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MOLECULAR BIOLOGY

Detection of Enhanced Green Fluorescent Protein DNA in Pink Bollworm through Polymerase Chain Reaction

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INTERPRETIVE SUMMARY

Indelible marking of sterile insects released in a control program allows pest management and quarantine or regulatory personnel to distinguish definitively between released insects and indigenous, non-sterile pest insects. Additionally, genetic manipulation through DNA-mediated transformation of pink bollworm demands such a genetic marker.

Ideally, there should be multiple, independent methods for detection of the marker. To this end we are investigating an indelible genetic marker, enhanced green fluorescent protein, that can be distinguished by a variety of methods.

Definitive detection of enhanced green fluorescent protein fluorescence in pink bollworm can be difficult; so, there is a need for additional detection methods to confirm such detection. Here we describe an alternative to optical detection of flourescent proteins through polymerase chain reaction (PCR) amplification of the DNA that encodes the enhanced green fluorescent protein.

We also describe conditions for detection of enhanced green fluorescent protein DNA by diagnostic PCR, and demonstrate the technique's utility in identifying marked adult insects. Amplification via PCR provides additional confirmation of the presence of the enhanced green fluorescent protein genetic marker to that provided by fluorescent microscopy and other techniques.

ABSTRACT

Indelible marking of pink bollworm (*Pectinophora gossypiella*) would assist in implementation of sterile insect technique-based pest control of this insect. Additionally, genetic manipulation through DNA-mediated genetic transformation also requires a reliable genetic marker. To this end, we injected DNA plasmids encoding the enhanced green fluorescent protein gene into pink bollworm pre-blastoderm embryos and analyzed *in-vivo* the expression of DNA that encodes the enhanced green fluorescent protein. As a confirmatory technique for detection of the protein-encoding DNA, in addition to visual detection of the protein, we used the polymerase chain reaction (PCR) to amplify the expected 579 bp enhanced green fluorescent protein DNA fragment from DNA extracted from green fluorescent protein-positive pink bollworm. We did not amplify such a 579 bp DNA fragment from negative control DNA templates or from DNA isolated from pink bollworm that did not exhibit green fluorescence from the enhanced protein.

Pest management and quarantine decisions are properly based on the detection and numbers of a pest in an area. These decisions are costly to implement and always result in inconvenience and disruption of agricultural practices. One of many possible pest management methods, sterile insect technique is an important, effective and popular pest control technology (Krafsur and Lindquist, 1996).

Sterile insect technique relies on release of sterilized conspecific pest insects into an infested area. Subsequent mating with endemic pests leads to pest population reduction. It would be of great help in determining the need for pest control procedures if plant protection and quarantine personnel could

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Abbreviations: PCR, polymerase chain reaction; DNA, deoxyribonucleic acid; Tris, (tris [hydroxymethyl]aminomethane); EDTA, ethylenediamine tetraacetic acid.

distinguish the harmless, sterile-insect-technique insects from the fertile pest insects. It is now possible to consider indelible genetic marking of sterile insects through transgenic introduction of marker genes (Ashburner et al., 1998).

Depending on the marker gene(s) selected, detection methods can vary. Previously successful efforts toward producing transgenic insects relied on introduction of genes rescuing a mutant eye color in the recipient strains (Ashburner et al., 1998; Coates et al., 1998; Handler, 1993; Handler et al., 1998; Jasinskiene et al., 1998).

Green fluorescent protein and derivatives, first cloned from the jellyfish *Aequorea victoria* (Chalfie et al., 1994; Cody et al., 1993; Heim et al., 1994; Prasher et al., 1992), also have been proposed and used as genetic markers (Ashburner et al., 1998; Handler et al., 1998). Enhanced green fluorescent protein is detected most directly by its characteristic green fluorescence.

Immunological, biochemical, and molecular genetics methods can augment, confirm, or replace direct visualization where such visualization is difficult. This is the case where expression of the green fluorescent protein is minimal, or if protein is inconveniently localized within the insect body, or if there is too much interfering autofluorescence.

