Differential Expression of Thaumatin-Like Proteins in Sorghum Infested with Greenbugs

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This study was designed to quantitatively analyze the expression of thaumatin-like protein (TLP) at the transcriptional level in different sorghum lines when they were infested with greenbugs. Three sorghum lines, Tx7000, PI550607, and PI550610, were used. RNAs were isolated from the different sorghum lines that were infested with greenbugs at different infestation times. The resultant mRNA was reverse transcribed into cDNA, and the RT-PCR products were separated by agarose gel. Then, real-time PCR data of the TLP gene expression were analyzed in comparison with the β-actin gene as a reference. The expression levels of the TLP gene were also compared between samples. The results showed that the transcripts of the TLP were induced by greenbug feeding and the increased levels were time-dependent. In the susceptible line, the TLP's transcripts increased several thousand-fold at 120 hours post infestation, while for the two resistant sorghum lines the TLP expression level increased less than one hundred-fold compared to the controls. This is the first demonstration that thaumatin-like proteins are involved in plant defense response against insects.

Key words: Greenbug, Sorghum, Thaumatin-Like Protein

Introduction

Plants have their inherent mechanisms for resistance to pathogens and many factors are involved in these mechanisms: some are preformed and some are inducible (Hammerschmidt, 1999). Pathogenesis-related (PR) proteins like chitinase, osmotins, and β-1,3-glucanase are defined as proteins that are encoded by the plant genome and induced specifically in response to infections by pathogens such as fungi, bacteria, or viruses, or by adverse environmental factors (Breiteneder, 2004). PR proteins are divided into several families. The PR-5 family has amino acid sequence similarities to thaumatin proteins, which are sweet and were first found as a mixture of proteins isolated from the katemefe fruits (Van der Wel and Loeve, 1972). These thaumatin-like proteins (TLPs) belong to the PR-5 family and some are involved in plant resistance mechanisms. There are three classes of thaumatin-like proteins: proteins produced in response to pathogen infection, osmotic proteins, and plant antifungal proteins (AFPs) which are constitutive in plants, especially seeds (Breiteneder, 2004).

TLPs, which are involved in pathogen resistance, can be induced by a large spectrum of pests (not only insects, but also viruses, bacteria, and fungi) and stimuli-like chemicals, wounding, cold stress etc. (Bol *et al.*, 1990; Lotan and Fluhr, 1990; Trudel *et al.*, 1998). For example, *Rhizoctonia solani*, the sheath blight fungus, caused the induction of TLPs in rice, based on molecular analysis, and two different TLPs involved in this mechanism were revealed by Western blotting (Velazhahan *et al.*, 1998). A recent study showed that, salicylic acid (SA) and jasmonic acid (JA) could induce TLPs and β-1,3-glucanases in wheat plants, and cause system-acquired resistance (SAR) leading to enhanced resistance to bacterial diseases. The induction level was time-dependent (Jayaraj *et al.*, 2004).

An antifungal TLP was isolated from sorghum leaves in 2002 (Velazhahan *et al.*, 2002). The

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aphid greenbug (*Schizaphis graminum*), one of the major pests of sorghum (*Sorghum bicolor* L. Moench), might be an inducer of this TLP in sorghum (Hammerschmidt, 1999; Porter *et al.*, 1997). Although many studies are about TLP's production when plants encounter outside stimuli, no study has quantitatively compared the time-dependent expression of TLPs in different sorghum cultivars, especially between cultivars resistant and susceptible to greenbugs.

A recent report showed that PR proteins were differentially expressed among different cultivars when barley plants interacted with their bacteria pathogen (Geddes *et al.*, 2008). It is reasonable to expect a similar differential expression of TLPs in sorghum at the cultivar level. Real-time PCR is a quantitative and time-dependent technique that is widely used in the molecular world. It is a promising tool for the in-depth study of TLP-related pathogen responses (Klein, 2002). For the present study, RT (reverse transcription)-PCR and relative real-time PCR techniques were used to quantitatively analyze the expression of the TLP at the transcription level of greenbug in the resistant lines PI550607 and PI550610, and one greenbug-susceptible line, Tx7000, when they were infested with greenbug biotype I.

