

Analysis of ascorbate peroxidase genes expressed in resistant and susceptible wheat lines infected by the cereal cyst nematode, *Heterodera avenae*

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Abstract Changes in ascorbate peroxidase (APX) enzyme activity in response to nematode (*Heterodera avenae*) attack were studied in roots of three hexaploid wheat lines carrying *Cre2*, *Cre5*, or *Cre7* nematode resistance genes and the susceptible *Triticum aestivum* cv. Anza. A spectrophotometric analysis was carried out with root extracts of infected plants 4, 7, 11, and 14 days after nematode inoculation using uninfected plant as control. APX induction in infected resistant genotypes was similar and higher than in the susceptible control. The introgression wheat/*Aegilops ventricosa* H-93-8 line, carrying the *Cre2* gene, and its parental line H-10-15 as susceptible control were used to analyze whether this increase of activity was correlated with the induction of APX gene expression. Genes encoding cytosolic forms of APX were induced in roots of both lines in response to nematode infection. This induction took place both earlier and with greater intensity in the resistant line than in the susceptible one, and it was also higher in the root area at the site of nematode attachment.

Keywords *Aegilops* · Cereal cyst nematode (CCN) · Host–pathogen interaction · Reactive oxygen species (ROS) · *Triticum aestivum* · Ascorbate peroxidase

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Abbreviations

APX	Ascorbate peroxidase
AsA	Ascorbic acid
Cre	Cereal root eelworm
CCN	Cereal cyst nematode
dai	Days after inoculation
EST	Esterase
GSH	Reduced form of glutathione
PRX	Plant class III peroxidases
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

The cereal cyst nematode (CCN) (*Heterodera avenae* Woll.) is the most widely distributed and damaging species on cereals cultivated in less temperate regions. This nematode species has been detected in many countries and it is responsible for yield losses in wheat of up to 30%. The CCN induces syncytial feeding sites in the roots of its hosts. Infective second-stage juveniles (J2) enter the plant roots at the level of the differentiation zone and penetrate intracellularly towards the vascular cylinder. Once there, they select and pierce with their stylet a single cell where they release esophageal secretions. In the following hours, the affected plant cells start to develop feeding structures (Das et al. 2008). Approximately 2 weeks after infection, the nematode reaches maturity and it is associated with a syncytium, typically including a great number of cells (Golinowski et al. 1996). Many different methods are available to control nematodes, but one of the most effective is the use of host resistance, which is defined as the reduction in nematode multiplication in the presence of

resistant plants. Globally, this is acknowledged as one of the most cost-effective, environmentally sustainable, and accessible methods to control CCN. There has been considerable progress in identifying resistance to *H. avenae*, with all reported genes exhibiting a major effect (i.e., controlled by a single dominant gene), making their use in a breeding program very amenable (Nicol and Rivoal 2007). Sources of *H. avenae* resistance (named *Cre* genes) have been found predominantly in wild relatives of wheat, with six of the eight coming from four *Aegilops* spp. Most of these genes have already been introgressed into hexaploid wheat backgrounds for bread wheat breeding purposes (Delibes et al. 1993; Eastwood et al. 1994; Romero et al. 1998; Seah et al. 2000; Ogonnaya et al. 2001). However, the effectiveness of *Cre* resistance genes toward *H. avenae* depends on the nematode pathotype in conferring total or partial resistance to different CCN populations. For example, it has been demonstrated that *Cre3* has the greatest impact on reducing the Ha13 Australian population, followed by *Cre1* and *Cre8* (Safari et al. 2005), but it is ineffective against European pathotypes (Montes et al. 2008; Rivoal et al. 2001). In contrast, *Cre2*, which confers a high level of resistance to several European CCN populations, including the preponderant Spanish pathotype, Ha71, used in this study (Delibes et al. 1993), has no effect on the Australian pathotype (Ogonnaya et al. 2001). In a previous study, the efficacy of *Cre* genes against Spanish pathotype Ha71 was rank-ordered as $Cre1 \geq Cre4 \geq Cre5 \geq Cre2 \geq Cre7 \gg Cre8 > Cre3 > Cre6$ (Montes et al. 2008).

