Analysis of Class III Peroxidase Genes Expressed in Roots of Resistant and Susceptible Wheat Lines Infected by *Heterodera avenae*

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The response of resistant wheat–*Aegilops ventricosa* introgression line H-93-8 and its susceptible parent, *Triticum aestivum* H-10-15, to Ha71 Spanish population of *Heterodera avenae* was studied to determine the changes in peroxidase gene expression during incompatible and compatible wheat–nematode interactions. Twenty peroxidase genes were characterized from both 211 expressed sequence tags and 259 genomic DNA clones. Alignment of deduced amino acid sequences and phylogenetic clustering with peroxidases from other plant species showed that these enzymes fall into seven different groups (designated TaPrx108 to TaPrx114) which represent peroxidases secreted to the apoplast by a putative N-terminal peptide signal. TaPrx111, TaPrx112, and TaPrx113 were induced by nematode infection in both genotypes but with differing magnitude and timing. TaPrx112 and TaPrx113 groups increased more in resistant than in susceptible infected lines. In addition, in situ hybridization analyses of genes belonging to TaPrx111, TaPrx112, and TaPrx113 groups revealed a more intense signal in cells close to the vascular cylinder and parenchyma vascular cells of resistant than susceptible wheat when challenged by nematodes. These data seem to suggest that wheat apoplastic peroxidases, because of their different expression in quantity and timing, play different roles in the plant response to nematode infection.

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, including Australia, Canada, South Africa, Japan, most European countries, and Asia, 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 (J2s) enter the plant roots at the level of the differentiation zone and penetrate intracellularly toward the vascular cylinder. Here, they select and pierce with their stylet a single cell where they release oesophageal secretions. In the following hours, the affected plant cells start to develop the feeding structures (Das et al. 2008). The central vacuoles disintegrate into several small vesicles, nuclei enlarge, and the number of cytoplasmic organelles increases. Cell walls are dissolved locally along plasmodesmata, thus giving rise to the formation of a syncytium. Approximately 2 weeks after infection, the nematode reaches maturity and is associated with a syncytium, typically including a great number of cells (Golinowski et al. 1996).

Many different methods are available to control nematodes such as chemical, cultural, genetic (resistance/tolerance), and biological control and their effects are aimed at decreasing population densities below damage thresholds so as to maintain or reach an attainable yield. One of the most effective control measures is the use of host resistance, which is defined as the reduction in the multiplication of the nematode in the presence of resistant plants. Globally, this is acknowledged as one of the most cost effective, environmentally sustainable, and accessible methods of controlling CCN. As reviewed by Nicol and Rivoal (2007), there has been considerable progress in identifying resistance to *H. avenae*, with all reported genes to have a major effect (i.e., controlled by a single dominant gene), making their use in a breeding program very amenable. These sources have been found predominantly in wild relatives of wheat in the *Aegilops* genus, with six of the eight named *Cre* genes for *H. avenae* resistance 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; Ogbonnaya et al. 2001; Romero et al. 1998; Seah et al. 2000). However, the effectiveness of these *Cre* genes 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 susceptible to European pathotypes (Montes et al. 2008; Rivoal et al. 2001). In contrast, the dominant resistance gene *Cre2*, which confers a high level of resistance to several European populations of CCN, including the Ha71 Spanish pathotype used in this study (Delibes et al. 1993), was ineffective against the Australian pathotype (Ogbonnaya et al. 2001). In a previous work, the efficacy of *Cre* genes against Spanish patho-

Nucleotide sequence data are available under the following accession numbers: TaPrx111-A, EU542600; TaPrx111-E, EU595572; TaPrx111-H, EU595575; TaPrx111-L, EU595573; TaPrx112-A, EU595569; TaPrx112-C, EU567314; TaPrx112-D, EU595568; TaPrx112-M, EU595567; TaPrx113-A, EU595576; TaPrx113-B, EU595577; TaPrx113-F, EU595578; TaPrx114-A, EU595579; TaPrx114-B, EU595580; TaPrx114-C, EU595581; TaPrx108, EU595582; TaPrx109-A, EU725463; TaPrx109-B1 to B5, EU725464 to 68; TaPrx109-C, EU725469; TaPrx110-A, EU725470; TaPrx110-B, EU725471; and TaPrx110-C, EU725472.

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*The e-Xtra logo stands for “electronic extra” and indicates that three supplemental tables are published online.

type 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 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 because some of the products of these genes are directly responsible for promoting nematode parasitism or for fending off the nematode attack. 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 is functional against a wide spectrum of pathogens (Nürnberg 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 the plant to overcome the parasite (Zhao et al. 2005).

Plant 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 ($OH^\bullet$), and hydroperoxyl ($OOH^\bullet$) radicals (Bindschedler et al. 2006; Bolwell et al. 2002), depending on whether the peroxidative (catalytic) or the hydroxylic cycles of the enzyme are operating (Kawano 2003; Passardi et al. 2005). During the hydroxylic cycle, peroxidases use mainly the superoxide anion (O_2^-) to generate $OH^\bullet$ while, in the peroxidative cycle, these enzymes catalyze the reduction of H_2O_2 by oxidizing various substrates. PRX, by using both these cycles, are known to participate in many different plant processes from germination to senescence (i.e., auxin metabolism, cell wall elongation, and stiffening) or protection against abiotic and biotic stresses (Cosio et al. 2009; Kawano 2003; Liu et al. 2005).

