INTRODUCTION
Leaves of the privet tree, *Ligustrum obtusifolium* (Oleaceae), contain a large amount (3% to fresh weight) of oleuropein, a phenolic secoiridoid glycoside (Fig. 1), as a precursor of a defense agent against herbivores (Konno et al., 1998). When the leaves are damaged by herbivorous insects, the activating enzymes, *b*-glucosidase and polyphenol oxidase, activate oleuropein into a strong protein denaturant with a structure closely related to glutaraldehyde (Fig. 1) (Konno et al., 1999). Activated oleuropein exhibits a very strong alkylating activity against amino residues in the side chains of lysine molecules. Proteins treated *in vitro* with activated oleuropein become denatured, loses lysine (Konno et al., 1997, 1999), and thus become innutritive, since lysine is one of the essential amino acids for insects (Konno et al., 1997). Since glutaraldehyde selectively binds to amino residues, we hypothesized that enzymatically activated oleuropein binds to the amino residue of the side chain of lysine, thereby decreasing available lysine (Konno et al., 1999). We previously found that several privet-specialist lepidopteran larvae, but not non-privet feeders, retain high concentrations of free glycine in their digestive juices (Konno et al., 1997). For example, the glycine concentrations of larvae of two privet-specialist species, *Dorbina tancrei* (Sphingidae) and *Brahmaea wallichii* (Brahmaidae), exceeded 50 mM. An injection experiment with 15N-labeled glycine showed that glycine is

Glycine addition improves feeding performance of non-specialist herbivores on the privet, *Ligustrum obtusifolium*: *In vivo* evidence for the physiological impacts of anti-nutritive plant defense with iridoid and insect adaptation with glycine

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Abstract
The leaves of the privet tree, *Ligustrum obtusifolium* (Oleaceae), retain a strong lysine-decreasing activity caused by enzymatically-activated oleuropein, an iridoid glycoside. Protein treated with this activity become innutritive to insects because of the loss of lysine. We previously found that several privet specialist caterpillars secrete high concentrations of glycine in their digestive juices. Previous *in vitro* experiments showed that glycine inhibits lysine-decreasing activity of oleuropein. Therefore, we hypothesized that the lysine-decreasing activity acts to defend privet tree against herbivores and that glycine secretion by insects is an adaptive trait to counter the privet defense. In the present study, we aimed to examine whether these assumptions hold true under physiological conditions, and performed *in vivo* bioassays and physiological analyses using the Eri silkworm, *Samia ricini* (Saturniidae), a non-privet specialist. Significant decreases in larval growth and lysine concentration in the midgut lumen were observed when larvae were fed intact privet leaves compared to when they were fed heat inactivated privet leaves. These decreases were inhibited when larvae were fed intact privet leaves together with glycine, indicating that the privet defense with oleuropein and the specialist adaptation with glycine do function under physiological conditions. This study thus provides a rare view into the detailed physiological impacts of anti-nutritive plant defense and insect physiological adaptation *in vivo*.

Key words: Plant-herbivore interactions; anti-nutritive plant defense; coevolution; essential amino acids; chemical ecology
actively transported from the hemolymph to the midgut lumen against the concentration gradient (Konno et al., 2001), suggesting that the glycine secreted in digestive juice has physiological functions. Because glycine completely inhibited the lysine decreasing activity of privet leaf extract and enzymatically activated oleuropein in vitro (Konno et al., 1997, 1998, 1999), it was suggested that glycine secretion in the digestive juice of several privet specialists is a convergent adaptive trait against the chemical defense of the privet trees by oleuropein (Konno et al., 1997). We further hypothesized that glycine neutralizes lysine-decreasing activity of enzymatically activated oleuropein by competing with the side chain of lysine for the glutaraldehyde-like structure of enzymatically activated oleuropein (Konno et al., 1999) (Fig. 1). However, all these assumptions are made based on in vitro experiments, by treating protein with enzymatically activated oleuropein or with privet extract in the presence or absence of glycine in the test tubes. It has not been determined whether the lysine-decreasing activity of privet leaves and the glycine of privet specialists function as a plant defense and insect adaptation, respectively, under physiological condition in the insect midgut in vivo, in the manners suggested from in vitro experiments. In order to clarify these points, we performed in vivo bioassays using the Eri silkworm, Samia ricini (Saturniidae), an oligophagous insect which never utilizes the privet tree as a host plant under natural conditions. In the in vivo bioassays, Eri silkworms were fed privet leaves together with additional free glycine and then the larval growth and the change in glycine and lysine concentrations in the midgut contents were analyzed.

