Antibiotic Effect of Insect-resistant Soybean on Common Cutworm (Spodoptera litura) and its Inheritance

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The common cutworm (Spodoptera litura Fabricius; Lepidoptera: Noctuidae) is a menace to soybean (Glycine max (L.) Merr.) production in southwestern Japan. We have been evaluating soybean germplasm for resistance to common cutworm in order to develop resistant cultivars and have found a cultivar named ‘Himeshirazu’, which is distinguished by its high level of resistance. We compared the antibiosis of Himeshirazu with those of ‘Fukuyutaka’ (susceptible) and ‘Sodendaizu’ (resistant). Himeshirazu depressed the weights of individual common cutworm larvae and prolonged the duration of the instar stage, compared with the other two cultivars. We analyzed the inheritance of this antibiosis in an F$_2$ population derived from a cross between Fukuyutaka and Himeshirazu. The broad-sense heritability of the antibiosis was estimated as 71.3%. The segregation pattern in the F$_2$ progeny suggested that a recessive factor controlled a considerable part of the antibiosis. From the relationship between the antibiosis and the genotype of a simple sequence repeat marker, Satt220, in the F$_2$ progeny, we inferred that the locus of the putative recessive factor was located on linkage group M.

Key Words: soybean, antibiosis, common cutworm, resistant cultivars, heritability, simple sequence repeat, quantitative trait loci.

Introduction

In southwestern Japan, damage to soybean plants caused by herbivorous insects is a serious problem. The common cutworm (CCW, Spodoptera litura Fabricius) is a major pest. Plant resistance to insects can contribute considerably to integrated pest management.

In the early 1970s, three soybean cultivars (PI171451: ‘Kosamame’; PI227687: ‘Miyako White’; PI229358: ‘Sodendaizu’) were reported to have resistance to Mexican bean beetle (Epilachna varivestis Mulsant) (Van Duyn et al. 1971). They were also found to have resistance to other herbivorous insects, including Spodoptera species (Hatchett et al. 1976, Lambert and Kilen 1984a). The resistance of the three cultivars to Mexican bean beetle is inherited quantitatively, but two or three major genes are responsible for it (Sisson et al. 1976). Kilen and Lambert (1986) reported that each of the three cultivars differs by at least one resistance gene from the other two. Quantitative trait loci (QTL) analysis has allowed researchers to investigate this resistance in detail. In total, four QTLs for antixenosis resistance have been detected from the three insect-resistant cultivars and one from a susceptible cultivar (Rector et al. 1998, 1999). In addition, three QTLs for antibiosis resistance have been detected from the three resistant cultivars, one located in linkage group (LG) M has been suggested to be associated with antixenosis resistance by its location in LG-M (Rector et al. 2000). Narvel et al. (2001) reported that at least 13 of the 15 insect-resistant cultivars developed from Sodendaizu have the allele of Sodendaizu on the QTL of LG-M, and the locus is tightly linked to the simple sequence repeat (SSR) marker Satt536. Narvel et al. (2001) inferred that the QTL on LG-M had a considerable effect but the other QTLs on LG-D1b, G and H had minor effects or no effect, from the genotypes of the SSRs flanking the QTL in the 15 insect-resistant cultivars. By using a backcrossed line, the QTL on LG-M derived from Sodendaizu was confirmed to have a detrimental effect on the development of lepidopteran larvae and defoliation by them (Walker et al. 2002). QTL analysis has made it possible to investigate insect resistance even in progeny derived from crosses between non-resistant lines. With regard to larval weight reduction, in total nine QTLs were detected in non-resistant cultivars and two of the nine were obviously distinguishable from the QTLs of insect-resistant cultivars because they were located on different linkage groups (Terry et al. 1999, 2000). Such genetic information about resistance to herbivorous insects makes it possible to breed elite insect-resistant soybean lines.

However, resistance can break down, as happened with brown planthopper (Nilaparvata lugens Stål) resistance in rice (Claridge and Den Hollander 1980), and some insect species can appear as new pests with the spread of resistant cultivars. For that reason, we have been searching for new CCW-resistant soybean cultivars.

We found that the cultivar ‘Himeshirazu’ had the high resistance to CCW. It is a forage cultivar that was developed in the early 1960s as a line resistant to the soybean beetle (Anomala rufocuprea Motschulsky), but its poor agronomic qualities—small-seededness and excessive vegetative...
growth—were obstacles to breeding. Genetic study of its insect resistance is required to make use of Himeshirazu in soybean breeding programs.

