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Insertion of a yeast metallothionein gene into the model insect *Drosophila melanogaster* (Diptera: Drosophilidae) to assess the potential for its use in genetic improvement programs with natural enemies

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Abstract

The use of copper-containing fungicides, such as Kocide, negatively affects nontarget beneficial insects in Florida citrus. To determine if the insertion of the yeast metallothionein gene (*CUP1*) into the genome of beneficial insects could produce populations resistant to copper, the model insect *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) was transformed with the *Drosophila* metallothionein promoter *Mtn* fused to the *CUP1* open reading frame. Two transformed lines, confirmed by high-fidelity polymerase chain reaction and Southern blot analysis, were assessed for tolerance to copper hydroxide (CuOH). Transgenic lines 8 and 14 exhibited 1.5- to 2-fold increases in tolerance when held at 24 °C, ~75% RH, and 14L:10D. In the absence of copper, lines 8 and 14 exhibited fitness costs as compared to the *ww* control line. In addition, each line (*ww* control, lines 8 and 14), when pretreated with 2.5 mM CuOH and held at ~22–24 °C, ~50–75% RH, and 14L:10D, was more tolerant to copper, with line 14 exhibiting the greatest increase. The increased copper tolerance of the pretreated transgenic lines could be a result of induced transcription of both the *CUP1* and native metallothionein (MT) genes whereas the increase in the pretreated *ww* control could be a due to induced transcription of native MT genes. Both transgenic lines exhibited reduced fitness compared to the *ww* control line, which could be due to the effects of inbreeding or to the insertion of the *CUP1* construct into the genome. The data suggest that *CUP1* may be useful for the genetic improvement of natural enemies where copper is applied.

Keywords: *Drosophila melanogaster*; Transgenesis; *P*-element mediated transformation; Copper resistance; Yeast metallothionein gene; Fitness

1. Introduction

The success of Integrated Pest Management (IPM) programs is strengthened by the compatibility of chemical and biological control methods used in the system (Hoy, 1990a, 1992). IPM is a challenge for complex agricultural systems where it is not possible to control key pests by methods other than chemical control (Hoy, 1985, 1995, 2000). For example, copper-containing fungicides, such as Kocide, are applied to large amount of citrus acreages in Florida to control several fungal diseases (Childers, 1994; Lapointe et al., 2004; McCoy et al., 2004), but may limit the success of biological control management strategies due to negative impacts on nontarget beneficial insects (Childers et al., 2001). This was shown in both field and laboratory studies where copper fungicides decreased fecundity and survival rate of beneficial insects and increased secondary pest populations (Childers, 1994; Childers et al., 2001; Michaud, 2001; Villanueva-Jiménez and Hoy, 1998). Genetic modification by recombinant DNA methods may provide new strategies for biological control (Beckendorf and Hoy, 1985). In this system, beneficial natural enemies genetically transformed with a gene that increases resistance to copper could potentially improve the compatibility of fungal disease control and biological control in Florida citrus.

Another reason for studying chemical resistance genes is that these genes may provide selectable markers to detect

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genetic transformation in insects lacking other marker genes. Transformation vectors are being developed for insects in a variety of orders for which there are no available genetic markers. A chemical resistance gene that could function in insects across many orders may provide a useful tool for insect transgenesis.

Metallothioneins belong to a family of metal-binding proteins found in all eukaryotes and many prokaryotes and function to detoxify metals, including copper (Palmiter, 1998). The genes that code for these proteins could facilitate resistance to copper-containing fungicides in natural enemies. The yeast metallothionein gene *CUP1* codes for a 53-residue polypeptide including 12 cysteine sites that can cumulatively bind up to eight copper ions (Tohoyama et al., 1995). The CUP1 protein functions in yeast to protect cells against toxic levels of copper and to repress the transcription of *CUP1* when external levels of copper are low (Winge et al., 1985).