In situations where the character of enhanced green fluorescent protein expression in a given organism is unknown, as when this protein is used as a marker for the expression and maintenance of foreign DNA, green fluorescence alone may not be sufficient to reliably confirm the presence of foreign fluorescent protein or DNA.

Additionally, fluorescence alone, even if due to the enhanced green fluorescent protein, cannot prove that the DNA that encoded this foreign protein is still present, intact, or integrated in the genome of the insects at the time of the observations.

Further confusing the issue is the fact that pink bollworm larvae show substantial yellowish-green autofluorescence when illuminated with wavelengths at the optimum excitation peak for enhanced green fluorescent protein. Such false fluorescence might be confused by an inexperienced observer. Older pink bollworm larvae also have a pinkish cuticle color that can absorb green light emitted from the enhanced-green protein, further reducing the protein's signal-to-noise ratio.

Finally, there can never be too much evidence to support an hypothesis, particularly the hypothesis that a heterologous gene is present and expressed in an insect species that heretofore had not been shown to express fluorescent proteins.

Demonstration of foreign DNA in the subject organism is part of the definition of genetic transformation of an organism and, as such, the work presented here comprises part of our efforts toward genetic manipulation of pink bollworm.

This situation is not unique to pink bollworm. Analysis via PCR is commonly used to confirm the presence of transforming DNA constructs. It has been used to demonstrate fluorescent protein genes in putative transgenic organisms that were first distinguished by a visual screen for fluorescence in green fluorescent protein (Aigner and Brem, 1995; Ikawa et al., 1995; Lee et al., 1998; Meyer, 1995; Reue and Rehnmark, 1994; Yin et al., 1998).

Additionally, PCR identification of enhanced green fluorescent protein-positive transgenic animals requires significantly less capital-intensive, specialized equipment in organisms that emit autofluorescence, and therefore can confound visual detection of the green-fluorescent protein. To eliminate autoflourescence close to the emission wavelengths of enhanced green fluorescent protein, optical filtering devices are needed. But even these expensive and specialized filters are not always sufficient to remove confusing signals.

Though the use of the PCR is not trivial, it does not demand expensive optical filtering devices and specific illumination wavelengths. PCR requires only a thermal cycler, consumables, and reagents. Unfortunately, an inexpensive, reliable, and routine immunochemical test for enhanced green fluorescent protein and related transgenic proteins is not available.

Here we report annealing and reaction conditions for PCR amplification of enhanced green fluorescent protein-encoding DNA fragments from pink bollworm template DNA extracted from insects that exhibited enhanced green fluorescent protein fluorescence.

Of more than 450 individual insects visually screened for absence or presence of the desired fluoresence, 58 possessed fluorescence due to the enhanced green fluorescent protein. Conditions were determined to minimize false positives and maximize

detection of the desired protein's target gene fragments. If enhanced green fluorescent protein were introduced into an insect colony and subsequently bred to homozygosity, all insects in the colony would have the desired DNA.

Because enhanced green fluorescent protein is not present naturally in pink bollworm (or as far as is known any organism other than *Aequorea* jellyfish species) detection of enhanced green fluorescent protein in a captured animal would identify that insect as having come from a colony that was positive for the enhanced green fluorescent protein. If this were done with sterile-insect-technique colonies, the sterilized insects released would be indelibly marked. As stated above, this distinction between colony and wild insects is important to sterile-insect-technique programs.

MATERIALS AND METHODS

The DNA construct used to introduce genes of enhanced green fluorescent protein into pink bollworm, pB(BmA3EGFP plasmid DNA), was isolated from lysates of transfected *Escherichia coli* bacteria by NucleoBond (ClonTech Laboratories, Inc., Palo Alto, CA) plasmid-purification affinity columns and reagents.

Genomic DNA from wild-type pink bollworms was purified en masse by a cetyltrimethylammonium bromide method (Ausubel, 1987) or a rapid DNA prep method (Davis et al., 1986). The DNA from individual insects was prepared as follows: a total of 450 individual pink bollworm adults, pupae, or larvae were frozen, then individually crushed in separate 1.5 mL microcentrifuge tubes with tissue grinders (Pellet Pestles, Lida Manufacturing Corporation/Kontes Glass Co., Vineland, New Jersey) chilled by dry ice (CO_2). Lysis buffer (10 m*M* EDTA, 200 m*M* NaCl, 10 m*M* Tris HCl, pH 8.0, 0.5% SDS, 0.06% Anti-Foam B, 10 g/mL RNAse A) (300 µL) was added to each tube and the mixture further ground followed by 30 min incubation at 37 ˚C. Proteinase K was added (10 g mL^{-1}) and the mixtures incubated 2 to 6 h at 55 to 65 °C.

