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Differential Induction of Distinct Glutathione-*S*-Transferases of Wheat by Xenobiotics and by Pathogen Attack[1]

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We have previously characterized a pathogen-induced gene from wheat (*Triticum aestivum* L.) that was named *GstA1* based on sequence similarities with glutathione-S-transferases (GSTs) of maize (R. Dudler, C. Hertig, G. Rebmann, J. Bull, F. Mauch [1991] Mol Plant Microbe Interact 4: 14-18). We have constructed a full-length *GstA1* cDNA by combinatorial polymerase chain reaction and demonstrate by functional expression of the cDNA in *Escherichia coli* that the *GstA1*-encoded protein has GST activity. An antiserum raised against a GstA1 fusion protein specifically recognized a protein with an apparent molecular mass of 29 kD on immunoblots of extracts from bacteria expressing the *GstA1* cDNA and extracts from wheat inoculated with *Erysiphe graminis*. The *GstA1*-encoded protein was named GST29. RNA and immunoblot analysis showed that *GstA1* was only weakly expressed in control plants and was specifically induced by pathogen attack and by the GST substrate glutathione, but not by various xenobiotics. In contrast, a structurally and antigenically unrelated GST with an apparent molecular mass of 25 kD that was detected with an antiserum raised against GSTs of maize was expressed at a high basal level. This GST25 and an additional immunoreactive protein named GST26 were strongly induced by cadmium and by the herbicides atrazine, paraquat, and alachlor, but not by pathogen attack. Compared with the pathogen-induced GST29, GST25 and GST26 showed a high affinity toward glutathione-agarose and were much more active toward the model substrate 1-chloro-2,4-dinitrobenzene. Thus, wheat contains at least two distinct GST classes that are differentially regulated by xenobiotics and by pathogen attack and whose members have different enzymic properties. GST25 and GST26 appear to have a function in xenobiotic metabolism, whereas GST29 is speculated to fulfill a more specific role in defense reactions against pathogens.

GSTs (EC 2.5.1.18) are multifunctional dimeric proteins that catalyze the conjugation of the tripeptide GSH to a large variety of hydrophobic compounds containing electrophilic centers. The conjugation to GSH usually results in the detoxification of these potentially cyto- and genotoxic compounds (for overviews, see Ketterer et al., 1988; Mannervik and Danielson, 1988). In addition to their enzymic activity, GSTs are also known for their capacity to bind a large number of lipophilic compounds that serve as ligands but not as substrates (Jakoby and Habig, 1980).

That the role of GSTs is vital is supported by their ubiquitous occurrence in bacteria, fungi, animals, and plants (Frear and Swanson, 1970; Lamoureux and Frear, 1979; Jakoby and Habig, 1980; Ketterer et al., 1988; Piccolomini et al., 1989). GSTs have been most intensively studied in relation to their role in xenobiotic metabolism in mammals, where they are encoded by a gene family consisting of at least three major classes (Ketterer et al., 1988; Pickett and Lu, 1989). GST enzymes have also been identified and partially characterized from various plant species (Frear and Swanson, 1970; Diesperger and Sandermann, 1979; Mozer et al., 1983; Hunaiti and Bassam, 1990; Dudler et al., 1991; Edwards and Dixon, 1991; Meyer et al., 1991; Singhal et al., 1991; Kutchan and Hochberger, 1992). In maize, at least three distinct GST genes were described (Moore et al., 1986; Shah et al., 1986; Grove et al., 1988; Timmerman, 1989), and it was suggested that plant GSTs might be encoded by a multigene family similar to that found in mammals (Grove et al., 1988; Timmerman, 1989).

Plant GSTs were intensively studied with regard to their role in herbicide detoxification. In many plant species, GST activity has been shown to increase in response to herbicide and safener treatments (Mozer et al., 1983; Wiegand et al., 1986; Gronwald et al., 1987; Edwards and Owen, 1988; Dean et al., 1990; Hunaiti and Bassam, 1990). Moreover, detoxification of various herbicides by GSH conjugation has been demonstrated to be a major factor in herbicide tolerance of maize (Frear and Swanson, 1970; Shimabukuro et al., 1971; Edwards and Owen, 1986, 1988; Timmerman, 1989) and other plants (Lamoureux and Frear, 1979; Gronwald et al., 1987; Dean et al., 1990; Hunaiti and Bassam, 1990).

Besides their involvement in xenobiotic metabolism, little is known about the biological function of GSTs in plants. One of the few endogenous substrates identified is cinnamic acid (Diesperger and Sandermann, 1979; Edwards and Dixon, 1991). Only a few reports exist on the regulation of GST expression by compounds of biological origin. An auxin-inducible GST transcript has been described in protoplast cultures of tobacco (Takahashi and Nagata, 1992). In carnation, a senescence-related mRNA was shown to encode an ethylene-regulated GST (Meyer et al., 1991), and in suspension cultures of French bean GST activity was found to be induced by a fungal elicitor (Edwards and Dixon, 1991). However, the structural and functional interrelationship between GSTs regulated by compounds of biological origin and those regulated by xenobiotics remained unclear.

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione-S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction.