Materials and Methods

Plant material and growth conditions

Three sorghum (*Sorghum bicolor* L. Moench) lines were used in this study: Tx7000, PI550607 and PI550610. Tx7000 is a greenbug-susceptible line, while PI550607 and PI550610 are greenbug-resistant lines. Each sorghum line was grown in five individual pots, which were designed for the time-dependent study. For each pot, 15 to 20 seeds were added into the soil and covered with a thin layer of soil. Sorghum plants were grown in pots at 20–25 °C for one week in a greenhouse.

Greenbug infestation

Aphid greenbug (*Schizaphis graminum)* biotype I was used in this study. Each pot of plants was infested with 15 to 20 greenbugs at the same age and at the same time except the control plants. Each infested plant was covered with a plastic cage. Control plants which were not infested with greenbugs [0 hpi (hours post infestation)] were collected as soon as the other sorghum seedlings were infested with greenbugs. The collected leaves were covered with foil paper and put into liquid nitrogen, then stored at –80 °C. The infested plants were collected at different post-infestation times: 12, 24, 72, and 120 hpi. The greenbugs were brushed off the leaves before storage.

RT-PCR and real-time RT-PCR

Extraction of mRNA

RNAs of the different sorghum lines from the different infestation durations were isolated. Tissue was homogenized with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and RNAs were separated by chloroform, precipitated, washed, and dissolved in diethylpyrocarbonate-treated (DEPC) water.

RT-PCR

5 μg of the total cellular RNA of each sample were heat-denatured at 65 °C for 5 min with 10x buffer (Invitrogen) and used as template for RT. RT reactions were performed using 50 U Superscript II reverse transcriptase (Invitrogen) at 42 °C for 60 min in the presence of 5x first strand buffer (Invitrogen), 0.5 mM dNTP, 10 mM DTT, 40 U RNaseOut (Invitrogen), and 12.5 ng random primers (Invitrogen). A 1:5 dilution of the RT reaction product was used for quantitative RT-PCR (qRT-PCR) analysis.

The 1:5 diluted cDNA products were amplified by PCR. The PCR running mix was 18 μl H_2O, 2.5 μl 10× PCR buffer, 1.5 μl 25 mM $MgCl_2$, 1.0 μl 2.5 mM dNTP, 0.5 μl forward primer, 0.5 μl reverse primer, 0.2 μl *Taq* enzyme, 1.0 μl cDNA. PCR was run with the following program: 3 min at 95 °C, 5 min at 94 °C, 0.5 min at 58 °C, 1 min at 72 °C, and 5 min at 72 °C for 30 cycles. The PCR fragments were fractionated on a 0.3% agarose gel.

Quantitative real-time PCR and threshold cycle analysis

Quantitative real-time PCR was carried out in a final volume of 15 μl with the RT-PCR mix from Takara Bio Inc (Japan). cDNA was diluted to 1:10, TLPs and β-actin forward and reverse primers were used to further quantitatively analyze the transcriptional levels of the TLP gene. The reaction mix was as follows: 7.5 μl Takara mix, 3.0 μl cDNA (cDNA dilution 1:10), 1.5 μl 25 μM forward primer, 1.5 μl 25 μM reverse primer. Annealing temperature was 58 °C for both the actin

gene and the TLP gene. The running cycle was 40 times and set up as follows: 95 °C for 10 s, 55 °C (or appropriate temperature) for 30 s, 72 °C for 30 s, repeated for 40 times, then kept at 4 °C. The threshold cycle data were collected and analyzed using the $2^{-\Delta\Delta Ctn}$ method (Livak and Schmittgen, 2001).

