Research Article

Received: 16 May 2012 Revised: 8 August 2012 Accepted article published: 21 August 2012 Published online in Wiley Online Library: 28 November 2012

(wileyonlinelibrary.com) DOI 10.1002/ps.3407

Abdominal color of the Asian citrus psyllid, *Diaphorina citri*, is associated with susceptibility to various insecticides

Siddharth Tiwari,[a] Nabil Killiny,[a] Rajinder S Mann,[a] Erik J Wenninger[b] and Lukasz L Stelinski[a]*

Abstract

BACKGROUND: Color morphs of an insect species are known to vary in activities of detoxifying enzymes and associated susceptibility to insecticides. In *Diaphorina citri*, three color morphs are known to occur. In the present study, susceptibility to four insecticides was compared among gray/brown, blue/green and orange/yellow color morphs of field-collected *D. citri*.

RESULTS: The orange/yellow morph was significantly more susceptible to fenpropathrin than the blue/green morph, and imidacloprid and chlorpyrifos caused higher mortality in the orange/yellow morph than in the blue/green and gray/brown morphs. To confirm the genetic basis of variable levels of susceptibility, the relative expression of five *CYP4* genes was compared among the color morphs. *CYP4C67* was expressed at significantly higher levels in the blue/green than in the orange/yellow and gray/brown morphs. *CYP4DA1*, *CYP4C68*, *CYP4G70* and *CYP4DB1* were expressed at significantly higher levels in the blue/green and gray/brown morphs than in the orange/yellow morph. Lower expression of *CYP4* genes in the orange/yellow morph as compared with the others was correlated with reduced signal of 45 kD cytochrome P450 proteins, as determined by the western blot.

CONCLUSIONS: The results indicate differential susceptibility of *D. citri* color morphs to insecticides, which will need to be accounted for in future insecticide monitoring programs and may affect management programs.

Keywords: *CYP4* genes; cytochrome P450 monooxygenase; gene expression; huanglongbing; citrus greening

1 INTRODUCTION

In insects, individuals of a species commonly have variations in body color.[1] The range of color variation plays an important role in mate selection,[2] deterring predators,[3] regulating body temperature[4,5] and habitat selection.[6] These color variations are possibly the result of phenotypic plasticity,[7] nutritional status,[8] bacterial symbionts,[9–11] genetic factors or a combination of two or more of the above factors.[12] The diversity of body color at an individual level within a species – often referred to as color polymorphism[13,14] – can also be the result of allelic variation at polymorphic loci or environmental factors.[15]

Color polymorphism is common in the Asian citrus psyllid, *Diaphorina citri*; this species occurs in three distinct color morphs: gray/brown, blue/green and orange/yellow.[8,16–18] The abdominal color of *D. citri* may reflect sexual maturity or indicate the oviposition period of females.[17,19] In *D. citri*, gray/brown adults are characterized by lower body mass than other color morphs, and orange/yellow adults exhibit the greatest body mass.[8] When transferred to new citrus seedlings, gray/brown adults show an increase in body mass, which suggests that body color may be indirectly related to plant quality.[18]

Color morphs are known to vary in activities of detoxifying enzymes and associated susceptibility to insecticides.[20–22] For example, red morph tobacco aphids, *Myzus nicotianae* Blackman, exhibit higher carboxylesterase activity than green morphs.[20] In addition, the red morph is characterized by a 1,3 autosomal translocation, which is less common in green morph aphids that exhibit high carboxylesterase activity.[20] The red morph of the green peach aphid, *Myzus persicae* (Sulzer), is more resistant to dimethoate, endosulfan and lambda-cyhalothrin than the green morph.[21] However, susceptibility to acephate, bifenthrin, imidacloprid or mevinphos is comparable among different color morphs in this species.[21] Likewise, the green morph of the tobacco-adapted form of the green peach aphid, *M. persicae*, exhibits lower esterase levels and LC_{50} values when exposed to acephate and methomyl than red and orange morphs. This highly resistant orange morph was characterized by both *E4* and *FE4* gene amplification.[22] The above results indicate that color polymorphism influences insecticide susceptibility at the gene level.

* *Correspondence to: Lukasz L Stelinski, Entomology and Nematology Department, Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850, USA. E-mail: stelinski@ufl.edu*

a *Entomology and Nematology Department, Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA*

b *Department of Plant, Soil and Entomological Sciences, Kimberly Research and Extension Center, University of Idaho, Kimberly, ID, USA*

Previously, the present authors reported that insecticide susceptibility and detoxifying enzymes are influenced by the presence of a bacterial endosymbiont, *Candidatus* Liberibacter asiaticus, in *D. citri*.[23,24] Bacterial endosymbionts are known to influence the development of color polymorphism by facilitating the synthesis of pigments in hemolymph.[9–11] The present results indicate that color polymorphism is associated with insecticide susceptibility of *D. citri*. In addition, color-morph-associated insecticide susceptibility was negatively correlated with the expression of five *CYP4* genes and 45 kD cytochrome P450 proteins in *D. citri*.

