Molecular changes in downy mildew-infected sunflower triggered by resistance inducers

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ABSTRACT

Benzothiadiazole (BTH), a plant defense activator, has recently been found to restrict downy mildew development in sunflower. To elucidate the background of this phenomenon, a research programme was started and some of our preliminary results are reported in this paper. The gene expressions of glutation S-tranferase (GST), defensin (PDF) and catalase (CAT) were the subject of investigation using compatible, incompatible and partially resistant sunflower – *Plasmopara halstedii* interactions, respectively. The accumulation of all three gene transcripts were found to be increased in the susceptible sunflower genotype following BTH treatment. Furthermore, in case of the resistant sunflower, HA 335, BTH enhanced GST and PDF accumulation, whereas with the partially resistant RHA 340 the results were ambiguous. It is hoped that our findings may contribute to a better understanding of the plant's own defense system triggered by chemical inducers.

Key words: benzothiadiazole – catalase – defensin – glutathion S-transferase – *Plasmopara halstedii* – sunflower.

INTRODUCTION

Although *Plasmopara halstedii* can be effectively controlled by using genetic resistant plants and seed treatment with fungicides, protection can be hindered by the genetic variability of the fungus (Albourie et al., 1998; Gulya, 2007). Thus, besides the traditional control strategies, there was a need to look for alternative methods to provide effective disease control. One solution can be the use of systemic induced resistance, i.e. the activation of the defense system of plants.

The plant activator BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester) has already been shown to induce activated resistance in many crops against a broad spectrum of diseases (Cohen et al., 1994; Kogel et al., 1994; Pajot et al., 2001). BTH appears to be able to restrict downy mildew symptoms in sunflower under greenhouse conditions (Bán et al., 2004). Microscopic observations show that BTH treatment significantly decreased the development of fungal structures associated with cell necrosis and H_2O_2 accumulation in the BTH-treated susceptible sunflower hypocotyls.

Glutathione S-transferase (GST) has a well defined role in plant detoxification reactions. It is capable of catalyzing the binding of various xenobiotics, like pathogens. Various abiotic stressors are the inducers of GST activity in plants (Dean et al., 1990). GST is also considered one of the antioxidative enzymes, because it plays an important role in the protection against oxidative membrane damage and necrotic disease symptoms. Enhanced GST activity has been found in plants after pathogen infection, for example in barley plants infected by powdery mildew (El-Zahaby et al., 1995), and tobacco plants infected by TMV (Fodor et al., 1997).

To protect themselves against pathogenic attacks, plants evolve diverse strategies, for example the synthesis of antimicrobial peptides, like defensin. Defensin is a small, cysteine-rich antimicrobial peptide, existing in a wide range of plants and animals. Urdangarin et al. (2000) described full length sunflower cDNA from *Helianthus annuus* flowers encoding for defensin, and the authors supposed there was a relationship between enhanced expression of a defensin gene and decreased susceptibility to *Sclerotinia sclerotiorum*. Solis et al. (2006) isolated a defensin gene from *Lepidium meyenii*, having activity against *Phytophthora infestans*.

Catalase is one of the main antioxidant enzymes; it catalyzes the dismutation of H_2O_2 into water and dioxygen. This enzyme is located in peroxisomes and glyoxisomes. Catalase activity is affected by abiotic stressors, like boron (Karabal et al., 2003), light and chilling (Gechev at al., 2003), and acid rain (Gabara et al., 2003). In sunflower, catalase activity was increased by UV-B radiation (Costa et al., 2002) and cadmium treatment (Azpilicueta et al., 2007). Niebel et al. (1995) demonstrated induction of catalase in potato upon nematode and bacterial infection as well. Several plants have multiple CAT isoenzymes. For

example, in sunflower at least eight isoforms (CAT1-CAT8) have been described (Azpilicueta et al., 2007).

MATERIALS AND METHODS

The USDA sunflower inbred lines RHA 274, RHA 340 and HA 335, as well as *Plasmopara halstedii* pathotype 700 were used to get one compatible, and two incompatible combinations. While HA 335 is characterized by total resistance, RHA 340 exhibits HLI (hypocotyl-limited) resistant type (Virányi and Gulya, 1996).

Pre-germinated seeds were soaked in an aqueous solution of BTH (160 mg/L) for at least 6 hours (first day), followed by their inoculation with *P. halstedii* sporangia (50000 sporangia/ml) using the whole seedling inoculation technique (Cohen and Sackston, 1973). Germlings were subsequently planted into pots filled with a commercial soil mixture and grown in the greenhouse (18/24ºC, 60 % RH, 16h light) for 3 weeks.

Samples were taken 3, 9, 13, 16 days after infection (dpi). The whole seedlings were frozen in liquid nitrogen and ground with mortar and pestle. Total RNAs were extracted using the Qiagen Plant Mini kit, and then the extracted RNA treated with RNase inhibitor to protect the extracted RNA and with DNase I to remove genomic DNA contamination. The extracted RNAs were measured with spectrophotometer and the RNA concentration of 1µg/µl adjusted. One µg of RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad).

