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Research Article

Resistance mechanisms of the two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae) populations to fenpyroxymate

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Abstract: The two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae) is one of the most important pests of field crops, orchard trees and ornamentals around the world. The short life cycle, high reproductive potential, accompanied by frequent acaricide applications have caused resistance development to wide range of acaricides. In this study the susceptibility of two populations, collected from Karaj and Mahllat, was investigated against fenpyroximate. The bioassay test was carried out by using the leaf-dip method. The results showed that the LC_{50} values for Karaj and Mahallat population were 2.1 and 92 (mg/ml), respectively. The resistance ratio was 43.8. The enzyme assay results revealed that the activity ratios of esterase in Mahallat to Karaj populations were 2.5 and 1.2 when α-NA and β-NA were used as a substrate, respectively. The activity of cytochrome P_{450} in Mahallat population was 1.37 times higher than the Karaj population. There was no significant difference in glutathion S-transferase activity between the two populations. The gene expression (qRT-PCR) results showed that the expression level of CYP392A11 in Mahallat population was 3.52 times higher than Karaj population. These results suggested that esterase and cytochrome P_{450} monoxygenase are probably involved in resistance of *T. urticae* to fenpyroximate.

Keywords: Biochemical assay, Cytochrome P_{450}, Detoxification enzymes, Glutathion S-transferase, qRT-PCR

Introduction

The two spotted mite *Tetranychus urticae* Koch is an important pest of crops, leading to considerable economic damage. Using of synthetic pesticides is one of the main methods of controlling this pest in Iran hence, acaricides are widely used against the pest. The high potential of reproduction and short life cycle combined with the frequent use of acaricides to keep the population under the economic threshold has fortified the mite's ability to develop resistance to acaricides. Therefore, the use of new pesticides intermittently, to prevent the pest resistance phenomenon, is inevitable (Herron *et al*., 1998). Although the registered acaricides in Iran are of different chemical groups, they comprise less than 20% of the proprietary produced acaricides in the world. This limitation on the variety of acaricides causes the continu-

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ous application of acaricides in a crop season and intensifies the resistance in the population of various crop-damaging mites. Resistance causes problems such as greater damage to crops, endangering human health, lower crop quality, increased spraying rates and increased pesticide use (Brown, 2003). Insecticide resistance is a decrease in the susceptibility of the pest population to the chemical, which has previously been effective in pest control. The high reproducibility (12-25) and multiple generations are the two most important features of the species which lead to resistance. Continued use of a specific pesticide activates enzymes in the body, which decompose and inactivate the pesticide, reducing its effect or preventing it from reaching the target site. Perhaps more than 90% of insect and mite resistance cases are caused by reduced target site sensitivity or increased pesticide detoxification. The three major enzyme groups involved in metabolic resistance to insecticides and killers include esterases, glutathione S-transferases and monooxygenases (Devorshak and Roe, 1998). In a study of three field populations of *T. urticae* on abamectin resistance, Stumpf and Nauen (2002) found that the increase in 1-dimethylation activity by the activity of cytochrome P_{450} monooxygenases was accompanied by an increase in the activity of glutathione S-transferase resistance factors. Kim *et al*., (2007) examined the resistance of *T. urticae* to dicofol and found that "this population" was highly resistant to dicofol with a 465 folds resistance rate. Van Asperen (1962) studied on the mechanisms of multiple resistances to abamectin in two-spotted spider mites and showed that the involvement of esterase and P_{450} monooxygenases in abamectin resistance is by synergistic bioassays, in which LT_{50} decreases significantly by pretreatment with triphenylphosphate and p-propyl butoxide. Assessment of detoxification enzymes suggested that enzymes esterase and P_{450} monooxygenases are metabolic factors in abamectin resistance, and triphenylphosphate acts as inhibitor of cytochrome P_{450} monooxygenases and esterase (Ay and Kara, 2011). Memarizadeh *et al.* (2013) investigated the resistance of two-spotted mites to abamectin in two populations in vitro. The enzymatic assay showed that using alpha naphthyl acetate and alpha naphthyl propionate substrates the esterase activity in the resistant population was 2.14 and 1.33 times higher than the susceptible, respectively. The results also showed that cytochrome P_{450} was 1.37 fold higher in the resistant population than the susceptible population. Mahdavi Moghadam *et al.* (2012) investigated the mechanism of fenazaquin resistance in Iranian populations of two-spotted mite and showed that the Isfahan, Yazd and Rasht populations were 3109, 439 and 10.53 times more resistant than the susceptible population, respectively. The enzymatic results showed that esterase activity in the Isfahan, Yazd and Rasht populations was 3.9, 1.8 and 1.5 times higher than the susceptible population, respectively. Zamani *et al.* (2014) investigated the toxicity of chlorpyrifos in three populations of two-spotted spider mites collected from Isfahan, Yazd and Guilan and showed that the population resistance rates of Isfahan and Yazd were 176.9 and 9.78 times higher than Guilan, respectively. Determination of esterase and glutathione S-transferase activity and kinetic parameters showed that monooxygenase was highest in Isfahan population. Riga *et al.* (2015) investigated the function of the CYP392A11 gene, cytochrome P_{450} in the hydroxylation of the two-spotted spider mite mitochondrial electron transfer chain system compared to the METI acaricides cyenopyrafen and fenpyroximate, and they found that the CYP392A11 and CYP392A12 genes of the cytochrome P_{450} strongly correlated with the phenotype. However, expression of CYP392A11 produced functional enzyme, with high activity. Khalighi *et al.* (2016) investigated the molecular basis of cyenopyrafen resistance in two-spotted spider mites and found that resistance of cyenopyrafen was strongly increased with biphenyl butoxide and was 15-fold higher than that of P_{450} in the resistant strain. Genome-wide gene expression analysis showed the overexpression of CYP392A11 and CYP392A12 genes coding for cytochrome P450 monooxygenases.