2 MATERIALS AND METHODS

2.1 *D. citri* cultures

Three color morphs of *D. citri* were collected using aspirators from a commercial citrus grove located in Polk County, Florida. Collected adults were transferred to the laboratory in coolers and released onto citrus plants in Plexiglas cages (40 × 40 × 40 cm) until treatment with insecticide, or gene and protein expression assays. Ten adults were randomly chosen from each morph to confirm the absence of Las using qPCR assay methods described by Tiwari *et al*.[23]

2.2 Insecticides

Three color morphs of adult *D. citri* were tested for susceptibility to four insecticides from various insecticide chemistry classes and modes of action. Commercially available formulations of each insecticide were used in bioassays, and each insecticide was tested at 5–6 concentrations, which were prepared in distilled water on the day of testing. The insecticides evaluated against *D. citri* adults included chlorpyrifos (Lorsban 4E; Dow AgroSciences, LLC, Indianapolis, IN), fenpropathrin (Danitol 2.4EC; Valent USA Corp., Walnut Creek, CA), imidacloprid (Provado 1.6F; Bayer CropScience LP, Research Triangle Park, NC) and spinetoram (Delegate WG; Dow AgroSciences, LLC, Indianapolis, IN).

2.3 Leaf-dip bioassay

Insecticide susceptibility was determined using a leaf-dip petri-dish bioassay method.[25,26] Plastic disposable petri dishes (35 mm diameter; Fisherbrand, Thermo Fisher Scientific, Waltham, MA) containing 3–4 mL of a 1.5% solidified agar solution constituted the bioassay arenas. The leaf discs (35 mm diameter) were made from citrus leaves collected from Valencia orange trees growing in a greenhouse at the Citrus Research and Education Center, Lake Alfred, Florida. Leaf discs were dipped in test (insecticide) solutions for 30 s and allowed to air dry in a fume hood for 1 h prior to bioassays. For the control treatment, leaf discs were dipped in distilled water alone. After 1 h, leaf discs were placed on agar beds, and 20–25 *D. citri* adults were transferred into each dish using a camel hair brush following a brief anesthetization with CO_2 to facilitate handling and transfer. Petri dishes were wrapped with parafilm (Pechiney Plastic Packaging, Chicago, IL) to prevent adults from escaping. Sealed petri dishes with *D. citri* were transferred into a growth chamber (Percival Scientific, Inc., Perry, IA) set at 25 ± 1 °C, 50 ± 5% RH and 14:10 (L:D) photocycle. Each concentration of an insecticide was replicated 3 times (n = 60–75 *D. citri* per concentration).

D. citri mortality was assessed 48 h after transfer into the growth chamber. *D. citri* found on their side or back that were unable to move when probed with a camel hair brush were considered dead. Mortality data were corrected for control mortality (<5%) using Abbott's formula[27] and analyzed separately for each color morph. Mortality data for each concentration and color morph were subjected to probit regression analysis to calculate the LC_{50} and LC_{95} for each insecticide with corresponding 95% confidence intervals and slopes of regression lines.[28] The LC_{50} and LC_{95} values among color morphs were considered significantly different ($P < 0.05$) if their 95% confidence intervals did not overlap.

2.4 Quantitative real-time PCR (qPCR) for quantifying *CYP4* gene expression

Field-collected *D. citri* of each color morph and an insecticide-susceptible gray/brown morph from the authors' laboratory culture were subjected to RNA isolation and cDNA synthesis. Prior to RNA isolations, ten adults were randomly chosen from each morph to confirm the absence of Las using qPCR assay methods described by Tiwari *et al*.[23] RNA isolations were performed using the SV total RNA isolation kit (Promega, Madison, WI). RNA isolation was performed on groups of 40–50 psyllids of each color morph. Each treatment was replicated 3 times. The quality and quantity of RNA from each sample was measured on a NanoDrop 1000 spectrophotometer using an A_{260} and A_{260}/A_{280} ratio respectively.[29] Additionally, the quality of RNA was assessed by visualizing the RNA samples on 1% agarose gel electrophoresis in TAE buffer with ethidium bromide staining. cDNA was synthesized from the RNA samples using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). In brief, cDNA was synthesized in a 20 µL reaction volume containing 4 µL of 5× iScript reaction mix, 1 µL of iScript reverse transcriptase, 8 µL of nuclease-free water and 7 µL of total RNA (100 ng μL^{-1}). Amplification cycles consisted of 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min, and subsequently the samples were held at 4 °C. The PCR products were verified by 1% agarose electrophoresis.

The relative expression of five *CYP4* genes in each color morph was detected by qPCR using SYBR Green PCR master mix in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Primers for the five *CYP4* genes and the reference gene, *Actin*, were used as described by Tiwari *et al*.[30] For all qPCR experiments, the production of gene-specific products and the absence of 'primer-dimers' was verified by 1% agarose electrophoresis in TAE buffer with ethidium bromide staining. In brief, qPCR was performed in a 20 µL reaction volume containing 10 µL of SYBR green PCR master mix, 1 µL of cDNA, 1 µL each of forward and reverse primers and 7 µL of nuclease-free water. Amplification cycles consisted of an initial denaturing step at 95 °C for 10 min, followed by 45 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, an extension step at 72 °C for 10 min and the final melting-curve step (90 cycles of temperature reduction from 90 to 50 °C at a rate of 0.5 °C 10 s^{-1}). Three biological replicates were performed for each gene and each color morph.