Current models of plant defense responses include peroxidase-generated radicals and ROS as catalysts of covalent cell-wall modifications (Østergaard et al. 2000; Ros Barcelo 1995) as well as signals for cell-death reactions (Passardi et al. 2005). NAD(P)H, indoleacetic acid, and saturated fatty acids represent the most important substrates oxidized by the peroxidase-mediated pathway (Hirada et al. 2001). In this regard, the peroxidases appear to act as bifunctional enzymes that can oxidize various substrates in the presence of H_2O_2 , but also generate H_2O_2 . Despite the correlative evidence for the involvement of peroxidase activity with pathogen resistance, regulation of PRX during nematode infection, on the isoenzyme basis, is largely unknown because many of these isoenzymes are simultaneously expressed in plants in response to pathogen infection and, moreover, some of them work as antioxidant enzymes (Zacheo et al. 1997). Involvement of peroxidases in wheat defense was suggested by selectively induced expression of the *pox2* peroxidase gene in leaves of hexaploid wheat (Båga et al. 1995) as well as other peroxidase genes in diploid wheat, *Triticum monococcum*, when challenged by powdery mildew fungus (Liu et al. 2005). In addition, hydrogen peroxide production is a known response of host plants to root-knot (Das et al. 2008; Melillo et al. 2006) and cyst (Waetzing et al. 1999) nematode invasion.

In previous studies, findings obtained by our group (Andrés et al. 2001; Montes et al. 2003, 2004) demonstrated that cyst nematode infection enhances PRX, esterase, and superoxide dismutase activity in wheat roots carrying *Cre2*, *Cre5*, or *Cre7* resistance genes. The highest response was found in the PRX system with an increase in both cationic and anionic isozymes. Therefore, to understand the precise role of each individual isoform in planta, it is imperative to study its function, and its regulation following nematode infection.

The objective of the present study was to assess the involvement of peroxidases in the defense response against nematode infection. We describe the expression patterns of these isoforms at the level of transcript accumulation and localization in resistant (H-93-8) and susceptible (H-10-15) wheat lines challenged by CCN. Indeed, although, in some cases, spatial and temporal localization or indirect evidence might indicate the involvement of peroxidases in specific mechanisms (Andrés et al. 2001), their in planta transcription has never been clearly characterized.

The data presented here suggest that a number of PRX transcripts might play a role in the constitutive and inducible defense mechanisms, as reported in other host–pathogen interactions (Liu et al. 2005; Schweizer 2008). Moreover, these findings provide a comprehensive response of accumulation of PRX gene transcripts and might help to understand their involvement in wheat roots challenged by CCN.

RESULTS

TaPrx genes and predicted proteins identified from nematode-infected roots.

Root cDNAs from H-93-8 at 4 and 7 days after nematode inoculation were used to identify the PRX genes involved in the response to the pathogen attack. Rapid amplification of cDNA ends (RACE)-3' and RACE-5' products were aligned in order to obtain complete transcript sequences. Specific primers were designed from low-homology areas of these peroxidase transcripts to amplify genomic DNA belonging to H-93-8 and its parents, *T. aestivum* (H-10-15), *T. turgidum* (H-1-1), and *Aegilops ventricosa* (AP-1). Sequence similarity analysis showed that the 470 sequenced clones (211 expressed sequence tags [EST] and 259 genomic) represented at least 17 peroxidase genes and three pseudogenes (Fig. 1; Table 1), all present in the four tested lines. Alignment of these peroxidase genes revealed changes in length and nucleotide sequence, in particular, in 5' (38 to 100 bp) and 3' (91 to 567 bp) untranslated regions (UTR) (Fig. 2).

The 20 *TaPrx* predicted protein sequences showed well-conserved B, D, and F domains, also found in *Arabidopsis* (AtPrxs) and rice (OsPrx). The phylogenetic tree generated by encompassing complete PRX amino acid sequences from wheat ($n = 17$), rice ($n = 10$), barley ($n = 2$), and *Arabidopsis thaliana* ($n = 1$) is represented in Figure 3. The PRX sequence of *Marchantia polymorpha* (Mp-Q8H958) was used to root the tree because it provided the maximum sequence length when compared with other outgroups. Clusters were strongly supported by bootstrap values. Six clusters, designated TaPrx108 to TaPrx114, were found: three members fit in the cluster TaPrx111 (TaPrx111-A, TaPrx111-E, and TaPrx111-L), four in TaPrx112 (TaPrx112-A, TaPrx112-C, TaPrx112-D, and TaPrx112-M), three in TaPrx113 (TaPrx113-A, TaPrx113-B, and TaPrx113-F), three in TaPrx114 (TaPrx114-A, TaPrx114-B, and TaPrx114-C), one in TaPrx108, and three in TaPrx109 (TaPrx109-A, TaPrx109-B, and TaPrx109-C). TaPrxs exhibited an identity different from the most similar selected rice PRX at protein levels ranging from 77% (OsPrx110 with TaPrx108) (Table 1) to 85% (OsPrx74 with TaPrx109-A). The closest homologue for clusters TaPrx112 and TaPrx113 came from barley (*Hordeum vulgare* peroxidase 1 precursor showing 85% identity with TaPrx112-D and TaPrx112-A and 96% with TaPrx113-F) (Table 1). The homology among the 17 full-length TaPrx-deduced amino acid sequences ranged from 34 to 99% (data not shown). Based on isoelectric point (pI) predictions (Table 1), mature proteins represented either acid (clusters TaPrx111, TaPrx113, TaPrx108, and TaPrx109) or basic (clusters TaPrx112 and TaPrx114) isoforms of PRX, with pI

ranging from 5.51 to 9.00. TaPrx113-A showed the highest identity to Pox3 (CAA59486, 96%) (Table 1) and TmPRX1 (AAW52715, 99%) (Table 1), two proteins deduced from powdery mildew inducible *PRX* genes isolated from bread wheat (*T. aestivum*) and *T. monococcum* leaves, respectively. TaPrx112-M was identical to TmPRX3 (Table 1) and showed very high identity to Pox2 (CAA59485, 99%), another protein sequence deduced from a *PRX* gene induced by powdery mildew in bread wheat (Båga et al. 1995) (Table 1). Other GenBank peroxidase proteins from different monocot plants (rice and barley) were found to be homologous (more than 72% identity) to the wheat *PRX* (Table 1).

Intron structure of wheat peroxidase genes.