MATERIALS AND METHODS

Insects and plants. Eri silkworms, S. ricini, maintained in our institute as experimental insects, were used to assay the biological effects of the lysine-decreasing activities in privet leaves on generalist caterpillars not specialized in feeding on privet leaves. Eri silkworms are oligophagous; their natural host plants include castor oil plants, ailanthus, cassava, kesseru, and plumeria. Moreover, Eri silkworms will eventually eat any kind of non-host plant leaves unless the leaves are too hard or hairy, and will also eat artificial diets containing extracts from various plants, with the result that they sometimes die from poisoning or experience inhibited growth depending on the nutrients or defense substances in the particular plant. For this reason, Eri silkworms have been successfully used in bioassays and analyses to evaluate the defense activities and defense levels of plants against herbivorous insects (Fukui et al., 2002; Konno et al., 2004, 2006; Hirayama et al., 2007; Wasano et al., 2009). Privet trees are not host plants of Eri silkworms under natural conditions. In the present study, Eri silkworms were reared on an L4M artificial diet (Nihon Nosan Kogyo Co., Japan) from hatching until the beginning of the experiments at 25°C under a 12-h light/12-h dark photoregime. Feeding experiments were carried out using the same temperature and photoregime. Leaves of the privet trees, L. obtusifolium, are collected from wild population growing in the forest in Tsukuba, Ibaraki, Japan (36°N, 136°E).

In vivo bioassay of plant defense and insect adaptation performed using the Eri silkworms, Samia ricini. Newly hatched larvae, and forth instar larvae of Eri silkmoth reared on an artificial diet, were used for the bioassays. Larvae were given either fresh privet leaves (treatment 1), privet

![Fig. 1. A proposed chemical model of relationships between the privet tree and privet specialists.](image)
leaves steamed for 4 min in 100°C steam (treatment 2), fresh leaves dipped in 0.5% glycerol solution (treatment 3) or fresh leaves dipped in 0.5% glycerol + 2% glycine solution (treatment 4). The dipping was performed with small twigs with leaves attached to them. After dipping, the leaf surfaces were dried in air for about 2 h. In order to prevent the leaves from getting dry, water was supplied to the twigs with wet cotton balls. Glycerol is added to make the glycine adhere to the surface of the privet leaves. Every other day, the larval weights were measured and the leaves were exchanged for new ones. The numbers of feces in the initial two day period (Day 0–2) were counted to confirm that the preference of larvae to leaves did not differ among treatments. RGRs (Relative Growth Rates) (mg/mg/day) of larvae between day A and day B were calculated as $RGR = \exp[(\ln W_B - \ln W_A)/(B - A)] - 1$, where $W_A$ and $W_B$ are the weight of a larva on days A and B. On day 6, fourth instar larvae were frozen and the midgut contents from the anterior half of the midgut were collected. Free glycine in the midgut contents was analyzed using an auto amino acid analyzer (Model L5000, Hitachi, Tokyo) based on HPLC and the ninhydrin reaction. The lysine content of the proteins in the midgut lumen was analyzed using the same auto amino acid analyzer after hydrolyzing the midgut contents in 6 M HCl in 110°C for 22 h. Concentrations were determined and described in wet weight basis. As a control for assays with privet leaves, leaves of the castor oil plant, *Ricinus communis* (Euphorbiaceae), which is one of the natural host plants of the Eri silkworm, were fed to them (treatment 5). Statistical analyses were performed on results of bioassays and chemical analyses comparing among the four different treatments on the privet leaves using Tukey-Kramer multiple comparison test.