We have previously isolated and characterized two wheat genes that were postulated to encode GSTs based on their sequence similarity to GSTs of maize (Dudler et al., 1991; Mauch et al., 1991). The expression of one of these genes, *GstA1*, was demonstrated to increase following an inoculation with the fungus *Erysiphe graminis* f.sp. *hordei* (barley powdery mildew). This fungus is not pathogenic on wheat but induces resistance to a subsequent infection with the wheat powdery mildew *E. graminis* f.sp. *tritici* (Schweizer et al., 1989). Therefore, *GstA1* belongs to a group of putative defense genes whose expression correlates with the onset of induced resistance (Rebmann et al., 1991a, 1991b; Bull et al., 1992).

In this article, we first establish by functional expression of the *GstA1* cDNA in *Escherichia coli* that the encoded protein has GST activity. Because plant GSTs have been shown to accumulate in different stress situations, we tested the specificity of *GstA1* induction and found that *GstA1* was induced only by fungal infection and by reduced GSH, one of the two GST substrates. Other compounds known to induce defense genes in dicots (Enyedi et al., 1992), such as salicylic acid, ethylene, and methyl jasmonate, as well as various herbicides, did not induce an increase in *GstA1* expression. In contrast, the expression of GST isozymes that are structurally and antigenically unrelated to the *GstA1*-encoded protein were found to be strongly induced by herbicides and other xenobiotics but not by pathogen attack. Thus, wheat contains at least two distinct types of GST genes that are differentially regulated and encode GST isozymes with different substrate specificities. This situation indicates that the individual GST isozymes might have specific functions that are possibly related to the inducing stress stimulus.

MATERIALS AND METHODS

Biological Material

Wheat plants (*Triticum aestivum* L. cv Fidel) were grown at 21°C with a 16-h photoperiod. Six- to 7-d-old seedlings were inoculated in a moist chamber with conidiospores of *Erysiphe graminis* f.sp. *tritici* or *Erysiphe graminis* f.sp. *hordei* by brushing plants infected 7 d earlier over the test plants. *E.g.* f.sp. *hordei* was maintained on barley (*Hordeum vulgare* L. cv Gerbel). Uredospores of *Puccinia recondita* f.sp. *tritici* were sprayed on wheat plants in a suspension containing mineral oil. Control plants were sprayed with mineral oil only.

Chemical Induction

The shoots of excised 7-d-old wheat seedlings were incubated in solutions of reduced GSH (1–5 mM), salicylic acid (1–3 mM), herbicides (0.5 mM), cadmium sulfate (0.1–0.3 mM), or the supernatant of autoclaved *E. graminis* spores (50 mg/mL). Treatment of wheat seedlings with methyl jasmonate (1–30 nL/L) or ethylene (100 nL/L) was performed in airtight plexiglass chambers. Incubation conditions were the same as those described above. All treatments have been repeated at least once.

RNA Gel-Blot Analysis

Total RNA was isolated as described (Dudler et al., 1991). Samples of total RNA (10 μg) were separated by electrophoresis through formaldehyde-agarose gels as described in the protocol of the λZAP cDNA synthesis kit (Stratagene, La Jolla, CA). Ethidium bromide was included in the loading buffer at a concentration of 60 μg/mL, which allowed photography under UV light after electrophoresis to confirm equal sample loading. The gels were blotted onto nylon membranes (GeneScreen, New England Nuclear, Boston, MA) and hybridized to a random-primed ^{32}P-labeled *GstA1* cDNA probe according to standard procedures. An RNA ladder (Pharmacia, Uppsala, Sweden) was used as a size standard.

Protein Analysis

Plant material was pulverized in liquid nitrogen and extracted in 50 mM Tris-Cl, 0.5 mM PMSF, 3 mM EDTA, 10 mM DTT, pH 7.5. After centrifugation (15 min at 15,000*g*), the supernatant was used for protein analysis. Protein concentration was determined as described (Bradford, 1976). Proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose (0.45 μm, Bio-Rad) with a semi-dry blotting apparatus (Pharmacia LKB, Uppsala, Sweden). For immunodetection, the nitrocellulose sheets were blocked in PBST (20 mM sodium phosphate buffer, 150 mM NaCl, 0.1% [v/v] Tween-20, pH 7.5) containing 10% (w/v) nonfat milk powder and incubated in primary antiserum diluted 1:1000 in PBST. The blots were washed and incubated with horseradish peroxidase- or alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. The peroxidase-labeled antigens were visualized with luminol followed by autoradiography (ECL kit, Amersham International, Amersham, UK). The phosphatase-labeled antigens were visualized with the colorigenic substrate 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium chloride.

GST Assay

GST activity was measured spectrophotometrically with the artificial substrate CDNB. The reaction mixture contained, in a volume of 1 mL, 100 μmol of sodium phosphate buffer, pH 6.5, 1 μmol of CDNB, 1 μmol of reduced GSH, and 50 μL of enzyme solution. The increase in A_{340} was measured for 10 min at 30°C. Other potential GST substrates were tested as described (Habig et al., 1974). The amount of enzyme producing 1 μmol min^{-1} of conjugated product was defined as one unit.