The *PRX* genes present the classical pattern of four exons and three introns shared by the majority of *PRX* genes (Tognolli et al. 2002). The *TaPrx111* group was also characterized by this pattern (Fig. 2), while the remaining *PRX* encoding genes consisted of three exons and two introns. Only these two models were found in all analyzed genomic sequences. Wheat introns exhibited a smaller size range (81 to 499 bp) than rice (38 to 9,974 bp), with *TaPrx114-A* to *-C* genes showing the largest size (385 to 499 bp) (Fig. 2). Sequences *TaPrx111-H*, obtained from line H-10-15, and *TaPrx114-C* (data not shown) were characterized by the presence of very large introns con-

Fig. 1. Multalin alignment of predicted amino acid sequences of wheat *TaPrx* genes. Gaps introduced for better alignment are shown by dashes. Vertical arrow indicates the separation between putative signal peptides and mature proteins. Horizontal lines represent conserved distal heme-binding (B), proximal heme-binding (F), and central conserved (D) domains of unknown function. A region presumed to be important for determining the specific function (see text) is indicated by a dashed line. Invariable eight-cysteine residues, which form four disulphide bridges, are marked with the diamond symbol. Putative active sites (such as N-glycosylated triplets) are labeled with an inverted 'V' symbol on the top of the sequences. A putative monocotyledon-specific motif, present in the *TaPrx109* group, is surrounded by a pointed line and two vertical arrows at the margins. *TaPrx110* amino acid deduced sequences were truncated at their 5' region.

taining the CACTA E/Spm-like DNA transposon and Mariner/Tc1 nonautonomous transposon, respectively. The position of introns in relation to the reading frame was totally conserved within all the wheat peroxidases characterized in this study. The nucleotide sequences of introns greatly diverged and almost showed no similarity. The second and third intron contained the classical GT/AG splice sites. In contrast, the processing of the first intron seems to be performed at a nonconsensus GC donor site in *TaPrx111*, *TaPrx112*, and *TaPrx114* groups (data not shown).

Expression of *TaPrx* genes is induced by *Heterodera avenae* infection.

In order to evaluate the expression of *TaPrx* genes during the process of wheat infection by *Heterodera avenae*, transcript levels were monitored by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) 4 and 7 days after nematode inoculation in both susceptible H-10-15 and resistant H-93-8 lines (Fig. 4). No significant differences were observed between the two methods used for data analysis (quantification assuming efficiencies were equal to two and real-time PCR Miner; discussed below); therefore, only results obtained by Miner analysis are reported in Figure 4.

TaPrx111, *TaPrx112*, and *TaPrx113* groups showed a similar expression pattern in response to nematode infection, reaching

their maximum levels at the fourth day postinoculation in the resistant line (Fig. 4). Higher induction of *TaPrx112* and *TaPrx113* groups (threefold) was observed in the H-93-8 resistant than in the H-10-15 susceptible line. It is interesting to note that constitutive expression of *TaPrx112* and *TaPrx113* genes in the uninfected H-10-15 line was lower than in the uninfected H-93-8 (3- and 10²-fold, respectively). In addition, *TaPrx111* group in the susceptible line showed a different kinetic of transcripts accumulation, with a greater increase of mRNA levels in 7-day infected roots, compared with the resistant one (Fig. 4). These findings suggest that this group of enzymes works in a different manner in compatible and incompatible interactions. The expression patterns of genes in *TaPrx114* and *TaPrx109* groups were similar in both interactions and did not show any transcript accumulation following nematode infection (data not shown). *TaPrx108* expression was not detected at the RNA level. The expression pattern of *TaPrx* genes in leaf tissue of both lines showed lower transcript accumulation than in roots (4- to 300-fold) (data not shown), and this behavior was not altered by nematode infection.

Localization of *PRX* transcripts after nematode infection.

In situ hybridization on serial sections of uninfected and 4-day-infected wheat roots was performed to analyze the locali-

Table 1. List of peroxidase genes isolated from *Triticum aestivum*–*Aegilops ventricosa* introgression line, H-93-8, resistant to *Heterodera avenae*^a