The fold change in expression of the TLP gene was calculated using the ΔΔCt method with the levels of the *β*-actin gene RNA as an internal control. The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The threshold cycle change is as follows: $\Delta Ct_n = \Delta Ct_{nTLP} - \Delta Ct_{nactin}$. At the time point of 0 hpi, the threshold cycle of the TLP gene is Ct_{nTLP}, the threshold cycle of the actin gene is Ct_{nactin}, and the threshold cycle difference of the TLP gene and the actin gene at n hpi is ΔCt_n. The threshold cycle change compared to the control sample is $-\Delta\Delta Ct_{n-0} = \Delta Ct_n - \Delta Ct_0$ at the time n hpi, *i.e.* the changes of the threshold cycle difference of the TLP gene and actin gene at n hpi compared to 0 hpi. The threshold cycle fold change is $2^{-\Delta\Delta Ctn}$, which describes the multiplication number of each sample compared to the control. The following formulas were used:

$$\Delta Ct_{target} = Ct_{control} - Ct_{treatment},$$
$$\Delta Ct_{reference} = Ct_{control} - Ct_{treatment},$$
$$\text{and ratio} = 2^{-\Delta\Delta Ct},$$
$$\text{where } \Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{target}.$$

Results

Three sorghum lines were infested with greenbugs and examined for the transcription level of the TLP and *β*-actin using the RT-PCR techniques. DNA agarose gel analysis of the real-time products revealed that the transcripts of the TLPs increased and those of *β*-actin decreased in all three sorghum lines. The TLP content of the susceptible line Tx7000 elevated much more than those of the resistant lines PI550607 and PI550610, while the content of *β*-actin of Tx7000 declined more when compared to those of the other two lines (Fig. 1).

The threshold cycle analysis using real-time PCR further confirmed the PCR results. Upon analysis of the real-time PCR threshold cycle data, the induction of the TLPs' transcriptional levels of three sorghum lines increased, which gave further confirmation of the relative real-time PCR results. The induction of the TLPs of

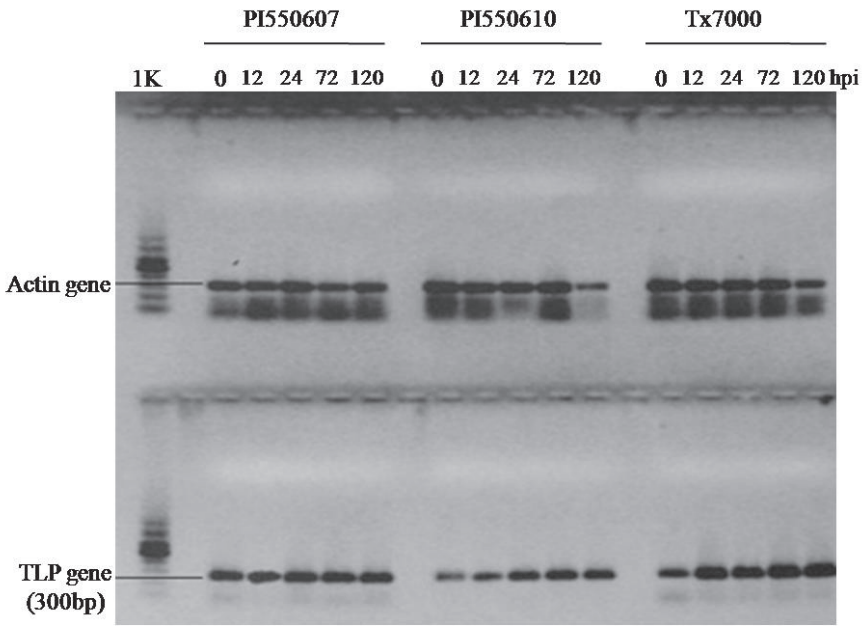

Fig. 1. RT-PCR DNA gel showing the transcriptional induction of thaumatin-like protein (TLP) of three sorghum lines when infested with greenbugs for different treatment times. The infestation times were 0, 12, 24, 72, 120 h. Tx7000, a greenbug-susceptible line; PI550607 and PI550610, greenbug-resistant lines.

the three sorghum lines was time-dependent; as the infestation time increased, the amount of the TLPs increased. At 120 hpi, the threshold cycle fold change $2^{-\Delta\Delta Ctn}$ of Tx7000 was several thousand compared to 0 hpi, while the threshold cycle fold changes of PI550607 and PI550610 were around 100.

