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Cloning and expression of a cDNA encoding a maize glutathione-S-transferase in *E. coli*

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ABSTRACT

The isolation and characterization of a family of maize glutathione-S-transferases (GST's) has been described previously (1). These enzymes are designated GSTs I, II and III based on size, substrate specificity and responsiveness to safeners. GST III has been shown to act on the herbicide alachlor as well as the commonly used substrate 1-chloro-2,4-dinitrobenzene (CDNB) (1,2). Clones were isolated from a maize cDNA library in λgt10. Three clones contained the entire coding region for GST III. The sequences of these clones were consistent with the known amino terminal GST III protein sequence. Moreover, expression of one of these clones in *E. coli* resulted in a GST activity as measured with both CDNB and alachlor, proving that at least one of the clones encodes an active GST III species. With the enzyme expressed in *E. coli* it will become possible to study enzyme structure-function relationships *ex planta*. While a number of different GST proteins are present in maize tissue the GST III gene is present in single or low copy in the genome.

INTRODUCTION

A family of enzymes termed glutathione-S-transferases (GST's) catalyze the conjugation of glutathione to a large variety of electrophilic, hydrophobic compounds. In plants, these enzymes appear to be involved in the detoxification of a number of chloroacetanilide herbicides. Certain types of GST activity are increased in response to chemical safeners, compounds which render some plants more resistant to certain herbicides. Several GST protein species have been isolated from maize, each with its own characteristic size, range of substrate specificities and responsiveness to safeners (1). Each exists as a dimer, GST I of subunits 29 kd in size, GST II, 27 kd and GST III, 26 kd.

We have been interested in the plant GST's because of their role in dealing with chemical stress and because a gene encoding GST might be useful as a selectable marker in plant cell transformation vectors. GST III was of interest because it has a relatively higher activity on alachlor than does GST I (Mozer *et. al.*, unpublished data). Herein, we describe the isolation

of cDNA clones from maize which encode the protein species designated GST III. Protein sequence was obtained from the amino terminus of GST III, from which we defined a synthetic 21-base oligonucleotide probe which we have used to isolate two classes of GST III cDNA clones. One of these clones has been expressed in *E. coli* and shown to encode a GST that does indeed act on alachlor.

MATERIAL AND METHODS

Preparation of RNA

Safener-treated etiolated maize tissue was obtained essentially as described previously and was used as the source for poly(A)RNA (1,2).

Preparation of cDNA library

Preparation of the cDNA library followed the scheme of Maniatis *et al.* (3) with slight modifications. Second strand synthesis was by reverse transcriptase followed by incubation with the Klenow fragment of DNA polymerase I. Following methylation of the cDNA with *Eco*R1 methylase to prevent digestion of internal *Eco*R1 sites and subsequent attachment of *Eco*R1 linkers, the cDNA was size selected on an agarose gel to be >500 bp and was then ligated into *Eco*R1 digested λgt10. The DNA was packaged and amplified as detailed by Huynh *et. al.* (4) to yield a cDNA library containing approximately 100,000 different hybrids.

Northern blot analysis using degenerate oligonucleotide probes.

RNAs were size fractionated by electrophoresis on a 1.5% agarose formaldehyde denaturing gel (5). The RNA was blotted onto nitrocellulose which was then divided into strips and hybridized as described (6,7). Hybridization with labeled probe was in 6XSSC, 10X Denhardts, 100 μg/ml tRNA at 40°C for 72 hours. Probe sequences were based on amino terminal sequencing of GST III which gave the following sequence; NH_2-Met-Ala-Pro-Leu- Lys-Leu-Tyr-Gly-Met-Pro-Leu-Ser. The underlined protein sequence was the basis for the synthesis of a 96X degenerate probe mix which contained all possible probes complementary to that mRNA sequence. Probes were synthesized as three different mixes, each 32X degenerate (8). We used 4.8 pmoles of each [^{32}P] labeled probe mix; either
mix 1 (5′-GG-CAT-NCC-RTA-YAG-YTT-3′) at $4x10^6$ cpm/pmole,
mix 2 (5′-GG-CAT-NCC-RTA-RAG-YTT-3′) at $3x10^7$ cpm/pmole or
mix 3 (5′-GG-CAT-NCC-RTA-YAA-YTT-3′) at $7x10^6$ cpm/pmole.
The blots were washed in 6xSSC at room temperature for 10 minutes 3 times, then at 51°C for 10 minutes. Blots were exposed to Kodak XAR film at -70°C for 19 hours with an intensifying screen.

Primer extension and screening of cDNA Library.