In general, two modes of resistance—antibiosis and antixenosis—are evaluated, and the bioassay procedures for each mode have been well established (Hatchett et al. 1976, All et al. 1989). Although these procedures have many advantages, it is difficult to apply them directly to our genetic study because of the limited amount of leaves that we can feed to CCW larvae for antibiosis evaluation of segregating progeny. To measure antibiosis we have therefore designed a new bioassay procedure whereby only limited numbers of sample leaves are fed to larvae. The objectives of this study are (1) to evaluate the antibiotic effect of Himeshirazu and (2) to analyze the inheritance of its antibiotic resistance by using our new bioassay procedure. In the inheritance study, we determined the broad sense heritability of antibiosis. In addition, we used an SSR marker to estimate the effect of the insect resistance QTL reported in LG-M in Sodendaizu and Kosamame (Rector et al. 1998, 1999, 2000).

Materials and Methods

Plant materials

We compared the antibiotic effects of three soybean cultivars—Fukuyutaka, Himeshirazu and Sodendaizu—to CCW. Fukuyutaka is a leading cultivar in southwestern Japan but is susceptible to CCW. Himeshirazu is a forage cultivar resistant to soybean beetle. Sodendaizu is well characterized as a multiple insect-resistant cultivar (Hatchett et al. 1976, Lambert and Kilen 1984a, 1984b). Seeds were planted in plastic pots on 8 October 2001. About 150 plants of each cultivar were grown in a greenhouse at the National Agricultural Research Center for Kyushu Okinawa Region for evaluation of their antibiotic effect.

To study the inheritance of antibiosis in Himeshirazu, we developed an F2 population by crossing Fukuyutaka and Himeshirazu. The parents, F1 and F2 seeds were sown in potting compost on 31 May 2001. Fourteen days later, the seedlings were transplanted to an experimental field which was covered with a vinyl house to protect them from pests. No chemical control was applied. We assayed 143 F1 plants, 1976, Lambert and Kilen 1984a, 1984b). Seeds were planted in plastic pots on 8 October 2001. About 150 plants of each cultivar were grown in a greenhouse at the National Agricultural Research Center for Kyushu Okinawa Region for evaluation of their antibiotic effect.

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Newly developed bioassay procedure for evaluating antibiosis

All experiments were performed in an air-conditioned room maintained at 25±1°C with a 16-h-light/8-h-dark photoperiod. A CCW colony was maintained all year round on an artificial diet (Insecta LF S; Nippon Nousan Kougyo Co., Yokohama, Japan). The larvae to be used for the bioassay were reared on the artificial diet until the 5th instar. At the end of the 5th instar, the width of the head capsule was measured. Larvae with head capsules at least 1.65 mm wide were selected to eliminate larvae of supernumerary instars (Morita and Tojo 1985). After measurement, each larva was transferred to a plastic cup 6.5 cm in diameter and 4.0 cm in height. About 16 h later, larvae that had finished ecdisys and weighed 180 to 300 mg were selected for the bioassay to eliminate experimental error as much as possible. The 16 h without food maximized their appetite without having an adverse effect on their growth (Morita and Tojo 1985). These last-instar larvae were fed on the soybean leaves, one or two leaflets were provided per day according to their appetite. At the 3rd feeding, sawdust was put in the bottom of each cup to encourage pupation. To measure the duration of the 6th instar, we checked for pupation every 8 h. The pupal weight was measured at the 4th day after pupation. The antibiotic effect was evaluated from the pupal weight and the duration of the 6th instar.

Evaluation of the antibiotic effect of the three cultivars began on 7 October 2001, when plants for the bioassay were at the V14–V16 (Himeshirazu and Sodendaizu) or V14–V15, R1 (Fukuyutaka) stage (Feht et al. 1971). At stage “V14–V16” the plants had 14 to 16 nodes on the main stems but no flowers, and at the “V14–V15, R1” stage plants had 14 to 15 nodes and at least one flower at any node. Larval sex was checked and about 40 male and 40 female larvae were used per cultivar.

The study of the inheritance of the antibiotic of Himeshirazu began on 13 August 2001. At that time, the F2 plants ranged from the V14–V16 stage to the V14–V16, R1 stage. For this experiment, leaves from one plant were fed to six larvae of undetermined sex. Larval sex was checked only after pupation.