Plants defend themselves from nematodes using a wide variety of mechanisms, including rapid induction of localized necrosis at the site of infection (the hypersensitive response: HR), increased expression of defense-related proteins, production of antimicrobial compounds, lignin formation, and the oxidative burst. The nature of the plant genes switched on or off during the nematode–host interaction is a subject of great interest as some of the products of these genes are directly responsible for promoting nematode parasitism or for fending-off the nematode attack in the resistant genotypes. Moreover, the timing in the surveillance system of receptor molecules that can recognize elicitor molecules of pathogen origin is fundamental for the host response and it is functional against a wide spectrum of pathogens (Nürnberger et al. 2004). Following this initial recognition, multiple biochemical pathways are activated and lead to a cascade of reactions, ultimately resulting in the formation and accumulation of secondary metabolites which help in the plant to overcome the parasite (Zhao et al. 2005). Among the altered biochemical pathways are those involving peroxidases, which comprise a large group of enzymes that use different peroxides (ROOH) as electron acceptors. According to Welinder

(1992), these enzymes are classified into three classes (I, II, and III). Within class I enzymes, the ascorbate peroxidases (APX, EC 1.11.1.11) are intracellular and characterized to show a high affinity for ascorbate (AsA) as electron donor to reduce H_2O_2 . This reaction gives rise to monohydroascorbate and H_2O , being highly unstable in the absence of AsA (Chen and Asada 1989). Through a wide series of reactions, AsA plays an important role in the neutralization of hydrogen peroxide (H_2O_2) in photosynthetic organisms. AsA is responsible for H_2O_2 detoxification in green leaves (Chaudie' re and Ferrari-Iliou 1999) and APX is considered as a key antioxidant enzyme in plants (Orvar and Ellis 1997). Thus, the reactions catalyzed by APX and the coupled reaction AsA–GSH prevents the accumulation of toxic levels of H_2O_2 , part of which is generated by class III peroxidases in photosynthetic organisms. APX activities are located in chloroplasts (chAPX), cytosol (cAPX), peroxisomes, or microbodies (pAPX) and mitochondria, each cellular compartment possessing one or several APX isoforms. In *Arabidopsis*, the same protein is dually targeted to mitochondria and chloroplast stroma (Chew et al. 2003). The different APX isozymes are very similar sharing 50–70% of sequence homology between them, so that a common evolutionary route for different APX genes has been proposed (Teixeira et al. 2004). Recent studies have focused on changes in activity and gene expression for APX isozymes in plants subjected to environmental and pathogen stress (Shigeoka et al. 2002; Chen et al. 2006).

Class II peroxidases are secreted by fungi while class III peroxidases are plant enzymes that target the secretory pathway via their signal peptide. Class III peroxidases (PRX) have been proposed as key regulators of the level of extracellular H_2O_2 and as producers of the extremely reactive oxygen species (ROS), hydroxyl (OH^\cdot), and hydroperoxyl (OOH^\cdot) radicals (Bolwell et al. 2002; Kawano 2003; Passardi et al. 2005; Bindschedler et al. 2006). The three described classes of peroxidases, in combination with other systems, regulate H_2O_2 levels that act as a systemic signal (Shigeoka et al. 2002).

Previous studies from our group (Andrés et al. 2001; Montes et al. 2003, 2004) demonstrated that cyst nematode infection enhances PRX, esterase (EST), and superoxide dismutase (SOD) activity in wheat roots carrying *Cre2*, *Cre5*, or *Cre7* resistance genes. The highest response was found in the PRX system. More recently, we have identified three groups of PRX genes greatly induced by the CCN *H. avenae* in a resistant wheat line carrying the *Cre2* gene (Simonetti et al. 2009a), which was accompanied by an increase in H_2O_2 synthesis (Simonetti et al. 2009b). All these findings suggest that apoplastic PRX from wheat plays a role in triggering some defense-related responses regulating H_2O_2 levels, a process in which APX activity would also be involved through H_2O_2 detoxification.

Therefore, to understand the precise role of APX in the wheat response to *H. avenae* infection, it is imperative to study its activity and regulation following nematode attack. The aim of the present study was to assess the involvement of APX in the defense response against nematode infection. Here, we describe the changes produced in APX enzyme activity and in its gene expression due to infection by the Ha71 pathotype of *H. avenae* in wheat lines carrying *Cre* resistance genes. The accumulation of cytosolic APX gene transcripts in wheat roots challenged by CCN suggests that the different ROS neutralization systems are co-regulated.

Materials and methods

Plant material and nematodes

The wheat introgression hexaploid lines, H-93-8 and TR-3531, carriers of *Cre2* and *Cre7* CCN resistance genes transferred from *Aegilops ventricosa* and *Aegilops triuncialis*, respectively, have been previously described (Delibes et al. 1993; Mena et al. 1993; Romero et al. 1998; Ogonnaya et al. 2001; Montes et al. 2003). The substitution line 6N^v/6D, carrying *Cre5* gene from *Ae. ventricosa* #10, was provided by Dr. Jahier from INRA, France (Jahier et al. 2001). *Triticum aestivum* cvs Anza and Almatense, H-10-15 (parent of H-93-8 and TR-3531) were used as susceptible controls (compatible interaction). Hereafter, abbreviations H-93-8, TR-3531, 6N^v/6D, Anza and H-10-15 will be used.