Twenty-five micrograms of poly(A)+RNA was hybridized to 7 pmole of [^{32}P] labeled probe mixture 1 (1.2x10^{7} cpm/pmole) for 3 hours at 40°C. Hybridized primers were extended with reverse transcriptase (9). The extended products were then separated by size on a 20% acrylamide gel containing 7 M urea in TBE. Following autoradiography each band was excised from the gel and sequenced by the Maxam-Gilbert technique (9,10). The primer extension sequence permitted definition of a unique 21 base oligonucleotide specific to GST III (Fig. 1). This unique probe was then used to screen our cDNA library by a method described previously (11).

DNA subcloning and sequencing.

cDNA inserts were released from the unique *Eco*R1 site of λgt10 DNA and the fragment that bound the probe was identified. Purified inserts were subcloned into M13 mp9 and sequenced (12,13).

Site directed mutagenesis was performed as described previously (14). Screening for the correct mutation was by dot blot hybridization using the mutagenesis primer as a [^{32}P] labeled probe.

Genomic Southern blot analysis.

DNA samples (10 µg) from corn hybrid line 3780A were digested with various restriction enzymes and electrophoretically resolved on a 0.7% agarose-gel. The gel was blotted (15) and the filter probed with a 700bp *Eco*R1/*Ava*I GST IIIA fragment nick-translated with [^{32}P]dCTP to a specific activity of 2 x 10^{8} cpm/µg. Hybridization was at 48°C for 48 hours in a 10 ml volume of 50% formamide, 5X SSPE, 5X Denhardts, 0.1% SDS, 300 µg/ml tRNA and 3 x 10^{6} cpm of probe. The blot was washed in 1X SSPE, 0.2% SDS 30 minutes at 25°C and 30 minutes at 50°C, then washed twice in 0.1X SSPE, 0.2% SDS at 50°C. Exposure to Kodak XAR film was for 6 days at -70°C with an intensifying screen.

GST Enzyme assays.

Preparation of Extract. Cells transformed (16) with control or GST-expression plasmids were pelleted in a 1.5 mL microcentrifuge tube and the supernatant discarded. 0.5 ml of 100 mM Tris (pH 7.5), 1 mM DTT and glass beads were added to the pellet. The tube was vortexed vigorously for 20-30 seconds to break the cells. Cell debris was pelleted by centrifugation for 5 minutes at 4°C. The extract was collected and the pellet re-extracted one time as before. The extracts were combined and samples tested for GST activity.

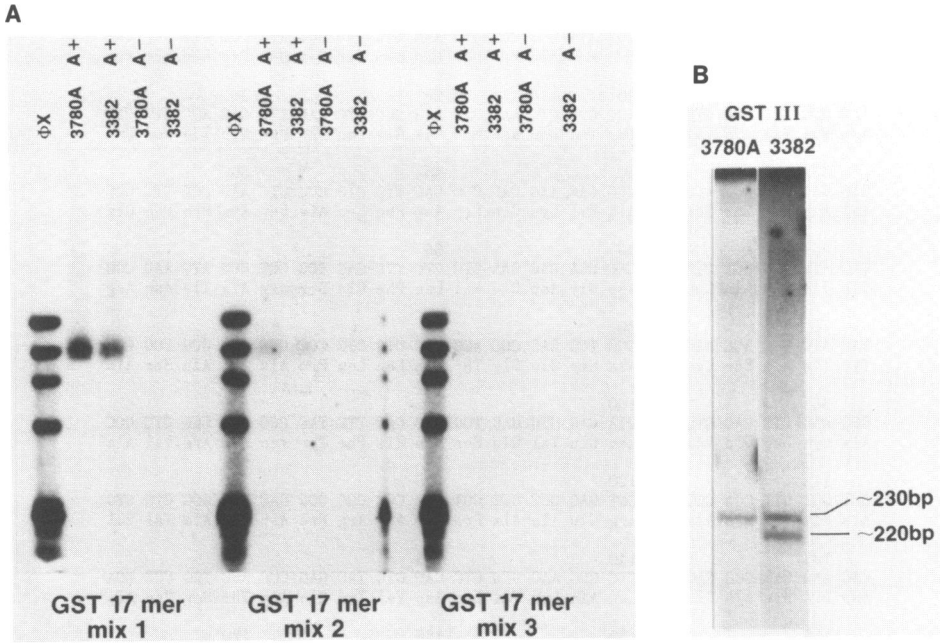
CDNB assay. 50-200 µl of cell extract and 33 µl 100 mM CDNB (1-chloro-2,