Affinity Adsorption of GSTs

S-Hexyl-GSH-agarose and cibacron blue 3GA-agarose (Sigma) were used as an affinity matrix to remove specifically GST isozymes from plant extracts. Both affinity resins were washed five times in 50 mM Tris-Cl, pH 7, prior to use. One milliliter of the affinity matrix corresponding to about 0.4 mL of swollen bed volume was added to 1 mL of crude extract from plants inoculated with *E. graminis*. The suspension was incubated at 4°C for 2 h on a shaker. The sediment formed

after centrifugation was washed twice with 50 mM Tris-Cl, pH 7 and twice with the same buffer containing 0.5 M NaCl, followed by a final wash with 50 mM Tris-Cl, pH 7. Proteins attached to the affinity matrix were eluted with the same buffer containing 10 mM reduced GSH. After determination of the protein concentrations and GST activities in the various fractions, the proteins were precipitated with acidic methanol and dissolved in SDS sample buffer.

Antiserum Production against a GstA1 Fusion Protein

A bacterial fusion protein containing amino acids 111 to 228 of the *GstA1*-encoded protein was produced in *E. coli*. A 353-bp *Sca*I fragment corresponding to the gene sequence between positions 1120 and 1473 was subcloned into the *Sma*I site of pIJ2922. A *Bgl*II/*Bam*HI fragment was cut out from the resulting plasmid and subcloned into the *Bgl*II site of the expression vector pDHFRS-(-1/-1)-6xHis (Stüber et al., 1990). A construct with the right orientation was selected and the reading frame was verified by sequencing over the insert junctions. The recombinant expression vector was transformed into *E. coli* strain M15 (Stüber et al., 1990). Production of the recombinant protein (DHFR-GstA1 peptide-6xHis) and its purification by nickel chelate affinity chromatography were performed as described (Stüber et al., 1990). Antiserum against the purified fusion protein was raised in rabbits. The antiserum was affinity purified on a column containing the fusion protein bound to a 1:1 mixture of Affi-Gel 10 and Affi-Gel 15 (Bio-Rad). Bound antibodies were eluted from the column with 0.1 M Gly-HCl, pH 2.8. Antiserum against GSTs of maize was a gift of Dr. K. Kreuz (Ciba-Geigy, Ltd., Basel, Switzerland). The antiserum was raised in rabbits against a combination of two maize GST isozymes with subunit masses of 25 and 26 kD.

Construction of a *GstA1* cDNA

The exon/intron boundaries of the *GstA1* gene have been determined previously (Dudler et al., 1991). To construct a full-length cDNA, introns were removed from the *GstA1* gene by specifically combining the PCR products of the three exons (using the cloned *GstA1* gene as a template) through complementary overlaps created by addition of 5′ sequences to the PCR primers (Fig. 1). Exon 1 was amplified with primer 1 (5′-TTCTAGAGCATCCATCACG-3′; bases 4–19 correspond to the gene sequence between positions 544 and 558) and primer 2 (5′-CGCAAACGGGTTTAGCTGGACGTGTTG-3′; bases 10–27 are complementary to the gene sequence between positions 709 and 726 [3′ end of exon 1], and the add-on bases, 1–9, are complementary to the gene sequence between positions 817 and 825 [5′ end of exon 2]). Exon 2 was amplified with primer 3 (5′-CAGCTAAACCCGTTTGCGAAGATGCCT-3′; bases 10–27 correspond to the gene sequence between positions 817 and 834 [5′ end of exon 2], and the add-on bases, 1–9, correspond to the gene sequence between positions 718 and 726 [3′ end of exon 1]) and primer 4 (5′-GCGCGACTCGAACAGGACGAGATCG-3′; bases 9–25 are complementary to the gene sequence between positions 849 and 865 [3′ end of exon 2], and the add-on bases, 1–8, are complementary to the gene sequence between positions 988 and 995 [5′ end of exon 3]). Exon 3 was amplified with primer 5 (5′-TCCTGTTCGAGTCGCGCGCCATCGC-3′; bases 10–25 correspond to the gene sequence between positions 988 and 1003 [5′ end of exon 3], and the add-on bases, 1–9, correspond to the gene sequence between positions 857 and 865 [3′ end of exon 2]) and primer 6 (5′-TGGATCCAGTTATCCCTGT-3′; bases 6–19 are complementary to the gene sequence between positions 1824 and 1837).

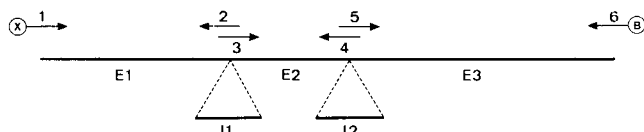

Figure 1. Schematic representation of the *GstA1* cDNA construction. The two introns (I1 and I2) of the *GstA1* gene were removed by specifically combining the PCR products of the three exons (E1-E3) through complementary overlaps created by 5′ sequences added to the PCR primers. Details are explained in "Materials and Methods." →, PCR primers with a sequence corresponding to the coding strand of the gene. ←, PCR primers with a sequence complementary to the coding strand of the gene sequence. X, *Xba*I. B, *Bam*HI. Not drawn to scale.

The amplification products of exon 1 and exon 2 were mixed, denatured, and annealed. Heteroduplex forms consisting of DNA strands that overlap at their 3′ ends were amplified by PCR using primers to the 5′ end of the strands (primer 1 and primer 4). The product of this reaction and the amplification product of exon 3 were mixed, denatured, annealed, and then PCR amplified with the outside primers 1 and 6. The 5′ add-on restriction sites *Xba*I and *Bam*HI included in primers 1 and 6 allowed the cloning of the final PCR product into pBluescript SK$^+$ (Stratagene). The sequence of the subcloned PCR product was verified on both strands with the dideoxy-chain termination method (Sanger et al., 1977) using specific oligonucleotides as sequencing primers.