**RESULTS**

**In vivo bioassay of the effect of the lysine-decreasing activity in privet leaves on herbivores**

We assayed the biological effect of the lysine-decreasing (denaturing) activity of privet leaves *in vivo* using larvae of the Eri silkworm. When we fed fresh privet leaves to the first (Fig. 2A, B; treatment 1) and the fourth (Fig. 2C; treatment 1) instar larvae of Eri silkworms, the growth was very slow (RGR in first instar larvae $= 0.063 \pm 0.028$), although the larvae eagerly ate the leaves throughout the experimental period. Then, we steamed the privet leaves in order to heat-inactivate oleuropein-activating enzymes, and fed them to the larvae (Fig. 2A–C; treatment 2). Although oleuropein is stable to heat (Konno et al., 1997), the lysine-decreasing activity of privet leaves is completely lost by heat treatment due to the heat inactivation of activating enzymes (Konno et al., 1997). Both the first and fourth instar larvae fed heat-inactivated leaves (Fig. 2A–C; treatment 2) grew very well (RGR $= 0.336 \pm 0.087$ in the first instar larvae) and significantly faster ($p < 0.001$; Tukey-Kramer test) than those fed untreated fresh privet leaves (Fig. 2A–C; treatment 1). The number of feces produced by the first instar larvae fed steamed privet leaves in the first two day period of the bioassay was 24.1 (/larva/day), which was close to the number of feces produced by the first instar larvae fed fresh privet leaves (21.1/larva/day), suggesting that the initial feeding preference of the larvae did not differ between fresh and steamed leaves. Further, the lysine content of the proteins in the midgut lumen of larvae fed fresh leaves (2.560 ± 0.899 μmol/g midgut content) (Fig. 2D; treatment 1) was significantly lower ($p < 0.01$; Tukey-Kramer test) than that of the larvae fed steamed leaves (4.751 ± 0.231 μmol/g midgut content) (Fig. 2D; treatment 2). The results show that the lysine-decreasing activity of enzymatically activated oleuropein defends the privet tree against herbivorous insects by decreasing the lysine amount of foliar proteins *in vivo* under physiological condition in the midgut lumen.

**In vivo bioassay of the adaptive role of glycine**

When glycine (and glycerol) were fed together with the fresh privet leaves, both the first and fourth instar larvae grew significantly ($p < 0.001$; Tukey-Kramer test) faster (RGR $= 0.206 \pm 0.033$ in first instar larvae) (Fig. 2A–C; treatment 4) than when fed fresh leaves coated only with glycerol (RGR $= 0.061 \pm 0.019$ in first instar larvae) (Fig. 2A–C; treatment 3). The number of feces produced by the first instar larvae fed glycine-coated leaves was 22.5 (/larva/day), and were close to the number of feces produced by the first instar larvae fed leaves coated only with glycerol (24.0/larva/day), suggesting that the initial preference of larvae did not differ between leaves with the two treatments.
The concentration of free glycine in the midgut lumen of fourth instar larvae fed glycine (29.013 ± 3.539 mM) was as high as that in the midgut of privet-specialists (ca. 50 mM in D. tancrei and B. wallichii) (Konno et al., 1997), and the lysine content of the protein in the midgut lumen of larvae fed fresh leaves with glycine (+glycerol) (4.169 ± 0.616 μmol/g midgut content) (Fig. 2D; treatment 4) was significantly (p<0.01; Tukey-Kramer test) higher than that of larvae fed fresh leaves coated only with glycerol (2.174 ± 0.256 μmol/g midgut content) (Fig. 2D; treatment 3). These results showed that even non-specialist insects without a high concentration of glycine can show an improved performance when glycine is added to the privet leaves. Our results provide clear evidence that the glycine found in the digestive juices of privet-specialist insects plays an adaptive role by neutralizing the lysine-decreasing activity of privet leaves under physiological conditions, and thereby allowing them to feed on privet trees.