The curves of PI550610 and PI550607, which are greenbug-resistant lines, were more reduced than that of Tx7000 and almost the same (Fig. 2).

The preformed amount ΔCt_{nTLP} of the TLP of Tx7000 was lower than those of PI550607 and PI550610, while the induced amount was much higher than for the other sorghum lines. The $-\Delta\Delta Ct_{n-0}$ value of PI550607 was almost the same but slightly higher than that of PI550610. The induced level of PI550607 was slightly higher than that of PI550610 (Table I). Infestation of sorghum plants with greenbugs increased the transcriptional level of TLPs in three sorghum lines when β-actin was used as the reference gene; as the infestation time increased, the induction level increased.

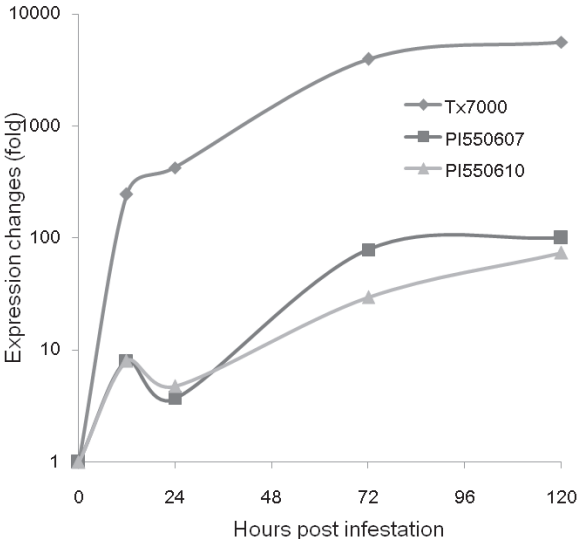

Fig. 2. Threshold cycle fold changes of thaumatin-like protein at the transcriptional level in three sorghum lines when infested with greenbugs. Tx7000 is greenbug-susceptible, while PI550607 and PI550610 are greenbug-resistant lines. The *Y*-axis values are the calculation amounts of $2^{-\Delta\Delta Ctn}$ of each sample.

Table I. Real-time PCR threshold cycle analysis of three sorghum lines when infested with greenbugs. The times of collection since infestation are indicated on the top; Tx7000 is a greenbug-susceptible line, while PI550607 and PI550610 are greenbug-resistant. Ct is the threshold of the fractional cycle number at which the fluorescence passes the fixed threshold. Thaumatin-like proteins and β-actin gene primers were used in this study. Ct_n is the threshold cycle value of each sample. ΔCt_n is the threshold cycle difference of the TLP gene and the β-actin gene of each sample. $-\Delta\Delta Ct_{n-0}$ means the decreased amount of threshold cycle of each sample compared to the control plant (0 hpi).

Change of gene expression in different lines		0 hpi	12 hpi	24 hpi	72 hpi	120 hpi
Tx7000	Ct_{nTLP}	28.26	20.85	21.25	19.27	18.94
	Ct_{nactin}	23.98	24.51	25.70	26.94	27.12
	$\Delta Ct_n = Ct_{nTLP} - Ct_{nactin}$	4.28	-3.66	-4.45	-7.67	-8.18
	$-\Delta\Delta Ct_{n-0} = \Delta Ct_n - \Delta Ct_0$	0	7.94	8.73	11.95	12.46
	$2^{-\Delta\Delta Ctn}$	1	245.51	424.61	3956.48	5634.22
PI550607	Ct_{nTLP}	25.07	24.48	22.26	23.65	20.88
	Ct_{nactin}	22.72	25.14	21.79	27.60	25.18
	$\Delta Ct_n = Ct_{nTLP} - Ct_{nactin}$	2.35	-0.66	0.47	-3.95	-4.30
	$-\Delta\Delta Ct_{n-0} = \Delta Ct_n - \Delta Ct_0$	0	3.01	1.88	6.30	6.65
	$2^{-\Delta\Delta Ctn}$	1	8.055	3.68	78.79	100.43
PI550610	Ct_{nTLP}	26.96	23.19	23.87	22.98	24.69
	Ct_{nactin}	23.32	22.55	22.64	24.23	27.26
	$\Delta Ct_n = Ct_{nTLP} - Ct_{nactin}$	3.64	0.64	1.23	-1.25	-2.57
	$-\Delta\Delta Ct_{n-0} = \Delta Ct_n - \Delta Ct_0$	0	3.00	2.41	4.89	6.21
	$2^{-\Delta\Delta Ctn}$	1	8	4.73	29.65	74.03

