# Tandemly Duplicated Safener-Induced Glutathione S-Transferase Genes from *Triticum tauschii* Contribute to Genome- and Organ-Specific Expression in Hexaploid Wheat[1]

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Glutathione S-transferase (GST) gene expression was examined in several *Triticum* species, differing in genome constitution and ploidy level, to determine genome contribution to GST expression in cultivated, hexaploid bread wheat (*Triticum aestivum*). Two tandemly duplicated tau class GST genes (*TtGSTU1* and *TtGSTU2*) were isolated from a single bacterial artificial chromosome clone in a library constructed from the diploid wheat and D genome progenitor to cultivated wheat, *Triticum tauschii*. The genes are very similar in genomic structure and their encoded proteins are 95% identical. Gene-specific reverse transcriptase-polymerase chain reaction analysis revealed differential transcript accumulation of *TtGSTU1* and *TtGSTU2* in roots and shoots. Expression of both genes was induced by herbicide safeners, 2,4-dichlorophenoxyacetic acid and abscisic acid, in the shoots of *T. tauschii*; however, expression of *TtGSTU1* was always higher than *TtGSTU2*. In untreated seedlings, *TtGSTU1* was expressed in both shoots and roots, whereas *TtGSTU2* expression was only detected in roots. RNA gel-blot analysis of ditelosomic, aneuploid lines that are deficient for 6AS, 6BS, or 6DS chromosome arms of cultivated, hexaploid bread wheat showed differential genome contribution to safener-induced GST expression in shoots compared with roots. The GST genes from the D genome of hexaploid wheat contribute most to safener-induced expression in the shoots, whereas GSTs from the B and D genomes contribute to safener-induced expression in the roots.

Glutathione S-transferases (GSTs) belong to multigene families common to all plants (Edwards et al., 2000; McGonigle et al., 2000). They are well known for their responses to numerous endogenous and xenobiotic stresses, and glutathione conjugation of toxic electrophilic molecules. The roles of GST proteins in endogenous plant metabolism, as well as their role in stress tolerance, have yet to be clearly defined. GST gene expression is induced after exposure to many stresses, including biotic stresses such as pathogen attack and fungal elicitors, and abiotic stresses such as heat shock, cold, high salt, UV light exposure, heavy metals, and herbicides. Phytohormone treatments such as ethylene, auxins, abscisic acid (ABA), methyl jasmonate, and salicylic acid have also been shown to induce expression of GSTs (for review, see Marrs, 1996; Dixon et al., 1998; Edwards et al., 2000). Induction of GST expression by so many diverse stimuli implies that plant GSTs are critical in plant response to stress, either by participating in the signal transduction process and/or detoxifying harmful compounds produced in response to or as a result of a given stress. It is likely that GST gene expression is induced by conditions that lead to oxidative stress (Polidoros and Scandalios, 1999). The encoded GST proteins play an important but poorly understood role in plant response to stress, possibly through the central role of antioxidant function. GST enzymatic activity could involve direct glutathione conjugation to toxic electrophilic molecules, or glutathione-dependent peroxidase activity, using glutathione as reductant for the detoxification of toxic oxygen species, oxygen radicals, and lipid peroxides formed during or after plant stress (Dixon et al., 1998; Edwards et al., 2000). One biochemical function of GST proteins that is well defined is their role in herbicide metabolism in crops. GSTs are the predominant detoxification enzymes in maize (*Zea mays*) and cereal crops that are responsible for metabolism of triazine herbicides, acetamide herbicides, and certain graminicides, such as fenoxaprop-ethyl in wheat (Edwards and Cole, 1996; Riechers et al., 1996b, 1997b; Cummins et al., 1997). Herbicide-detoxifying GSTs have been well characterized in maize and soybean (*Glycine max*; Fuerst et al., 1993; Irzyk and Fuerst, 1993, 1997; Jepson et al., 1994; Andrews et al., 1997; Droog, 1997; Dixon et al., 1998), but have also been identified and partially characterized in wheat (Cummins et al., 1997; Riechers et al., 1997b).

Herbicide safeners are chemical compounds that increase the tolerance of certain grass crops (e.g.

