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A SENSITIVE AND SPECIFIC POLYMERASE CHAIN REACTION TECHNIQUE FOR EVALUATION OF *LYGUS* EGG PARASITISM BY *ANAPHES IOLE* GIRAULT (HYMENOPTERA: MYMARIDAE)

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Abstract

Specific and early monitoring and evaluation of *Lygus* egg parasitization by *Anaphes iole* Girault has become a reality. We developed a molecular approach to detect the presence of this minute wasp developing within *Lygus lineolaris* (Palisot de Beauvois) and *L. hesperus* Knight eggs. Early and specific detection was achieved by amplification of parasitoid DNA fragment even at a trace level within its host. One pair of primers were designed based on cloned ribosomal DNA sequences of *A. iole.* These primers specifically flanked to wasp genomic DNA and generated a unique 661-bp band visualized on an agarose gel after polymerase chain reaction (PCR) amplification. This 661-bp DNA fragment was amplified only from those samples containing wasp DNA. With this technology, parasitism could be detected at a trace DNA level as low as 1 pg of genomic DNA (equivalent of 10^{-6} total DNA from a adult wasp), and could be detected as early as the egg stage (less than 24 h after oviposition).

Introduction

The tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois), has a wide range of host plant species including cotton, *Gossypium hirsutum* L., and its economic importance has become prominent in areas where lepidopteran insects are effectively suppressed by widespread use of cotton genetically modified to produce *Bacillus thuringiensis* (Bt) protein. Tarnished plant bugs are controlled in cotton almost exclusively with insecticides and resistance to pyrethroids and organophosphates has been found in many tarnished plant bug populations in the mid-south (Snodgrass, 1996; Snodgrass and Scott, 2000). Increased environmental concerns coupled with the growing prevalence of insecticide resistance and outbreaks of secondary pests have led to an increased interest in biological control (Ruberson and Williams 2000). *Anaphes iole* Girault is an egg parasitoid of plant bugs (Jackson and Graham, 1983). Present methods of rearing for assessing parasitism require a relatively long time to maintain the parasitoids and their host insect. Development of quick and sensitive technique for evaluation of egg parasitism would facilitate accurate and rapid assessment of *A. iole* parasitism rate.

Polymerase chain reaction allows detection of parasitoid DNA at an extremely low level, and detection specificity can be achieved by the careful design of primers specific to the subject organism. Herein we describe the results of a study using a sensitive PCR-based technique employing variability in the ITS2 region, for detecting immature stages of *A. iole* developing within *L. lineolaris* eggs.

Materials and Methods

Procedures used in this study were modified version of Zhu and Greenstone (1999) and Zhu et al. (2000). To design specific primes for detection of parasitoid DNA, wasp DNA was extracted and subjected to PCR amplification of DNA fragments of the ITS2, located between the 5.8 S and 28 S ribosomal DNAs. This DNA fragment was isolated from agarose gel, and was cloned and sequenced. Specific primers, the forward primer 58SF (5'-GTGAATTCTGTGAACTGCAGGACACATGAAC-3') and reverse primer WaspITSR (5'-CAAGCAGACTGCCGTTTCTTC-3'), for the parasitoid were designed based on ITS2 DNA sequence. PCR reactions (25 µl) contained 10 mM Tris-HCl at pH 9, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 50 mM KCl, 0.1 mM of each dNTP, 0.05 unit/µl of *Taq* DNA polymerase, and 5 µl of DNA template (~12 ng), and were performed in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). DNA was initially denatured for 3 min at 94°C, and the PCR amplification was conducted for 45 cycles, with 30 s denaturing at 94°C, 30 s annealing at 60°C, and 1 min extension at 72°C. PCR products (5-8 µl) were separated on 1% agarose gel, stained with 0.5 µg/ml ethidium bromide, and photographed under UV light.

DNAs of different combinations were isolated and subjected to PCR amplification to verify the specificity of the primers. These included parasitoid DNA as the positive control, host insect DNA as the negative control, insect host plant (alfalfa and cotton) DNA as the negative control, and different combinations of parasitoid, host insect, and host plant DNAs. Primers were also tested for detection sensitivity by adding different amount of DNA template (10 concentrations from 0.03 to 1000 pg with 3-3.3 increment) to each PCR reaction.

Results and Discussion

Specificity of the Detection

From the cloned ribosomal DNA ITS2 sequences, specific primers were designed to distinguish parasitoid *A. iole* from the insect hosts *L. lineolaris* and *L. hesperus*, and host plant alfalfa and cotton DNA. The primers 58SF and WaspITSR flanked a sequence region of 661 bp nucleotides of the wasp ITS2 fragment. PCR amplification using these two primers generated a high-intensity 661 bp fragment only from positive control (wasp) DNA and from other samples containing wasp DNA. No DNA fragment was amplified with these two primers from treatments not containing wasp DNA. The presence of host insect DNA and host plant DNA had no negative influence on the amplification of DNA from *A. iole*. By using primers specific to *L. lineolaris* and *L. hesperus*, 1277 bp and 1274 bp DNA fragments were amplified from *L. lineolaris* and *L. hesperus*, respectively, but not from parasitoid DNA.

Results from specificity trials indicated that this technique can be used to distinguish between DNA from *A. iole*, *L. lineolaris*, and host plants. Unsuccessful PCR amplification represented mismatches between the primers and DNA from different origins, and can be used for differentiating parasitoid DNA from that of both *L. lineolaris/L. hesperus* and host plants. Our results are especially important because they indicated that plant tissue can be included in analysis without complicating the verification of parasitism. This eliminates the difficult and time-consuming dissection of host eggs from all plant tissue. These studies suggest that molecular approaches may have general application for detection and identification of parasitoids developing inside hosts.

Sensitivity of the Detection

Detection sensitivity was indicated by a minimum level of wasp DNA template in the PCR reaction to generate a visible 661-bp band on gel. Results showed that the 661-bp band intensity completely correlated to the wasp DNA concentrations tested. The band decreased as wasp DNA decreased from 1000 pg to 1 pg (1 pg equal to 10^{-6} of total DNA extracted from an adult wasp). The band was no longer visible when the wasp DNA was reduced to 0.3 pg or below.

Detection Stages

PCR amplifications of parasitized *L. lineolaris* egg DNA resulted in 661-bp wasp DNA fragments from both egg and larval stages of *A. iole*. Approximately 74.5±8.82% stung *L. lineolaris* eggs were detected to be parasitized (to contain wasp DNA) at the egg stage of the wasp (host insect eggs were frozen right after female wasp finished oviposition). The detection rate was similar to that (87.5±4.33%) of the larvae stage (host insect eggs were incubated for 48 h to allow parasitoids to develop to larval stage). These results confirmed that PCR can be used to detect *A. iole* eggs and larvae developing within hosts. Using the PCR technique, host eggs can be analyzed soon after parasitization, allowing less time for developing parasitoids to die, and thus providing a more accurate assessment of parasitism. A limitation of the PCR technique is that DNA extraction from immature stages of *A. iole* may be impeded due to their minute size, and may lead to underestimation of parasitism rates. This may be particularly true of eggs, and would explain the lower detection level observed for eggs as compared to larvae. Future refinements in the PCR technique may lead to improved detection of *A. iole* developing within host eggs.

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