The CCN (*H. avenae*) population from a naturally infested plot at “La Poveda” Experimental Station in Arganda (Madrid) has been previously characterized as belonging to Ha71 pathotype (López-Braña et al. 1996). Seeds of resistant and susceptible wheat lines were germinated on wet paper after surface sterilization with 5% sodium hypochlorite and sowed in 5-cm diameter plates containing 10 ml of wet sterilized sand and maintained at $15 \pm 2^\circ\text{C}$ in a growth chamber. At 48 h, seedlings were divided into two groups: one was inoculated with 100 J2s of Ha71 pathotype per plant and the second one (uninfected seedlings) was used as a control as described by Andrés et al. (2001). RNA was extracted from root sections, with and without nematode, and leaves of infected plants, harvested 4 and 7 days after inoculation (dai), as well as from the uninoculated tissues used as control. Whole infected roots 4, 7, 11 and 14 dai and an equal part of uninfected material were used for enzyme analysis. Root and leaf strips were flash frozen in liquid nitrogen and then stored at -80°C for further use.

APX enzyme activity

To analyze quantitative changes in APX enzyme after wheat inoculation with pathotype Ha71 of *H. avenae*,

global enzyme activity of root extracts was measured spectrophotometrically in resistant (introgression lines carrying *Cre2*, *Cre5*, or *Cre7* genes) and susceptible (*T. aestivum* cv. Anza) genotypes. The supernatant used for enzyme assays was obtained by grinding 100 mg of root tissue with 200 μl of extraction buffer (25 mM potassium phosphate pH 7.0, 1 mM EDTA and 5 mM ascorbic acid) in a small mortar in the presence of glass beads, followed by centrifugation at 13,000 rpm for 15 min at 4°C . Global APX activity was determined spectrophotometrically measuring the decrease in absorbance at 290 nm wavelength of ascorbate in 0.5 ml of a reaction mixture, containing 25 mM potassium phosphate buffer pH 7.0, 0.5 mM ascorbic acid and enzyme extracts (100 μl), as previously described (Nakano and Asada 1981). Then, the peroxide reaction was started by the addition of 100 μl of 30% H_2O_2 (v/v). Enzyme activity was calculated as the concentration of ascorbic acid oxidized $\text{min}^{-1} \text{mg}^{-1}$ protein using the molar extinction coefficient ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). Concentration of total protein in samples was determined using the Bradford method (1976). Each activity value was the average of three assays.

RNA purification

Total RNA was isolated according to Båga et al. (1995). Concentration of each RNA sample was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Only the RNA samples with a 260/280 ratio between 1.9 and 2.1 and with a 260/230 ratio greater than 2.0 were used. Validation of RNA quality was performed using the RNA 6000 Nano Assay on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Primers design and real-time PCR analysis

First-strand cDNA was synthesized by reverse transcribing 2 μg of total RNA in a final reaction volume of 20 μl using a high-capacity cDNA reverse transcription kit (Applied Biosystems) following the manufacturer's instructions and using a poli-T oligonucleotide.

Specific primers for each APX group were designed from 3' low homology region sequences of different groups of APX available in the databases of the NCBI website (National Center for Biotechnology Information; Altschul et al. 1997). The APX sequences of the different cereal species were aligned according to their subcellular location (cytosol, chloroplast and peroxisome) by MULTALIN program (Corpet 1988) and then manually adjusted using the GeneDoc program version 2.7.000 (Nicol and Rivoal 2007). Primers were designed using Primer Express program version 2.0 (Applied Biosystems). The primers

selected from a preliminary list are shown in Table 1. This selection was carried out by picking primer sequences that were present in all APX of the same location (cytosol, chloroplast, or peroxisome), but absent in those of different location. The sizes of the amplified fragments (amplicons) obtained by PCR were in all cases 101 base pairs in length (except actin that was 151 base pairs).