Gene name	GenBank accession no. ^b	AA ^c	pI ^c	Molecular mass (Da)	Signal peptide length	Closest GenBank rice or barley PRX (identity %)	Closest GenBank <i>Triticum</i> PRX (identity %)
<i>TaPrx111-A</i>	EU542600	295	5.78	31,156.8	21	<i>Oryza sativa japonica</i> OsPrx112 (73) ^d	<i>T. aestivum</i> Pox1 (97) ^e
<i>TaPrx111-E</i>	EU595572	295	6.08	31,200.8	21	<i>O. sativa japonica</i> OsPrx112 (72) ^d	<i>T. aestivum</i> Pox1 (99) ^e
<i>TaPrx111-L</i>	EU595573	295	5.51	31,164.9	21	<i>O. sativa japonica</i> OsPrx112 (72) ^d	<i>T. aestivum</i> Pox1 (95) ^e
<i>TaPrx112-A</i>	EU595569	291	8.44	30,433.1	23	<i>Hordeum vulgare</i> peroxidase 1 precursor (85) ^f	<i>T. monococcum</i> TmPRX3 (98) ^g
						<i>T. aestivum</i> Pox2 (97) ^e	<i>T. aestivum</i> WP2 (95) ^h
<i>TaPrx112-C</i>	EU567314	291	8.44	30,503.2	23	<i>H. vulgare</i> peroxidase 1 precursor (83) ^f	<i>T. aestivum</i> WP2 (99) ^h
						<i>O. sativa japonica</i> OsPrx114 (77) ^d	<i>T. monococcum</i> TmPRX3 (97) ^g
<i>TaPrx112-D</i>	EU595568	291	8.82	30,514.2	23	<i>H. vulgare</i> peroxidase 1 precursor (85) ^f	<i>T. aestivum</i> Pox2 (97) ^e
						<i>O. sativa japonica</i> OsPrx114 (77) ^d	<i>T. monococcum</i> TmPRX3 (96) ^g
<i>TaPrx112-M</i>	EU595567	291	8.44	30,461.1	23	<i>H. vulgare</i> peroxidase 1 precursor (85) ^f	<i>T. aestivum</i> Pox2 (96) ^e
						<i>O. sativa japonica</i> OsPrx112 (77) ^d	<i>T. aestivum</i> WP2 (94) ^h
<i>TaPrx113-A</i>	EU595576	290	5.52	30,637.2	22	<i>H. vulgare</i> subsp. <i>vulgare</i> peroxidase (93) ⁱ	<i>T. monococcum</i> TmPRX3 (100) ^g
						<i>O. sativa japonica</i> OsPrx114 (78) ^d	<i>T. aestivum</i> Pox2 (99) ^e
<i>TaPrx113-B</i>	EU595577	294	5.79	30,889.5	22	<i>H. vulgare</i> peroxidase 1 precursor (96) ^f	<i>T. aestivum</i> WP2 (97) ^h
						<i>O. sativa japonica</i> OsPrx 114 (77) ^d	<i>T. monococcum</i> TmPRX1 (99) ^g
<i>TaPrx113-F</i>	EU595578	294	5.79	30,932.5	22	<i>H. vulgare</i> peroxidase 1 precursor (96) ^f	<i>T. aestivum</i> Pox3 (96) ^e
						<i>O. sativa japonica</i> OsPrx114 (77) ^d	<i>T. aestivum</i> TaPrx103 (95) ^j
<i>TaPrx114-A</i>	EU595579	296	8.88	31,380.2	23	<i>O. sativa japonica</i> OsPrx109 (80) ^d	<i>T. monococcum</i> TmPRX2 (99) ^g
<i>TaPrx114-B</i>	EU595580	298	9.00	31,375.4	23	<i>O. sativa japonica</i> OsPrx109 (76) ^d	<i>T. aestivum</i> Pox4 (100) ^e
<i>TaPrx114-C</i>	EU595581	298	9.00	31,373.4	23	<i>O. sativa japonica</i> OsPrx109 (77) ^d	<i>T. aestivum</i> Pox4 (88) ^e
<i>TaPrx108</i>	EU595582	293	6.09	31,106.8	20	<i>O. sativa japonica</i> OsPrx110 (77) ^d	<i>T. aestivum</i> Pox4 (85) ^e
							<i>T. monococcum</i> TmPRX5 (99) ^g
<i>TaPrx109-A</i>	EU725463	304	6.08	33,458.0	22	<i>O. sativa japonica</i> OsPrx74 (85) ^d	<i>T. monococcum</i> TmPRX4 (98) ^g
<i>TaPrx109-B1 to B5</i>	EU725464 to 68	304	6.26	33,443.0	23	<i>O. sativa japonica</i> OsPrx74 (84) ^d	...
<i>TaPrx109-C</i>	EU725469	304	6.08	33,465.0	25	<i>O. sativa japonica</i> OsPrx74 (82) ^d	...

^a Predicted mature protein characteristics and percent identity with closest GenBank proteins. Closest homologous protein, accession number, percent identity, and reference were obtained by BLAST at the National Center for Biotechnology Information server. PRX = peroxidase.

^b Genomic sequences, except *TaPrx109-C* that corresponds to mRNA isolated from roots of H-93-8 line.

^c Amino acids number (AA); isoelectric point (pI) for mature protein was estimated by Swiss-Prot/TrEMBL.

^d Passardi et al. 2004.

^e Båga et al. 1995.

^f Thordal-Christensen et al. 1992.

^g Liu et al. 2005.

^h Hertig et al. 1991.

ⁱ Green 1991.

^j Rebmann et al. 1991.

zation of each peroxidase group transcripts during CCN infection. Sense and antisense probes spanning 3' UTR of two transcripts (PRB-111-L and PRB-111-A) of group *Taprx111*, three (PRB-112-F, PRB-112-A, and PRB-112-Ab) of group *Taprx112*, and one (PRB-113-F) of group *Taprx113* were used (Fig. 5). The choice of probes (discussed below) in each group, three clones from *Taprx112* and two from *Taprx111*, was done with the aim of unraveling different behaviors, if any, in the same group.

In resistant infected roots, each probe gave positive results by means of brown staining of cells injured by nematode migration. The most reactive sites were the cortical cells penetrated by the nematode and endodermal and pericycle cells injured by nematode feeding action. Surprisingly, syncytial cells did not give any reaction.

In particular, sections hybridized with the PRB-111-L probe showed a strong reaction just in cells delimiting nematode entrance (Fig. 6A) whereas, in the syncytium, no reaction was

detected (Fig. 6B). In contrast, a very faint signal, only restricted to cells broken by the juvenile entrance and suggesting a wounding response, was observed in sections hybridized with PRB-111-A (Fig. 6C). In susceptible infected roots, PRB-111-L and PRB-111-A probes induced more or less the same reaction observed in the resistant line probed with PRB-111-A and previously suggested to be a wounding response (Fig. 6A', B', and C'). No signal was detected in the controls with both sense probes on resistant and susceptible wheat lines (Fig. 6D and D').

Sections of resistant H-93-8 hybridized with probes PRB-112-F, PRB-112-A, and PRB-112-Ab showed the strongest signals. PRB-112-F probe gave heavy staining on cortical, endodermal, and vascular parenchyma cells directly injured by the nematode and appeared as amorphous stained aggregates (Fig. 6E). Cells of the same line challenged by nematode penetration strongly reacted also to probes PRB-112-A and PRB-112-Ab (Fig. 6F and G). A ring of hyper-reacting cells entrap-

Fig. 2. Genomic organization of *TaPrx* gene groups. Coding regions (gray arrows), introns (double lines), untranslated regions (UTR) (striped boxes), and their sizes in terms of sequences (bp) are indicated.

ping the nematodes at their feeding sites were observed in root sections probed with PRB-112-A (Fig. 6F). In contrast, a number of cortical cells affected by nematode penetration were labeled when probed with PRB-112-Ab (Fig. 6G). The PRB-112-F probe showed heavy staining of collapsed cells bordering the endoderma in the susceptible root (Fig. 6E'), where

reaction with probes PRB-112-A and PRB-112-Ab was poorly detectable (Fig. 6F' and G'). Each sense probe did not give a signal in either resistant or susceptible lines, as can be seen in Figure 6H and H'.