In *Drosophila melanogaster* (Meigen), copper has been shown to reduce viability and growth rate (Maroni and Watson, 1985); however, natural populations of *D. melanogaster* containing multiple copies of the *Drosophila* metallothionein gene, *Mtn*, exhibited a 5- to 6-fold increase in accumulation of mRNA and exhibited a 1.5-fold increase in resistance to copper (Maroni et al., 1987; Theodore et al., 1991). The CUP1 and MTN proteins have similar structure and function suggesting that the *CUP1* gene may be effective in increasing copper resistance in this model insect and, subsequently, in agriculturally important insects. Although *D. melanogaster* usually are not agriculturally important pests, many of the same genetic and biochemical mechanisms that underlie resistance in pest insects are seen in *Drosophila* (Wilson, 2001). *P*-element-mediated transformation can be used to transform *D. melanogaster* (Spradling and Rubin, 1982) and may be an effective technique to determine if the yeast metallothionein gene *CUP1*, regulated by the *Drosophila* metallothionein promoter, *Mtn*, could increase tolerance to copper in this model insect. The use of *CUP1* allows transformation of *D. melanogaster* to be detected readily because it is easily distinguished from the endogenous *Drosophila* metallothionein gene *Mtn*.

There are two methods for inserting exogenous DNA into the germline cells: egg microinjection and maternal microinjection. Egg microinjection involves injecting the posterior end of each individual egg prior to pole cell (germline) formation (Spradling and Rubin, 1982). Maternal microinjection involves injecting the abdomen of a female with the objective that the injected DNA will be taken up by multiple oocytes or eggs and insert into the nuclear genome. Maternal microinjection has been demonstrated effective for the transformation of the western predatory mite, *Metaseiulus occidentalis* (Nesbitt) (Acari: Phytoseiidae) (Li and Hoy, 1996; Presnail and Hoy, 1992; Presnail et al., 1997) and a braconid endoparasitoid, *Cardiochiles diaphaniae* Marsh (Presnail and Hoy, 1996), and may provide an easier method of achieving transformation than egg microinjection.

Inserting exogenous genes into the chromosomes of insects may reduce fitness, possibly due to expression of the exogenous gene, mutations caused by its insertion or laboratory rearing of the insect resulting in genetic bottlenecks, inbreeding, drift and laboratory adaptation (Beckendorf and Hoy, 1985; Catteruccia et al., 2003; Hoy, 1985; Irvin et al., 2004; Kaiser et al., 1997; Levis et al., 1985; Lenski and Nguyen, 1988; Mackay, 1989). In addition, fitness costs have been associated with resistance genes in unpolluted environments. *Drosophila melanogaster* strains artificially selected for heavy-metal resistance exhibited reductions in fecundity and growth rate in unpolluted environments (Shirley and Sibly, 1999). Therefore, after putatively transformed flies are screened for the selectable marker and assessed for transgene expression, they should be assessed for fitness costs. Fitness has been defined as the ability of an organism to perpetuate itself as measured by its reproductive success (Pianka, 1983). Indirect measures of relative fitness include developmental rate, fecundity, fertility, adult longevity, sex ratio, and viability between two or more distinct populations. Studies regarding transgene expression, stability and transmission to progeny, and fitness of transgenic insects are key steps in determining the feasibility and efficacy of employing transgenic insects in pest management programs (Beckendorf and Hoy, 1985; Catteruccia et al., 2003; Hoy, 1990b; Irvin et al., 2004).

2. Materials and methods

2.1. Drosophila melanogaster

The w[m] strain, which contains a recessive white-eyed (*w*) mutant gene in the homozygous condition (*ww*), was obtained from the Bloomington Stock Center (Bloomington, IN). Stocks were reared in 250-ml milk bottles containing a diet of 1:1 Ward's Instant Fly media (Ward's Natural Science Establishment, Rochester, NY) to sterile water and maintained at 24 °C, ~75% RH, and a 14L:10D photoperiod. The fly stocks were transferred to fresh bottles every two weeks using an air-pump aspirator, and each bottle was kept no longer than one month.

2.2. Constructing the P-element plasmid pAJ171

Drosophila melanogaster Mtn promoter sequences were amplified using two primers designed from sequences in GenBank (M12964); forward primer (MHO70, 5′-CTT TGC TGC AGG CCG TCC TAT CCT CTG GTT-3′) and reverse primer (MHO89, TCC GCA TGG GCA AGG CAT CTT GAT TGA GTT G-3′). Standard PCR was performed in a 25-μl volume containing 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each of dATP, dGTP, dTTP, dCTP, 200 pM of primers, and 0.8 units of *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The PCR profile used consisted of 35 cycles, with each cycle consisting of denaturation at 94 °C for 30 s and extension at 72 °C for 1 min. A 0.4-kb DNA fragment was ampli-