maize, grain sorghum [*Sorghum bicolor*], wheat, rice [*Oryza sativa*]) to herbicides (Hatzios and Hoagland, 1989). Herbicide safeners protect the crop plant by increasing herbicide metabolism and detoxification pathways (Fuerst and Gronwald, 1986; Hatzios, 1991; Farago et al., 1994; Riechers et al., 1996a; Davies and Caseley, 1999). The increase in metabolism results from an increase in the activity of herbicide detoxification enzymes, such as GSTs, cytochrome P450-dependent monooxygenases, and glucosyltransferases (Gronwald et al., 1987; Hatzios, 1991; Cole, 1994; Kreuz et al., 1996). Despite the widespread agronomic use of safeners and information about their effects on GST and P-450 enzymatic activity, there is little information on the precise molecular mechanism for safener induction of the genes that encode these herbicide-detoxifying enzymes. A safener-binding protein and its activity have been characterized in maize seedlings, along with its gene expression patterns (Walton and Casida, 1995; Scott-Craig et al., 1998). An area that is especially lacking in information is the identification and characterization of important regulatory sequences present in the promoters of safener-responsive GST genes, and the transcription factors that bind to these DNA sequences in agronomically important grasses such as maize, rice, and wheat. Molecular analyses of plant GST genomic sequences have focused mainly on dicot species, and have examined GST gene expression in response to pathogen attack, plant hormones, or stress treatments. Promoter regulatory elements that confer increased GST gene expression in response to auxins, ethylene, salicylic acid, hydrogen peroxide, heat shock, heavy metals, and plant pathogens have been identified in several dicot species including potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), Arabidopsis, soybean, and carnation (*Dianthus caryophyllus*; Martini et al., 1993; Itzhaki et al., 1994; Ulmasov et al., 1994; Droog et al., 1995; Maxson and Woodson, 1996; Strittmatter et al., 1996; Chen and Singh, 1999; Johnson et al., 2001). Safener-responsive expression of GSTs and their cDNA sequences have been reported for maize, wheat, and rice (Jepson et al., 1994; Irzyk and Fuerst, 1997; Riechers et al., 1997a; Wu et al., 1999). However, among these safener-responsive genes in monocot crops, only the maize *GST-27* promoter has been partially characterized (Robertson et al., 2000).

Our studies have utilized the diploid wheat *Triticum tauschii* (synonymous with *Aegilops tauschii* and *Aegilops squarrosa*) as a model plant and genome to understand regulation of GST expression in grass crops with large and/or polyploid genomes (Keller and Feuillet, 2000). Previous research focused on a herbicide safener-induced GST isozyme that was purified from *T. tauschii* using anion-exchange and affinity chromatography, and was biochemically characterized (Riechers et al., 1997b). This safener-inducible GST isozyme can use the chloroacetamide

herbicide dimethenamid as a substrate (Riechers et al., 1997b), where its conjugation with reduced glutathione results in metabolic detoxification of the herbicide (Dixon et al., 1998). In subsequent studies, a corresponding cDNA was isolated from *T. tauschii* and was used to map the homoeologous GST genes to a chromosome arm in cultivated, hexaploid bread wheat (*Triticum aestivum*) and to a linkage group in barley (*Hordeum vulgare*; Riechers et al., 1997a, 1998). Here, we report the analysis of genomic sequences for the safener-inducible, tau class GST genes (*TtGSTU1* and *TtGSTU2*) and characterization of their expression profiles in *T. tauschii*. We also utilized ditelosomic, aneuploid wheat lines and various wheat species that differ in genome constitution and/or ploidy level to determine genome contributions to expression patterns in cultivated, hexaploid bread wheat. Our results provide novel evidence for differential genome contributions to constitutive and inducible gene expression in organs of hexaploid wheat, and differences in expression between closely related gene family members that are organized as a tandem repeat in a large grass genome.

## RESULTS

### Isolation and Sequence Analysis of GST Genes from *T. tauschii*

Three different GST genes were isolated by screening a *T. tauschii* large DNA insert genomic library (Moullet et al., 1999). The genomic library was screened with the safener-inducible *GST TSI-1* (renamed *TtGSTU1*; Edwards and Dixon, 2000) cDNA that had been isolated previously from *T. tauschii* (Riechers et al., 1997a). A total of four positively hybridizing genomic clones were obtained and further analyzed. DNA gel-blot analysis of the four bacterial artificial chromosome (BAC) clones digested with different restriction enzymes showed that BAC 1 (insert size of 150 kb) and BAC 4 (insert size of 130 kb) contained overlapping genomic fragments, and each BAC clone appeared to contain at least two GST genes. Subsequent experiments focused on analyzing only BAC 1. BAC 1 contained an approximately 14-kb *Eco*RI/*Kpn*I-hybridizing fragment. An *Xho*I site within the 14-kb fragment was utilized to separate this fragment into two smaller fragments of 8.5 and 5.3 kb. Each smaller fragment contained one of two tandemly repeated GST genes, *TtGSTU2* and *TtGSTU1* (accession no. AY013753; Fig. 1). These results are consistent with DNA gel-blot analysis (using *Xba*I as the restriction enzyme) of the cultivated, hexaploid bread wheat genome (Riechers et al., 1998), where it was hypothesized that the homoeologous GST genes were represented by at least two copies in each of the three wheat genomes. Sequence analysis of the entire 14-kb fragment showed that the coding region and untranslated regions (UTRs) of *TtGSTU1* are identical to the *TtGSTU1* cDNA (Riec-
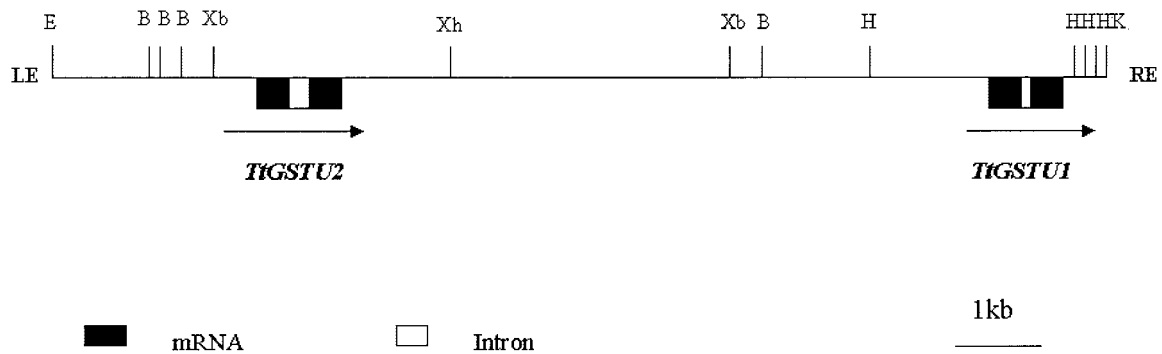
**Figure 1.** Restriction map of the 14-kb region of the BAC 1 clone from *T. tauschii*, containing the two tandemly duplicated GST genes. Restriction sites are: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; Xb, *Xba*I; and Xh, *Xho*I. LE, Left end (position 0); RE, right end (position 13, 710).