In Vitro Transcription/Translation

The *GstA1* cDNA was cloned into pSP64poly A (Promega, Madison, WI) as an *Xba*I/*Bam*HI fragment. Plasmids were linearized 3′ of the open reading frame with *Eco*RI and transcribed with Sp6 RNA polymerase following the manufacturer's instructions (Promega). Approximately 100 ng of the in vitro-transcribed RNA were in vitro translated in a rabbit reticulocyte system (Promega) in the presence of [^{35}S]-Met (1070 Ci/mmol) according to the manufacturer's instructions. In vitro translation products were subjected to SDS-PAGE followed by fluorography with Enlightning (Dupont, Boston, MA). Dried gels were exposed to x-ray film Fuji RX at −80°C. ^{14}C-Labeled molecular mass markers were from Amersham International (Amersham, UK).

Expression of *GstA1* in *E. coli*

The expression plasmid pDS56/RBS II, *Sph*I, belonging to the pDS family (Stüber et al., 1990), was a gift from Dr. D. Stüber (F. Hoffman-La Roche Ltd., Basel, Switzerland). It is

now commercially available as pQE-7 (Quiagen Inc., Chatsworth, CA). The plasmid contains a regulatable promoter/operator element that is controlled by the *lac* operator. The plasmid has an *Sph*I site that is part of the translational initiation codon for bacterial expression. The initiation codon of the *GstA1* cDNA, coincidentally, is part of an *Nsp*HI site. This situation allowed the in-frame cloning of an *Nsp*HI/*Bam*HI fragment containing the complete *GstA1* coding sequence into the *Sph*I/*Bam*HI-digested expression plasmid. The resulting chimeric expression plasmid pGstA1 was transformed into *E. coli* strain SG 13009 containing the repressor plasmid pDMI,1 (Stüber et al., 1990). Bacteria were grown to an A_{600} of 0.7 at 37°C in 2× tryptone-yeast extract medium containing 100 μg/mL of ampicillin and 25 μg/mL of kanamycin. Expression was then induced by adding IPTG to a final concentration of 2 mM and the cultivation was continued. The cells were harvested by centrifugation and extracted as described (Tabor and Richardson, 1985); this procedure included a 20-s sonication step. After centrifugation (15 min at 15,000*g*), the resulting supernatant was used for analysis.

RESULTS

Functional Expression of *GstA1* in *E. coli*

We have previously isolated and characterized a pathogen-induced gene from wheat that was named *GstA1* based on its sequence homology with GSTs of maize (Dudler et al., 1991). To demonstrate enzyme activity of the *GstA1*-encoded protein, a full-length cDNA was constructed and functionally expressed in *E. coli*.

The cDNA was prepared by removing the three introns from the *GstA1* gene with combinatorial PCR (see "Materials and Methods," Fig. 1). Sequence analysis showed that the sequence of the constructed cDNA matched exactly the sequence of the coding region of *GstA1*. In vitro transcription/translation of the *GstA1* cDNA produced a protein with an apparent molecular mass of 29 kD (Fig. 2A). The *GstA1* cDNA was cloned into the bacterial expression vector pDS56/RBSll. The resulting plasmid, pGstA1, allowed the expression of the authentic protein in *E. coli* under the control of an IPTG-inducible promoter.

SDS-PAGE analysis of extracts from bacteria containing pGstA1 showed the IPTG-induced accumulation of a protein with an apparent molecular mass of 29 kD (Fig. 2B). Immunoblot analysis with anti-GstA1 fusion protein antiserum labeled a single protein with an apparent molecular mass of 29 kD that was present only in extracts of IPTG-treated bacteria containing pGstA1 (Fig. 2C).

Bacterial extracts were analyzed for GST activity with the artificial substrate CDNB. No increase in CDNB activity upon addition of IPTG was observed in bacteria transformed with the original pDS56/RBSll plasmid. In contrast, bacteria containing pGstA1 reacted with a 10-fold increase in CDNB activity upon IPTG addition (Fig. 2D). The increase in enzyme activity appeared to be small compared with the results of the immunoblotting experiments. This discrepancy was due to the presence of a constitutive bacterial GST activity (2 milliunits/mg protein), unrelated to the *GstA1*-encoded protein, that was not IPTG inducible and that was present in the

