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Detection of the A2'N mutation in the RDL GABA receptor subunits of fipronil-resistant pests *Sogatella furcifera* and *Laodelphax striatellus* using a PCR-RFLP assay

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Sogatella furcifera and *Laodelphax striatellus* are serious pests of rice plants that have developed fipronil resistance in Asia. The A2'N mutation in the RDL γ -aminobutyric acid (GABA) receptors conferred fipronil resistance and was suggested to be a heterozygous mutation. We have developed a rapid PCR-RFLP assay to detect the A2'N mutation in individual insects. This assay is useful for monitoring *S. furcifera* and *L. striatellus* carrying the A2'N mutation in field populations and may contribute to managing these pests. © Pesticide Science Society of Japan

Keywords: GABA receptor, *Sogatella furcifera*, *Laodelphax striatellus*, fipronil resistance, PCR-RFLP.

Introduction

Fipronil is a phenylpyrazole insecticide that shows high insecticidal activity against a wide spectrum of pests. However, extensive use of fipronil has caused the development of fipronil resistance among various pests and several mechanisms, such as decreased penetration and/or elevated levels of detoxification enzymes, have been suggested to be involved in fipronil resistance.^{1–3)}

Fipronil and cyclodienes are noncompetitive antagonists of the ionotropic γ -aminobutyric acid (GABA) receptor that act on the pore formed by the M2 membrane-spanning region. Although A2'S and A2'G mutations (index number for the M2 membrane-spanning region⁴⁾) conferred resistance to cyclodienes,^{5–12)} these mutations showed low levels of cross-resistance to fipronil.⁹⁾

In a previous study, the A2'N mutation was associated with fipronil resistance and was suggested to be a heterozygous muta-

tion in the white-backed planthopper *Sogatella furcifera*¹³⁾ and small brown planthopper *Laodelphax striatellus*¹⁴⁾. The membrane potential assay showed that the A2'N mutation in the *S. furcifera* and *L. striatellus* RDL GABA receptor subunits (SF-RDL and LS-RDL, respectively) decreased fipronil sensitivity profoundly in *Drosophila* Mel-2 cells expressing wild-type genes with A2'N mutant SF-RDL¹⁵⁾ genes or LS-RDL genes.¹⁴⁾

As *S. furcifera* and *L. striatellus* are major pests that cause serious damage to rice plants in Asia, management of fipronil-resistant *S. furcifera*- and *L. striatellus*-expressing A2'N mutant GABA receptors is quite important. For this reason, there is a critical need to establish monitoring methods.

In this study, we have developed a rapid PCR-RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) assay to detect the A2'N mutation in individual insects. This assay is useful for monitoring *S. furcifera* and *L. striatellus* expressing the A2'N mutant RDL GABA receptor subunits in field populations and may contribute to managing fipronil-resistant pests.

Materials and Methods

1. Insects

A population of fipronil-resistant *S. furcifera* was collected from a paddy rice field in Fukuoka Prefecture, Japan, in August 2007, as described previously.¹³⁾ Another population of fipronil-resistant *S. furcifera* was collected from a paddy rice field in Kumamoto Prefecture, Japan, in 2008.

A population of fipronil-resistant *L. striatellus* was collected from a wheat field in Fukuoka Prefecture, Japan, in May 2009.¹⁴⁾ We used the laboratory strain maintained at the Agrochemicals Research Center, Mitsui Chemicals Agro (Mobara, Chiba, Japan) as a fipronil-susceptible strain of *L. striatellus*,¹⁴⁾ which was collected in Kanagawa Prefecture, Japan, in 1981.

Insects were reared on rice seedlings at 25°C under a 16:8 hr light:dark regime and frozen until RNA and genomic DNA were isolated.

2. DNA extraction and PCR-RFLP assay for the A2'N mutation in SF-RDL and LS-RDL

Genomic DNA was extracted from individual *S. furcifera* and *L. striatellus* using the DNAzol Genomic DNA Isolation Reagent (Invitrogen, Carlsbad, CA, USA) at one-fifth the recommended rate, as described previously.^{13,14)} To amplify PCR products of the M2 region of the SF-RDL gene, primary PCR was performed in a reaction volume of 20 μ L using the primers SF-5'-2 (AAC TAC AGC CGA CTG GCG TGC GAG ATC CA) and SF-3'-2 (GAG CAA CGA CGC GAA GAC CAT GAC GAA AC), followed by a secondary nested PCR in a reaction volume of 50 μ L using the primers SF-5'-2 and SF-3'-Sal-XhoI (GTT CCT CGA GGA CAT CAG TGT).

