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Determination of acaricide resistance allele frequencies in field populations of *Tetranychus urticae* using quantitative sequencing

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ABSTRACT

Resistances to monocrotophos, fenpropathrin and abamectin in *Tetranychus urticae* are primarily conferred by reduced sensitivities of respective target sites [i.e., acetylcholinesterase (TuAChE), voltage-sensitive sodium channel (TuVSSC) and glutamate-gated chloride channel (TuGluCl)], which are due to point mutations (G228S and F439W in TuAChE; L1022V in TuVSSC; G323D in TuGluCl). As a population-based genotyping technique, a quantitative sequencing (QS) protocol was developed for the determination of the resistance-associated mutation frequencies in *T. urticae*. Standard prediction equations revealed high correlation coefficients (r^2 = 0.993–0.999), demonstrating that the resistant nucleotide signal ratio is highly proportional to the resistance allele frequencies. The lower and higher detection limits for the four resistance mutations were 3.7–13.1% (7.8 ± 3.3%) and 89.4–97.3% (93.3 ± 3.2%), respectively, suggesting that QS can be employed as a preliminary monitoring tool for the detection of resistance allele frequencies, which ranged approximately 7.8–93.3% at the 95% confidence level. The QS was successfully employed for the determination of resistance allele frequencies in 26 *T. urticae* populations. The two TuAChE mutations responsible for monocrotophos resistance were almost saturated in most field populations. The TuVSSC L1022V mutation tentatively associated with fenpropathrin resistance was also found in 9 field populations. However, the TuGluCl G323D mutation conferring abamectin resistance was found only in one field population, suggesting that abamectin resistance is not yet widespread. The QS protocol, as an alternative to traditional bioassays, will greatly facilitate resistance monitoring of *T. urticae*.

Introduction

The two-spotted spider mite, *Tetranychus urticae* Koch, is a notorious pest of vegetable crops, fruit trees and ornamental plants worldwide. Over 90 active ingredients of acaricides have been used for the control (Whalon et al., 2008) and resulted in emergence of resistant population to almost all types of acaricides in *T. urticae* (Croft and Van De Baan, 1988; Houck, 1994; Koh et al., 2009; Van Leeuwen et al., 2010).

Early resistance monitoring is an essential step for the efficient pest management. Traditionally, several bioassay methods, such as spray and leaf dipping, have been widely employed for resistance monitoring in *T. urticae*, but they require a large commitment of time and resources. As alternatives of traditional bioassay-based resistance monitoring, various molecular techniques have been developed for the detection of resistance allele frequency at the individual and population levels (Clark et al., 2001; Clark, 2009). PCR-based methods, such as PASA (PCR Amplification of Specific Alleles) (Sommer et al., 1989) and bi-PASA (Liu et al., 1997), have been introduced for the detection of several point mutations that are associated with insecticide resistance, such as the A302S mutation (*rdl*) of GABA receptor channel associated with cyclodiene resistance (Ffrench-Constant et al., 1994; Ffrench-Constant et al., 2000) and the L1014F and T929I mutations of the voltage-sensitive sodium channels associated with pyrethroid resistance (Jamroz et al., 1998; Clark et al., 2001; Kwon et al., 2004a). The PASA-based diagnostic technique is a rapid and accurate method for individual genotyping, but it requires a large number of sample analyses for reliable estimation of resistance allele frequencies. Population genotyping methods based on pooled DNA samples (Amos et al., 2000), such as real-time PCR amplification of specific allele (rtPASA) (Germer et al., 2000), real-time PCR with allele-specific TaqMan probes (Livak, 1999) and quantitative sequencing (QS) (Wilkening et al., 2005), have been developed to reduce the time and cost associated with individual genotyping. These methods have been applied for the determination of insecticide resistance allele frequencies (Kwon et al., 2004b). Among these, QS was evaluated to be the simplest and highly accurate in population genotyping. QS has been successfully applied for the determination of resistance allele frequencies in the head louse (Kwon et al., 2008), the bed bug (Seong et al., 2010) and *T. urticae* (Kwon et al., 2010b).

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Reduced target site sensitivity caused by various point mutations has been linked to resistance in *T. urticae* to a variety of acaricides. Several mutations (G228S, A309S, G436A and F439W) in the acetylcholinesterase (TuAChE) are known to confer resistance to organophosphate (OP) acaricides (Khajehali et al., 2010; Kwon et al., 2010b). More recently, mutations (1022V, A1366D and F1538) in the voltage-sensitive sodium channel (TuVSSC) (Tsagkarakou et al., 2009; Kwon et al., 2010a), the G323D mutation in the glutamate-gated chloride channel (TuGluCl) (Kwon et al., 2010e) and several mutations (G126S, I136T, S141F and P262T) in the mitochondrial cytochrome b gene (Van Leeuwen et al., 2008) have been reported to be responsible for resistances to pyrethroid, avermectin and carbazate acaricides, respectively.

In this study, we established prediction equations for the estimation of the TuVSSC L1022V and TuGluCl G323D mutation frequencies based on QS and estimated the resistance allele frequencies to monocrotophos, fenpropathrin and abamectin in field-collected *T. urticae* populations.

Material and methods

Mite strains and rearing conditions

Field populations of *T. urticae* were collected from various host plants from 25 different localities between the years 2002 and 2008 and stored at −80 °C until use (Table 1). Some *T. urticae* field strains were maintained on soybean as a host in rearing room at 25 °C and 50–60% humidity.