The qPCR mixture contained 1 µl of fivefold diluted cDNA, 10 µl of Power SYBR Green PCR Master Mix (Applied Biosystems), and 300 nM each gene-specific primer in a final volume of 20 µl. PCR without template was also performed for each primer pair as controls. Data acquisitions were carried out with an ABI Prism 7300 Sequence Detection System and Software (Applied Biosystems). Cycling conditions for all reactions were 10 min at 95°C (denaturation), 40 cycles of 15 s at 95°C, and 1 min at 60–68°C in 96-well optical reaction plates (Applied Biosystems). The specificity of amplicons was verified by melting curve analysis (60–95°C) after 40 cycles and agarose gel electrophoresis. All genes were analyzed in triplicate from two independently isolated RNA samples. The cycle threshold (Ct) values were normalized to a conventional reference gene (actin). Two different approaches were used to process qRT-PCR data: (1) the relative quantification method which assumes that amplification efficiencies are equal to 2 for all reactions (Pfaffl 2001) and (2) the method based on the kinetics of individual PCR reactions. In the latter case, raw fluorescence values, as a function of cycles, were exported into the real-time PCR Miner program (Zhao and Fernald 2005) to calculate the efficiency and Ct values for individual PCR reactions.

Results

APX activity

APX specific activity showed a significant increase in roots of wheat lines following the inoculation with juveniles of the Ha71 *H. avenae* pathotype (Fig. 1). The maximum activity level was seen 11 dai in all assayed genotypes. Enzyme levels were quite similar in the resistant lines and, in all cases, higher than those in the susceptible one. The

pre-infection levels of APX were not significantly different between resistant lines carrying *Cre2*, *Cre5*, or *Cre7* and the susceptible control (data not shown) and no differences were detected 4 dai. There was a significant increase in the activity in resistant lines as compared to the susceptible control 7 dai, reaching the maximum activity approximately 11 dai. This increase was 1.8–2.0 fold in resistant genotypes, over the uninfected controls. The activity of all resistant lines 14 dai declined significantly to levels observed in the susceptible control.

TaAPx genes expression

To evaluate the expression of the different groups of *TaAPx* genes during the process of wheat infection by *H. avenae*, transcript levels were monitored by quantitative reverse transcription polymerase chain reaction (qRT-PCR) 4 and 7 dai in both susceptible H-10-15 and resistant H-93-8 lines. Different sets of *TaAPx* genes analyzed in this study are named according to the nomenclature proposed in the PeroxiBase database (Passardi et al. 2007) with the letters “*Ta*” for *Triticum aestivum* and “*APx*” for APX, preceded by different letters according to their subcellular location (*cTaAPx* for cytosol, *chTaAPx* for chloroplast, and *pTaAPx* for peroxisome or microbody). In Figs. 2, 3, 4 we have differentiated attacked plants (+) from control plants without attack (–) and in Fig. 2 also the root zone in which the nematodes have attached (+N) from the areas without nematodes (+).

There were no significant differences between the two methods used for data analysis (relative quantification assuming efficiencies of two and real-time PCR Miner). The results obtained using the second method are reported in the corresponding figures.

The largest increase in *cTaAPx* expression levels (Fig. 2) took place 4 dai in roots of line H-93-8, especially near the area where the nematode had established its feeding site (+N). The changes in expression of *cTaAPx* after infection in the susceptible line H-10-15 were much less pronounced, although, there was also a slight increase at 4 dai. The expression levels, at this time, were found to be three times higher in the resistant line than in the susceptible under all conditions. However, higher values were

Table 1 Primer sequences used in quantitative real-time polymerase chain reaction analysis

APX cluster	Sense primer	Antisense primer
cAPX	GCCATGGCTAAGAAGTACCCC	CGCAGTTCTTCTCGGCGAT
chAPX	CGTGGCAACTGAAGAGAAGG	AAGAGTATTTGGCGGCAACG
pAPX	GTCTGGGTTTGACGGTGCAT	GGAGCTTCAGAAGACCCCTCAG
Actin	GAAGCTGCAGGTATCCATGAGACC	AGGCAGTGATCTCCTTGCTCATC

The nomenclature cAPX, chAPX and pAPX is used for cytosol, chloroplast and peroxisomes APXs location, respectively