The mixtures were phenol/chloroform extracted and ethanol precipitated. Purified DNA was resuspended in 100 µL TE (10 m*M* Tris HCl, 1 m*M* EDTA, pH 8.0) pH 8.0. DNA concentration was estimated by spectrophotometery (Sambrook et al., 1989).

The DNA from enhanced green fluorescent protein-negative insects was extracted individually or en masse from wild-type pink bollworm strain insects that never were exposed to enhanced green fluorescent protein-encoding DNA constructs.

Visually enhanced green fluorescent protein-positive insects had been either injected as embryos with the fluorescent protein-encoding plasmid or descended from insects that had been so injected. Special attention was taken to identify individuals by a unique number and to correlate that number with visual examination and pedigree.

Visual detection of enhanced green fluorescent protein was performed with a Leica MZ12 stereoscope modified for fluorescence excitation illumination and visualization (McBain Instruments, Chatsworth, CA). Illumination was by a 100-W mercury vapor lamp in a Leica (ser. no. 307-672-067) lamp house attached to the stereoscope. The lamp was powered by a Leica HBO-100 power source. A Leica green fluorescent protein-plus (ser. no. 1046143) optical filter set was used to filter both the excitation light and the light returned from the specimen to optimize the visualization of enhanced green fluorescent protein emissions.

All PCRs were performed in an Eppendorf Mastercycler gradient thermal cycler (Eppendorf-Netheler-Hinz, Hamburg, Germany). Specific ssDNA PCR primers for enhanced green fluorescent protein were custom made (DNAgency, Malvern, PA). Enhanced green fluorescent protein 1: $^{5'}$GGCCACAAGTTCAGCGTG$^{3'}$ was designed to anneal to the 5' end of enhanced green fluorescent protein cDNA; whereas, enhanced green fluorescent protein 2: $^{5'}$GATCGCGCTTCTCGTTGG$^{3'}$ was designed to anneal to the 3' end. PCRs (20 µL) were performed in 0.2 mL thin wall reaction tubes (Fisher Brand 05-407-3b). For each 20 µL reaction, 2 µL Taq Polymerase buffer (1.5 m*M* $MgCl_2$, 50 m*M* KCl, 10.0 m*M* Tris–HCl pH 9.0 @25 °C, 0.1% Triton x-100), 2 µL 2 m*M* dNTPs, 1 ng each enhanced green fluorescent protein 1 and enhanced green fluorescent protein 2 primers and 2 units of Taq Polymerase (Promega) were combined along with ultrapure water to make up 19 µL total volume. Both water and buffer had been irradiated on a UV transilluminator for 15 min to destroy any

contaminating DNA. Then 1 µL of template DNA solution (roughly 10–100 ng of DNA) was added to each reaction.

In addition to PCRs that received pink bollworm DNA from enhanced green fluorescent protein-positive insects, positive controls were performed. These contained 10 pg of enhanced green fluorescent protein-encoding plasmid template plus up to 1 μg of enhanced green fluorescent protein-negative pink bollworm DNA.

Negative controls containing only wild-type pink bollworm DNA as template and an additional negative control set of reactions with no template also were run in addition to the enhanced green fluorescent protein-positive experimental reactions and positive controls. Reaction conditions were as follows:

1 cycle of 1 min @ 95 °C;
35 cycles of 30 s @ 95 °C;
    30 s @ annealing temperature;
    30 s @ 72 °C;
1 cycle of 5 min @ 72 °C;
    Soak @ 4 °C.