The relative expression of each gene among the treatments was compared using the $2^{-\Delta\Delta C_t}$ method.[31] Initially, normalization was performed using *Actin* (i.e. the reference gene) gene expression, followed by normalization to the treatment with the lowest gene expression (insecticide-susceptible greenhouse gray/brown morph). A separate one-way analysis of variance (ANOVA) was performed for each gene to compare the relative gene expression among the various color morphs, followed by Fisher's protected LSD tests for mean separation (PROC GLM).[28]

Table 1. Insecticide toxicity in relation to color polymorphism in *Diaphorina citri*

Color morph	95% CI at LC_{50}	95% CI at LC_{95}	Slope ± SEM	χ^2
Fenpropathrin				
Blue/green	0.85–1.57	29.21–306.46	0.90 ± 0.10	3.42
Gray/brown	0.05–6.67E16	11.32–1.08E222	0.49 ± 0.15	15.83
Orange/yellow	9.70E-10–0.13	0.57–9.26E17	0.77 ± 0.22	22.05
Imidacloprid				
Blue/green	0.40–0.55	1.84–6.36	2.04 ± 0.27	0.72
Gray/brown	0.41–0.52	1.59–3.68	2.38 ± 0.24	4.19
Orange/yellow	0.18–0.25	1.09–3.25	1.87 ± 0.26	2.40
Spinetoram				
Blue/green	2.06–32.17	28.82–55 3124.00	0.98 ± 0.19	13.01
Gray/brown	3.47–12.47	51.71–1856.00	1.12 ± 0.12	6.87
Orange/yellow*	—	—	—	—
Chlorpyrifos				
Blue/green	3.93–4.74	12.12–22.48	2.95 ± 0.31	5.98
Gray/brown	3.62–11.17	11.62–919.60	2.32 ± 0.47	15.50
Orange/yellow	0.92–1.56	2.67–5.36	3.81 ± 0.71	5.37

* Bioassays were not performed owing to the lack of a sufficient number of field-collected *D. citri* adults.

2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

The greenhouse (insecticide-susceptible) gray/brown, field-collected gray/brown and field-collected orange/yellow morphs were subjected to extractions and quantifications of subcellular protein fractions. Prior to protein analysis, ten adults were randomly chosen from each morph to confirm the absence of Las using qPCR assay methods described by Tiwari *et al*.[23] Protein concentration was determined by the Bradford method[32] using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with ovalbumin as the standard. Subcellular protein fractions were extracted using methods described by Wheeler *et al*.[33] Each protein fraction dissolved in potassium phosphate buffer was loaded on a 10% polyacrylamide gel separately and subjected to electrophoresis using methods described by Mouchés *et al*.[34] Protein fractions were visualized by staining with colloidal Coomassie blue G250. In order to determine the presence of cytochrome P450 proteins, a western blot was performed on various subcellular protein fractions using the greenhouse population. In brief, after electrophoresis, subcellular proteins were transferred to a PVDF membrane for 60 min at 10 V using a semi-dry transfer apparatus (Bio-Rad). For renaturation of proteins and blocking of unoccupied spaces, the membrane was incubated overnight at 4 °C in TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 6% non-fat dry milk (Sigma-Aldrich, St Louis, MO). After blocking, the membrane was used immediately for western blot assays. The membrane from the blocking solution was transferred to the primary antibody solution (polyclonal rabbit antibody, anticytochrome P450 19A1, Sigma-Aldrich, with a concentration of 1:1000 in TBS), placed on a shaker for 1 h, rinsed with TBS-Tween-20 3 times and then transferred to a secondary antibody solution (antirabbit IgGs-alkaline phosphatase with a concentration of 1:10 000 in TBS) and placed on a shaker for 1 h. After three rinses with TBS-Tween-20 buffer, the membrane was placed into the BCIP/NBT solution. After developing color, the membrane was transferred to fresh nanopure water, placed on a clean filter paper and photographed as soon as possible. As cytochrome P450 proteins were clearly detected in the microsomal protein, subsequent electrophoresis and western blot were performed using microsomal proteins from each morph by the methods described above.

3 RESULTS

3.1 Leaf-dip bioassay

Susceptibility to four insecticides differed among field-collected gray/brown, blue/green and orange/yellow color morphs of *D. citri* (Table 1 and Figs 1B and C). Based on non-overlapping 95% confidence intervals at the LC_{50}, the orange/yellow morph was significantly more susceptible to fenpropathrin than the blue/green morph. Likewise, the orange/yellow morph was significantly more susceptible to chlorpyrifos and imidacloprid than the blue/green and gray/brown morphs, and the blue/green and gray/brown morphs were equally susceptible to spinetoram. Moreover, based on non-overlapping 95% confidence intervals at the LC_{95}, the orange/yellow morph was significantly more susceptible to chlorpyrifos than the blue/green and gray/brown morphs.

3.2 *CYP4* expression levels in *D. citri* color morphs

Relative expression levels for the five *CYP4* genes differed among three field-collected morphs and one greenhouse (insecticide-susceptible) morph (Fig. 2). The expression levels of *CYP4C67* ($F = 13.29$; df = 3, 8; $P = 0.0018$), *CYP4C68* ($F = 52.44$; df = 3, 8; $P < 0.0001$), *CYP4DA1* ($F = 876.92$; df = 3, 8; $P < 0.0001$), *CYP4G70* ($F = 6.33$; df = 3, 8; $P = 0.0166$) and *CYP4DB1* ($F = 99.91$; df = 3, 8; $P < 0.0001$) were significantly different among the three field-collected morphs and the greenhouse morph. Expression levels of *CYP4C68*, *CYP4DA1*, *CYP4G70* and *CYP4DB1* were significantly lower in the orange/yellow morph than in the blue/green and gray/brown morphs. The expression level of *CYP4C68* was 45- and 67-fold lower for the orange/yellow morph than for the blue/green and gray/brown morphs respectively. The expression level of *CYP4DA1* was 70 and 125 times higher in the blue/green and gray/brown morphs, respectively, than in the orange/yellow morph. Likewise, the expression level of *CYP4G70* in both blue/green and gray/brown morphs was 11-fold higher than in the orange/yellow morph. The expression level of *CYP4DB1* was 64 and 101 times higher in the blue/green and gray/brown morphs, respectively, than in the orange/yellow morph.