4-dinitrobenzene) were added to 1 ml of 100 mM $KHPO_4$ (pH 6.5), 10 mM glutathione in a 1.5 ml cuvette. The reaction was run at 25°C. The change in A_{340}/minute was measured at 5 minute intervals over a 25 minute period. Alachlor assay. The assay mix consisted of 1 ml of 400 mM $KHPO_4$ (pH 6.5), 8 mM glutathione, 0.2 mM alachlor and 1 µCi [^{14}C]-alachlor. Assay mix (40 µL) was added to 60 µL of cell extract and incubated at 34°C for 1 hour. To the reaction tube was then added 0.5 ml H_2O and 0.5 ml $CHCl_3$ and the tube was vortexed vigorously for 30 seconds. The glutathione-alachlor conjugate remains in the aqueous phase while unconjugated alachlor goes into the organic phase. The aqueous phase was removed and counted. We found that crude *E. coli* extracts inhibited this assay, so it was necessary to dialyse samples against 50 mM KPO_4 (pH 7.5), 1 mM DTT prior to assay.

RESULTS AND DISCUSSION

Northern analysis and primer extension.

Three samples containing equal amounts of maize poly(A)RNA were size-fractionated by electrophoresis on one gel. The gel samples were blotted to nitrocellulose and the nitrocellulose divided into three sections. Each section was hybridized with one of three oligonucleotide mixtures which collectively represent all possible codon combinations complementary to the corresponding mRNA. The washing conditions of the blots discriminated between perfectly matched duplexes and mismatched duplexes. One member contained within mix 1 apparently formed a perfect match with a polyadenylated RNA species approximately 1100 bases long that is present in both hybrid maize lines 3780A and 3382 (Fig. 1). The mRNA was present at highest concentration in corn line 3780A, which was used as the source of RNA to construct the cDNA library. The other two probe mixures showed slight hybridization, consistent with the lower efficiency pairing of mismatched probes. GST 17-mer mix 1 was next used in a primer extension experiment with maize poly(A)RNA. An identical primer extension product, approximately 230 bp in size, was obtained with RNA from both corn lines 3382 and 3780A. In addition, a smaller primer extension product was obtained only in corn line 3382. (Fig. 1B). All three bands gave identical nucleotide sequence (Fig. 1C) through the region sequenced by us. The smaller extension product may have been due to differences beyond the region we sequenced, or may have resulted from premature termination by the reverse transcriptase. In all cases the sequence obtained was consistent with the amino acid sequence upstream from the probe region: met-ala-pro-leu. A



C

NH_2-Met-Ala-Pro-Leu-Lys-Leu-Tyr-Gly-Met-Pro

5'-AGACTTGACCAAGCAGCAGCAGGG-ATG-GCG-CCT-CTG-AAG-CTG-TAC-GGA-ATG-CC-3'

Unique 21 base probe 3'-A-GAC-TTC-GAC-ATG-CCT-TAC-GG-5'

Figure 1. (A) Hybridization of oligonucleotide probes to maize poly(A)RNA. RNA was fractionated on a formaldehyde-agarose gel, transferred to nitrocellulose and hybridized with three different 32x degenerate probe mixtures as described in the Materials and Methods. (B) Primer extension products using 32x degenerate oligonucleotide mix 1 as a primer with maize poly(A)RNA from two corn lines. 5'- end labeled probe mix 1 was hybridized to poly(A)RNAs and was extended with reverse transciptase. End labeled products were electrophoresed on acrylamide-urea gels as detailed in the Materials and Methods. Extension products were excised from gels and sequenced (10). A portion of the sequence of one extension product that confirms the GST III protein sequence and was used to design a longer 21 base unique probe used to screen cDNA libraries is provided in (C).

unique 21 base oligonucleotide probe was made based on this sequence (Fig. 1C).

Screening of the cDNA library.

Screening of about 50,000 plaques from our cDNA library in λgt10, distributed over 30 plates, was carried out (6, 11). Three independent

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                                                5                   10
                    GGATAGGG ATG GCG CCT CTG AAG CTG TAC GGG ATG CCG CTG TCC CCC
                             Met Ala Pro Leu Lys Leu Tyr Gly Met Pro Leu Ser Pro

    15                  20                  25                  30
AAC GTG GTG CGC GTG GCC ACC GTG CTC AAC GAG AAG GGC CTC GAC TTC GAG ATC GTC CCC
Asn Val Val Arg Val Ala Thr Val Leu Asn Glu Lys Gly Leu Asp Phe Glu Ile Val Pro