In this study, the mechanism of resistance of the Iranian populations of two-spotted mites was investigated using bioassay, biochemical and molecular tests.

Materials and Methods

Mite strains

The resistant strain to fenpyroximate was collected from infected Beans in plant pests and disease research institute of Mahalat, Markazi, Iran. Susceptible strain was collected from the research greenhouse in Department of Plant Protection, University of Tehran, Iran, which had no previous exposure to pesticides. The mites were reared routinely on leaves of common beans under greenhouse conditions at 25 ± 2 °C, 60 ± 5 RH and 16:8 (L:D) h.

Pesticide and Chemicals

Fenpyroximate was used as commercial formulation in the bioassay (Ortus® 5% SC) and was purchased from Gyah Corporation, Iran.

Alpha-naphthyl acetate, beta-naphthyl acetate, reduced glutathion, CDNB, TMBZ, Triton X100, fast blue RR salt, methanol, potassium phosphate and sodium acetate were purchased from Merck company (Germany).

Bioassay

The toxicity of fenpyroximate to two-spotted spider mite on the two collected population was assayed by the dipping method. Five serial dilutions of fenpyroximate were prepared in distilled water. The leaf disk (diameter 3.5 cm) was immersed in the serial dilutions for 45 seconds. After drying, adult mites were placed on each treated leaf disk on wet cotton in a Petri dish. Ten adult female two-spotted spider mites were placed on each leaf disk. Mortality was recorded after 48 hours. Bioassay data was analyzed for LC_{50} by 95% confidence intervals using the POLO-PC computer program (LeOra Software 1987). Resistance factor (RF) was calculated by dividing the LC_{50} value of the resistant strain to the LC_{50} value of the susceptible strain.