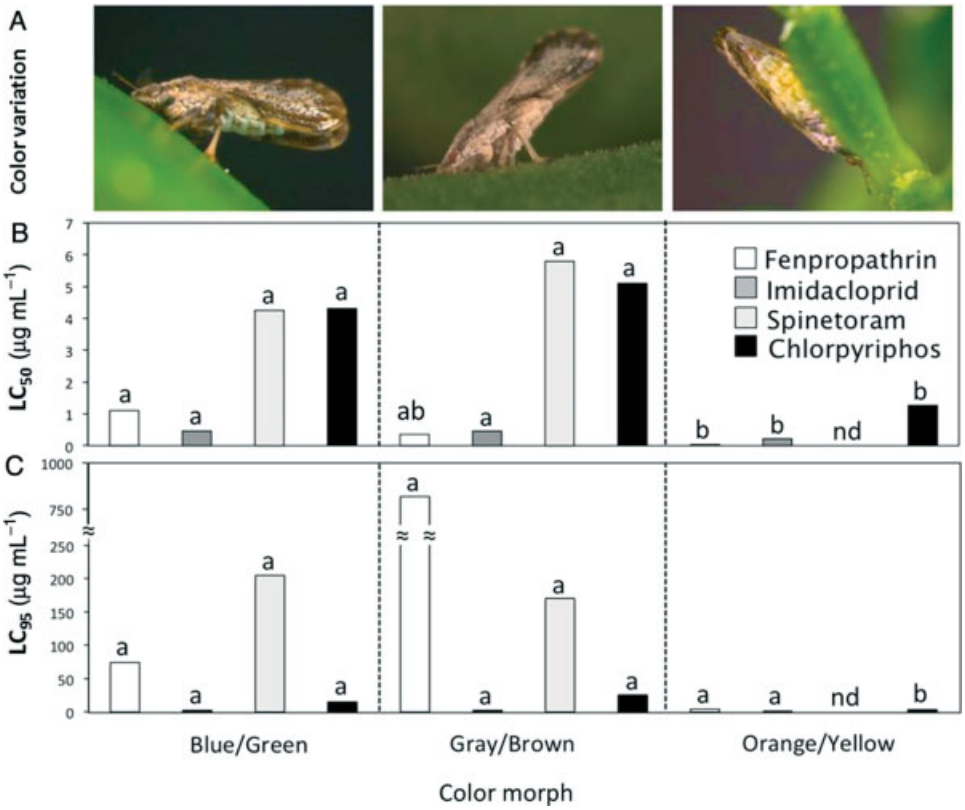

Figure 1. Differential toxicity of four insecticides as a function of color polymorphism in *Diaphorina citri*. (A) *D. citri* color morphs. (B) LC_{50} (mg AI L^{-1}) values obtained on the basis of color morphs of *D. citri*. Significant differences in LC_{50} values among the morphs are indicated by different letters, based on non-overlapping 95% confidence intervals. The 95% confidence intervals, slope and χ^2 obtained from probit analysis are presented in Table 1. (C) LC_{95} (mg AI L^{-1}) values obtained on the basis of color morphs of *D. citri*. Significant differences in LC_{50} values among the morphs are indicated by different letters, based on non-overlapping 95% confidence intervals. The 95% confidence intervals, slope and χ^2 obtained from probit analysis are presented in Table 1.

3.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

SDS-PAGE was performed using subcellular protein fractions prepared from the greenhouse (insecticide-susceptible) gray/brown morph of *D. citri* (Fig. 3A). SDS-PAGE indicated varying levels of proteins in various subcellular fractions. Subsequently, western blot was performed with the different subcellular fractions to locate cytochrome P450 proteins (Fig. 3B), which revealed the presence of a band corresponding to a 45 kDa protein in the microsomal protein fraction that cross-reacted with the primary antibody of cytochrome P450 protein. Subsequently, microsomal fractions were used to perform a western blot for comparing cytochrome P450 protein levels among field-collected gray/brown, greenhouse (insecticide-susceptible) gray/brown and field-collected orange/yellow *D. citri* morphs (Fig. 3C). Three quantities of microsomal proteins for each morph were used to perform the western blot. The field-collected orange/yellow morph exhibited lower levels of cytochrome P450 proteins than the field-collected or greenhouse (insecticide-susceptible) gray/brown morphs (Fig 3C).

