A Pathogen-Induced Wheat Gene Encodes a Protein Homologous to Glutathione-S-Transferases

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Winter wheat (Triticum aestivum) f. sp. tritici following exposure to the nonpathogen E. g. f. sp. hordei. The onset of this resistance has been shown to be correlated with the activation of putative defense genes, and cDNA clones representing transcripts of induced genes have been obtained (P. Schweizer, W. Hunziker, and E. Mosinger, Plant Molecular Biology 12:643-654, 1989). We have cloned and sequenced a gene corresponding to one of these cDNAs, WIR5. Sequence analysis indicated that this gene contains three exons and encodes a protein of 229 amino acids. S1 mapping showed that transcripts homologous to this gene are at least 20 times more abundant in leaves infected 14 hr earlier with E. g. f. sp. hordei than in control leaves. Sequence comparison showed that the WIR5 gene product is highly homologous to glutathione-S-transferases (GSTs; EC 25.1.18) of maize. This, together with the fact that the intron positions of both the wheat gene and the maize GST1 gene are conserved, suggests that the cloned pathogen-induced gene, named GstA1, encodes a wheat glutathione-S-transferase.

Additional keywords: disease resistance.

Wheat (Triticum aestivum L.) shows the phenomenon of induced resistance to Erysiphe graminis DC. f. sp. tritici Ém. Marchal (wheat powdery mildew). The success of an infection with this pathogen was found to be remarkably reduced locally by a previous infection with E. g. f. sp. hordei Ém. Marchal, which is not pathogenic to wheat (Schweizer et al. 1989). The onset of this induced resistance is associated with the activation of host genes. To study this phenomenon at a molecular level and to determine the function and regulation of putative defense genes, we cloned genes corresponding to pathogen-induced cDNA clones obtained previously by Schweizer et al. (1989). Here we report the sequence of a gene corresponding to one of the induced cDNA clones, WIR5. From the sequence information we conclude that this gene probably encodes a glutathione-S-transferase (GST; EC 2.5.1.18). These enzymes, which occur ubiquitously in the animal and plant kingdoms, catalyze the conjugation of the tripeptide glutathione to electrophilic centers of lipophilic compounds, thereby detoxifying them (for an overview, see Sies and Ketterer 1988). In plants, GSTs have been implicated in the detoxification of herbicides (for a review, see Timmerman 1989). A possible role for GSTs in pathogen defense has so far not been described.

MATERIALS AND METHODS

Plant material and infection. Growth and cultivation conditions of winter wheat (cultivars Fidel and Cheyenne)

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overnight and used for Southern blotting.

Total RNA from leaves of 5- to 7-day-old wheat plants, which were infected or not infected with spores of *E. g. f. sp. hordei* 14 hr earlier, was isolated by the hot phenol method. One to 2 g of tissue were powdered in liquid nitrogen and added immediately to 1:1 mixture of phenol, and 2× NETS (200 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl, pH 7.5, 1% SDS) was preheated to 80°C. The mixture was thoroughly vortexed, cooled on ice, and centrifuged. After two more phenol extractions at room temperature, the nucleic acids were precipitated with ethanol and redissolved in water. RNA was then precipitated by adding 1 volume of 5 M LiCl and incubating on ice for 4 hr. The RNA was pelleted and dissolved in water.

**S1 probe and S1 mapping.** A 371-base pair (bp) *SacI* fragment (between *SacI* sites at position 298 and 664; Fig. 1) was cloned into a *SacI*-digested pBluescript KS+ vector. This construct was multiplied as a single-stranded phage that was used as a template for the synthesis of a uniformly 32P-labeled single-stranded probe by extension from the universal sequencing primer. The product was cut with *EcoRI*, which cleaves in the cloning box after the insert, denatured, and run on a sequencing gel, from which the newly synthesized radioactive strand was eluted and used as a probe. S1 mapping procedures were performed with 10 µg of LiCl-precipitated RNA exactly as described previously (Dudler and Travers 1984), except that 220 units of S1 nuclease (Boehringer, Mannheim, Germany) were used per reaction.