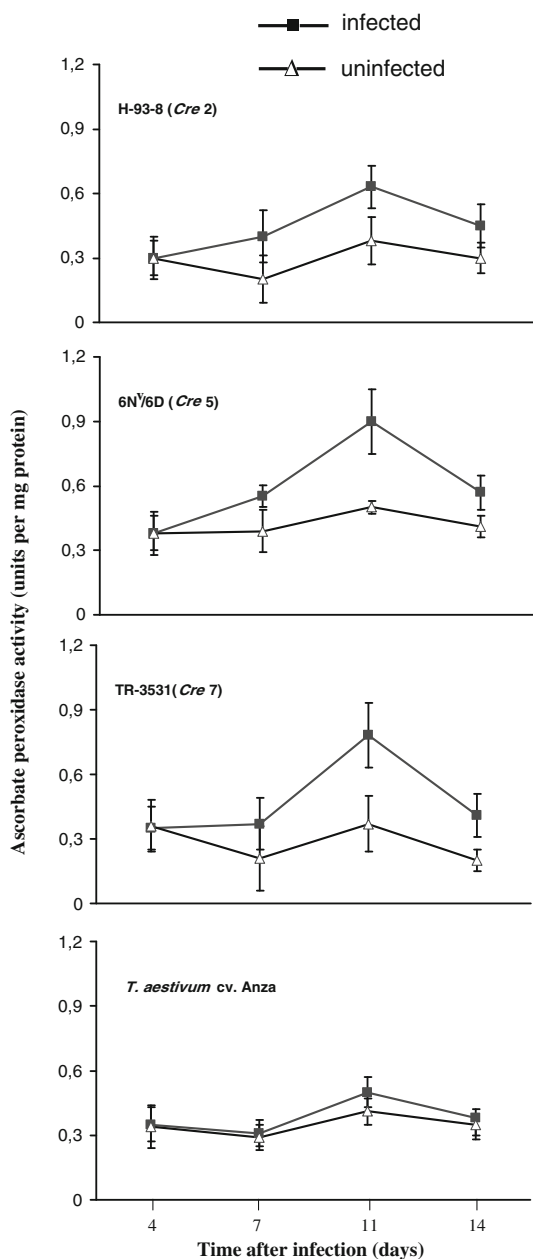


Fig. 1 Ascorbate peroxidase activity at different time intervals post-infection (4, 7, 11 and 14 days) in roots of cereal cyst nematode. (1) Resistant (H-93-8, 6N/6D and TR-3531) or (2) susceptible (*T. aestivum* cv Anza) genotypes, infected with juveniles of *H. avenae* (filled square). Open triangle indicate their respective uninfected controls. Values represent the mean ± standard errors of three assays

detected 7 dai in the susceptible wheat, in the region where the nematodes were attached (+N). Differences between regions of infected plants in the susceptible wheat disappeared 7 dai, but showed an increase in the levels in infected root areas without nematode (+). It can be seen in resistant wheat that the mRNA levels 7 dai were similar in all conditions regardless of infection (Fig. 2).

The expression levels were lower in leaves than in roots of both lines (Fig. 3), the differences being 8–tenfold in

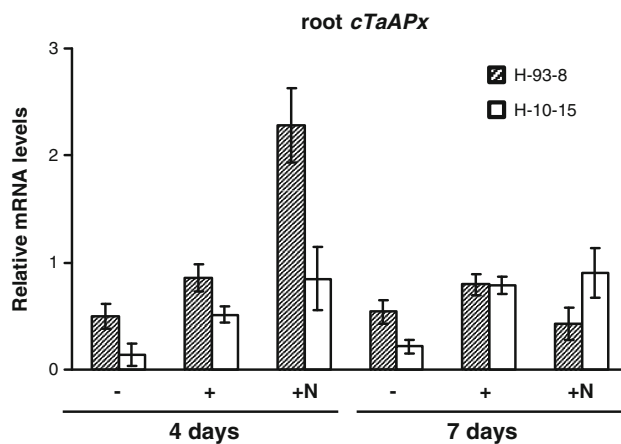


Fig. 2 Expression of *cTaAPx* genes in wheat roots following *Heterodera avenae* infection. Steady-state mRNA levels of resistant (H-93-8) and susceptible (H-10-15) wheat were normalized with respect to *actin*. All data are mean ± standard error of three replicates from two biological samples. – Uninfected root section, + infected root section without nematode, +N infected root section with nematode 4 and 7 days, respectively, after nematode infection

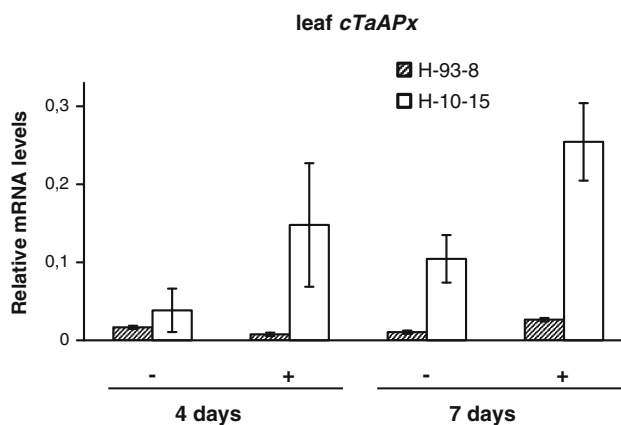


Fig. 3 Expression of *cTaAPx* genes in wheat leaves following *Heterodera avenae* infection. Steady-state mRNA levels of resistant (H-93-8) and susceptible (H-10-15) wheat were normalized with respect to *actin*. All data are mean ± standard error of three replicates from two biological samples. – Leaves of uninfected plants, + leaves of infected plants 4 and 7 days, respectively, after nematode infection