4 DISCUSSION

Several factors may influence the development of color in *D. citri*, including the contents of the abdomen, mating, developmental stage, sex, feeding, reproductive potential and the presence of various pigments.[8,17,18,35] In other insect species, color polymorphism is influenced by juvenile hormones, selection pressures imposed by natural predators, symbiotic bacteria and lateral gene transfer related to carotenoid synthesis.[11,36–38] Irrespective of the role or underlying mechanism responsible for color polymorphism, all insects that display multiple color morphs exhibit the presence or absence of specific pigments. One of the most commonly occurring pigments in insects are carotenoids, which occur in various forms and influence insect color.[9,11,36–38] However, the mechanism explaining color polymorphism in *D. citri*, including the effect of carotenoids, has not been explored. In general, after mating, *D. citri* females become orange/yellow in color, which indicates the presence of eggs in the female's abdomen.[8,19,35] No such change occurs in males after mating. The orange/yellow color morph in *D. citri* may be associated with the presence of carotenoids and blue bile pigments.[8]

In the present study, insecticide susceptibility was compared among three color morphs of *D. citri*, and expression of five *CYP4* genes and proteins related to insecticide susceptibility in this species was analysed. Insecticide susceptibility and activity of related enzyme systems are known to vary among color morphs in other insect species,[20–22,39] and color morphs also vary in their susceptibility to natural enemies.[37,40] In the present study, the orange/yellow morph was more susceptible to chlorpyrifos, fenpropathrin and imidacloprid than the blue/green and gray/brown morphs. Similar results were found with *M. persicae*, where the orange morph was characterized by higher esterase activity and LC_{50} values (to methomyl and acephate) than red and green morphs.[22] However, the red morph of *M. persicae* was

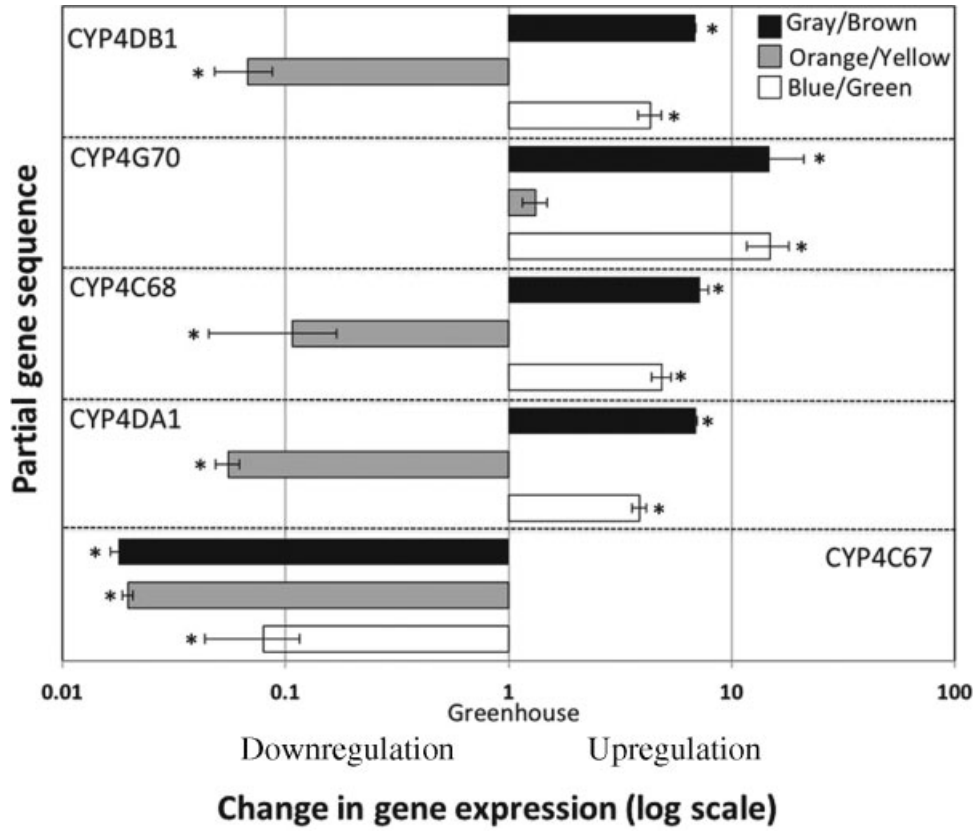

Figure 2. Quantification of the transcription level for five *CYP4* genes in three field-collected morphs and one greenhouse (insecticide-susceptible) morph of *Diaphorina citri*. C_t values were first normalized to the endogenous control gene, *Actin*, followed by normalization to the treatment giving the lowest gene expression using the $2^{-\Delta\Delta C_t}$ method. Standard errors were calculated on the basis of three independent experiments with three technical replicates each. Asterisks indicate statistically different expression as compared with the greenhouse gray/brown morph ($P < 0.05$).

more resistant to dimethoate, endosulfan and lambda-cyhalothrin than the green morph.[21] Variation in insecticide susceptibility among color morphs has also been reported for the tobacco aphid, *M. nicotianae*. In this species, the red morph is less susceptible to malathion, monocrotophos, acephate and the esterase inhibitor *S,S,S*-tributyl phosphorotrithioate than the green morph.[39] Lower carboxylesterase activity was reported in the green morph of *M. nicotianae* than in the red morph.[20] The above results confirm that insecticide susceptibility varies among insect color morphs, and, in a few instances, this is related to variation in enzymatic activity among different morphs.