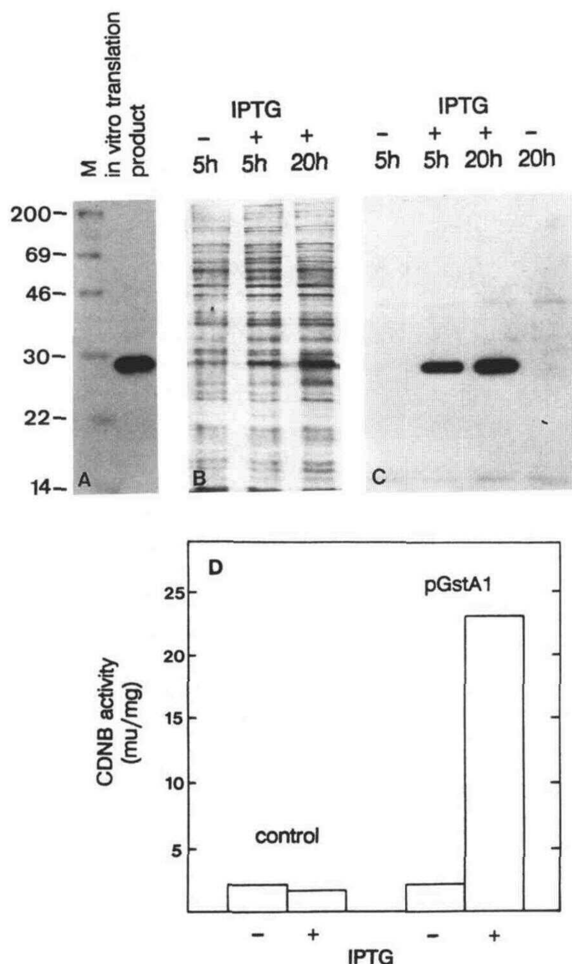

Figure 2. Analysis of the *GstA1*-encoded protein by in vitro transcription/translation and by functional expression in *E. coli*. A, SDS-PAGE of the protein produced by in vitro transcription/translation of the *GstA1* cDNA. The molecular mass of the markers (M) in kD is indicated on the left. B, SDS-PAGE analysis of extracts from bacteria containing pGstA1 after incubation in the absence (−) or presence (+) of IPTG for 5 and 20 h. The gel was silver stained. C, Immunoblot analysis of extracts from bacteria containing pGstA1 after incubation in the absence (−) or presence (+) of IPTG for 5 and 20 h. The blot was labeled with antiserum raised against a GstA1 fusion protein and was developed with the light-based ECL detection system. D, GST activity of extracts from control bacteria containing the empty plasmid vector pDS56/RBSll and from bacteria transformed with pGstA1. The bacteria were grown for 20 h in the absence (−) or presence (+) of IPTG. The measurements represent the mean value of two independent experiments.

bacteria independent of the plasmid they contained. Taken together, the results demonstrate that the *GstA1* gene encodes a GST with an apparent molecular mass of 29 kD. This protein was named GST29.

Induction of *GstA1* Expression by Pathogens and Abiotic Inducers

To analyze the expression of mRNA corresponding to *GstA1*, gel blots of RNA samples were probed with the *GstA1*

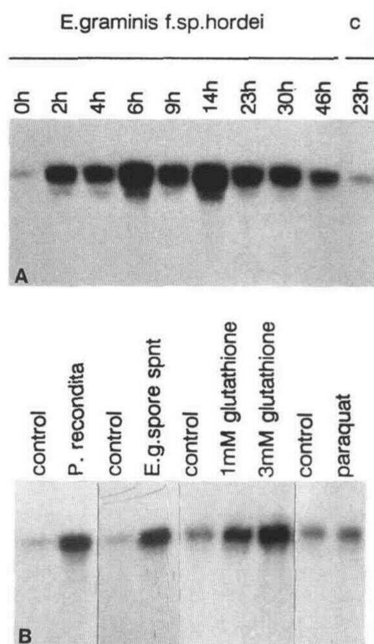

Figure 3. Accumulation of mRNA corresponding to *GstA1* in response to fungal infection and abiotic inducers. Time course of *GstA1* mRNA accumulation after inoculation with *E.g.* f.sp. *hordei* (A) and after a 24-h treatment with other agents (B). The RNA blots were probed with the ^{32}P-labeled *GstA1* cDNA.

cDNA. The time course of induction was examined in wheat seedlings inoculated with *E.g.* f.sp. *hordei* (Fig. 3A) or *E.g.* f.sp. *tritici* (data not shown). *GstA1* mRNA was present at a low basal level in healthy control plants. Within 2 h after inoculation, the *GstA1* mRNA level increased dramatically and remained elevated for at least 2 d. The level and time course of expression of *GstA1* in the incompatible and compatible interaction were similar, indicating that the induction of *GstA1* expression is a general response to infection. This conclusion was supported by the finding that inoculation with another fungal pathogen, *P. recondita* f.sp. *tritici*, also induced the accumulation of the *GstA1* mRNA (Fig. 3B).

Induction of *GstA1* expression did not require the presence of living fungal material. The cell-free supernatant of autoclaved *E. graminis* conidiospores was sufficient to induce expression of *GstA1* (Fig. 3B). This, together with the fact that *GstA1* induction occurred well before the attempted penetration of the host cells by *E. graminis*, suggests that the induction of *GstA1* by *E. graminis* might be mediated by a soluble factor.

To test the specificity of the *GstA1* induction, different abiotic stimuli were tested for their ability to induce *GstA1* in excised wheat seedlings (Fig. 3B). The only active compound found was GSH. Excision of the seedlings as well as wounding by squeezing, or treatment with ethylene, methyl jasmonate, salicylic acid, or the herbicide paraquat had no measurable effect on *GstA1* expression. In conclusion, *GstA1* is specifically induced by fungal infection and by the GST substrate GSH but not by abiotic compounds known to induce GST activity in other systems or by compounds described as elicitors of defense genes in dicotyledonous plants.