**cDNA cloning by polymerase chain reaction (PCR) amplification.** As amplification primers we chose 23mer oligonucleotide with the sequence 5'-TTGATCCATATGCTGACCCTCTTC (oligo 1; bases 6 to 23 correspond to the gene sequence between positions 514 and 531 in Fig. 1, whereas the 3' end creates a BamHI site for later cloning) and a 21mer oligonucleotide with the sequence 5'-TTGATCCAAACACAGGTCCGGG (oligo 2; bases 6 to 21 are complementary to the gene sequence between positions 1136 and 1151; the 3' end creates a *SacI* site) were synthesized. The reactions were done essentially as described by Kawasaki and Wang (1989). Briefly, first strand cDNA synthesis was primed with 50 pmol of oligo 2 using 2 µg of LiCl-precipitated RNA extracted from infected wheat leaves (cultivar Cheyenne) as a template in a 20-µl reaction mixture containing 2 µl of 10× PCR buffer (500 mM KCl, 200 mM Tris-HCl, pH 8.4, 25 mM MgCl2, 1 mg/ml bovine serum albumin), 1 unit per micro of RNasin, 1 mM of each dNTP, and 200 units of M-MLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). The reaction was first incubated for 10 min at room temperature and then for 1 hr at 37°C. After heat inactivation (3 min at 90°C), a 10-µl aliquot of the reaction product (continued next column)
was directly amplified by the PCR in a volume of 100 μl containing 9 μl of 10× PCR buffer, 1.25 mM of each dNTP, 1 μM each of oligo 1 and 2, and 2 units of Taq polymerase (Perkin-Elmer, Norwalk, CT) through 25 cycles (1 min at 94° C, 30 sec at 50° C, and 1 min at 72° C). The product was extracted with phenol, precipitated, and cloned into a pBluescript KS+ vector.

RESULTS

The isolation of the cDNA clone WIR5 by differential screening of cDNA libraries has been described previously (Schweizer et al. 1989). This clone represents a wheat (cultivar Fidel) transcript that is induced upon infection of wheat with *E. graminis*. The clone was reported to contain an insert of about 700 bp and, therefore, does not represent the complete mRNA of 1.4 kilobases (kb) as determined by northern blots (Schweizer et al. 1989). We have used the WIR5 cDNA clone as a probe to screen a λ EMBL3 wheat genomic library (cultivar Cheyenne). Three positive λ clones were obtained, one of which, λ WIR56, was analyzed further. Digestion of this clone with restriction enzymes yielded a 2.2-kb BamHI-EcoRI fragment hybridizing with the WIR5 cDNA clone. This genomic fragment, as well as the WIR5 cDNA, was completely sequenced on both strands. The analysis of these sequences and the experiments described below led us to the conclusion that the fragment contains a gene with three exons which encode a protein of 229 amino acids with a calculated relative molecular mass of 25,828. The sequence of this gene and the encoded protein are shown in Figure 1. This conclusion is based on the following data:

1) The WIR5 cDNA sequence, which consists of 634 nucleotides, not counting the poly(A) tail, contains an open reading frame corresponding to part of exon 3. The 5' end of the truncated cDNA corresponds to nucleotide position 1071 of the gene sequence (Fig. 1). The cDNA contains a poly(A) tail 35 nucleotides 3' of the polyadenylation signal (corresponding to position 1700, overlined in Fig. 1). The coding part is 98% identical to the gene sequence, the eight base substitutions resulting in two conservative and one nonconservative amino acid changes (Fig. 2). Except for several small (1 to 10 bp) insertions and deletions, the nontranslated trailer sequence is also very similar.

2. To confirm the exon-intron structure shown in Figure 1, we cloned the cDNA parts bridging the exon-intron boundaries. cDNA was synthesized from mRNA of wheat plants (cultivar Cheyenne) infected with powdery mildew, and the relevant species was amplified in a PCR using synthetic oligonucleotides corresponding to sequences in the first and third exons as primers, respectively (see Materials and Methods). The product was cloned and one clone was sequenced. In this way a cDNA sequence was obtained starting in exon 1 and extending to exon 3. The exon-intron boundaries as shown in Figure 1 were confirmed. This cDNA sequence and the exon sequences of the gene are 99% identical to the coding regions, the three base substitutions resulting in one conservative amino acid change (Fig. 2).