Though more temperature cycles were performed (35) than are customary to amplify a given template in diagnostic PCRs, we did this to increase the chance that false positives would appear and thus to test more rigorously the specificity of the diagnostic PCRs. After completion of the reactions, 10 µL of each 20 µL reaction were loaded with bromophenol-blue-containing loading buffer and separated by electrophoresis in 1.25% agarose, 0.5x Tris borate buffer (44.5 m*M* Tris borate, 1.0 m*M* EDTA, pH 8.3, 100 ng/mL ethidium bromide) submarine horizontal electrophoresis gels. Due to the large number of samples screened in this study (more than 450 individual insects in addition to controls), two sets of 40 wells were cast in each 20-by-20-cm gel. Electrophoresis was continued until the bromophenol blue dye band was 2.5 cm from the end of the gel.

RESULTS AND DISCUSSION

The PCRs loaded in lanes 1 through 12 of Fig. 1 failed to amplify DNA of the size expected for enhanced green fluorescent protein DNA. This result was expected as these reactions lacked any template

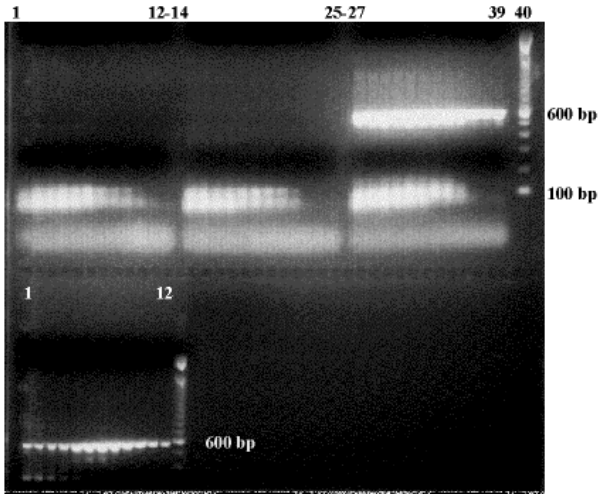

Fig.1. Digital photo of a 1.25% 0.5x TBE agarose gel with electrophoretically separated, PCR amplified DNA, showing two rows of 40 lanes on the same gel. Numbers refer to the first and last lane of each of four sets of 12 reactions plus 1 μg DNA standards (Gibco-BRL catalog no. 15628-050) in lanes 40 and 14 (lower row of wells). The 100 bp, and 600 bp bands of the DNA standards are labeled and appear brighter. Each set of 12 PCRs differed as follows: Lanes 1 to 12 contained PCRs with no template DNA. PCRs loaded in lanes 14 to 25 had 100 ng enhanced green fluorescent protein-negative pink bollworm template DNA. PCRs in lanes 27 to 39 contained 20 ng enhanced green fluorescent protein-positive pink bollworm template DNA. PCRs in lanes 1 to 12 lower row had 250 pg of enhanced green fluorescent protein-encoding plasmid as positive control template. Annealing temperatures in each set increased from left to right as follows: 50.6, 50.6, 51.6, 53.4, 55.8, 58.5, 61.4, 64.2, 66.7, 68.7, 69.7, 70.1 °C.

DNA. The PCRs loaded in lanes 14 through 26 (Fig. 1) had only wild-type pink bollworm DNA (with no DNA encoding for enhanced green fluorescent protein) as template and, as expected, also failed to amplify enhanced green fluorescent protein DNA. The PCRs containing template DNA from insects identified visually as positive for enhanced green fluorescent protein (and, thus, at least putatively possessing enhanced green fluorescent protein DNA), amplified an enhanced green fluorescent protein fragment (Fig. 1, lanes 28–40). The PCRs containing 250 pg of pB(BmA3EGFP) as template amplified a DNA fragment of approximately 579 base pairs.

This is the fragment size expected from a PCR using primers enhanced green fluorescent protein 1

and 2 and an enhanced green fluorescent protein DNA template (Fig. 1, bottom row). The PCRs also show the presence of encoding DNA for enhanced green fluorescent protein in isolates of DNA from individual green fluorescent insects (Fig. 2). In this figure, the expected enhanced green fluorescent protein DNA fragment was not PCR-amplified from any animals that were negative for enhanced green fluorescent protein, whereas a fragment of the expected size was amplified from enhanced green fluorescent protein fluorescent insects.