Analysis of data on the inheritance of antibiosis

We estimated the broad-sense heritability (H) of the antibiosis. As an index of resistance to CCW, we developed a special numerical value—standardized insect-growth index (SII)—to minimize the effects of CCW sex on the evaluation, because when the 6th instar female is fed on a high-quality diet she becomes heavier and has a longer instar duration than the male (Itoyama et al. 1999). SII is calculated as (pupal weight) / (duration of 6th instar for each larva).

The formula reported by Kenty et al. (1996) was used to calculate H, with modifications: $H = (V_g/V_p) \times 100\%$. $V_g$ is genetic variance and $V_p$ is phenotypic variance. The variance of the F2 population was used as the estimated phenotypic variance, $V_p$. The estimated genetic variance ($\hat{V}_g$) was calculated as $\hat{V}_g = \hat{V}_p - \hat{V}_e$, where $\hat{V}_e$ is estimated environmental variance. $\hat{V}_e$ was the weighted average of the variance of the F1 and parent populations: $\hat{V}_e = (n_1s_1^2 + n_2s_2^2 + n_3s_3^2) / (n_1 + n_2 + n_3)$. $n_1$, $n_2$ and $n_3$ are the degrees of freedom for the variance estimation of the two parental and one F2 populations, respectively. $s_1^2$, $s_2^2$ and $s_3^2$ are the estimated SII variances obtained from all larvae used for resistance evaluation of the two parental and one F2 populations, respectively. All insect data were used to calculate $\hat{V}_e$, instead of the
SSR analysis of insect resistance QTL on linkage group M

To estimate the effect of the insect resistance QTL region on LG-M, we classified the F₂ individuals into three genotypes (homozygous for Fukuyutaka, homozygous for Himeshirazu and heterozygous) with an SSR marker linked to the QTL and the SIIs of each genotype were compared. In addition, we classified the F₂ into 11 grades with the SII and compared the segregating ratios of the SSR locus in each grade. Satt220 was used as the SSR linked to the QTL on LG-M because the Satt220 was expected to be tightly linked to the QTL (Narvel et al. 2001).

DNA was extracted individually from the F₂ progeny and the parents by the method reported by Doyle and Doyle (1990). PCR amplifications were performed with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, USA). The PCR reaction mixture was composed of 0.25 μM of primers, 200 μM of each dNTP, 10 mM of Tris-HCl (pH 9.0), 50 mM of KCl, 1.5 mM of MgCl₂, and contained 20 ng of Triton X-100, 30 ng of template DNA and 0.5 U of Taq polymerase in total volume of 20 μL. The Taq polymerase was obtained from Promega K.K. Japan (Tokyo, Japan). The primers for Satt220 were obtained from Proligo Japan K.K. (Kyoto, Japan). The sequence information for the primers was obtained from Soybase (http://soybase.org). The reaction program was as follows: 30 cycles at 94°C for 45 s, 53.5°C for 45 s and 72°C for 45 s and after the cycles, 72°C for 5 min as a final extension step. The amplified products were denatured at 95°C for 5 min and electrophoresed in a sequencing gel consisting of 6% acrylamide (acrylamide : bis-acrylamide 19 : 1), 7 M of urea, 90 mM of Tris, 90 mM of boric acid and 2 mM of EDTA (pH 8.0). The electrophoregrams were visualized by the silver stain method with a Silver Sequence DNA Sequencing System (Promega K.K. Japan, Tokyo, Japan).

Results

Antibiosis of the three soybean cultivars

Frequently, the evaluation of antibiosis is based on the growth of larvae of all instars (Hatchett et al. 1976, Lambert and Kilen 1984a, 1984b, Beach et al. 1985). For CCW larvae this takes around 20 days and fresh leaves must be provided continuously. Obviously, a single plant cannot provide the entire diet. However, in our inheritance study, we had to evaluate the antibiotic effects of each F₂ plant individually. For this purpose, we designed a new bioassay procedure that uses 6th instar CCW larvae. The procedure therefore requires less food and six larvae can be fed on the leaves of a single plant.

The results of the bioassay are shown in Table 1. CCW pupal weight was significantly different among the three cultivars, being greatest on Fukuyutaka and least on Himeshirazu for both females and males. The instar duration was significantly shorter on Fukuyutaka than on Sodendaizu and Himeshirazu.