Resistance conferred by cytochrome P450 monooxygenases is the most frequent form of metabolism-based resistance, followed by resistance conferred by esterases and glutathione *S*-transferases.[41] Cytochrome-P450-dependent monooxygenases have been implicated in resistance to pyrethroid, neonicotinoid, organophosphate and spinosyn insecticides.[42–46] In the present study, the expression levels of five *CYP4* genes were compared among three field-collected morphs and one greenhouse (insecticide-susceptible) morph to determine whether there was a relationship between insecticide susceptibility levels and expression levels of these *CYP4* genes. All five of these *CYP4* genes were induced in response to varying doses of imidacloprid and therefore may be associated with insecticide detoxification in *D. citri*.[30] Field populations of *D. citri* exhibiting more than 30-fold resistance to imidacloprid are also characterized by greater expression of cytochrome-P450-dependent monooxygenases.[26] The present results indicated lower expression levels of *CYP4C68*, *CYP4DA1*, *CYP4G70* and *CYP4DB1* in orange/yellow than in blue/green and gray/brown morphs. These results are consistent with the hypothesis that expression of *CYP4* genes is related to insecticide susceptibility levels in *D. citri*.

The western blot indicated lower expression of cytochrome P450 proteins in the orange/yellow morph than in the other morphs examined, which was congruent with the observed expression levels of the *CYP4* genes examined. Previously, the partial sequences of these genes in *D. citri* were identified.[30] Given partial sequencing, the molecular masses of the corresponding proteins are unknown. As expected, lower expression of *CYP4* genes was correlated with lower expression of corresponding protein(s) in the orange/yellow morph than in the other morphs examined. Increased quantity of cytochrome-P450-dependent monooxygenases, corresponding to increased *CYP* gene transcripts, is associated with lower insecticide susceptibility.[47–49] The present protein analysis suggests a variation in cytochrome P450 protein levels among the three color morphs that relates to their susceptibility to insecticides. Further investigations are needed to synthesize the antibody-based amino acid sequences of *CYP4* genes and to conduct a western blot assay for each individual protein.

The present results indicate that insecticide susceptibility of *D. citri* is associated with color polymorphism. This variation in color-related insecticide susceptibility may be explained by differential expression of *CYP4* genes, which code for cytochrome P450 enzymes/proteins involved in insecticide detoxification. However, changes in gene expression levels over time as a function of

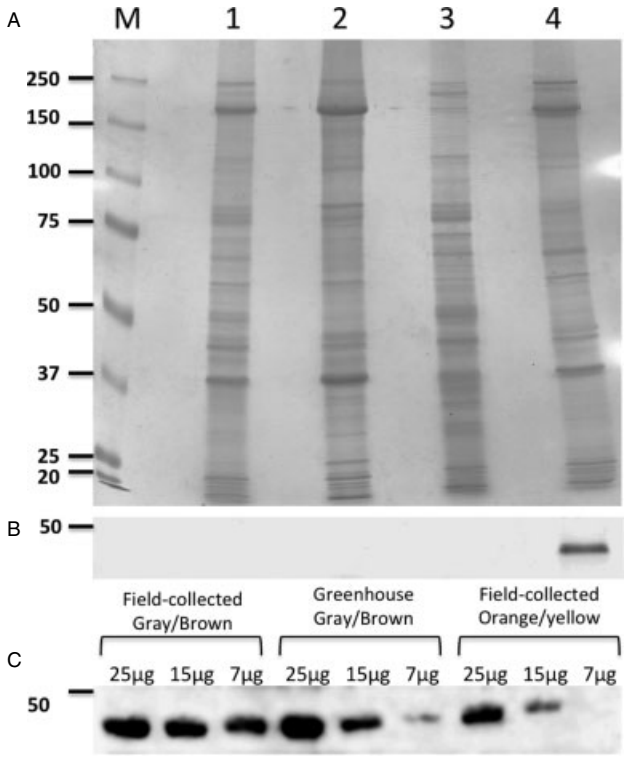

Figure 3. Immunodetection of cytochrome P450. (A) Subcellular protein fractions of the greenhouse (insecticide-susceptible) morph of *Diaphorina citri* analyzed by SDS-PAGE using 10% polyacrylamide gel stained with colloidal Coomassie blue G250. Protein fractions were prepared using methods described by Wheeler *et al.*[33] A quantity of 25 µg of protein was loaded from each fraction, represented by 1, 2, 3 and 4 as total proteins, mitochondrial proteins, cytosolic proteins and microsomal proteins, respectively. M refers to the molecular mass marker. (B) Western blot performed using the four protein fractions described in (A). The primary antibody consisted of a commercial polyclonal rabbit antibody (anticytochrome P450 19A1). Alkaline-phosphatase-conjugated goat antirabbit IgGs were used as a secondary antibody, and detection was performed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT). (C) Western blot performed using three quantities of microsomal proteins prepared from the three color morphs of *D. citri*.

insecticide susceptibility will require further investigation. Studies are also needed to determine the role of carotenoids or other pigments in color development of the three *D. citri* morphs. The color trait in various morphs of *D. citri* may be related to the differential expression of several other genes, including additional *CYP4* genes, or it may simply be an incidental correlation with insecticide susceptibility. Future microarray analysis should provide a more comprehensive description of gene expression associated with insecticide susceptibility in *D. citri*. The present results indicate that future monitoring of insecticide resistance for *D. citri* will need to account for the color morph trait. It is also possible that these data may have implications for *D. citri* management. If a specific color morph dominates the population during certain points of the season,[8] it may influence the choice of insecticide chemistry applied for *D. citri* management. In addition to the color morph trait, the presence of Las infection in *D. citri* may influence management. Previously, the present authors reported reduced insecticide susceptibility to chlorpyrifos and spinetoram in Las-infected when compared with uninfected *D. citri* adults.[23] Therefore, both the rate of Las infection and the prevalence of specific color morphs may be factors to consider in management decisions for *D. citri*.