Probe PRB-113-F heavily labeled resistant cells injured by nematode invasion (Fig. 6I and J). All cortical cells challenged

Fig. 3. Phylogenetic relationships based on predicted protein sequences from *Triticum aestivum* (TaPrx), representative rice (OsPrx) (Passardi et al. 2004), *Arabidopsis* (AtPrx52) (Tognolli et al. 2002), and *Hordeum vulgare* (Hv) (Green 1991; Thordal-Christensen et al. 1992) peroxidases. The tree was constructed by the neighbor-joining method and rooted with the peroxidase sequence of *Marchantia polymorpha* (Mp). Values at nodes indicate bootstrap supports. All branches are drawn to scale and bar represents 0.1 substitutions per site.

by nematode penetration were strongly stained (Fig. 6I). Interestingly, parenchyma vascular cells selected by the nematode as its feeding site and developing into a syncytium showed great staining of the cytoplasm and partially digested walls (Fig. 6J). The same probe in the susceptible root induced a lighter reaction, where only the walls of cortical cells along the nematode entrance were brown stained (Fig. 6I' and J'). No signal at all was detected in the negative control (Fig. 6K and K').

Fig. 4. Expression of *TaPrx* gene groups 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 means $\pm$ standard error of three replicates from four biological samples. Symbols: –, uninfected root; +, infected root at 4 and 7 days (4d and 7d, respectively) after nematode inoculation.

Sections of uninfected roots of both resistant and susceptible genotypes did not show any signal no matter what probe was used (Fig. 6L and L').

DISCUSSION

Although several hypotheses have been formulated, the function of peroxidases in cereals has not been clearly assessed. The first aim of this study was to isolate and characterize the *PRX* genes in wheat. Moreover, expression and localization of different genes to get more insights into their putative role, if any, in the defense response of wheat roots to *H. avenae* were investigated.

Identification and characterization of root peroxidases.

PRX are synthesized as preproteins with N-terminal signal peptide and sometimes with C-terminal extension which are removed to produce the mature protein (Buffard et al. 1990; Johansson et al. 1992; Welinder et al. 2002). Precursors are targeted either to the cell wall or vacuole, probably sorted by the N terminal and C terminal, respectively (Kristensen et al. 1999; Matsui et al. 2003). None of the PRX analyzed exhibited C-terminal extension, suggesting that they may be secreted to the apoplast and associated with ROS production as previously hypothesized for a group of *T. monococtum* peroxidases (TmPRX1 to TmPRX6) by Liu and associates (2005). Alignment of deduced amino acid sequences from *TaPrx* genes revealed that they contain conserved structural features and activity sites of class III PRX such as the predicted N-terminal signal peptide; the highly conserved B, D, and F domains essential to enzyme catalysis and protein folding (Welinder 1992); eight cysteine residues involved in the formation of four disulfide bridges; N-glycosylated triplets; and a variable region 40 to 50 amino acids toward the carboxyl end of the proximal heme-binding site (Fig. 1). It has been suggested that these variable sequences are correlated with the substrate specificity of the protein (Chittoor et al. 1999). The *TaPrx109* cluster was notably divergent because it possesses distinct amino acid sequences (Fig. 1) and structural features, which suggest a more ancient differentiation (Fig. 3). Furthermore, a 16-amino-acid domain, considered to be monocotyledon specific (Passardi et al. 2004), is only present in the *TaPrx109* cluster (Fig. 1).

Sequences of the *TaPrx109* group in the wheat genome were characterized for the first time in this study. All these genes showed high identity to rice peroxidase *OsPrx74* at the DNA level (62 to 68%), although they lacked the third intron characteristic of this gene. Additionally, this group exhibited the highest variability in its 3' UTR, which may be related to alternative and regulated 3' end processing. BLAST of *TaPrx109* group sequences showed high identity to GenBank EST isolated from wheat plants under abiotic stress; however the bio-

Fig. 5. Sequence alignment of gene-specific probes used for in situ hybridization. These sequences represent reverse-transcription polymerase chain reaction products spanning 3' untranslated regions corresponding to *TaPrx111* (PRB-111-L and PRB-111-A), *TaPrx112* (PRB-112-F, PRB-112-A and PRB-112-Ab), and *TaPrx113* (PRB-113-F) peroxidase groups. Gaps introduced for better alignment are shown by dashes.

logical function of this group is still unknown. All 5'-RACE products isolated from the *TaPrx110* group gave truncated sequences lacking the 5' region, indicating that they might be nonfunctional. Indeed, a complete wheat peroxidase sequence of this group has not been described to date, although a great number of sequences are available in the GenBank. The strongest homologous gene in rice, *OsPrx89*, was characterized by a very large third intron and detected at the mRNA level, despite containing retrovirus sequences (Passardi et al. 2004). DNA sequence alignment of the *TaPrx* groups showed more variants, mainly in introns and 5' and 3' UTR regions, than those previously described for *T. aestivum* (Båga et al. 1995). It is possible that some of these variants originated from the three different ancestral wheat genotypes while others may have arisen from duplication or transposition events. The DNA sequences *TaPrx113-A*, *pox3* (Båga et al. 1995), *TmPRX1* (Liu et al. 2005), and *pox381* (Rebmann et al. 1991) were also found to be very similar (identity of 93 to 98%) (data not shown). All these genes were isolated from different wheat species, suggesting that they are variants of the same gene. Notable DNA sequence similarities were found between *TaPrx112-M*, *TmPRX3* (Liu et al. 2005), and *pox2* (Båga et al. 1995) as well as between *TaPrx111-E* and *pox1* (Båga et al. 1995), which may also indicate a common origin. On the other hand, deduced protein sequences from *TaPrx108*, *TaPrx111*, *TaPrx112*, and *TaPrx113* grouped with rice peroxidases *OsPRX110*, *OsPRX112*, and *OsPRX114*, whereas *TaPrx114* fell into the same group with *OsPRX109*. All these rice PRX were assigned to group IV (Passardi et al. 2004), which contained peroxidases possibly involved in plant-pathogen interaction (Chittoor et al. 1997).