3. The methionine codon at position 577 (Fig. 1) is likely to be the translation initiation codon, because six codons upstream of it there is an in-frame TGA stop codon (position 559). Between this initiation codon and an upstream potential transcription initiation site defined by the TATA box at position 445 (Fig. 1, overlined), there is no other initiation codon. That transcription does indeed start in the expected region (Fig. 1, broken arrow) was confirmed by S1-mapping the 5' end of the transcripts. For this purpose, a 371-bp SacI fragment of the cloned gene (between SacI sites at positions 298 and 664, Fig. 1) was subcloned, and a continuously labeled single-stranded probe was prepared representing the antisense strand of the gene. This probe was hybridized to equal amounts of total RNA extracted from plants (cultivar Cheyenne), which were or were not infected with *E. graminis*. As can be seen in Figure 3, the major protected fragment has a length of about 190 bases, indicating that transcription is initiated around position 479, that is about 30 nucleotides downstream of the putative TATA box. It is evident from Figure 3 that mRNA homologous to the cloned gene is indeed pathogen-induced. RNA extracted from infected plants gives a signal at least 20 times stronger than RNA from control plants, as determined by densitometry of appropriately exposed autoradiograms. The weaker bands with sizes between 110 and 136 nucleotides map to the nontranslated leader sequence. We interpret these signals to result most likely from transcripts of homologous genes with slightly diverged leader sequences. The presence of several related WIR5 genes in wheat is evident from the Southern blot experiment shown in Figure 4. About six to 10 bands hybridize to the WIR5 cDNA.
in HindIII and EcoRI digests of wheat (cultivar Fidel) genomic DNA.

The sequence of the putative protein encoded by the λ WIR56 gene was compared to the Swiss-Prot protein sequence data base. Significant similarity to the GSTs (GST, EC 2.5.1.18) of maize was detected. Figure 2 shows the optimized alignment of the wheat protein with the two published maize GST sequences (GSTI and GSTIII). The wheat protein shows 51% identical and 13.5% conserved amino acids compared to GSTI, and 40% identical and 14.5% conserved amino acids compared to GSTIII of maize, respectively (Moore et al. 1986; Shah et al. 1986; Grove et al. 1988). The sequence similarity between the two maize GSTs is about the same: 50% identical and 14% conserved amino acids. We conclude from this analysis that the λ WIR56 clone contains a gene which is likely to encode a GST. We name this gene Gsta1.

DISCUSSION

In this study we present evidence that pathogen attack of wheat results in a strong increase of mRNA coding for a protein homologous to GST. We have not shown the enzyme activity of this protein. However, the following considerations strongly suggest that the protein is indeed a GST. First, the wheat protein and the maize GSTs are similar over their entire sequence. With 40–50% sequence identity, the wheat protein sequence is as similar to the maize GSTs as these are to each other. Second, both the wheat Gsta1 and the maize GSTI (Shah et al. 1986) genes are interrupted by two introns, the positions of which are exactly conserved in the two species. This suggests that these genes are homologous.

Our S1-mapping experiments show that transcripts homologous to the Gsta1 gene are about 20 times more abundant in wheat leaves infected with powdery mildew than in uninfected ones. This increase results, at least in part, from transcriptional induction (Schweizer et al. 1989). Our S1-mapping data are compatible with the assumption that the cloned Gsta1 gene is activated. However, this can only be proven by a promoter analysis in appropriate assay systems.

We have measured GST activity in leaves 24 and 48 hr after infection with E. g. f. sp. hordei in a spectrophotometric assay using 1-chloro-2,4-dinitroacetohydrozone as a substrate. Compared to control plants, GST activity increased by a factor of two after 48 hr (unpublished results). Although this confirms that GSTs are induced by pathogen attack, the factor of induction measured in this assay is much lower than the one implied by the S1-mapping experiments. This might be due to the relatively high background GST activity in noninduced leaves, or due to the possibility that 1-chloro-2,4-dinitroacetohydrozone may be a poor substrate for the induced isozyme(s), as has been reported for the GST isozymes reactive with metolachlor in sorghum (Gronwald et al. 1987) and with atrazine in maize (Timmerman and Tu 1987), respectively. Clarification of this point will have to await production of specific antibodies directed against fusion proteins and the availability of the Gsta1-encoded protein expressed in Escherichia coli.