The optimum annealing temperatures for detection of enhanced green fluorescent protein DNA in the presence of pink bollworm DNA were between 55.8 and 61.4 °C. Below these temperatures, much non-specific product was amplified that could interfere with diagnosis and detection. Above this temperature range, the amount of enhanced green fluorescent protein-specific product amplified was reduced, compromising the sensitivity of the assay (Fig. 1).

We thus have demonstrated the efficacy of PCR for identification of enhanced green fluorescent protein DNA in pink bollworm. This technique will be a helpful adjunct in efforts to control pink bollworm via the sterile insect technique and will boost genetic manipulation of this insect pest. By allowing identification of transgenic animals that are positive for enhanced green fluorescent protein, it should remain a useful tool of confirmation even after the development of effective and fast immunochemical detection technology for enhanced green fluorescent protein.

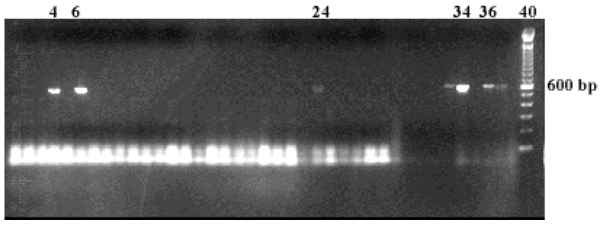

Fig. 2. Digital photo of 1.25% 0.5x TBE agarose gel showing electrophoretically separated, PCR-amplified ethidium-bromide stained DNA fragments amplified by diagnostic PCRs. PCRs loaded in lanes 4, 6, 24, 34, 35, 37, and 38 had template DNA from enhanced green fluorescent protein-positive insects and are numbered. Lane 40 (also numbered) contains DNA size standard (Gibco-BRL catalog no. 15628-050). The 600 bp band is so marked. Template DNA in all other PCRs was from enhanced green fluorescent protein-negative insects. PCRs were annealed at 56 °C.

CONCLUSIONS

Polymerase chain reaction has been widely used to identify specific human and animal DNA. It is used in legal proceedings to establish the identity and origin of forensic specimens, and to distinguish transgenic animals from untransformed siblings (Aigner and Brem, 1995; Ikawa et al., 1995; Lee et al., 1998; Meyer, 1995; Reue and Rehnmark, 1994; Yin et al., 1998).

We investigated a PCR assay for detection of the genetic marker, enhanced green fluorescent protein. This gene soon will be introduced into the sterile-insect-technique colony in Phoenix, AZ, to mark insects produced for the California Department of Food and Agriculture-USDA pink bollworm sterile-insect-technique program. Additionally, PCR technology will be used to confirm the presence of enhanced green fluorescent protein-encoding DNA in initial genetic transformations of pink bollworm.

After introduction of the enhanced green fluorescent protein genetic trait into sterile-insect-technique colonies, PCRs will be used to detect the DNA that encodes enhanced green fluorescent protein in field-collected pink bollworm. Pest survey personnel could then quickly identify bollworm colonies that are positive for enhanced green fluorescent protein collected in their monitoring programs.

This particular diagnostic technique requires less than 3 h for PCR (depending on the thermal cycler used) and less than 2 h for electrophoresis and subsequent documentation.

Though it would require proprietary supplies (Qiagen Inc., Valencia, CA, for example), rapid DNA extraction and preparation techniques used to prepare DNA from intact tissues (mouse tails, plant tissue, etc.) could be employed. With the shortening of time for DNA isolation, the complete analysis of a sample could be performed within a working day of 8 h or less.

With one thermal cycler designed for high throughput and sufficient gel electrophoresis apparatuses, one could screen 384 samples (or more, as high throughput technology improves) simultaneously for the DNA that encodes enhanced green fluorescent protein. The facilities used to screen pink bollworm could also be used to screen samples from other sterile-insect-technique programs with little or no modification.

The rate-limiting step in our assay is the time needed to prepare samples properly for PCR. This may also be improved because it may be possible to screen samples by putting insect tissue, such as a leg, wing scales, or similar small body parts directly into a PCR (Crabtree et al., 1995; Crabtree et al., 1997).

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