H-10-15 and 20–50 fold in the H-93-8 line. Moreover, *cTaAPx* mRNA levels in leaves were higher in the susceptible line, H-10-15, than in the resistant one, in all conditions, with and without attack, and both 4 and 7 dai. The H-10-15 line showed a slight induction in leaves in response to infection, both 4 and 7 dai, the levels being higher in the latter. The H-93-8 line exhibited very low levels in all cases.

The expression levels of *chTaAPx* in the H-10-15 line were higher than in the H-93-8 line, except in leaves of resistant plants 7 dai, which exhibited a light induction in response to the infection (Fig. 4). The H-10-15 line did not show significant differences in expression levels at 4 and

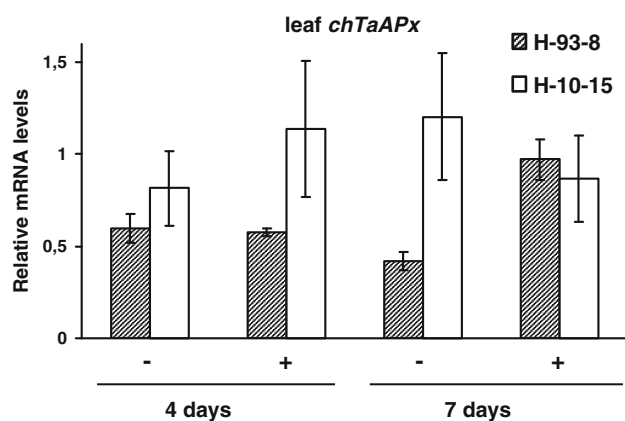


Fig. 4 Expression of *chTaAPx* genes in wheat leaves following *Heterodera avenae* infection. Steady-state mRNA levels of resistant (H-93-8) and susceptible (H-10-15) wheat were normalized with respect to *actin*. All data are mean \pm standard error of three replicates from two biological samples. – Leaves of uninfected plants, + leaves of infected plants 4 and 7 days, respectively, after nematode infection

7 dai, regardless of whether they were attacked or not. The expression levels in roots (data not shown) were, in all the cases, 20–30 times lower than in leaves and also lower than those of *cTaAPx* (≈ 40 times) and the changes observed could not be considered significant.

Relative expression levels of *pTaAPx* in leaves were lower than those of *chTaAPx* (≈ 20 – 50 times), in both the H-10-15 and H-93-8 lines. Furthermore, the expression levels did not show differences that can be considered significant at 4 and 7 dai, except in attacked leaves of resistant plants 7 dai, which exhibited a very slight induction (Fig. 5). It is worth mentioning that levels were lower in roots (≈ 4 times) than in leaves (data not shown) and could be considered negligible both in the resistant and the susceptible lines.

Discussion

The results reported here are consistent with our previous work in which changes in SOD, EST and PRX were detected in roots of the same biological material (H-93-8, 6N^v/6D and TR-3531), inoculated with juveniles from the same *H. avenae* pathotype (Montes et al. 2003, 2004). Increase in the activity of these enzymes was detected 4 dai in lines carrying *Cre2*, *Cre5*, or *Cre7* genes, being yet more evident 7 dai. We have previously observed that induction in PRX activity was accompanied by an increase in levels of some mRNAs belonging to this enzyme system (Simonetti et al. 2009a).

High levels of H₂O₂ are produced as a consequence of PRX action (Simonetti et al. 2009b), which may have several important effects on the host–pathogen interaction.