**DISCUSSION**

In the present study, we succeeded in demonstrating with a simple bioassay that the privet leaves, which are not nutritive to ordinary non-specialist feeding insects, can be made nutritive simply by coating their surface with free glycine (Fig. 2A–C). This simple result alone is a very strong and direct piece of evidence that glycine, which is often found in high concentrations in privet-specialists (Konno et al., 1997), has an adaptive function against the defense of privet trees with oleuropein. Further, our physiological analyses show evidence that both the defense of privet trees with oleuropein and the adaptation of privet-specialists with glycine is functioning in the manner shown in our previous in vitro experiments (Konno et al., 1997) and presented in our chemical model (Fig. 1). The lysine content of the proteins in the midgut lumen was significantly smaller in larvae fed intact privet leaves than in those fed heat-inactivated privet leaves (Fig. 2D), although oleuropein was stable to
heat treatment and existed in heat inactivated leaves (Konno et al., 1998). The results indicated that enzymatically activated oleuropein in privet leaves not only decreases lysine \textit{in vitro} but also decreases the lysine content of the proteins in the midgut lumen and reduces the growth \textit{in vivo} in non-specialist insects. Since lysine is one of the essential amino acids for insects, its loss reduces the growth of insects (Felton and Gatehouse, 1996; Konno et al., 1997). This result also proves that not only oleuropein but also its activation by foliar enzyme is also indispensable for the defense activities of privet trees under physiological conditions. When glycine was fed together with fresh privet leaves, the decreases both in the amount in lysine and in larval growth were inhibited (Fig. 2). This result also suggested that free glycine inhibits the decrease of lysine caused by oleuropein not only \textit{in vitro} but also \textit{in vivo} under physiological conditions. What is important in our results is that the concentration of glycine in the midgut content of the larvae fed privet leaves coated with glycine reached ca. 30 mM (Fig. 2D), which is close to the range of the glycine concentrations in privet specialists (ca. 50 mM in \textit{D. tancrei} and \textit{B. wallichii}) (Konno et al., 1997). This indicates that the concentrations of glycine found in the digestive juice of privet specialists are sufficient for glycine to improve the performance of privet specialists on privet leaves by preventing the loss of dietary lysine in foliar protein.

Our present study shows a very clear example of an anti-nutritive plant defense in which essential amino acids of nutritive proteins are targeted and destroyed by the plant itself (Felton and Gatehouse, 1996; Zhu-Salzman et al., 2008). It has been well demonstrated that the phenolics oxidized by oxidative enzymes such as polyphenol oxidase destroy cysteine by reacting with its side chain both \textit{in vitro} and \textit{in vivo} (Felton et al., 1989; Felton and Gatehouse, 1996). Another recent example of such an anti-nutritive plant defense through the targeting of amino acids by the plant comes from a recent study about the defense of the tomato, \textit{Solanum lycopersicum} against the tobacco hornworm, \textit{Manduca sexta} (Chen et al., 2005). It has been shown \textit{in vivo} experiments that when tomatoes are damaged by tobacco hornworms, arginase and threonine deaminase are induced in the tomato leaves, which, upon ingestion, destroy arginine and threonine, respectively, in the midgut lumen of the worms (Chen et al., 2005). These enzyme activities result in a significant reduction of insect growth, and therefore, function in the defense of tomatoes against herbivorous insects (Chen et al., 2005). Our present results on the privet defense, in which lysine in proteins was the specific target to be destroyed, show another type of anti-nutritive plant defense targeting a particular essential amino acid, whose physiological consequences (i.e., the loss of particular target amino acids in the midgut lumen and reduction in growth) are well established \textit{in vivo}. These present results, along with the finding of the two previous studies described above, indicate that anti-nutritive plant defenses targeting particular essential amino acids are widespread and important among plant species. Although not strongly toxic enough to kill herbivores in short time, plants with anti-nutritive defenses, such as privet trees, are in nutritious to non-specialists, and such plants will not allow non-specialists to grow and reproduce efficiently on them. Therefore, it is suggested that the traits of herbivorous insects that lead to feeding on these plants, such as oviposition preference on these plants, will be less likely to evolve in oligophagous and polyphagous insects without any adaptive mechanisms against anti-nutritive defenses. It is possible that in this way, plants with anti-nutritive defense are avoided and well defended from most herbivorous insects except for a few specialists with adaptive mechanisms such as glycine secretion in digestive juice.