ACKNOWLEDGEMENTS

This project was supported by a grant to LLS from the Citrus Research and Development Foundation. The authors acknowledge R Blanco, Y Cruz-Plemons, D Diaz, M Flores, S Holladay, A Hoyte and I Jackson for *D. citri* collection and technical assistance. Thanks to Paul Clayson and Wendy Meyer for reviews of earlier versions of this manuscript.

REFERENCES

1 Clarke CA, Dickson CGC and Sheppard PM, Larval color pattern in *Papilio demodocus*. *Evolution* **17**:130–137 (1963).
2 Oneill KM and Evans HE, Alternative male mating tactics in *Bembecinus quinquespinosus* (Hymenoptera, Sphecidae) – correlations with size and color variation. *Behav Ecol Sociobiol* **14**:39–46 (1983).
3 Sandre S, Tammaru T and Mänd T, Size-dependent colouration in larvae of *Orgyia antiqua* (Lepidoptera: Lymantriidae): a trade-off between warning effect and detectability? *Eur J Entomol* **104**:745–752 (2007).
4 Jong P, Gussekloo S and Brakefield P, Differences in thermal balance, body temperature and activity between non-melanic and melanic two-spot ladybird beetles (*Adalia bipunctata*) under controlled conditions. *J Exp Biol* **199**:2655–2666 (1996).
5 Forsman A, Thermal capacity of different colour morphs in the pygmy grasshopper *Tetrix subulata*. *Ann Zool Fenn* **34**:145–149 (1997).
6 Ahnesjo J and Forsman A, Differential habitat selection by pygmy grasshopper color morphs; interactive effects of temperature and predator avoidance. *Evol Ecol* **20**:235–257 (2006).
7 Marriott CG and Holloway GJ, Colour pattern plasticity in the hoverfly, *Episyrphus balteatus*: the critical immature stage and reaction norm on developmental temperature. *J Insect Physiol* **44**:113–119 (1998).
8 Wenninger EJ and Hall DG, Daily and seasonal patterns in abdominal color in *Diaphorina citri* (Hemiptera: Psyllidae). *Ann Entomol Soc Am* **101**:585–592 (2008).
9 Weisgraber K, Lousberg R and Weiss U, The chemical basis of color dimorphism of an aphid, *Macrosiphum liriodendri* (Monell), and a locust, *Amblycorypha sp.* novel carotenoids. *Cell Mol Life Sci* **27**:1017–1018 (1971).
10 Jenkins RL, Loxdale HD, Brookes CP and Dixon AFG, The major carotenoid pigments of the grain aphid, *Sitobion avenae* (F.) (Hemiptera: Aphididae). *Physiol Entomol* **24**:171–178 (1999).
11 Tsuchida T, Koga R, Horikawa M, Tsunoda T, Maoka T, Matsumoto S, *et al*, Symbiotic bacterium modifies aphid body color. *Science* **330**:1102–1104 (2010).
12 Lambers DHR, Polymorphism in aphididae. *Annu Rev Entomol* **11**:47–78 (1966).
13 Rowell CHF, The variable coloration of the acridoid grasshoppers. *Adv Insect Physiol* **8**:145–198 (1971).
14 Karlsson M and Forsman A, Is melanism in pygmy grasshoppers induced by crowding? *Evol Ecol* **24**:975–983 (2010).
15 Langley SA, Tilmon KJ, Cardinale BJ and Ives AR, Learning by the parasitoid wasp, *Aphidius ervi* (Hymenoptera: Braconidae), alters individual fixed preferences for pea aphid color morphs. *Oecologia* **150**:172–179 (2006).
16 Tsai JH and Liu YH, Biology of *Diaphorina citri* (Homoptera: Psyllidae) on four host plants. *J Econ Entomol* **93**:1721–1725 (2000).
17 Skelley LH and Hoy MA, A synchronous rearing method for the Asian citrus psyllid and its parasitoids in quarantine. *Biol Control* **29**:14–23 (2004).
18 Wenninger EJ, Stelinski LL and Hall DG, Relationships between adult abdominal color and reproductive potential in *Diaphorina citri* (Hemiptera: Psyllidae). *Ann Entomol Soc Am* **102**:476–483 (2009).
19 Halbert SE and Manjunath KL, Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. *Fla Entomol* **87**:330–353 (2004).
20 Harlow CD, Southern PS and Lampert EP, Geographic-distribution of two color forms, carboxylesterase activity, and chromosome configuration of the tobacco aphid (Homoptera, Aphididae) in North Carolina. *J Econ Entomol* **84**:1175–1179 (1991).
21 Kerns DL, Palumbo JC and Byrne DN, Relative susceptibility of red and green color forms of green peach aphid to insecticides. *Southwest Entomol* **23**:17–24 (1998).
22 Srigiriraju L, Semtner PJ, Anderson TD and Bloomquist JR, Esterase-based resistance in the tobacco-adapted form of the green peach

aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), in the Eastern United States. *Arch Insect Biochem Physiol* **72**:105–123 (2009).
23 Tiwari S, Pelz-Stelinski K and Stelinski LL, Effect of *Candidatus* Liberibacter asiaticus infection on susceptibility of Asian citrus psyllid, *Diaphorina citri*, to selected insecticides. *Pest Manag Sci* **67**:94–99 (2011).
24 Tiwari S, Pelz-Stelinski K, Mann RS and Stelinski LL, Glutathione transferase and cytochrome P450 (general oxidase) activity levels in *Candidatus* Liberibacter Asiaticus-infected and uninfected Asian citrus psyllid (Hemiptera: Psyllidae). *Ann Entomol Soc Am* **104**:297–305 (2011).
25 Prabhaker N, Castle S, Byrne F, Henneberry TJ and Toscano NC, Establishment of baseline susceptibility data to various insecticides for *Homalodisca coagulata* (Homoptera: Cicadellidae) by comparative bioassay techniques. *J Econ Entomol* **99**:141–154 (2006).
26 Tiwari S, Mann RS, Rogers ME and Stelinski LL, Insecticide resistance in field populations of Asian citrus psyllid in Florida. *Pest Manag Sci* **67**:1258–1268 (2011).
27 Abbott WS, A method of computing the effectiveness of an insecticide. *J Econ Entomol* **18**:265–267 (1925).
28 *SAS User's Guide*. SAS Institute, Cary, NC (2004).
29 Scharf ME, Zhou X and Schwinghammer MA, Application of RNA interference in functional genomics studies of a social insect, in *Methods in Molecular Biology: siRNA, shRNA and miRNA Protocols*, ed. by Barik S. Humana Press, Totowa, NJ, pp. 205–229 (2008).
30 Tiwari S, Gondhalekar AD, Mann RS, Scharf ME and Stelinski LL, Characterization of five *CYP4* genes from Asian citrus psyllid and their expression levels in *Candidatus* Liberibacter asiaticus-infected and uninfected psyllids. *Insect Mol Biol* **20**:733–744 (2011).
31 Livak KJ and Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**:402–408 (2001).
32 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**:248–254 (1976).
33 Wheeler MM, Tarver MR, Coy MR and Scharf ME, Characterization of four esterase genes and esterase activity from the gut of the termite *Reticulitermes flavipes*. *Arch Insect Biochem Physiol* **73**:30–48 (2010).
34 Mouchés C, Vignault J, Tully J, Whitcomb R and Bové J, Characterization of spiroplasmas by one- and two-dimensional protein analysis on polyacrylamide slab gels. *Curr Microbiol* **2**:69–74 (1979).
35 Husain MA and Nath D, The citrus psylla (*Diaphorina citri*, Kuw.) [Psyllidae: Homoptera]. *Mem Dep Agric India Entomol Ser* **10**:1–27 (1927).
36 Nijhout HF and Wheeler DE, Juvenile hormone and the physiological basis of insect polymorphisms. *Q Rev Biol* **57**:109–133 (1982).
37 Losey JE, Ives AR, Harmon J, Ballantyne F and Brown C, A polymorphism maintained by opposite patterns of parasitism and predation. *Nature* **388**:269–272 (1997).
38 Moran NA and Jarvik T, Lateral transfer of genes from fungi underlies carotenoid production in aphids. *Science* **328**:624–627 (2010).
39 Harlow CD and Lampert EP, Resistance mechanisms in two color forms of the tobacco aphid (Homoptera: Aphididae). *J Econ Entomol* **83**:2130–2135 (1990).
40 Harmon JP, Losey JE and Ives AR, The role of vision and color in the close proximity foraging behavior of four coccinellid species. *Oecologia* **115**:287–292 (1998).
41 Scott JG, Cytochromes P450 and insecticide resistance. *Insect Biochem Molec Biol* **29**:757–777 (1999).
42 Komagata O, Kasai S and Tomita T, Overexpression of cytochrome P450 genes in pyrethroid-resistant *Culex quinquefasciatus*. *Insect Biochem Molec Biol* **40**:146–152 (2010).
43 Bass C, Carvalho RA, Oliphant L, Puinean AM, Field LM, Nauen R, *et al*, Overexpression of a cytochrome P450 monooxygenase, *CYP6ER1*, is associated with resistance to imidacloprid in the brown planthopper, *Nilaparvata lugens*. *Insect Mol Biol* **20**:763–773 (2011).
44 Shang QL, Pan Y, Fang K, Xi JH and Brennan JA, Biochemical characterization of acetylcholinesterase, cytochrome P450 and cross-resistance in an omethoate-resistant strain of *Aphis gossypii* Glover. *Crop Prot* **31**:15–20 (2012).
45 Scott JG, Toxicity of spinosad to susceptible and resistant strains of house flies, *Musca domestica*. *Pestic Sci* **54**:131–133 (1998).
46 Sial AA, Brunner JF and Garczynski SF, Biochemical characterization of chlorantraniliprole and spinetoram resistance in laboratory-selected obliquebanded leafroller, *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae). *Pestic Biochem Phys* **99**:274–279 (2011).
47 Pittendrigh B, Aronstein K, Zinkovsky E, Andreev O, Campbell B, Daly J, *et al*, Cytochrome P450 genes from *Helicoverpa armigera*: expression in a pyrethroid-susceptible and -resistant strain. *Insect Biochem Molec Biol* **27**:507–512 (1997).
48 Berge JB, Feyereisen R and Amichot M, Cytochrome P450 monooxygenases and insecticide resistance in insects. *Phil Trans R Soc B* **353**:1701–1705 (1998).
49 Kasai S, Weerashinghe IS, Shono T and Yamakawa M, Molecular cloning, nucleotide sequence and gene expression of a cytochrome P450 (CYP6F1) from the pyrethroid-resistant mosquito, *Culex quinquefasciatus Say*. *Insect Biochem Molec Biol* **30**:163–171 (2000).