Discussion

Thaumatin-like proteins are reported to be associated with plant defense systems. When plants encounter chemicals, wounding, pathogens, or other kinds of challenges, the production level of the TLP will increase (Velazhahan *et al.*, 1999). Our study showed that the TLP could also be induced when sorghum plants are infested with greenbugs; and as the infestation duration increases, more TLP transcripts are produced (Fig. 2).

According to the present study, the insects act as an inducer of the resistance system, and the induction levels vary based on cultivars. It is well known that insect viruses, bacteria and fungi are inducers of various resistance systems (Bol *et al.*, 1990; Lotan and Fluhr, 1990). The aphid greenbug (*Schizaphis graminum*), which is one of the major pests of sorghum (*Sorghum bicolor* L. Moench) since 1868 (Porter *et al.*, 1997), is shown also to be an inducer of the TLP in sorghum.

The induction levels of the TLP have cultivar differences (Fig. 1, Table I). PI550607 possesses a high level of resistance to greenbug biotypes C and E (Katsar *et al.*, 2002). While PI550610 has shown a little higher level of antibiosis, its TLP induction level is just a little different from that of PI550607 (Bowling and Wilde, 1996). Our results indicated that with a more susceptible sorghum line, Tx7000, compared to a more resistant line more TLP transcripts are produced to protect itself against greenbug damage. There was a small difference between the resistant lines PI550607 and PI550610; the induction level of PI550610 was higher than that of PI550607, which is consistent with the previous hypothesis that the more resistant a line is, the less TLP is induced.

TLPs are not only constitutive or preformed (seed permatins and fruit proteins), but also stress-induced (PR-5 proteins and osmotins) (Trudel *et al.*, 1998). Our data indicated that the preformed amounts of TLP are different among three sorghum lines (Table I). The resistant sorghum lines' ΔCt_0 values are lower than that of the susceptible line, which indicates that the preformed amount of TLPs of a resistant sorghum line is higher than that of a susceptible line. 12 hpi, the ΔCt_{12} value indicated that the TLP amount of Tx7000 surpassed those of PI550607 and PI550610. In the more susceptible sorghum line Tx7000, more TLP transcripts were induced to protect itself against greenbug damage compared to a more resistant line (Fig. 1, Table I).

Plants have many natural mechanisms to protect themselves from pathogens (Hammerschmidt, 1999). Our results showed that the TLP apparently plays a role in attenuating the insect-pathogen response, but does not overcome the natural weak resistance and defense system of a susceptible sorghum line. There were more TLP transcripts produced in the susceptible line, and the TLP transcription level went higher as the infestation duration went longer.

When insects bite plant leaves, they cause wounding damage to the plants; metal files can mimic the insects' damage to the plants which can help us to study the insect-induced pathogen system. Other pathogens or stimuli could be used in the future, like bacteria inoculation (*Pseudomonas andropogoni*), SA or JA stimuli, and wounding damage to study the effect of TLP levels.

Based on differences in the induction levels of different sorghum cultivars, we could investigate TLP expression in different species in the future. For example, since the wheat plant has been reported to acquire system resistance (Jayaraj *et al.*, 2004), we could explore the differences of TLP's insect induction between sorghum and wheat.