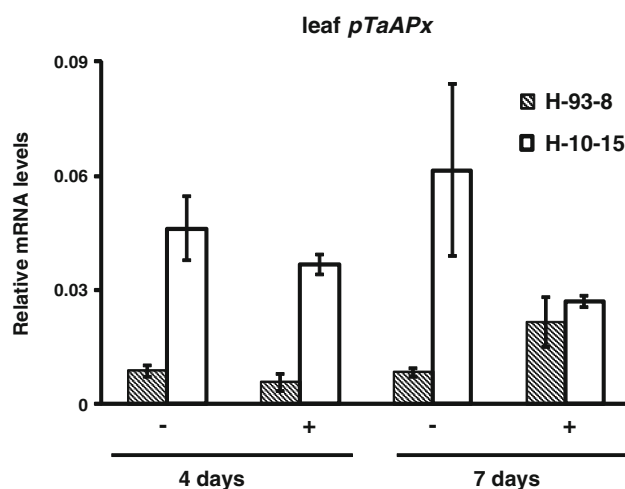


Fig. 5 Expression of *pTaAPx* genes in wheat leaves following *Heterodera avenae* infection. Steady-state mRNA levels of resistant (H-93-8) and susceptible (H-10-15) wheat were normalized with respect to *actin*. All data are mean \pm standard error of three replicates from two biological samples. – Leaves of uninfected plants, + leaves of infected plants 4 and 7 days, respectively, after nematode infection

APX and CAT (catalase) are some of the key enzymes of the plant antioxidant system, playing a central role in H₂O₂ scavenging and therefore its action is considered to be later than PRX. On the other hand, Alkharouf et al. (2004) noticed an early response of APX genes in a 12 h library made from mRNA extracted from roots of a resistant soybean inoculated with *H. glycine*, but the expression of these genes was not so abundant at 6–8 days. The induction of APX activity as a result of subjecting various plant species to different types of stress such as drought (Mittler and Zilinskas 1994), presence of salts (Seckin et al. 2009), high temperatures (Shi et al. 2001), excess light (Mullineaux and Karpinski 2000) and infection by pathogens (Mittler et al. 1998; Chen et al. 2006) has also been described.

Given this background, we were interested to study the changes in APX enzyme activity as a result of nematode infection in wheat lines carrying *Cre* resistance genes. In our first approach, we tried to detect changes in APX activity by means of conventional electrophoresis or isoelectrofocusing in wheat roots infected with *H. avenae*, following the methods described by Mittler and Zilinskas (1994) and Bai et al. (2009). However, these techniques were not sensitive enough to detect APX activity in roots. For this reason, we decided to carry out the study by spectrophotometry, which detects activity at lower concentrations.

The nematode infection produced an increase in APX activity (Fig. 1) in extracts from roots of the resistant genotypes as well as in the susceptible control *T. aestivum* cv. Anza. The activity was always higher 11 dai and with a greater increase ($\times 2$) in the infected lines carrying

resistance genes. This finding was similar to that previously obtained by isoelectrofocusing for SOD, PRX and EST enzyme systems in roots of the same biological material (H-93-8, 6N^v/6D and TR-3531) inoculated with juveniles of the Ha71 *H. avenae* pathotype (Andrés et al. 2001; Montes et al. 2003, 2004).

The induction of APX activity as a result of nematode attack led us to study the accumulation of messenger RNA (transcripts) for *TaAPx* genes. This enzyme group, as it has been previously mentioned, is located in cytosol, chloroplasts and peroxisomes. Accumulation of *cTaAPx* transcripts over their normal basal level was produced by nematode infection in roots of both resistant and susceptible genotypes (Fig. 2). In contrast, changes in *chTaAPx* and *pTaAPx* groups were lower and not significant, suggesting that these two gene groups might not be directly involved in the early defense response to *H. avenae*. Furthermore, the *cTaAPx* genes were induced in roots of the resistant line in the zone in which the nematode establishes its feeding site (+N, Fig. 2) and this induction was greater than in the same area (+N) of the susceptible line. Unlike the spectrophotometric data, this value was higher 4 dai and then it fell 7 dai. These results are coherent if we keep in mind that mRNA expression precedes the enzymatic activity of the product that it encodes. The susceptible line showed a lower response to infection in *cTaAPx*, constant over time, in agreement with the results obtained by Sobczak et al. (2005). Transcription *cTaAPX* levels in leaves were lower than those registered in roots in both lines studied (Fig. 3). In addition, *cTaAPx* genes were induced in leaves of susceptible line 7 dai (Fig. 3). The expression of *chTaAPx* was much lower (about 10² times, data not shown) in roots, in both lines, and was not affected by nematode attack, suggesting that these genes would be not directly involved in the plant defense response against *H. avenae*. As predicted, the levels of *chTaAPx* were higher in leaves than in roots, in both lines. Moreover, *pTaAPx* expression was very low in leaves and roots as to be insignificant. The higher levels of *cTaAPx* in roots and *chTaAPx* in leaves agree with previous findings in *Arabidopsis* (Pnuelli et al. 2003), rice (Teixeira et al. 2006) and potato (Pereira et al. 2005). Nevertheless, it has been observed in *Arabidopsis* that only one of the cytosolic APX (*AtAPx1*) increases in response to diverse types of stress (Pnuelli et al. 2003; Rizhsky et al. 2004). Induction of another cytosolic APX (*APx2*) has been detected in roots of resistant soybean, during *H. glycine* infection, but this increase took place prior, at 12 h post-inoculation (Alkharouf et al. 2004). In contrast, Chen et al. (2006) described induction of *pTaAPx* in leaves of a resistant wheat cultivar in response to infection by *Blumeria graminis*. In our case, the levels of *pTaAPx* were very low and without significant differences in response to nematode