Esterase activity

Mite extracts were prepared by homogenizing 500 adult females in 100 µl phosphate buffer (0.1 M, pH 7.0 with 0.2% Triton X100). The homogenates were centrifuged at 10000 × g for 10 min at 4 °C. The evaluation of alpha or beta esterase activities was carried out by spectrophotometric assay method. The enzyme activity was recorded by absorbance changes of solution due to the formation of a diazo dye complex with substrate. Alpha-naphthyl acetate (alpha-NA) or beta-naphthyl acetate (beta-NA) were used as substrates for assaying alpha or beta esterase activities respectively. Briefly, 30 mM substrates (alpha-NA or beta-NA) were diluted in phosphate buffer 0.02 M in the ratio of (1:99). The samples (15 or 10 µl for alpha or beta esterase respectively), were added to 200 µl alpha-NA or beta-NA substrates. Then 50 µl, 0.075% Fast Blue RR salt (a diazonium salt) which was dissolved in distilled water in the ratio of 1:10 v/v poured in vials. The substrates were coupled to diazonium salt and formed a diazo dye complex. Finally, absorbance of produced dye complex was recorded at 450 nm for alpha-NA and 540 nm for beta-NA every 2 min for 10 minutes, continuously (Van Asperen, 1962). 1 U ($\mu mol.min^{-1}$) is determined as the amount of the enzyme that catalyzes the change of one µmol of substrate per minute under the specified conditions of the assay method. So, the $U.ml^{-1}$ activity of the enzyme was calculated based on the following formula:

$$\frac{(\Delta A)/\min \times (ml) \times dil}{\varepsilon \times V_{enz}(ml)} = \mu mol.ml^{-1}.\min^{-1} = U.ml^{-1}$$

Where ΔA/min = the initial slope of absorbance change, ε = molar absorption coefficient, and V, V_{enz} are the total volume and enzyme volume. The specific activity is the ratio of the enzyme activity divided by the protein concentration ($\mu mol.mg^{-1}.\ min^{-1} = U.mg^{-1}$).

Glutathione S-transferase (GST)

Assessment of glutathione S-transferase activity was performed based on Habig *et al.* (1974) method with little modifications. The samples were placed in each microplate (20 µl) plus 10 µl

of 1-chloro-2, 4-dinitrobenzene mixture (CDNB; 100 mM dissolved in methanol) and reduced glutathione (GSH; 25 mM) in the ratio of 1:50, giving final concentrations of 0.4 and 4mM of CDNB (or DCNB) and GSH, respectively. The CDNB coupled by GSH substrate and produced a dye complex. Then, the absorbance of the dye complex was recorded at 340 nm every 120 seconds for 20 minutes (Habig *et al.* 1974). The GST unit.ml^{-1} was calculated same as the formula presented for esterase activity.

Monooxygenase content

The amount of general oxidase was measured based on heme-proxidase method (Brogdon *et al.*, 1997). Briefly, each microplate contained the enzyme samples (20 µl), 80 µl of potassium phosphate buffer 0.625 M (pH 7.2), 200 µl of TMBZ (dissolved in methanol and sodium acetate buffer 0.25 M) and 25 µl of H_2O_2 (3%). After two hours of incubation at 25 °C (in the dark), absorbance was recorded at 450 nm (Brogdon *et al.*, 1997). The concentration of monooxygenase was achieved by comparing the absorbance data with a standard curve of purified cytochrome C. To measure the samples protein content, the Bradford (1976) method was used with BSA as standard. Hence, the concentration of monooxygenase was reported as Cytochrome C (mg)/Total protein content (mg).

RNA extraction and cDNA synthesis

For RNA extraction from the adult female of *T. urticae,* TRI Reagent kit (Sigma, Aldrich) was used with according to the manufacturer's protocol. The RNA quality was assessed by electrophoresis on 1% agar gel. Samples were treated with DNase I to remove DNA from the total RNA.cDNA was synthesized by reverse transcription using a cDNA synthesis kit AccuPower® RocketScript™ (Takara, Shinga, Japan).

Gene expression analysis

Real-time PCR was employed to determine the expression of CYP392A11. The reactions were performed with Eva green master mix 5 × HOT Gene-specific primers (Table 1) were designed using Primer Blast. PCR amplification using forward and reverse primers resulted in an approximately 100 bp fragment within the coding region of each gene. The transcript level of the ribosomal 18S of *T. urticae* was used as a reference to normalize the expression levels of CYP392A11. The expression of the cytochrome P_{450} gene was carried out in two susceptible and resistant populations of the two-spotted spider mite. The real-time PCR analysis was independently repeated three times for each sample. Temperature profile was 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s, 57 °C for 20 s and a final extension step at 72 °C for 20 s.