Real-time PCR is used in the comparison of different genes involved in the same or different mechanisms. Many factors are involved in the plant's natural resistance system (Hammerschmidt, 1999); the TLP is just one factor involved in the induced resistance mechanisms. Our results indicate that greenbugs induce TLP production, and the levels are negatively associated with the plant's natural resistance level. In the future, we should study other protein factors combined with TLP, like chitinase or glucanase, as well.

To summarize, from the one-time results, the TLP is related to the sorghum-greenbug defense system, and the more susceptible a sorghum line is, the more TLP transcripts are produced. However, the TLP still may not overcome the weak resistance of the susceptible lines.

Bol J., Linthorst H., and Cornelissen B. (1990), Plant pathogenesis-related proteins induced by virus infection. Annu. Rev. Phytopathol. **28**, 113–138.

Bowling R. and Wilde G. (1996), Mechanism of resistance in three sorghum cultivars resistant to greenbug (Homoptera: Aphididae) biotype-I. J. Econ. Entomol. **89**, 558–561.

Breiteneder H. (2004), Thaumatin-like proteins – a new family of pollen and fruit allergens. Allergy **59**, 479–481.

Geddes J., Eudes F., Laroche A., and Selinger L.B. (2008), Differential expression of proteins in response to the interaction between the pathogen *Fusarium graminearum* and its host, *Hordeum vulgare*. Proteomics **8**, 545–554.

Hammerschmidt R. (1999), Induced disease resistance: How do induced plants stop pathogens? Physiol. Mol. Plant Pathol. **55**, 77–84.

Jayaraj J., Muthukrishnan S., Liang G. H., and Velazhahan R. (2004), Jasmonic acid and salicylic acid induced accumulation of β-1,3-glucanase and thaumatin-like proteins in wheat and enhanced resistance against *Stagonospora nodorum*. Biol. Plant. **48**, 425–430.

Katsar C. S., Paterson A. H., Teetes G. L., and Peterson G. (2002), Molecular analysis of sorghum resistance to the greenbug (Homoptera: Aphididae). J. Econ. Entomol. **95**, 448–457.

Klein D. (2002), Quantification using real-time PCR technology: applications and limitations. Trends Mol. Med. **8**, 257–260.

Livak K. and Schmittgen T. (2001), Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Meth. Mol. Biol. **25,** 402–408.

Lotan T. and Fluhr R. (1990), Function and regulated accumulation of plant pathogenesis-related proteins. Symbiosis **8**, 33–46.

Porter D. R., Burd J. D., Shufran K. A., Webester J. A., and Teetes G. L. (1997), Greenbug (Homoptera: Aphididae) biotypes: selected by resistant cultivars or readapted opportunists. J. Econ. Entomol. **90**, 1055–1065.

Trudel J., Grenier J., Potvin C., and Asselin A. (1998), Several thaumatin-like proteins bind to β-1,3-glucans. Plant Physiol. **118**, 1431–1438.

Van der Wel H. and Loeve K. (1972), Isolation and characterization of thaumatin I and II, the sweet-tasting proteins from *Thaumatococcus daniellii* Benth. Eur. J. Biochem. **31**, 221–225.

Velazhahan R., Chen-cole K., Anuratha C. S. and Muthukrishnan S. (1998), Induction of thaumatin-like proteins (TLPs) in *Rhizoctonia solani*-infected rice and characterization of two new cDNA clones. Physiol. Plant. **102**, 21–28.

Velazhahan R., Datta S. K., and Muthukrishnan S. (1999), The PR-5 Family: Thaumatin-Like Proteins. CRC, Boca Raton.

Velazhahan R., Jayaraj J., Jeoung J. M., Liang G. H., and Muthukrishnan S. (2002), Purification and characterization of an antifungal thaumatin-like protein from sorghum (*Sorghum bicolor*) leaves. J. Plant Dis. Protect. **109**, 452–461.