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To amplify the PCR products of the M2 region of the LS-RDL gene, PCR was performed in a reaction volume of 50 μ L using the primers LS-5'-1 (TACAGCCGACTG GCGTGC GAGATC CAG TTC GTG CGC TCG ATG GGC TA) and LS-3'-1 (CGA CGCGAA GACC ATGAC GAA ACA GGT GCCCAG).

All PCR reactions contained 0.2 mM dNTPs, 0.4 μ M of each primer, and 0.0525 units/ μ L of Expand High Fidelity enzyme mix (Roche, Mannheim, Germany). The DNA in the PCR reaction mixture was initially denatured for 2 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 30 sec at 52°C, and 3 min at 72°C. The resulting PCR products were purified using MinElute Reaction Cleanup Kit (Qiagen, GmbH, Hilden, Germany). The purified PCR products were digested with the restriction enzyme *HincII* (Takara, Shiga, Japan) according to the manufacturer's protocol. Digestion products were subjected to electrophoresis at 100 V for 30 min using 3% agarose gel with 1 $\times$ TAE and stained with ethidium bromide.

3. Isolation of cDNA clones encoding SF-RDL

Total cellular RNA was isolated from adult *S. furcifera* insects using the RNeasy Midi Kit following the manufacturer's instructions (Qiagen). Degenerate primers were designed on the basis of the amino acid sequences of GABA receptor subunits, as described previously.¹³⁾ DNA fragments of ca. 0.9 kbp encoding SF-RDL were amplified by reverse transcription-PCR (RT-PCR) using the degenerate primer sets, LS-5'-514[CA(A/G)TA(C/T)TT(C/T)CC(A/C/G/T)ATGG(C/T)]/LS-3'-1409[AG(A/G)TA(A/G/T)AT(A/G/T)ATCCA(A/G)TACAT]. The PCR products were cloned into the plasmid pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) and the sequences were determined.

Results and Discussion

In a previous study, we found that the A2'N mutation in the SF-RDL and LS-RDL genes conferred fipronil resistance and it was suggested to be a heterozygous mutation.^{13–15)} The purpose of this study was to develop a rapid monitoring method for identifying the A2'N mutation in the SF-RDL and LS-RDL genes.

To achieve this, we developed a PCR-RFLP assay. The two point mutations (GCC to AAC) that cause the A2'N mutation create a *HincII* restriction site in the M2 region of the SF-RDL and LS-RDL genes. We took advantage of this *HincII* site for developing the PCR-RFLP assay.

One possible problem with the SF-RDL gene is that the M2 region of the wild-type SF-RDL gene also contains a *HincII* restriction site created by a synonymous polymorphism (TCC to TCG) (Fig. 1A). To simplify the interpretation of the PCR-RFLP assay, the PCR products were amplified using the primers SF-5'-2 and SF-3'-2, followed by a nested PCR using the primers SF-5'-2 and SF-3'-Sal-XhoI, which removed the *HincII* restriction site created by the synonymous polymorphism (Fig. 1A). After amplification of the 198-bp fragment using nested PCR, *HincII* restriction endonuclease cleaved the PCR fragments containing the A2'N mutation allele to generate 144-bp and 54-bp fragments, whereas the PCR fragments containing the wild-type

Fig. 1. PCR-RFLP assay for detecting the A2'N mutation in the SF-RDL gene. (A) S-S.fur1 and S-S.fur2 indicate wild-type alleles with synonymous polymorphism. R-S.fur indicates the A2'N mutant allele. The locations of the primers for PCR amplification are indicated by arrows. The locations and sequences of *HincII* restriction sites are indicated by boxes. (B and C) PCR products of *S. furcifera* collected from Fukuoka Prefecture in 2007 before (B) and after (C) digestion with *HincII* and separated on 3% agarose gel. Lane M is a 100-bp ladder. Controls A/A and N/N are PCR products amplified from pENTR1A-Sf-RDL¹⁵⁾ and pENTR1A-Sf-RDL (A2'N)¹⁵⁾ respectively. In *S. furcifera* collected from Fukuoka Prefecture in 2007, A/N shows the heterozygous A2'N mutant allele and A/A shows the homozygous wild-type allele. All genotypes were consistent with the results determined by direct sequencing in a previous study.¹³⁾

allele were not cleaved by *HincII* and only one band of 198 bp was observed (Fig. 1A). The heterozygous mutant showed three bands (198 bp, 144 bp and 54 bp) (Fig. 1A).