Accumulation of GST29 upon Fungal Infection

The accumulation of *GstA1*-encoded protein upon fungal infection was monitored by SDS-PAGE and immunoblotting with the anti-GstA1 fusion protein antiserum. Immunoblot analysis revealed a strongly reacting 29-kD protein that was present at low levels in control plants and accumulated in seedlings inoculated with *E. graminis* (Fig. 4A). A second protein of an apparent molecular mass of 28 kD also reacted with the anti-GstA1 fusion protein antiserum. This minor protein did not increase in abundance upon fungal infection, and its relationship to GST29 is at present not clear.

Compared with uninoculated seedlings, GST activity toward CDNB was increased by a factor of 1.2 or less in wheat plants 48 h after inoculation with *E. graminis* or *P. recondita* (Table I). Although this result did indicate a slight increase in GST activity upon infection, the level of increase was much lower than expected from the results of the immunoblot analysis. In addition, the CDNB activity of wheat extracts could not be inhibited by immunotitration with anti-GstA1 fusion protein antiserum, indicating the presence of additional, immunologically unrelated GSTs that were masking the induction of GST29 at the enzymic level.

Constitutively Produced GST Isozymes

To test for the presence of constitutive GST isozymes unrelated to GST29, an immunoblot of wheat extracts was probed with antiserum raised against a mixture of two GST isozymes of maize (see "Materials and Methods"). As shown in Figure 4B, this antiserum did not cross-react with GST29 but specifically recognized a protein with an apparent molecular mass of 25 kD that was already present at high levels in control plants and did not accumulate further after inoculation with *E. graminis*. This protein was named GST25.

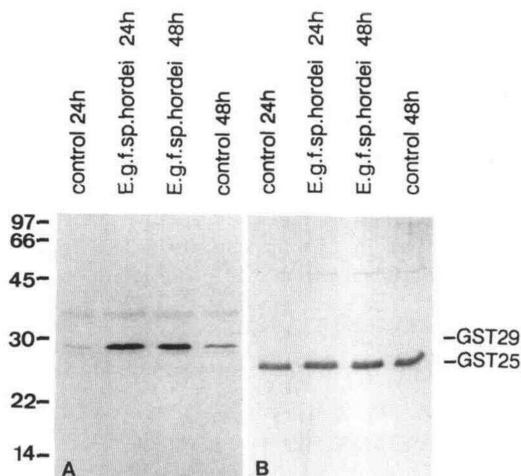

Figure 4. Immunoblot analysis of wheat seedlings at different time points after inoculation with *E.g.* f.sp. *hordei*. The blots were labeled with anti-GstA1 fusion protein antiserum (A) or anti-maize GST antiserum (B) and developed with a light-based detection system. Six micrograms (A) and 3 μg (B) of protein were loaded per lane. The GST isoforms are labeled on the right. The molecular mass of the markers in kD is indicated on the left.

Table I. *Induction of GST activity and individual GST isozymes by fungal infection, GSH, and herbicides*

Wheat seedlings were inoculated with spores of *E.g.* f.sp. *hordei* or *P. recondita* f.sp. *tritici* and incubated for 48 h (Intact Plants). Excised wheat seedlings were incubated for 48 h in a solution of 5 mM GSH or 0.5 mM herbicide (Excised Plants). Estimation of the expression of the individual GST isozymes was based on the results of RNA blot and immunoblot analysis.

Treatment	Specific CDNB Activity	GST Induction	GST29[a]	GST25[a]	GST26[a]
	milliunits/mg	*-fold*			
Intact plants					
None	58	1.0	−	+	−
E. graminis	70	1.2	++	+	−
P. recondita	65	1.1	++	+	−
Excised plants					
Water	61	1.0	−	+	−
GSH	82	1.3	++	+	−
Paraquat	260	4.2	−	++	++
Atrazine	250	4.1	−	++	++
Alachlor	234	3.8	−	++	++
Metolachlor	183	3.0	−	++	++

[a] −, Low constitutive expression; +, high constitutive expression; ++, strongly induced.

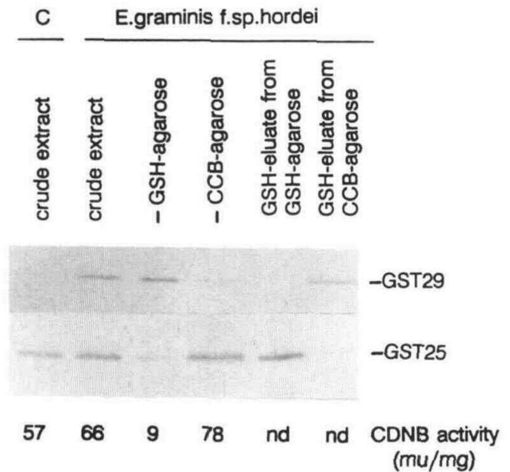

Figure 5. Differential adsorption of GST isozymes to different affinity resins. Immunoblot analysis of wheat extracts before (crude) and after removal of individual GST isozymes by S-hexyl-GSH-agarose (-GSH-agarose) or by cibacron blue 3GA-agarose (-CCB-agarose). GST29 was detected with anti-GstA1 fusion protein antiserum and GST25 and GST26 were detected with anti-maize GST antiserum. The blots were developed with a chromogenic substrate for alkaline phosphatase. Fifty micrograms of protein were loaded per lane except for those lanes containing the GSH eluates. The specific CDNB activity of the various fractions is indicated at the bottom. The specific CDNB activity of the GSH eluates could not be determined (nd) because the amount of protein eluted was too low to be measured. GST activity is expressed in milliunits (mu)/mg of protein.