    35                  40                  45                  50
GTC GAC CTC ACC ACC GGC GCC CAC AAG CAG CCC GAC TTC CTC GCC CTC AAC CCT TTC GGC
Val Asp Leu Thr Thr Gly Ala His Lys Gln Pro Asp Phe Leu Ala Leu Asn Pro Phe Gly

    55                  60                  65                  70
CAG ATC CCG GCT CTC GTC GAC GGA GAC GAA GTC CTC TTC GAG TCC CGT GCG ATC AAC CGG
Gln Ile Pro Ala Leu Val Asp Gly Asp Glu Val Leu Phe Glu Ser Arg Ala Ile Asn Arg

    75                  80                  85                  90
TAC ATC GCC AGC AAG TAC GCG TCG GAG GGC ACG GAC CTG CTC CCC GCG ACG GCG TCG GCG
Tyr Ile Ala Ser Lys Tyr Ala Ser Glu Gly Thr Asp Leu Leu Pro Ala Thr Ala Ser Ala

    95                  100                 105                 110
GCG AAG CTG GAG GTG TGG CTA GAG GTG GAG TCG CAC CAC TTC TAC CCG AAC CGG GTC GCC
Ala Lys Leu Glu Val Trp Leu Glu Val Glu Ser His His Phe Tyr Pro Asn Arg Val Ala

    115                 120                 125                 130
GCT GGT GTT CCA GCT GCT CGT GAG GCC GCT CCT GGG CGG CGC CCC GAC GCG GCG GTG GTG
Ala Gly Val Pro Ala Ala Arg Glu Ala Ala Pro Gly Arg Arg Pro Asp Ala Ala Val Val

    135                 140                 145                 150
GAC AAG CAC GCG GAG CAG CTC GCC AAG GTG CTC GAC GTG TAC GAG CGC ACC TCG CCC GCA
Asp Lys His Ala Glu Gln Leu Ala Lys Val Leu Asp Val Tyr Glu Arg Thr Ser Pro Ala

    155                 160                 165                 170
ACA AGT ACC TCG CCG GGG ACG AGT TCA CGC TCG CCG ACG CCA ACC ACG CGC TCC TAC CTG
Thr Ser Thr Ser Pro Gly Thr Ser Ser Arg Ser Pro Thr Pro Thr Thr Arg Ser Tyr Leu

    175                 180                 185                 190
CTC TAC CTC AGC AAG ACC CCA AGG CCG GCT CGT CGC CGC CCG CCC CAC GTC AAG GCC TGG
Leu Tyr Leu Ser Lys Thr Pro Arg Pro Ala Arg Arg Arg Pro Pro His Val Lys Ala Trp

    195                 200                 205                 210
TGG GAG GCC ATC GTC GCC CGC CCC GCG TTC CAG AAG ACC GTC GCC GCC ATC CCC TTG CCC
Trp Glu Ala Ile Val Ala Arg Pro Ala Phe Gln Lys Thr Val Ala Ala Ile Pro Leu Pro

    215                 220
CCG CCG CCC TCC TCC TCG GCT TGA CCTCGCCTTGCGTTGCGCCGTTGCCTGGGTCGCGGATGCTCGGAGCC
Pro Pro Pro Ser Ser Ser Ala STOP

CCGAGTCGAATAAAAGAGGCAGCATCCTGTCTTGCATTTGCTCGTGCGCCATGTGTTAACAGCCTGTGTAATAAACACT

GTTGCCTTTCGTGTGTGTTCATTGCCTTTTGGTTGGTCTTTGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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FIGURE 2. Nucleotide sequence of the GST IIIA cDNA clone. The coding strand nucleotide sequence and predicted amino acid sequence of GST IIIA are displayed in the 5' to 3' direction. Two conserved AATAAA sequences near the 3' end of the cDNA are underlined. GST IIIB is identical except that the 5' untranslated sequence is 5'-AGACTTGACCAAGCAGCAGCAGCAGGG-3' instead of 5'-GGATAGGG-3' and the poly (A) tract is not present.

isolates, putatively encoding GST III survived three successive rounds of screening, at which point they were plaque-purified.

Characterization of the putative GST III cDNA clones.