Genomic DNA extractions

The pooled genomic DNA (pooled gDNA) of *T. urticae* was extracted from 50 to 100 female adults using the DNeasy Blood & Tissue kit (Qiagen GmBH, Germany) following the manufacturer's instructions. Briefly, the mites were ground using a 0.1-ml glass-polypropylene homogenizer (Thomas Scientific, Swedesboro, NJ, USA) in tissue lysis buffer and incubated with 10 μl proteinase K (10 mg/ml) at 56 °C for 1 h. The gDNA was eluted using Mini-spin columns and stored at −20 °C until use.

Establishment of quantitative sequencing protocols

We newly designed QS linear regressions for L1022V in TuVSSC and G327D on TuGluCl following the previously published procedures (Kwon et al., 2008, 2010b). QS linear regression for G228S and F439W was previously reported by Kwon et al. (2010b).

Briefly, 390- and 400-bp genomic DNA fragments of *TuVSSC* and TuGluCl, respectively, were amplified to obtain the susceptible and resistant templates following PCR conditions: a thermal cycler program of 35 cycles of 94 °C/30 s, 58 °C/30 s, and 72 °C/70 s. The sequences of resulting PCR products were each analyzed by nested primers, 3′ TSSMscgQS(N) and 5′GluGC3′RACE(N), respectively (Table 2 and Fig. 1). All primers for QS analysis were designed from the highly conserved exon regions of gDNA to avoid amplification errors due to the nucleotide variation usually found in intron region. The PCR fragments obtained from respective reference templates were TA-cloned into a pGEM-T easy vector® (Promega, Madison, WI) according to the manufacturer's protocol to isolate individual DNA fragment with reference genotypes. The reference PCR products were mixed in the following molar ratios: 0:10, 0.5:9.5, 1:9, 3:7, 5:5, 7:3, 9:1, 9.5:0.5 and 0:10 (resistant allele:susceptible allele at each mutation site) to produce the standard DNA template mixtures after normalization by using the Low DNA mass ladder (Invitrogen) on agarose gel electrophoresis. All other procedures for the construction of QS were the same as those reported previously (Kwon et al., 2008, 2010b).

Table 1
Tetranychus urticae collection sites.

No.	Strains	Collection sites	Host plants	Date	Remarks
1	UD	Ulleung-eup, Ulleung-gun, Gyeongbuk	Greater celandine	Jun., 2006	Laboratory strain
2	PyriF	Susceptible laboratory strain	Soybean	Jul., 2005*	
3	GSS	Susceptible laboratory strain	Soybean	Nov., 2005*	
4	KRICT	Susceptible laboratory strain	Soybean	Apr., 2004*	
5	SW2006	Gweonseon-gu, Suweon-si, Gyeonggi-do	Apple tree	Sept., 2006	Field-collected
6	YS2006	Oga-myeon, Yesan-gun, Chungnam	Apple tree	Sept., 2006	
7	CJ2006	Noeun-myeon, Chungju-si, Chungbuk	Apple tree	Sept., 2006	
8	SW2004	Gweonseon-gu, Suweon-si, Gyeonggi-do	Apple tree	Oct., 2004	
9	YS2004	Oga-myeon, Yesan-gun, Chungnam	Apple tree	Oct., 2004	
10	CJ2004	Noeun-myeon, Chungju-si, Chungbuk	Apple tree	Oct., 2004	
11	SW2002	Gweonseon-gu, Suweon-si, Gyeonggi-do	Apple tree	Sept., 2002	
12	YS2002	Oga-myeon, Yesan-gun, Chungnam	Apple tree	Sept., 2002	
13	CJ2002	Noeun-myeon, Chungju-si, Chungbuk	Apple tree	Sept., 2002	
14	N1	Sangweol-myeon, Nonsan-si, Chungnam	Strawberry	Apr., 2006	
15	N2	Sangweol-myeon, Nonsan-si, Chungnam	Strawberry	Apr., 2006	
16	N3	Sangweol-myeon, Nonsan-si, Chungnam	Strawberry	Apr., 2006	
17	N4	Boojeok-myeon, Nonsan-si, Chungnam	Strawberry	Apr., 2006	
18	N5	Yeonsan-myeon, Nonsan-si, Chungnam	Strawberry	Apr., 2006	
19	CA	Dongnam-gu, Cheonan-si, Chungnam	Strawberry	Apr., 2006	
20	TA	Taean-eup, Taean-gun, Chungnam	Strawberry	Apr., 2006	
21	SW1	Jangan-gu, Suweon-si, Gyeonggi	Strawberry	Apr., 2006	
22	GC	Daesan-myeon, Gochang-gun, Jeonbuk	Watermelon	Jun., 2008	
23	GS	Gyeongsan-si, Gyeongbuk	Melon	Aug., 2008	
24	SJ	Seongjoo-si, Gyeongbuk	Melon	Aug., 2008	
25	YJ	Yeongjoo-si, Gyeongbuk	Melon	May, 2008	
26	SW2(= AbaR)	Jangan-gu, Suweon-si, Gyeonggi	Rose	Oct., 2007	

Asterisk represents the date of acquisition from research institutions.

Allele frequency determination from field strains

The partial fragment containing each point mutation from the pooled gDNA of 26 strains was PCR-amplified by using sequence-specific primer sets (Table 2) following the same conditions mentioned above. The signal ratio at each mutation site was substituted to corresponding prediction equation for the estimation of resistance allele frequency of each point mutation.

Results and discussion

Establishment of QS-based frequency prediction equations for the TuVSSC and TuGluCl mutations

In the previous report, equations for the prediction of the TuAChE mutation (G228S, A391T and F439W) frequencies were established with