The two enzymes, GST29 and GST25, could be separated from each other based on their different affinity for S-hexyl-GSH-agarose (Fig. 5). Selective removal of GST25 with this affinity matrix led to a reduction of CDNB activity in the remaining extract from 66 to 9 milliunits/mg of protein, thus demonstrating that GST25 was responsible for the high CDNB activities in wheat extracts. This conclusion was supported by the high CDNB activity measured in the protein fraction eluted from the affinity matrix by reduced GSH.

In a similar, but reciprocal, experiment, GST29 was removed from the extract by addition of cibacron blue 3GA-agarose without much effect on the GST25 level (Fig. 5). The removal of GST29 had no negative effect on the CDNB activity in the remaining extract. In fact, the specific CDNB activity increased slightly because more than 50% of the proteins, but not GST25, were removed by cibacron blue 3GA-agarose. Taken together, the results demonstrate that GST25 is highly active toward the substrate CDNB, whereas the pathogen-induced GST29 has a comparatively low CDNB activity.

Differential Induction of GST Isozymes by Herbicides

Synthesis of GST25, in contrast to the pathogen-induced GST29, was strongly induced by herbicide treatment (Fig. 6, Table I). The treatment of excised wheat seedlings with paraquat, atrazine, or alachlor led to the accumulation of GST25 and the appearance of an additional immunoreactive protein with an apparent molecular mass of 26 kD that was not detectable in control plants. Both proteins reacted with anti-maize GST antiserum and bound to S-hexyl-GSH-agarose, thus indicating that they were GSTs (data not shown). Concomitant with the induction of GST25 and GST26 synthesis, the specific CDNB activity in herbicide-treated plants increased up to 4-fold above the already high CDNB activity level in control plants (Table I). This increase in the specific GST activity was due to the accumulation of GST protein and not to a decline in protein content, since the herbicide treatments had no measurable effect on the protein concentration of wheat extracts. Treatment of excised wheat seedlings with the GST substrate CDNB or with cadmium also induced the accumulation of GST25 and GST26 (data not shown), indicating that the two isozymes are induced by a broad spectrum of toxic compounds.

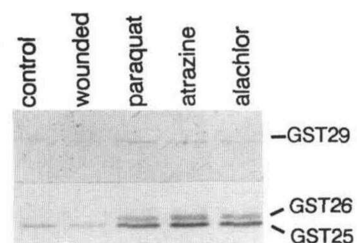

Figure 6. Immunoblot analysis of the GST isozymes induced by herbicides (0.5 mM) and wounding. GST29 was labeled with anti-GstA1 fusion protein antiserum (upper) and GST25/GST26 were labeled with anti-maize GST antiserum (lower). Fifty micrograms of protein were loaded per lane. The blots were developed with a chromogenic substrate for alkaline phosphatase.

To learn more about the mechanism leading to the induction of GST25/GST26 synthesis, herbicide treatments were also performed in the dark (data not shown). The induction of CDNB activity by metolachlor and alachlor was light independent. In contrast, the herbicides paraquat and atrazine, which interfere with electron transport in the photosystem, had only a small effect on the expression of GST25/GST26 in the dark. The induction of GST25/GST26 synthesis by paraquat and atrazine is apparently not due to the herbicide per se but is more likely the result of the light-dependent oxidative damage caused by these herbicides.

DISCUSSION

In this article, we demonstrate the presence of two distinct classes of GSTs in wheat. The first GST class is exemplified by GST29, a protein that is encoded by the previously cloned *GstA1* gene (Dudler et al., 1991). The second GST class has at least two members, which were named GST25 and GST26. With the exception of their capacity to catalyze the conjugation of GSH to electrophilic centers of lipophilic compounds, those members of these two classes that have been characterized appear to have little in common. First, they share little sequence identity, as indicated by the lack of cross-hybridization of the *GstA1* cDNA with the mRNA(s) encoding GST25/GST26. Second, GST29 and GST25/GST26 are antigenically unrelated. Antiserum raised against GST29 did not cross-react with GST25/GST26, and antiserum raised against GST25/GST26 of maize did not cross-react with GST29. Third, expression of Gst29 and GST25/GST26 is differentially regulated by pathogen attack and herbicide treatment, respectively. Fourth, Gst29 and GST25/GST26 differ in their enzymic properties.

The *GstA1* gene encodes a protein of 229 amino acids with a calculated molecular mass of 25.828 kD (Dudler et al., 1991). The in vitro translation product of the full-length *GstA1* cDNA and the *GstA1*-encoded protein produced in wheat have the same apparent molecular mass of 29 kD, indicating that the encoded protein, GST29, is not modified posttranslationally.

Functional expression of the *GstA1* cDNA in bacteria demonstrated that GST29 has GST activity. However, compared with the amount of GST29 present in bacteria expressing the *GstA1* cDNA, the specific GST activity toward the model substrate CDNB appeared to be rather low. CDNB is apparently not a very attractive substrate for GST29. In this regard, CDNB has been previously shown to be a poor substrate for a few GSTs from mammals (Jakoby and Habig, 1980; Ketterer et al., 1988) and plants (Gronwald et al., 1987; Timmerman, 1989; Dean et al., 1990; Edwards and Dixon, 1991; Meyer et al., 1991). Our search for a more suitable artificial substrate was not successful. The GST29 protein produced in bacteria was inactive against a number of other potential GST substrates such as 1,2-dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride, and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (data not shown), and against the herbicides metolachlor and atrazine (D. Adams, personal communication). GST29 appears to have a well-defined substrate requirement, which is atypical but not unique for a GST. Another unusual feature that GST29 shares with only a few of the GSTs that have been described (Ketterer et al., 1988; Ketterer and Coles, 1991) is its lack of affinity toward GSH-agarose.