Most plant *PRX* genes studied are found to be interrupted by two or three introns. *TaPrx111* genes carry three introns as the characterized *PRX* genes of horseradish (Fujiyama et al. 1990) and *Arabidopsis thaliana* (Tognolli et al. 2002) within the *Brassicaceae* family. The three-intron model was found in wheat, rice, and *Arabidopsis* in a significant proportion and it could be the ancestral intron organization pattern. The other isolated *PRX* genes from wheat (*TaPrx108* [not shown], *TaPrx109*, *TaPrx112*, *TaPrx113*, and *TaPrx114* groups) carried only two introns which are analogous to monocot *PRX* (Båga et al. 1995; Passardi et al. 2004).

TaPrx111-H and *TaPrx114-C* genes were characterized by the presence of a very large intron containing nonautonomous transposons. It has been proposed that the rice introns could play a role as viral genomic insertion "buffers" protecting the plant against gene inactivation phenomena (Passardi et al. 2004). However, this defense system against insertions is, apparently, not always efficient (Casacuberta and Santiago 2003) because truncated proteins may be produced, as happens with the *TaPrx110* group (Fig. 1).

The presence of GC nucleotides in place of the invariant GT at the 5' splice site of intron 1 in *TaPrx111*, *TaPrx112*, and *TaPrx114* seems not to affect the splicing process. Identical splice site patterns were also found in peroxidase genes of other plants (Mika et al. 2008; Xue and Rask 1995). GC-5' splice sites occur rarely and they were often found in genes coding for proteins involved in oxygen reactions (Jackson 1991a).

Expression analyses of *PRX* genes

in wheat roots subjected to *H. avenae* infection.

Accumulation of *TaPrx111*, *TaPrx112*, and *TaPrx113* peroxidase transcripts over their normal basal level was induced by nematode infection in both resistant and susceptible genotypes. In contrast, expression in *TaPrx114* and *TaPrx109* groups was not affected by nematode infection in both genotypes, suggesting that these two gene groups might not be directly involved

in the defense response to *H. avenae*. Båga and associates (1995) reported that the expression level of the *pox1* gene, a peroxidase highly similar to *TaPrx111*, was not affected by powdery mildew infection. This may reflect changes in gene expression mediated by the two different ways of pathogen penetration, intracellularly in CCN and appressorium-mediated in *Blumeria graminis*, with and without wounding, respectively. This is supported by in situ hybridization results, in which *TaPrx111* peroxidase probes gave a brown staining just in cells delimiting nematode entrance, thus suggesting a wounding response. Indeed, all *TaPrx111* sequences analyzed in this study carried a 10-bp inverted repeat located 17 bp upstream of the assigned TATA box (data not shown) that was previously proposed as a putative wound-induced regulatory sequence (Båga et al. 1995). In addition, the slightly higher accumulation of *TaPrx111* transcripts at 4 days in the resistant than in the susceptible line, but not at 7 days, suggests a putative role in the context of a general plant response, more related to wounding than to a specific reaction to nematode attack.

The highest expression level was detected for cluster *TaPrx112* independently of pathogen attack and genotype. These findings agree with previous results obtained for two similar wheat genes, *poxwht* and *pox2*, constitutively expressed in roots (Båga et al. 1995; Hertig et al. 1991) (Table 1). Moreover, the expression levels of clusters *TaPrx112* and *TaPrx113* were always much higher in the resistant H-93-8 line (3 and 10²-fold, respectively) (Fig. 4) than in the susceptible H-10-15, and may be related to a constitutive mechanism of defense in the resistant wheat. The mode of action of some of these peroxidases would be similar to that described for *TaPrx103* (previously named *TaPERO*) (Rebmann et al. 1991), which conferred enhanced resistance to *B. graminis* f. sp. *tritici* when it was constitutively or transiently expressed in transgenic wheat epidermis (Altpeter et al. 2005). Epidermal cells of transgenic lines were found to respond to *B. graminis* f. sp. *tritici* attack more frequently with hypersensitive cell death and generation of hydrogen peroxide (Schweizer 2008). Furthermore, a higher level of induction upon nematode infection was detected in H-93-8 wheat for *TaPrx112* and *TaPrx113* clusters. All in situ probes from these groups gave a strong reaction in cells injured by nematode migration in the resistant roots, suggesting that they may also be regulated by wounding. Possible regulatory G-box-like sequences were identified for *pox2* and *pox3* (Båga et al. 1995) and these seem to be required for wound-induced expression (Kawaoka et al. 1994). Whether any of these regulatory sequences is involved in gene regulation during nematode-plant interactions remains to be elucidated. Interestingly, infected roots from resistant wheat lines showed a heavy reaction in cells close to the vascular cylinder and parenchyma vascular cells when probed with PRB-112-F and PRB-113-F. These peroxidases could, then, play a role in the early resistance response by leading to membrane degradation and related death of cells selected as feeding sites and affected by nematode oesophageal secretions. This seems to be confirmed by the accumulation of the PBR-113-F probe signal just in the cells where the nematode, starting its feeding, is inducing the syncytial development. The stronger signal in the resistant than in the susceptible line, totally in agreement with qRT-PCR results, might suggest an early and heavy response of resistant cells to signals triggered by glandular nematode secretions. Our data also provide evidence that each *TaPrx* group responds in a different way to signals from the nematode and, as a whole, cooperate to limit root disease.