As controls for homozygous wild-type allele (A/A) and homozygous A2'N mutant allele (N/N), PCR fragments were amplified from plasmids pENTR1A-Sf-RDL¹⁵⁾ and pENTR1A-Sf-RDL (A2'N)¹⁵⁾ using the primers SF-5'-2 and SF-3'-Sal-XhoI, respectively (Fig. 1B). PCR products of the control A2'N mutant allele (N/N) were digested by *HincII*, but those of the control homozygous wild-type allele (A/A) were not (Fig. 1C).

Next, DNA extracted from four individual *S. furcifera* insects collected from Fukuoka Prefecture, Japan, in 2007, which were genotyped by direct sequencing in a previous study,¹³⁾ was analyzed using the PCR-RFLP assay. PCR products of 198 bp were amplified from the DNA extracted from each of these four insects (Fig. 1B). An additional thin band was observed above the 200-bp band (Fig. 1B), but it was not cleaved by *HincII* (Fig.

Fig. 2. PCR-RFLP assay of DNA extracted from *S. furcifera* collected from Kumamoto Prefecture in 2008. PCR products were digested with *Hinc*II and separated on 3% agarose gel. Lane M is a 100-bp ladder. Controls A/A and N/N are PCR products amplified from pENTR1A-Sf-RDL¹⁵⁾ and pENTR1A-Sf-RDL (A2'N)¹⁵⁾ respectively. In *S. furcifera* collected from Kumamoto Prefecture in 2008, A/N shows the heterozygous A2'N mutant allele.

1C) and did not affect the results of the PCR-RFLP assay. After *Hinc*II digestion, DNA from two individuals that were genotyped as heterozygous mutants (A/N)¹³⁾ displayed three bands (198 bp, 144 bp and 54 bp) and DNA from two individuals that were genotyped as homozygous wild-types (A/A)¹³⁾ displayed one band (198 bp) (Fig. 1C). Thus, the results of the PCR-RFLP assay are consistent with those obtained by direct sequencing. In this assay, complete digestion of the PCR products was required to distinguish the heterozygous mutant allele from the homozygous mutant allele. Digestion of the control PCR products amplified from pENTR1A-Sf-RDL (A2'N)¹⁵⁾ is useful for judging whether the PCR products are completely digested.

The performance of the PCR-RFLP assay was assessed using five individual *S. furcifera* insects collected from Kumamoto Prefecture, Japan, in 2008. All five individuals were genotyped as heterozygous mutants (A/N) (Fig. 2). To confirm that the population of *S. furcifera* from Kumamoto Prefecture expressed the A2'N mutant SF-RDL gene, total cellular RNA was isolated and ca. 0.9-kbp DNA fragments encoding SF-RDL were amplified by RT-PCR. The PCR products were cloned and the sequences were determined. Three clones contained the wild-type sequence and two contained the A2'N mutation, suggesting that the population of *S. furcifera* collected from Kumamoto Prefecture in 2008 harbored the A2'N mutant SF-RDL gene.