Fungal infection leads to a rapid induction of *GstA1* expression. A strong increase in the level of mRNA corresponding to *GstA1* was already observed 2 h after inoculation with *E. graminis.* Clearly, *GstA1* is induced well before penetration of the host cells that begins about 6 to 10 h after inoculation. Because the cell-free supernatant of autoclaved *E. graminis* spores was also able to induce the accumulation of *GstA1* mRNA, we speculate that a soluble factor released from the spores might be involved in the elicitation process. This hypothesis would fit in with results obtained from studies of induced resistance in barley, where it was shown that the inducing *E. graminis* needed to be in contact with the plant for less than 1 h to establish induced resistance against a challenge infection (Cho and Smedegaard-Petersen, 1985). No differential induction of *GstA1* expression in response to *E.g.* f.sp. *hordei* and *E.g.* f.sp. *tritici* was observed. The induction of *GstA1* expression is apparently not directly connected to the differential resistance of wheat against these two potential pathogens, but appears to be a more general response to pathogen attack.

In contrast to GST29, GST25 was expressed at a high basal level in wheat seedlings, indicating that this enzyme has a function in normal metabolism. GST25 shows characteristics typical for GSTs. It binds to the affinity matrix GSH-agarose and is highly active on the conventional substrate CDNB. GST25 and the antigenically related GST26 were strongly induced by a number of xenobiotics. This is consistent with results obtained with GSTs of maize (Mozer et al., 1983; Wiegand et al., 1986; Timmerman and Tu, 1987; Edwards and Owen, 1988). Similar to their counterparts in maize (Frear and Swanson, 1970; Shimabukuro et al., 1971; Edwards and Owen, 1986, 1988; Timmerman, 1989), GST25/GST26 were shown to detoxify atrazine and metolachlor through GSH conjugation (K. Kreuz, personal communication). The conclusion that GST25 and GST26 have a general function in xenobiotic metabolism is supported by the finding that the accumulation of both enzymes was also induced by cadmium (our unpublished results). A role for GST in heavy metal detoxification has been proposed in mammals (Almar and Diericks, 1990).

Because the production of GSTs in general is described as being inducible by a broad spectrum of compounds, it was important to assess the specificity of the induction of *GstA1* expression by pathogens. Elicitors of putative defense genes of dicotyledonous plants did not induce *GstA1*; neither did wounding. More importantly, *GstA1* expression was not induced by various herbicides, indicating that it is not involved in xenobiotic metabolism. In addition, the negative results obtained with the peroxidizing herbicide paraquat implies that *GstA1* expression is not induced by oxidative stress. Thus, the expression of *GstA1* is remarkably tightly controlled. Besides fungal infection, the only stimulus found to trigger an accumulation of *GstA1* mRNA was the GST substrate GSH. Whether this finding has any physiological relevance is at present not clear. It is interesting that it was shown that GSH induces a number of putative defense genes in plant cell cultures, and it was suggested that GSH might play a

role in mediating the response of plant cells to biological stress (Dron et al., 1988; Wingate et al., 1988).

The specific induction of *GstA1* expression by pathogens and the fact that the encoded protein differs in many respects from the GSTs involved in xenobiotic metabolism imply that GST29 has specific functions possibly related to pathogen attack. In the absence of any knowledge about the biological substrates of GST29, it can only be speculated what the in vivo function of this protein might be.

In mammalian systems, GST-catalyzed GSH conjugation was shown to be involved in the cellular export of biologically active compounds (e.g. leukotrienes) and in the detoxification of the products generated from tissue damage (Slater, 1984). For example, lipid and DNA hydroperoxides have been found to be detoxified by GST-mediated conjugation to GSH (see Ketterer and Coles, 1991, for a review) and it was demonstrated that this function is restricted to a specialized subset of GST isozymes (Jensson et al., 1986; Danielson et al., 1987). Because the production of active oxygen species and the resulting lipid peroxidation are known to occur in plants in response to pathogen attack (Keppler and Novacky, 1987; Adam et al., 1989; Slusarenko et al., 1991), it is tempting to speculate that some GSTs could play a similar protective role in plants. By detoxifying the products originating from lipid peroxidation, the pathogen-induced GST29 might, after an initial lag period, prevent continued cell disruption caused by these highly toxic radicals and thereby localize the host response, as is seen, for example, in the hypersensitive reaction. Due to the multitude of peroxidation products generated in infected tissue, this hypothesis is not easily verified. Our first crude attempts to demonstrate directly a detoxifying activity of GST29 toward two cytotoxic lipid peroxidation products, *trans*-2-hexenal and 4-hydroxy-nonenal, were unsuccessful. A more promising way to test our hypothesis would require the manipulation of *GstA1* expression by techniques that are not yet practical in wheat. Therefore, we have initiated experiments in a heterologous system to test whether the constitutive expression of *GstA1* has an effect on the phenotype of the pathogen-induced hypersensitive response.

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