However, gustatory or odor effects of chlorophyll *b* were not completely eliminated in the Bioassay 1 method. In Bioassay 2, the gel containing chlorophyll *b* was inaccessible but visible to the larvae (Fig. 1). The result of this bioassay confirmed that larvae preferred the gel containing chlorophyll *b*, and could identify it through only visual stimulus.

Further studies have indicated that the larvae of *S. litura* do not respond to the lightness of the food source (Figs. 2B and 6). The reflectance spectra of three gray films were similar to each other but different in the reflectance level, therefore, those films were considered to differ with lightness. No significant relationship was found between the lightness of the gels and amounts consumed by the larvae. Therefore, the *S. litura* larvae were not considered to respond to the lightness. This proves that green color functions as a visual stimulus and causes an increases in consumption by both *S. litura* and *M. b. pryeri* larvae (Figs. 4 and 7).

Plant leaves that most lepidopteran larvae feed on are generally of green in color, which is derived from chlorophylls, flavonoids and other pigments. The authors have not encountered any reports that chlorophylls act as a feeding stimulant, attractant,

or other behavior-modifying stimulant for larvae. In the observation of Bioassay 2, the frequency of the first choice by *S. litura* larvae was equal between the chlorophyll *b* color (50%) and colorless (50%) gels (Fig. 5A). However, the larvae were observed more frequently on the gels with silhouettes of the diet gel containing chlorophyll *b* (46%) than that of the colorless gel (16%) (Fig. 5B). These results reveal that the behavioral response by *S. litura* larvae to chlorophyll *b* color is orthokinetic rather than tactic. This did not support the idea that the chlorophyll *b* color is attractive for *S. litura* larvae from a distance. Lengths of staying time on the gels were considered to reflect the varying levels of consumption of the gels. Since *S. litura* larvae was revealed to recognize the green color (Fig. 7), chlorophyll *b* color function as a visual stimulus could replace the green color function. Why does the larval response to the green (chlorophyll *b*) color is orthokinetic rather than tactic? Phytophagous insects generally depend on green plants as their food source. Our results indicate that not only polyphagous *S. litura* but also monophagous *M. b. pryeri* have a tendency to prefer green color. It is possible that this phenomenon will be observed in many leaf-eating lepidopteran larvae. Leaf-eating larvae may first identify their preferred food plants through olfactory cues. This may be followed by recognition of green color by vision during the day, and finally by gustation. This may explain why green color induces orthokinetic rather than tactic response.

Larvae of Lepidoptera usually have several laterally positioned stemmata on each side of the head. The functions of these stemmata (observed also in Coleoptera) have been revealed to appear very similar to those of compound eyes (Gillott, 1995). Both larvae of the moth *Trabala vishnou* Lefebur (Lepidoptera: Lasiocampidae) and larvae of the Swallowtail butterfly (Papilionidae) are reported to have three types of color receptors: UV, blue and green. This structure is the same as that of compound eyes (Ichikawa, 1987; Lin et al., 2002). The compound eye is sensitive to wavelengths of light, though the peaks of sensitivity differ among species (Gillott, 1995). In this study, the behavioral experiments have revealed that *S. litura* and *M. b. pryeri* larvae are able to distinguish bluish green, green, and yellowish green (Fig. 7). The difference of reflectance spectra among three green films was

significant at 380–500 nm and 570–700 nm. Spectral sensitivity of blue receptors was fit to 380–500 nm (Lin et al., 2002), therefore the larvae of *S. litura* and *M. b. pryeri* were considered to distinguish the favorite color using the blue receptor. The peaks of sensitivity differ among the two species. The reason for this is not clear but possibly due to the color of the host plant. The host plant leaf of monophagous *M. b. pryeri* is a deep, slightly bluish-green, while host plants of polyphagous *S. litura* are usually a light, slightly yellowish-green.

In this study, we focused on the green color, which appears on the food source for leaf-eating Lepidoptera. The most important result of this study is the lepidopteran larvae recognize the green color by visual stimuli. Further experiments will be needed to investigate the recognition of other colors and/or shapes, which may also be important for the survival of lepidopteran larvae, and for distinction between the responses of naive larvae being innately sensitive to a color stimulus and those of experienced larvae.

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