DNA was purified from all three hybrid phage. Restriction mapping

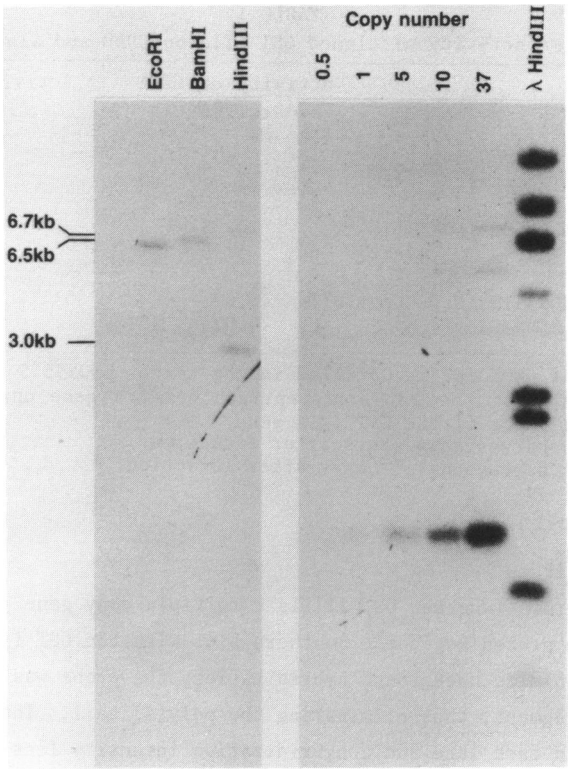

Figure 3. Genomic Southern blot analysis of the GST III structural gene. Lanes 1-3 contain 10 μg each 3780A DNA digested with the enzymes shown. Lanes 4-8 contain varying amounts of the GST III cDNA corresponding to different gene copy numbers in the genome.

and DNA sequencing allowed us to compare the three clones to each other and to the GST III sequence defined by primer extension. The inserts that hybridized to the probe used in screening were 930, 910, and 840 bp, respectively, in size. The three cDNA clones could be divided into two classes based on differences in their 5' untranslated regions (Fig. 2). Whereas the 840 bp clone has a 5' untranslated region identical to the primer extension sequence, the 930 bp and 910 bp clones have identical 5' untranslated regions which differ from the primer extension sequence. All three clones share identical sequence through the entire protein coding and 3' untranslated regions, with differences in the length of the poly(A) tract. We have designated the two larger clones GST IIIA and the 850 bp clone GST IIIB.

TABLE 1
Enzyme activity of cloned GST IIIA on CDNB and alachlor

Expression Construct	Activity on CDNB (ΔA_{340}/mg/min)	Activity on Alachlor (nmole/mg/hr)
pMON3502 (GST IIIA insert)	10.99_1	6.63_1
	5.23_2	2.84_2
pMON5515 (Neg. Control)	$<0.02_1$	$<0.65_1$
	$<0.02_2$	$<0.65_2$

The structure of pMON3502 is detailed in the text. pMON5515 is identical to pMON3502 except that the rat atriopeptigen gene, a gene unrelated to GST, was inserted in place of the GST IIIA gene.
1 Cells harvested 4 hours after induction.
2 Cells harvested 1.5 hours after induction.

Genomic blot analysis.

In order to determine whether GST III is a multiple copy gene in the maize genome, we probed a genomic Southern blot with the GST IIIA cDNA clone (Fig. 3). To minimize background hybridization, the probe was prepared as an *Eco*R1/*Ava*1 fragment, thus eliminating the poly(A) tail. The absence of multiple bands in each lane and a hybridization intensity less than the 5 copy control suggests that we are dealing with a low or single copy number gene that, at least at this relatively high level of stringency, does not appear to be a member of a larger gene family.

Expression of GST.

To show definitively that we had isolated a gene encoding a GST we introduced GST IIIA into an expression vector. An *Nco*I site was created at the start codon of GST IIIA by inserting two cytidine residues, thus allowing us to manipulate the gene as an *Nco*I/*Eco*R1 fragment free of 5′ untranslated sequences. This fragment was introduced into a pBR327 based expression plasmid, pMON3502, in which the *rec*A promoter is used to drive expression of the GST IIIA cDNA (P. Olins, manuscript in preparation). The vector also contains a ribosome binding site present in phage T7 gene 10 transcripts.

E. coli cells (strain JM101) transformed with pMON3502 were grown at 37°C. Duplicate cultures were induced to express GST III by the addition of nalidixic acid to 50 µg/ml and were incubated for an additional 1.5 or 4 hours. The cells were harvested and frozen at -80°C prior to assay.

Extracts of cells transformed with this construct contained GST activity using either CDNB or alachlor as substrate (Table 1). Expression of the GST IIIA clone in *E. coli* demonstrated the presence of an active GST enzyme with a substrate profile matching that of GST III isolated from maize tissue. The detection of enzyme activity *ex planta* will now allow detailed structure-function studies of the enzyme.

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