As for the LS-RDL gene, the wild-type LS-RDL gene does not contain the *Hinc*II restriction site created by the synonymous polymorphism (Fig. 3A). First, we compared the efficiency and specificity of PCR amplification from DNA extracted from *L. striatellus* individuals using three sets of primers, LS-5'-1/LS-3'-1, LS-5'-2(AAC TAC AGC CGA CTG GCG TGC GAG ATC CA)/LS-3'-2 (GAG CAA CGA CGC GAA GAC CAT GAC GAA AC), and Hime5'-3 (TAC CTG ATCCAG ATC TAC ATT CC)/Hime3'-3 (CGA CGC GAA GAC CAT GAC GAA). The primer set LS-5'-1/LS-3'-1 amplified the specific PCR products most efficiently (data not shown) and enough PCR products were amplified for RFLP analysis. So we used the primer set

A PCR-RFLP assay

B PCR products

C PCR products after *Hinc*II digestion

Fig. 3. PCR-RFLP assay for detecting the A2'N mutation in the LS-RDL gene. (A) S-L.Str indicates the wild-type alleles obtained from a laboratory strain of fipronil-sensitive *L. striatellus*. R-L.Str indicates the A2'N mutant allele of fipronil-resistant *L. striatellus* collected from Fukuoka Prefecture in 2009. The locations of the primers for PCR amplification are indicated by arrows. The locations and sequences of *Hinc*II restriction sites are indicated by a box. (B and C) PCR products of the laboratory strain and *L. striatellus* collected from Fukuoka Prefecture in 2009 before (B) and after (C) digestion with *Hinc*II, and separated on 3% agarose gel. Lane M is a 100-bp ladder. Controls A/A and N/N are PCR products amplified from pENTR1A-LS-rdl-V5¹⁶⁾ and pENTR1A-LSrdl(Asn)¹⁴⁾ respectively. In R-L.Str, A/N shows the heterozygous A2'N mutant allele. In S-L.Str, A/A shows the homozygous wild-type allele. All genotypes were consistent with the results determined by direct sequencing in a previous study.¹⁴⁾

LS-5'-1/LS-3'-1 for RFLP-PCR assay.

After amplification of a 271-bp fragment by PCR using the primers LS-5'-1 and LS-3'-1, *Hinc*II restriction endonuclease cleaved the PCR fragments containing the A2'N mutation allele to generate 141-bp and 130-bp fragments, but the PCR fragments containing the wild-type allele were not cleaved by *Hinc*II and a single band of 271 bp was observed (Fig. 3A). The heterozygous mutant showed both an undigested band (271 bp) and a digested band (141 bp+130 bp) (Fig. 3A).

As controls for a homozygous wild-type allele (A/A) and ho-

mozygous A2'N mutant allele (N/N), PCR fragments were amplified from plasmids pENTR1A-LS-rdl-V5¹⁶⁾ and pENTR1A-LSrdl(Asn)¹⁴⁾ using the primers LS-5'-1 and LS-3'-1, respectively (Fig. 3B). PCR products of the control A2'N mutant allele (N/N) were digested by *HincII*, but those of the control homozygous wild-type allele (A/A) were not (Fig. 3C).

Further, DNA extracted from two fipronil-susceptible and two fipronil-resistant *L. striatellus* individuals, which were genotyped by direct sequencing in a previous study,¹⁴⁾ was analyzed using the PCR-RFLP assay. PCR products of 271 bp were amplified from the DNA extracted from each of the four individuals (Fig. 3B). After *HincII* digestion, DNA from two individuals that were genotyped as heterozygous mutant (A/N)¹⁴⁾ displayed two bands (271 bp, 141 bp+130 bp), and DNA from two individuals that were genotyped as homozygous wild-type (A/A)⁷⁾ displayed one band (271 bp) (Fig. 3C).

Thus, the results of the PCR-RFLP assay are consistent with those obtained by direct sequencing. Digestion of the control PCR products amplified from pENTR1A-LSrdl(Asn)¹⁴⁾ is useful for judging whether the PCR products are completely digested.

In addition to the A2'N mutation, other mechanisms may confer fipronil resistance in *S. furcifera* and *L. striatellus*. To obtain information about the significance and prevalence of the A2'N mutation in fipronil-resistant *S. furcifera* and *L. striatellus*, a rapid monitoring method to identify the A2'N mutation is required.

In conclusion, we have developed a rapid monitoring method to identify the A2'N mutation in the SF-RDL and LS-RDL genes. Because the A2'N mutation appears to be a heterozygous mutation and the generation of these pests after collection seems to affect the frequency of the A2'N mutation, a rapid monitoring method is quite important. This assay provides information about the prevalence of the A2'N mutation in field

populations of these pests and may contribute to managing fipronil-resistant *S. furcifera* and *L. striatellus*.

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