The present study shows for the first time the physiological consequence of a plant defense employing iridoid glycoside, as well as the physiological mechanisms of the counteradaptation of insects against an iridoid-based plant defense. A large number of iridoid glycosides have been purified from plants, and their defensive roles against insects have been intensively studied (Bowers, 1991). However the molecular mechanisms by which iridoid glycosides exert their defensive activities have been obscure, as have the strategies by which the specialists that feed on iridoid-defended plants adapt themselves to iridoids. Our detailed results, both from \textit{in vitro} analyses in our previous study and from \textit{in vivo} analyses in the present study, elucidated these points for the first time. It is still unclear, however, whether similar mechanisms apply to iridoids other than oleuropein contained in
plants other than the privet tree. Since some iridoids, such as aucubin, have alkylating and lysine-decreasing activities similar to those of oleuropein (Konno et al., 1999), it is likely that in some iridoid-containing plants, iridoid glycosides may function as plant defenses in mechanisms similar that of the privet tree. Further investigations are needed to elucidate these points. Glutaraldehyde-like alkylators (di-aldehyde) are not only produced by the activation of iridoids by β-glucosidase (Konno et al., 1999), but also by the activation of caulerpenyne by esterase activity in an invasive sea green algae, Caulerpa taxifolia (Jung and Pohnert, 2001), and in both cases they exert lysine-decreasing activity in vitro (Konno et al., 1999; Weissflog et al., 2008), although the structures of the precursors, oleuropein and caulerpenyne, are very different. It would be interesting to examine whether caulerpenyne also functions in the defense of the sea algae by decreasing the nutritive value of the algae itself through a decrease in the amount of lysine, and to examine whether amino acid secretion also exists in the digestive juices specialist feeders of the sea algae in the marine ecosystem.

Our previous study showed that the high concentrations of glycine are not found exclusively in one privet specialist species, but rather exist in at least from three privet specialists from three different families (i.e., B. wallichii (Brahmaeidae); D. tancrei (Sphingidae); Pangrapta trimentesalis (Noctuidae)) (Konno et al., 1997), showing that glycine secretion convergently evolved several times in several different lineages. This fact implies that even chemical and physiological adaptation of herbivores with complicated physiological devices (e.g., secretion mechanism of amino acids) evolve more easily and rapidly than it appears. For convergent evolution in a particular trait to occur, it may be that the particular trait should be something that could easily evolve and be selected for without any special conditions. It may take considerable time for a complex physiological mechanism such as secretion of glycine to first appear. However, our in vivo result that the simple addition of glycine to the surface of privet leaves can dramatically improve the performance of non-specialist larvae without any further experimental conditions (Fig. 2A–C) suggests that if this trait (i.e., glycine secretion in the midgut) once emerged in a particular population of herbivorous insect species, it would readily function in the adaptation of the species to privet leaves without any special conditions, and would easily undergo positive selection and prevail in the population. To clarify the patterns and mechanisms of this convergent evolutionary trait, further analyses with an increasing number of privet specialists would be needed in the future.

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