Males weighed significantly less than females fed on Fukuyutaka (Table 1). A significant interaction between genotype and larval sex was found in both pupal weight and duration of 6th instar (Table 2). This result indicates that pupal weight and 6th instar duration, when used as an index of plant resistance to CCW, should be adjusted to minimize the error caused by sex-related differences when male and female larvae are used together. We contrived a simple index (standardized insect-growth index, or SII) by dividing pupal weight by the duration of the 6th instar. This index levels out the difference between females and males in weight and duration of the 6th instar. In SII, statistical differences related to larval sex and interaction of genotype × sex became non-significant, whereas the difference among the three cultivars remained significant (Table 1 and Table 2).

Inheritance of antibiosis in Himeshirazu

We used SII to evaluate Fukuyutaka, Himeshirazu, their F₁ progeny and 143 F₂ progeny. Leaves of each plant were fed to six CCW larvae (male and female), and the SII was calculated for all larvae. The mean SIIs on Himeshirazu, Fukuyutaka and their F₁ were 5.75, 13.22 and 10.22 respectively. SII on the F₂ plants ranged from 3.73 to 16.70. The F₂ progeny showed a bimodal SII distribution (Fig. 1). The H of SII was estimated as 71.3% on the F₂ progeny.
The role of insect resistance QTL region on LG-M in the antibiosis of Himeshirazu

The QTL on LG-M in Sodendaizu and Kosamame has been well studied in terms of its effect on herbivorous insects (Narvel et al. 2001, Walker et al. 2002). We estimated the effect of the QTL region of Himeshirazu. F$_2$ plants derived from the cross between Fukuyutaka and Himeshirazu were classified into three genotypes (homozygous for Fukuyutaka, homozygous for Himeshirazu and heterozygous), with the Satt220 tightly linked to the QTL.

The segregation ratio of Satt220 in the F$_2$ progeny was 41 : 61 : 41 (homozygous for Fukuyutaka: heterozygous: homozygous for Himeshirazu) and fitted to 1 : 2 : 1 statistically ($\chi^2 = 3.08$, 0.2 < $P$ < 0.3, Table 3). The SII of each genotype differed significantly from each other (Table 3). The mean SII of the plants homozygous for Himeshirazu was considerably lower than the others. The difference between plants homozygous for Fukuyutaka and the heterozygotes was small but statistically significant.

Figure 1 shows the distribution of each genotype in the F$_2$ progeny. The black, white and shaded bars indicate homozygous for Fukuyutaka and Himeshirazu and heterozygous, respectively. The F$_2$ progeny showed a bimodal distribution,

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of plants</th>
<th>SII</th>
<th>Mean</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous for Fukuyutaka</td>
<td>41</td>
<td>11.38 a$^{(1)}$</td>
<td>1.73</td>
<td></td>
</tr>
<tr>
<td>Heterozygous</td>
<td>61</td>
<td>10.27 b</td>
<td>2.16</td>
<td></td>
</tr>
<tr>
<td>Homozygous for Himeshirazu</td>
<td>41</td>
<td>7.41 c</td>
<td>2.09</td>
<td></td>
</tr>
</tbody>
</table>

$^{(1)}$ Values within a column followed by different letters are significantly different at 0.05 probability level by the Tukey-Kramer multiple test.

**Table 2.** Statistical analysis of sources of variation and their interactions in CCW larval growth and the standardized insect-growth index (SII)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Pupal weight (mg)</th>
<th>Duration of 6th instar (day)</th>
<th>SII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>MS</td>
<td>F</td>
</tr>
<tr>
<td>Genotype</td>
<td>2</td>
<td>202464.57</td>
<td>127.50**</td>
</tr>
<tr>
<td>Larval sex</td>
<td>1</td>
<td>1967.47</td>
<td>1.24</td>
</tr>
<tr>
<td>Genotype × Larval sex</td>
<td>2</td>
<td>17390.47</td>
<td>10.95**</td>
</tr>
</tbody>
</table>

* and ** indicate significant difference at 0.05 and 0.01 probability levels, respectively, based on an F-test.

**Table 3.** Standardized insect-growth index (SII) of each genotype of Satt220 in F$_2$ progeny derived from ‘Fukuyutaka’ × ‘Himeshirazu’
the lower peak of SII was predominated by plants homozygous for Himeshirazu and the higher peak by heterozygous plants and plants homozygous for Fukuyutaka.