Evaluating the function of each peroxidase isoform is quite difficult because of the redundancy of *PRX* genes in plants (Cosio and Dunand 2009). Therefore, it would be necessary to carry out further studies, such as the generation of transgenic

Fig. 6. In situ localization of peroxidase (*Prx*) transcripts in 4-day nematode-infected and uninfected H-93-8 (resistant) and H-10-15 (susceptible) wheat root sections. Cross sections of **A** through **D**, resistant and **A'** through **D'**, susceptible roots hybridized to digoxigenin (DIG)-labeled *Taprx111* group probes. **A**, **B** and **A'**, **B'**, PRB-111-L and **C**, **C'**, PRB-111-A antisense probes; and **D**, **D'**, control sense probe. **A**, PRB-111-L revealed a number of cortical and parenchyma vessel cells of H-93-8 roots strongly reacting to nematode penetration and its feeding action. **B**, Detail of root reacted cells and unlabeled syncytia (arrows). **A'**, In contrast, few cortical and parenchyma vessel cells in H-10-15 roots showed a light brown staining as a consequence of nematode penetration and its feeding action. **B'**, Nematode (arrowhead) in reacting parenchyma cells and completely unaffected syncytia (arrow). **C**, **C'**, Very faint signal, only in the cells broken by nematode entrance, is detectable in resistant and susceptible roots probed with PRB-111-A. **D**, **D'**, Negative controls were completely unstained. Cross sections of **E** through **H**, resistant and **E'** through **H'**, susceptible roots hybridized to DIG-labeled *Taprx112* group probes. **E**, **E'**, PRB-112-F, **F**, **F'**, PRB-112-A, and **G**, **G'**, PRB-112-Ab antisense probes; and **H**, **H'**, control sense probe. **E**, PRB-112-F probe gave the strongest signal in cortical, endodermal, and vascular parenchyma cells of H-93-8 as well as in **E'**, cortical cells bordering the endodermis in H-10-15. **F**, Resistant root probed with PRB-112-A showed a ring of hyper-reacting cells entrapping the nematode (arrowhead) at its feeding site while **F'**, susceptible root appeared to be less sensitive to the probe and showed reaction in very few parenchyma cells. **G**, PRB-112-Ab, which gave a strong labeling in cortical cells of H-93-8 endangered by nematode penetration, did not greatly affect **G'**, susceptible root tissues. **H**, **H'**, Sense probe did not give any staining in both resistant and susceptible root cells. Cross sections of **I**–**K**, resistant and **I'** through **K'**, susceptible roots hybridized to DIG-labeled PRB-113-F probes. **I**, **J** and **I'**, **J'**, PRB-113-F antisense probe and **K**, **K'**, control sense probe. **I**, Probe induced heavy reaction in cells of H-93-8 affected by nematode invasion and, in particular, in parenchyma vascular cells selected as nematode (arrowhead) feeding site where **J**, a developing syncytium showed strong staining of its cytoplasm and cell walls. **I'**, **J'**, PRB-113-F only labeled the walls of cortical cells at nematode (arrowhead) entrance but not cells of vascular cylinder in H-10-15. **K**, **K'**, No signal at all was detectable in the negative controls. View of **L**, resistant and **L'**, susceptible uninfected root sections which were not reactive to each probe used. Scale bar: **B**, **B'** and **J**, **J'** = 1.8 μ m; all others = 2.8 μ m.

plants overexpressing (Schweizer 2008) or silencing (Bind-schedler et al. 2006) different peroxidase genes, in order to analyze their specific involvement in plant resistance.

MATERIALS AND METHODS

Plant material and nematodes.

The experiments were performed on wheat line H-93-8, carrier of the *Cre2* CCN resistance gene derived from the cross (*T. turgidum* subsp. *turgidum* cv. *Rubrostratum*, H-1-1 × *Aegilops ventricosa* AP-1) × *T. aestivum* cv. *Almatense*, H-10-15 (previously described) (Delibes et al. 1993; Mena et al. 1993); its parents, *T. aestivum* H-10-15 and *T. turgidum* H-1-1, both without CCN resistance genes; and *A. ventricosa* AP-1. Hereafter, abbreviations H-93-8, H-10-15, H-1-1, and AP-1 will be used.

The CCN (*H. avenae*) population from a naturally infested plot at the La Poveda Experimental Station in Arganda (Madrid) had previously been characterized as belonging to the Ha71 pathotype (López-Braña et al. 1996; Romero et al. 1996). Seed of the resistant H-93-8 and susceptible H-10-15 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 ± 2°C in a growth chamber. At 48 h, seedlings were divided in two groups: one was inoculated with 100 J2s of the Ha71 *H. avenae* pathotype per plant and the second one (uninfected seedlings) was used as a control as described by Andrés and associates (2001). Roots and leaves of infected plants were harvested 4 and 7 days after inoculation, as well as uninoculated tissues utilized as the control. Root and leaf strips were flash frozen in liquid nitrogen and then stored at -80°C until further use.

RNA purification and RT-PCR analysis.

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

PRX cDNA syntheses were performed using the Superscript one-step RT-PCR kit for 3' RACE (Invitrogen Life Technologies, San Diego, CA, U.S.A.) and the SMART RACE cDNA Amplification Kit for 5' RACE (Takara Bio Company, Mountain View, CA, U.S.A.) in accordance with the manufacturers' recommendations. The primers used for amplification are shown in Supplementary Tables 1 and 2. RACE reactions were

carried out in a Mastercycler Gradient (Eppendorf HQ, Hamburg, Germany) with 0.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, U.S.A.), 150 ng of DNA, and 250 nM each primer in a 20-μl volume, with 10 min of initial denaturation at 94°C. Different combinations of annealing temperatures and extension times were tested to find the optimal conditions for each primer pair. PCR products were fractionated on a 1 to 2% agarose gel using Tris-acetate-EDTA buffer and stained with ethidium bromide. DNA bands were eluted with a QIAEX II gel extraction kit (Qiagen Inc., Valencia, CA, U.S.A.) and products were cloned in the pGEM-T Easy vector system (Promega Biotech Ibérica, SL, Alcobendas, Madrid,) following manufacturer's indications.