Primers for PCR amplifications were applied according to Radwan et al. (2005) and Azpilicueta et al. (2007) as shown in Table 1. Twenty-five µl of the PCR reaction mixture contained 1µl RNA, 1 unit of Taq DNA polymerase (Fermentas), 2.5 µl 10X Taq polymerase buffer, 1 µl 2.5mM dNTP mix, 1.5 µl 25mM $MgCl_2$, 2.5 µl 5 µM primers and 13.8 µl PCR water. PCR reactions were performed using a Gene Amp PCR System 2700 PCR machine. The amplification program included an initial step at 94°C for 3 min and 25-32 cycles (Ha-EF1a: 25; Ha-GST: 26; Ha-PDF: 30; CATA2: 31) of 15 sec at 94 °C, 15 sec at Tm °C (Ha-EF1: 58; Ha-GST, Ha-PDF: 61; CATA2: 50), 20sec at 72°C.

The PCR products were electrophorized through 1% agarose gel, visualized with ethidium bromide and photographed in a Molecular Imager Gel Doc system (Bio-Rad). The signals from gels were quantified using a Quantity One program with molecular mass ruler (Bio-Rad), and normalized over the signals from Ha-EF1α.

Table 1. Primer sequences and accession numbers used in this study

Gene[1]	Primer sequences	Accession number
Ha-EF-1α	Forward 5'-AGGCGAGGTATGATGAAATTGTCA-3' Reverse 5'-GTCTCTTGGGCTCATTGATTTGGT-3'	AAM19764
Ha-GST	Forward 5'-CCTCAGGATGCTTACGAGAAGG-3' Reverse 5'-GCAGAAATATCAACCAGGTTGATG-3'	AY667502
Ha-PDF	Forward 5'-ATGGCCAAAATTTCAGTTGCTTTCA-3' Reverse 5'-AAGACTTGCACTGGTCATCACAG-3'	AF364865
CATA2	Forward 5'-TTCCCGCTTGAATGTGAAG-3' Reverse 5'-CCGATTACATAAACCCATCATC3'	AF243517

[1]Ha-EF-1a: constitutive elongation factor 1a, Ha-GST: glutathione S-transferase, Ha-PDF: defensin, CATA2: catalase isoenzymes.

RESULTS

In general, Ha-GST transcript accumulation was higher in the untreated resistant sunflowers than in the susceptible ones. At 3 and 9 dpi the highest transcript accumulation was detected in the HA 335 plants. At 13 and 16 dpi, however, this accumulation was higher in the 'HLI resistant' RHA 340 plants as compared to HA 335. The BTH treatment increased Ha-GST transcript level in both the susceptible and totally resistant plants throughout the experiment. The effect of BTH treatment on this transcript accumulation in the HLI resistant plant was contradictory, because the treatment increased the transcript accumulation at 3 and 16 dpi, but appeared to reduce it at 13 dpi (Fig1).

HA-PDF transcript accumulation was found to be higher in the resistant sunflower lines than in the susceptible one, similar to the HA-GST transcript accumulation. The effect of BTH treatment on PDF activity was detectable in both the susceptible and totally resistant sunflowers. In case of the 'HLI resistant plants, Ha-PDF transcript accumulation was increased by BTH treatment on the second and the

last sampling days only. Among the untreated plants, the totally resistant plants showed Ha-PDF transcript accumulation at 3 dpi, whereas in all the BTH treated plants this transcript accumulation could be detected on the first sampling day. In the second sampling day the increase in transcript accumulation was observed in all plants examined, and there were no differences between the two resistant genotypes. At 13 and 16 dpi the maximum accumulation was evident in the 'HLI resistant' plants. It is interesting to note that there were no differences detected between the untreated 'HLI resistant' and the BTH-treated susceptible plants at 9, 13 dpi (Fig. 1).