**Discussion**

Reduction of pupal weight and extension of the larval period are often observed in many insect species fed on insect-resistant lines (Van Duyn *et al.* 1972, Hatchett *et al.* 1976, Lambert and Kilien 1984a, 1984b, Beach *et al.* 1985). The multiple insect-resistant Sodendaizu gave significantly less larval growth than Fukuyutaka, indicating that our bioassay using only the 6th instar was an appropriate method for evaluating the antibiotic effect of soybean on CCW. Himeshirazu gave significantly less larval growth than Sodendaizu, indicating its potential value as a genetic resource for the breeding of CCW-resistant soybean lines.

In relation to the difference between males and females fed on Fukuyutaka (Table 1), Itoyama *et al.* (1999) reported interesting differences in growth between male and female CCW larvae. In their report, females gained more weight and had a longer larval duration than males when fed on a high-quality artificial diet. Our 6th instar CCW larvae fed on Fukuyutaka showed a similar response. The significant interaction between genotype and larval sex in terms of pupal weight and 6th instar duration would be due to the different growth responses of males and females (Table 2).

This difference between males and females would be an obstacle to the evaluation of F2 progeny if the pupal weight or the instar duration were used directly as the index of antibiotic effect. However, by use of our simple SII index, which we obtained by dividing the pupal weight by the 6th instar duration, we could minimize these differences between male and female CCW larvae (Table 1 and Table 2). Since it was time-consuming and difficult to distinguish the sexes of 5th instar larvae before the bioassay, we used SII instead of checking larval sex before our study of the inheritance of antibiotic resistance in Himeshirazu.

Evaluation of the antibiotic effect of F2 progeny, their parents and the F1 gave an $H$ value of 71.3%. Rufenner *et al.* (1989) reported a value of 65% as the broad-sense heritability in a line developed from Sodendaizu that was resistant to Mexican bean beetle. Himeshirazu has more effective antibiotic resistance (Table 1) and a higher heritability than the resistant line developed from Sodendaizu. For those reasons, Himeshirazu could be an important pest-resistance donor in breeding programs.

The distribution of SII in the F2 progeny was continuous, but bimodality was detected (Fig. 1). As an example, if the distribution is divided at SII = 7.4, which seems to be the boundary between the two peaks in Fig. 1, the frequency ratio of the two parts does not deviate statistically from 1 : 3 ($\chi^2 = 1.69, 0.1 < P < 0.2$). Although resistance to CCW was inherited quantitatively, this distribution suggested that a principal recessive factor controlled a considerable part of the antibiotic resistance of Himeshirazu in the QTL has a major effect on the antibiosis of Himeshirazu (Table 3). The antibiosis of Himeshirazu was shown by the lowest mean SII and the differences of SII among the three genotypes were all significant. This revealed that the allele of Himeshirazu in the QTL had a detrimental effect on larval development. The mean value of the SII of the heterozygote was clearly close to that of Fukuyutaka, suggesting that the allele of Himeshirazu acted recessively. The SII distribution ratio of each genotype in the F2 progeny also confirms the effect of the QTL on the antibiosis of Himeshirazu (Fig. 1). Plants considered to have the insect resistance allele of Himeshirazu as homozygous predominated in the lower SII part of the F2 distribution.

The present results suggested the presence of a recessive factor from Himeshirazu located on the LG-M that controlled a considerable part of the antibiotic effect. These characteristics suggest that the factor is the same as the insect resistance QTL on the LG-M reported in Sodendaizu and Kosamame, from the three points as follows. At first, our SSR analysis was conducted using an SSR locus tightly linked to the QTL of LG-M (Rufener *et al.* 2001). Rector *et al.* (2000) also reported that the QTL located on the LG-M is partly recessive. In addition, Rufener *et al.* (2001) reported that the QTL on the LG-M had a major effect in the antibiotic resistance of Sodendaizu.

Nevertheless, the genetic cause of the difference in antibiotic resistance between Himeshirazu and Sodendaizu remains unidentified. The roles of the other QTLs differ from the QTL on the LG-M (Terry *et al.* 1999, 2000, Rector *et al.* 2000) in the antibiosis of Himeshirazu are also still unknown. In addition, the antixenosis of Himeshirazu was not investigated in this research because the focus of this research was restricted to the antibiosis of Himeshirazu. The antibiosis of Himeshirazu also needs to be analyzed. For gene pyramiding in breeding programs, more detailed genetic information about the resistance of Himeshirazu will be necessary. We have started a QTL analysis for the antibiosis to CCW using the results from the F2 progeny. The results will provide us with more detailed information on the resistance to CCW and will make the genetic basis of resistance to herbivorous pests in soybean clearer.

**Acknowledgments**

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Literature Cited