DNA extraction, amplification, and cloning.

Total genomic DNA was extracted according to the cetyltrimethylammonium bromide procedure described by Taylor and Powell (1982). Preferential amplification of sequences from individual PRX genes was obtained with primers shown in Supplementary Table 3. PCR products were treated as previously described for RT-PCR.

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. Primers specific for each PRX group were designed from 3' low-homology regions using Primer Express 2.0 software (Applied Biosystems). The primer sequences are shown in Table 2. The PCR 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 were 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 to 68°C in 96-well optical reaction plates (Applied Biosystems). The specificity of amplicons was verified by melting curve analysis (60 to 95°C) after 40 cycles and agarose gel electrophoresis. All genes were analyzed in triplicate from four independently isolated RNA samples. The cycle threshold (C_t) values were normalized to a conventional reference gene (actin) using the primer pairs showed in Table 2. Two different approaches were used to process qRT-PCR data: i) the relative quantification method which assumes that amplification efficiencies are equal to 2 and equal between all reactions (Pfaffl 2001) and ii) 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 C_t values for individual PCR reactions.

DNA sequencing and data analysis.

Sequencing was done on an Applied Biosystem ABI PRISM 3100 sequencer (Applied Biosystems). PRX homology sequences were searched with BLAST via the National Center for Biotechnology Information web server (Altschul et al. 1997). Amplified fragments were aligned to obtain full-length cDNA and genomic-DNA PRX sequences. We adopted the nomenclature used in rice (Passardi et al. 2004), with the letters "Ta" for *T. aestivum* and "Prx" for peroxidase, followed by a number, corresponding to clusters, and a letter, indicating variants inside each cluster. The splicing sites of introns were identified by the alignment of genomic DNA to EST or cDNA sequences. Transposable elements in genomic DNA introns

Table 2. Primer sequences used in quantitative real-time polymerase chain reaction analysis

Peroxidase cluster	Primer pair sequences	Size (bp) ^a
TaPrx111	GACTGCTTCGTCGATGGATGT TGCCTCGAATGGAATTGTTG	103
TaPrx113	ATTGACAAACGAGTTACTGCCTACTAG GATTGCTGCTGCTCGTACA	126
TaPrx112	CGGTGACACCAACATCAACACTG CAGGAGCCCTTTCTGTGACAG	146
TaPrx114	CAGTGCCGGTTTTTCAGGAG TATGCGTTGTCTGAACCCGTT	158
TaPrx109	TCTCCGACCGGCTCTACAACCT GCTGGCACACTTGCTCTTTAGC	104
Actin	GAAGCTGCAGGTATCCATGAGACC AGGCAGTGATCTCTTGCTCATC	151

^a Expected amplicon size.

were searched by comparison with Repbase through the Genetic Information Research Institute server (Kohany et al. 2006). The corresponding coding sequences were translated with the "translate" tool on the ExPASy Proteomics Server and controlled for specific peroxidase motifs. All DNA and protein sequence alignments were performed via the Plateforme Bioinformatique de la Génopole server with Multalin (Corpet 1988) and visually adjusted with the GeneDoc program (Nicholas et al. 1997). The N-terminal signal peptides of deduced amino acid sequences were predicted with the SIG-NALP 3.0 server (Bendtsen et al. 2004). The distance tree was constructed with the NEIGHBOR option under the JTT substitution frequency matrix, and 1,000 bootstrap replicates were carried out for this method with MEGA software version 3.1 (Tamura et al. 2007).

In situ hybridization.

Four-day nematode-infected and -uninfected roots from H-93-8 and H-10-15 wheat lines were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin wax as described by Jackson (1991b) with some modifications. To discriminate the three groups of peroxidases *TaPrx111*, *TaPrx112*, and *TaPrx113* (with a high conservation of sequences in the coding region), the nucleotide sequences corresponding to the 3' UTR were aligned. The selection of the probe was done, taking into account the sequence which was more representative, in terms of percentage of similarity of i) the entire group (PRB-113-F), ii) some intragroup clusters (PRB-112-F, PRB-112-A, PRB-112-Ab, and PRB-111-A), and iii) the uniqueness of a single member of the group (PRB-111-L). The specific cDNA fragments spanning 3' UTR were obtained by RT-PCR (Fig. 5). The peroxidase cDNAs were then cloned into pGEM-T Easy vector. Sense and antisense digoxigenin-labeled riboprobes were produced by in vitro transcription of linearized plasmid DNA using T7 and SP6 RNA Polymerase (Roche Diagnostic, Mannheim, Germany), respectively, according to the manufacturer's instructions. Hybridization of the samples was performed at 54°C overnight with 50 ng of riboprobe. Afterward, the slides were soaked twice in pre-warmed (54°C) 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min, rinsed twice for 5 min with NTE solution (500 mM NaCl; 10 mM Tris-HCl, pH 8; and 1 mM EDTA) at 37°C, and incubated for 30 min at 37°C in a pre-warmed NTE solution containing RNase A at 20 µg/ml. Slides were then rinsed for 5 min in NTE solution at 37°C twice and washed for 1 h in 0.2× SSC at 54°C and 5 min in PBS at room temperature. The hybridized probes were detected using an alkaline phosphatase-conjugated antibody (1:1,000). The color reaction, obtained by using NBT-BCIP (Roche Diagnostic) as substrate, was stopped by adding Tris-EDTA. Sections were mounted in Aquatext (Merck, Darmstadt, Germany) on glass slides and viewed under a bright-field microscope.

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