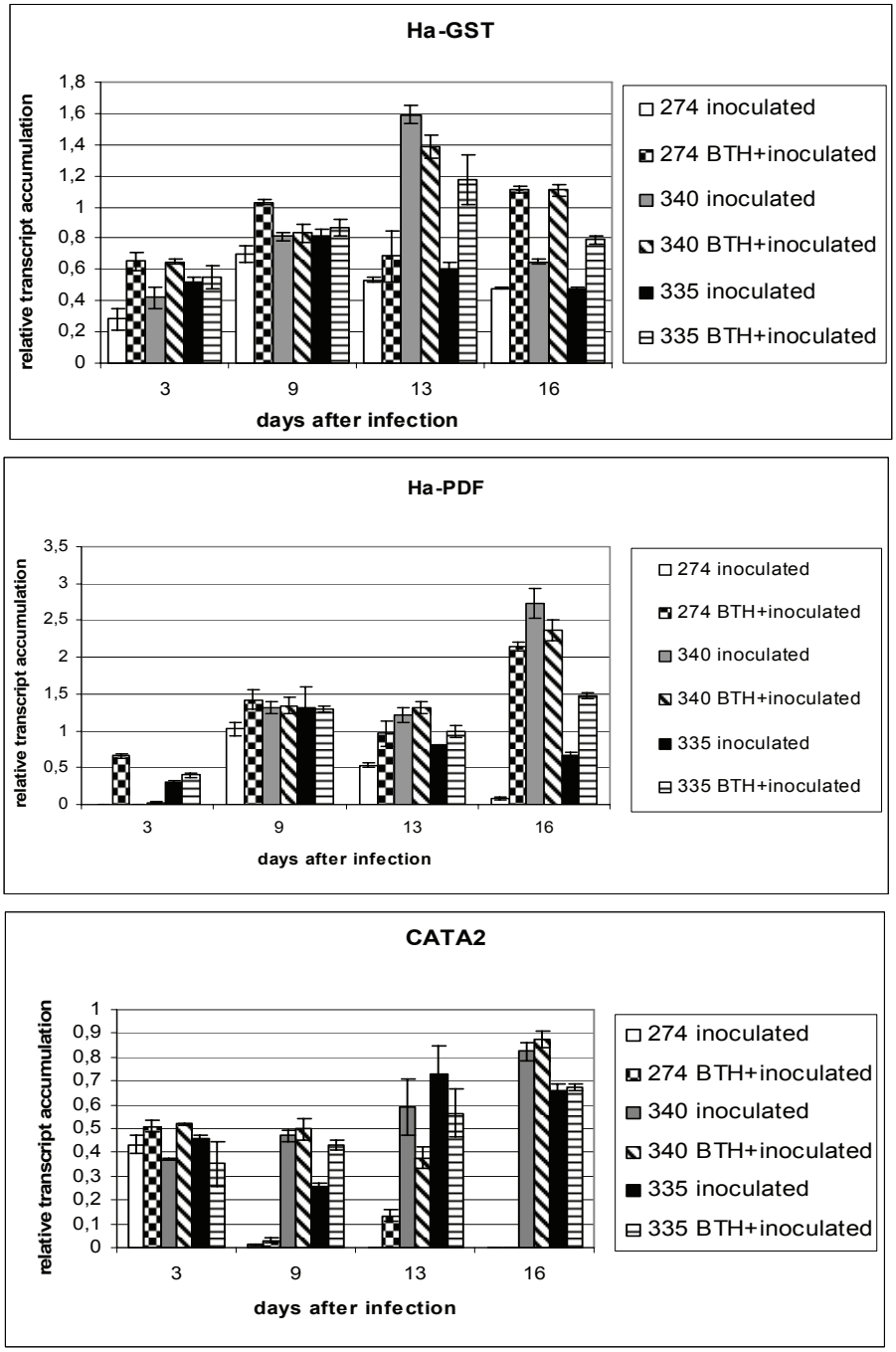

Fig. 1. Accumulation of gene transcripts in sunflower plants after BTH treatment and *Plasmopara halstedii* inoculation. 1. glutathione S-transferase (Ha-GST); 2. defensin (Ha-PDF) and 3. catalase (CATA2) gene expression in a susceptible (RHA 274), partially (HLI) resistant (RHA 340) and totally resistant (HA 335) sunflower line. Each value represents three replicates (±S.D.)

As for catalase activity, both resistant sunflowers exhibited higher CATA2 transcript level, than did the susceptible one, except for 3 dpi. After the first sampling day, catalase activity was not detectable in the untreated susceptible sunflower plants, but BTH-treatment considerably increased the level of CATA2 transcript. In case of untreated 'HLI resistant' plants, a continuous increase in transcript accumulation of CATA2 was found to reach its maximum at 16 dpi. With the exception of the third sampling day, the BTH treatment increased the transcript accumulation in the 'HLI resistant' plants as well. In the totally resistant untreated sunflowers the accumulation of CATA2 transcript reached its maximum at 13 dpi (Fig. 1).

DISCUSSION

In this study molecular changes in BTH-treated sunflowers were the subject of investigations associated with infection by *P. halstedii*. PCR was used in an attempt to describe induced resistance events in different sunflower genotypes.

Glutathione S-transferase usually detoxifies xenobiotics in plant tissues. We found an increased level of GST activity in the BTH-treated, susceptible sunflower and this increased activity resembled that detected in the 'HLI resistant plants'. Fodor et. al (1997) reported similar results with tobacco either treated or non-treated with salicylic acid. In contrast, El-Zahaby et al. (1995) found a significantly higher level of GST activity in susceptible barley plants than in resistant ones after powdery mildew inoculation. They assumed that the fungus itself contained GST enzyme, so that both the host and the pathogen might contribute to this increase in GST activity.

Defensins are a class of antimicrobial peptides found in several plants, including sunflower. In our experimental conditions defensin gene expression was induced by BTH treatment in the susceptible sunflower plants, and this enhanced level was equally found in the 'HLI-resistant' plants. Similar to Radwan et al. (2005), Ha-PDF transcript accumulation was lower in the non-treated susceptible plants, than in the resistant ones.

Catalase is usually considered to be one of the most important antioxidant enzymes. BTH treatment increased CATA2 transcript level in the susceptible sunflower plants but this effect was not evident in the resistant sunflowers.

In conclusion, the plant activator BTH had a positive effect on the natural defense system of sunflower by enhancing the expression of three genes that are considered to be associated with the chemically-induced host resistance to *P. halstedii*.

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