Data analysis

Data of bioassays were analyzed for calculating lethal concentrations by PoloPc and mean comparisons were performed using SAS software. Tukey's test ($P \leq 0.05$) was used to compare means in enzymes activity. After the PCR program and the data were analyzed with software Rest 2009. The threshold cycle (Ct) or the crossing points (CP) values were used to quantify the target gene expression for each sample (Pfaffl, 2001; Paim *et al.* 2012).

$$R = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. E_{target} is the real-time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; ΔCP_{target} is the CP deviation of control – sample of the target gene transcript; ΔCP_{ref} = CP deviation of control – sample of reference gene transcript (Pfaffl, 2001).

Results

Bioassays tests using leaf dip assay showed that LC_{50} of fenpyroximate on Karaj and Mahallat populations of *T. urticae* were 2.1 and 92 (mg/ml), respectively (Table 2). Comparison of

the 95% confidence interval of LC_{50} values in Karaj and Mahallat populations suggested that there was a significant difference between LC_{50} values of the populations which suggested the Mahallat population as the resistant population. Resistance ratio (RR) calculated as the ratio of Mahallat LC_{50}/Karaj LC_{50} was 43.8 folds. The line slopes of regression of the fenpyroximate dose-response in Karaj and Mahallat populations were 1.02 ± 0.24 and 6.3 ± 0.77, respectively.

The esterase activity in the Mahallat population was 2.5 and 1.2-fold higher than Karaj population when α-NA and β-NA was used as substrate, respectively (Table 3). Statistical analysis showed that there was no significant difference between esterase activity between Karaj and Mahallat populations when β-NA used as substrate, whereas there was significant difference between them when α-NA used as substrate.

Table 1 Primers used in the Real-time PCR reaction.

Primers	Forward	Reverse
re-18S	TTGCTTGTGTCTACGGATGC	GGCACCTCTTTCGAGGATTTG
Re-CYP392A11	CATGCAGCTTTTGTCCCTGG	TCAAGCGTGCTCGTACCAAG

Table 2 Comparison of Toxicity of fenpyroximate in Susceptible and Resistant Populations in *Tetranychus urticae.*

Population	Number	LC_{50} (95% CI) (mg/ml)	Slope ± SE	χ^2(df)	Rf
Susceptible	300	2.1(1.86-3.36)	1.02 ± (0.24)	0.39 (3)	43.8
Resistance	300	92.1 (91.2-92.8)	6.30 ± (0.77)	0.68 (3)	-

CI: Confidence interval, Rf: Resistant factor.

Table 3 Average activity of esterase in susceptible and resistant populations in *Tetranychus urticae*.

Substrate	Esterase activity ± SE (nmol/min/mg protein)		Activity Ratio (resistant/ susceptible)
	Resistant population	Susceptible population	
a-NA	0.78 ± 0.10	0.31 ± 0.05	2.5
β-NA	0.28 ± 0.04	0.23 ± 0.03	1.2

GST activity in the susceptible population was 0.032 ± 0.008 (nmol/min/mg protein) and for resistant population it was 0.038 ± 0.008 (nmol/min/mg protein) when CDNB was used as the substrate (Fig. 1). The results showed that there was no significant increase in value in the GST activity in the two populations.

The results of this assay, measuring the total amount of heme protein using a heme peroxidase assay, indicated that the heme content in the resistant population was 6.34 fold higher than that in the susceptible population (Fig. 2). This value in the susceptible population was 0.427 ± 0.07 (mg protein) and in the resistant population it was 2.71 ± 0.10 (mg protein).

Also, PCR results have been showed in fig. 3. The presence of a melting point reflects the specificity of the Real-Time PCR product. The expression of the cytochrome P_{450} gene in the susceptible and resistant populations of the two-spotted spider mite showed that the level of CYP392A11 transcript in the whole body of two-spotted spider mite in the resistant population was 3.52 more than the susceptible population ($P < 0.01$) (Fig. 3).