The sequences of the cDNAs are very similar, but not identical, to the sequence of the genomic clone. The sequence heterogeneities between the WIR5 cDNA and the Gsta1 gene may be attributable to the fact that they were obtained from different cultivars (Fidel and Cheyenne, respectively). However, the cDNA part cloned by PCR amplification originated from the same cultivar as the genomic clone. Thus, the sequence heterogeneities indicate the presence of several genes similar to Gsta1 in the wheat genome. Indeed, Southern analysis showed that between six to 10 bands are hybridizing to the WIR5 cDNA insert in both HindIII- and EcoRI-digested wheat DNA (cultivar Fidel). Since T. aestivum is an allohexaploid species, the number of bands seems compatible with the presence of one gene per monohaploid genome, that is six alleles in the allohexaploid genome, assuming heterozygosity (Fidel is a hybrid line). If this interpretation is correct, the situation in wheat parallels the one in the diploid maize, where both GSTI and GSTIII genes were reported to be present in one copy per haploid genome (Moore et al. 1986; Shah et al. 1986; Grove et al. 1988).

A widely assumed hypothesis is that pathogen-induced host genes encode for products which are involved in host defenses against pathogens. Our results strongly suggest that one such pathogen-activated gene encodes a GST. Assuming this to be true, the question arises as to how
these enzymes might be involved in the defense against pathogens. GSTs are widely found in animals and plants. They constitute a family of multifunctional dimeric enzymes catalyzing the conjugation of the tripeptide glutathione (γ-glutamyl-l-cysteinylglycine) to electrophilic centers of lipidic compounds, thereby detoxifying them (for a recent overview, see Sies and Ketterer 1988). In plants, GSTs have been shown to play a role in the detoxification of herbicides. Best characterized in maize (for a recent review, see Timmerman 1989), certain isozymes were found to detoxify atrazine and other herbicides. Moreover, treatment with safeners increased herbicide tolerance, and this correlated with an increase in GST activity. This increase was shown to be at least partly due to induction of gene transcription (Wiegand et al. 1986). Similar results were reported in sorghum (Sorghum bicolor (L.) Moench), where various herbicide antioxidants induced the de novo synthesis of GST isozymes reactive with the chloroacetanilide herbicide metolachlor (Gronwald et al. 1987; Dean et al. 1990). It is not known whether GST genes are induced by herbicides in these systems.

A possible connection between glutathione metabolism and pathogen defense of plants was recently revealed by the intriguing observation that glutathione (i.e. a substrate of GST) induces transcription of defense genes in cultured cells (Wingate et al. 1988; Dron et al. 1988). Furthermore, in animal systems, the highly toxic products of membrane lipid peroxidation have been found to be substrates of GST isozymes, which may thus contribute to protection from oxidative tissue damage (for a review, see Pickett and Lu 1989). Since formation of active oxygen species and membrane lipid peroxidation are known to occur in plants in response to tissue damage, elicitor treatment, and pathogen attack (Thompson et al. 1987; Rogers et al. 1988; Chai and Doke 1987; Kato and Misawa 1976; Keppler and Novacky 1987; Croft et al. 1990), it is conceivable that GST plays a similar protective role in plants. Thus, GST genes could be members of a class of general stress response genes, which are activated by many different stimuli. In addition to infection, we have tested one other stimulus: wounding of wheat leaves (by Carborundum treatment) does not result in increased abundance of GstA1 homologous mRNA, as determined by S1 mapping (data not shown), thus indicating a more specific role. However, whether or how GSTs contribute to the defense of wheat against E. graminis remains a matter of speculation at this time. Although genetic manipulation of wheat is as yet not possible, we hope that the cloned wheat GstA1 gene will help to attack experimentally some of the questions concerning GST and pathogen defense in other systems. It will also be interesting to investigate the relation between GST induction and herbicide resistance in wheat.

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LITERATURE CITED