attack. This may reflect changes in gene expression mediated by the two different ways of pathogen penetration, intracellularly in CCN and appressorium-mediated in *B. graminis*, with and without wounding. Nevertheless, our results are consistent with that obtained by analyzing transcriptomes of syncytia in *Arabidopsis* infected by *H. schachtii* (Szakasits et al. 2009) and also with studies carried out on whole galls and giant cells in tomato and *Arabidopsis* attacked by *Meloydogyne javanica* (Portillo et al. 2009; Barcala et al. 2010) that are more similar systems to that used in this work.

We have previously described an accumulation of *TaPrx111*, *TaPrx112*, and *TaPrx113* peroxidase transcripts over their normal basal level in response to nematode infection in both resistant, H-93-8, and susceptible, H-10-15, genotypes (Simonetti et al. 2009a), but the induction was not higher where the nematode was established. This was accompanied by an increase in H₂O₂ levels and PRX activity around nematode feeding site cells (Simonetti et al. 2009b), which again was higher on the resistant H-93-8 line than in the susceptible H-10-15. It could be suggested that *TaAPx* and *TaPrx* genes respond differently to signals from the nematode and, as a whole, they cooperate to limit root damage and to regulate hydrogen peroxide levels and such a defense mechanism in the resistant genotypes is activated immediately after infection by *H. avenae*. Probably the same mechanism could operate in the susceptible wheat cultivar, but it may function quite slowly and could reach lower levels. Such a mechanism has also been proposed for the wheat–Hessian fly interaction (Liu et al. 2010). Because two APX genes of cytoplasmic location have been described, and the existence of a functional specialization of each member has been suggested (Teixeira et al. 2006), it would be useful to study the response of the different wheat cytoplasmic APX genes to the infection by the nematode. Evaluating the function of each peroxidase isoform is quite difficult because of the redundancy of PRX and APX genes in plants (Cosio and Dunand 2009). Therefore, it would be necessary to carry out further studies such as the generation of transgenic plants overexpressing (Schweizer 2008) or silencing (Bindschedler et al. 2006) different peroxidase genes to analyze their specific involvement in the plant resistance response. Silenced transgenic plants exhibited a reduction in mRNA levels of two PRX genes accompanied by a greater susceptibility to different pathogens than that showed by wild plants (Bindschedler et al. 2006). Moreover, Johrde and Schweizer (2008) induced transient overexpression of the *HvPrx40* gene in epidermal cells of wheat and barley leaves and detected, in both cases, induction of resistance to *Bl. graminis*. These findings were complemented by transiently induced *HvPrx40* gene silencing showing hypersusceptibility of barley leaf epidermal cells to this fungus.

It would also be advisable to identify the tissue location *in planta* of each APX and PRX isoform by fusing the upstream region of each of the genes to a reporter gene such as β -glucuronidase (GUS) or green fluorescent protein (GFP). Using this method, in transgenic plants of *Arabidopsis thaliana*, one zucchini isoperoxidase was localized to the zone of elongation of the hypocotyl (Cosio et al. 2009).

In summary, *TaPrx* could be responsible for the HR as a result of increased levels of hydrogen peroxide, which are higher in the resistant genotype. The nematode could reduce its exposure to peroxide by inducing an increase in *cTaAPx* expression, particularly in the syncytium. Increased hydrogen peroxide would not affect catalase levels, because it is only involved when the amount of H_2O_2 is not very high (Ioannidi et al. 2009). A key role in the detoxification and maintenance of the ROS levels may be played by AsA, necessary for APX activity which is quickly inactivated in its absence (Chen and Asada 1989). The observed increased *cTaAPx* levels 7 dai in leaves of the susceptible line attacked by the nematode (Fig. 3) could be the result of systemic increase of H_2O_2 in the whole plant that would occur as a result of lower APX activity in roots of this line. Finally, our results suggest that APX present in wheat roots could play a role in *Cre*-mediated resistance to *H. avenae*, either directly or indirectly and that the biochemical basis of defense in hexaploid wheat against the Ha71 pathotype of *H. avenae* could be the same in all the resistant genotypes tested.

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