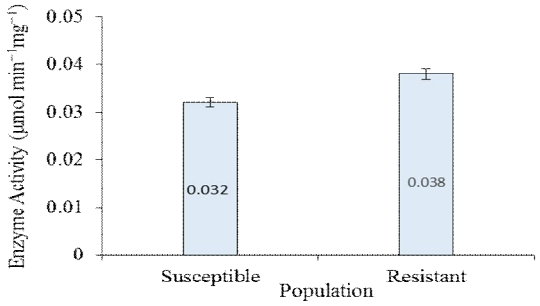

Figure 1 Comparison of activity of glutathione S-transferase enzyme in susceptible and resistant populations in two spotted mite.

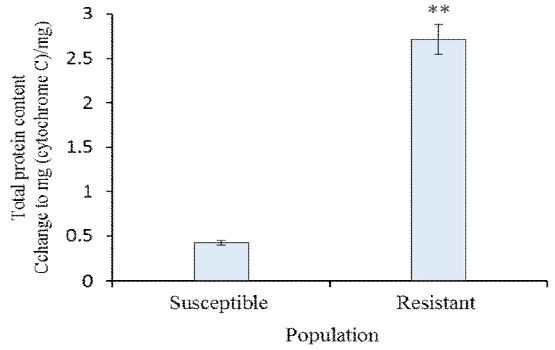

Figure 2 Cytochrome P_{450} monoxygenase levels in susceptible and resistant populations in two spotted mite.

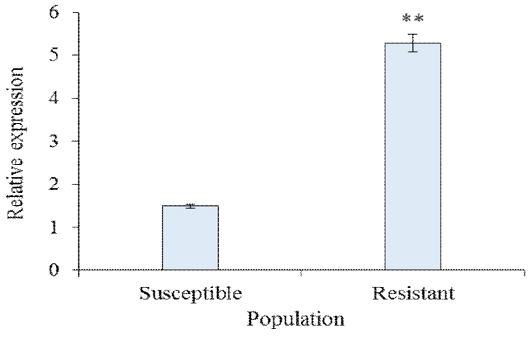

Figure 3 Comparison of relative expression of CYP392A11 gene in two susceptible and resistant populations in two spotted mite**.**

Discussion

Mahdavi Moghadam *et al.* (2012) investigated the mechanism of fenpyroximate resistance in Iranian populations of two-spotted mite and showed that the LC_{50} value of Isfahan, Yazd, Rasht and susceptible populations were 1541, 176, 5 and 0.4 (mg/ml) respectively, which is almost the same as our results. Zarei (2012) reported that the LC_{50} value of fenpyroximate on the two-spotted spider mite, *T. urticae*, was 132 µl/l that is relatively low in comparison with other studies. Previous studies have shown that the LC_{50} value of fenpyroximate was 111.81 mg/l (Hassan Poor, 2002). We have found some level of resistance in the mites collected from Mahallat; other studies have also reported resistance to other acaricides (Farahani *et al.* 2018). It seems that the mite resistance is beyond the laboratory level, because even the native farmers complain about the efficacy of a wide range of acaricides used against the *T. urticae* in Iran (Mohammadzadeh *et al.,* 2014).

Farahani *et al.* (2018) stated that there are several reasons for failure of chemical pest control including inappropriate timing of pesticide application, incorrect spraying techniques, undesirable quality of pesticides and pest resistance to pesticides. So every failure in pest control should not be considered as due to developing resistance to pesticides. The current study showed that Mahallat population of the *T. urticae* is resistant to fenpyroximate. Therefore, it seems that the main reason of failure in Mahallat population control is resistance development.

Our results showed that probably esterase plays a role in resistance of *T. urti*cae to fenpyroximate. These enzymes probably sequester or degrade insecticide esters before they reach their target sites in the nervous system (Ghadamyari and Jalali Sendi, 2009). Farahani *et al.*, (2018) reported that esterase activity rate in Mahallat population to Karaj populations was 1.91 times. They showed there was a significant difference in esterase activity between two populations. Mahdavi Moghadam *et al.* (2012) studied resistance mechanisms of Iranian populations of two-spotted spider mite to fenazaquin. Enzyme assay revealed that esterase activities in the Isfahan, Yazd and Rasht populations were 3.9, 1.8 and 1.5 more than that in the susceptible

population, respectively, while in our study α-naphthyl acetate result was not in conformity with those results. Mahdavi Moghadam *et al.* (2012) surveyed Resistance mechanisms to fenazaquin in Iranian populations of the two-spotted spider mite, and showed that Glutathione S-transferase (GST) activity in the Isfahan, Yazd and Rasht populations was 2.3, 2.4 and 1.4 times more than the susceptible population collected from Rasht, Iran, respectively. According to this result, it seems that cytochrome P_{450} has an important role in the observed resistance. Riga *et al.* (2015) revealed that cytochrome P_{450} had a crucial role in *T. urticae* resistance to fenpyroximate. It is not surprisingly because biochemical and synergism studies indicated that METI-resistance is, at least partially, associated with elevated cytochrome P450 monooxygenase (P450) activity in many different strains (Stumpf and Nauen, 2002; Tirrelo *et al*., 2012; Van Pottelberge *et al*., 2009).

Our study is consistent with previous reports showing a correlation between high P450 enzyme activities with fenpyroximate resistance (Khalighi *et al.* 2016). Riga *et al.* (2015) showed that the restructured functional monooxygenase complex of CYP392A11 is able of metabolizing fenpyroximate, a new acaricide that targets complex I. however, the strong association among the bioassays, molecular data and functional data powerfully advise a role of CYP392A11 in fenpyroximate detoxification, even though other mechanisms cannot be perfectly ignored at this stage. Our results demonstrated that the MFO is involved in the metabolism of fenpyroximate. MFO content in a resistant population was greater than in the susceptible population, also the high expression level of CYP392A11 in resistant population proved this claim. So, we conclude that MFO has an important role in developing resistance to fenpyroximate.

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مکانیسم‌های مقاومت کنه تارتن دولکه‌ای *Tetranychus urticae* (Acari: Tetranychidae) نسبت به کنه‌کش فن‌پیروکسی‌میت

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چکیده: کنه تارتن دولکه‌ای (Acari: Tetranychidae) *Tetranychus urticae* از آفات مهم درختان میوه، باغات و گیاهان زینتی در سراسر جهان می‌باشد. سیکل کوتاه زندگی، پتانسیل بالای تولیدمثل، همراه با کاربردهای مکرر آفت‌کش‌ها، منجر به توسعه مقاومت در برابر آفت‌کش‌های وسیع‌الطیف شده است. در این مطالعه میزان حساسیت دو جمعیت کنه تارتن دولکه‌ای جمع‌آوری شده از کرج و محلات به فن‌پیروکسی‌میت مورد بررسی قرار گرفت. آزمون‌های زیست‌سنجی به روش غوطه‌وری نشان داده که مقادیر LC_{50} در جمعیت کرج و محلات به‌ترتیب ۲/۱ و ۹۲ (میلی‌گرم/میلی‌لیتر) بود. نسبت مقاومت به فن‌پیروکسی‌میت در جمعیت‌های حساس و مقاوم ۴۳/۸ می‌باشد. نتایج سنجش آنزیمی نشان داد که با استفاده از سوبستراهای آلفانفتیل‌استات و بتانفتیل استات فعالیت استرازی در جمعیت محلات به‌ترتیب ۲/۵ و ۱/۲ برابر بیش‌تر از جمعیت کرج است. میزان سیتوکروم p_{450} در جمعیت محلات ۱/۳۷ برابر بیش‌تر از جمعیت کرج بود. فعالیت آنزیم گلوتاتیون اس-ترانسفراز تفاوت مشخص بین دو جمعیت را نشان نداد. نتایج کمّی PCR (qRT-PCR) نشان داد که میزان بیان ژن CYP392A11 در جمعیت محلات ۳/۵۲ برابر بالاتر از جمعیت کرج بود. این نتایج نشان می‌دهد که استراز و سیتوکروم p_{450} مونوکسیژناز احتمالاً در مقاومت کنه تارتن دولکه‌ای به فن‌پیروکسی‌میت نقش دارند.

واژگان کلیدی: سنجش بیوشیمیایی، سیتوکروم P_{450}، آنزیم‌های سم‌زدایی، گلوتاتیون اس-ترانسفراز، qRT-PCR