hers et al., 1997a). Both of the tandemly duplicated genes contain an intron that interrupts the coding region at the same location, although the length and sequence of the intron varies between the two genes. *TtGSTU1* has a single intron of 99 bp, whereas *TtGSTU2* has a 319-bp intron (Fig. 1), suggesting that these are tau class, or type III, GST genes (Droog, 1997; Edwards and Dixon, 2000; Edwards et al., 2000).

DNA gel-blot and sequence analysis of BAC 3 showed that it contained only the *TtGSTU1* gene, and was not analyzed further. DNA gel-blot analysis of the BAC 2 clone, digested with several restriction enzymes, showed a single, weakly hybridizing band (data not shown). BAC 2 was found to contain a related, yet divergent, GST-like sequence, which was named *TtGSTU3* (accession no. AY013754). The nucleotide sequence of the *TtGSTU3* gene's coding region is 76% identical to the corresponding region of the *TtGSTU1* gene, but this gene apparently does not contain an intron.
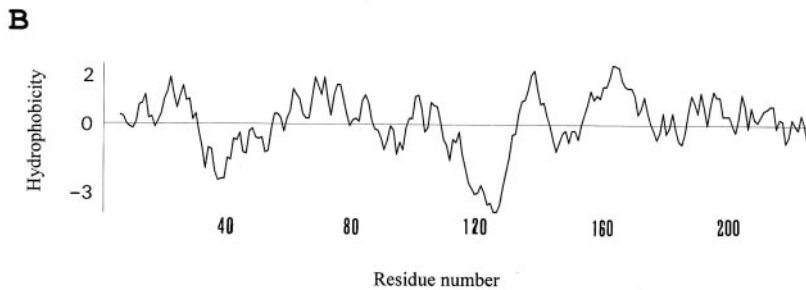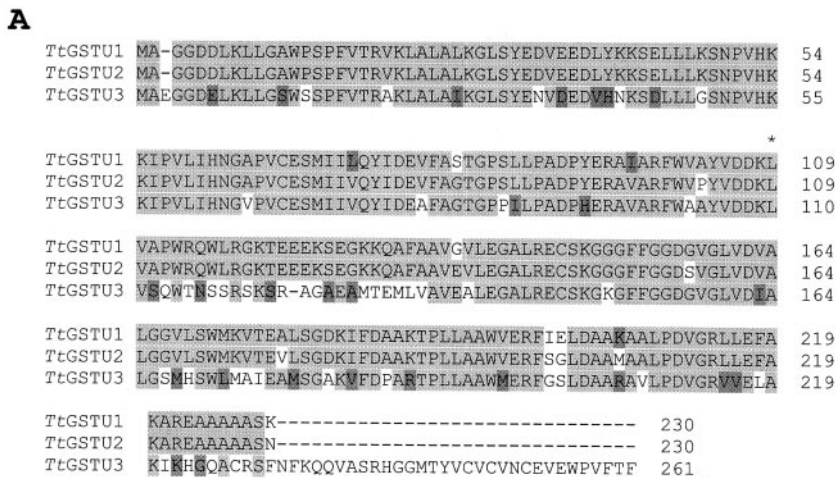
Comparison of the deduced amino acid sequences of the three GST genes showed that the *TtGSTU1* and *TtGSTU2* proteins are 95% identical and 96% similar, whereas the *TtGSTU1* and *TtGSTU3* proteins are 68% identical and 80% similar (Fig. 2A). There is a 31-amino acid residue extension at the C terminus of the *TtGSTU3*-encoded protein, compared with both *TtGSTU1* and *TtGSTU2* (Fig. 2A). These three GSTs belong to the tau class of plant GSTs, according to the classification system proposed by Droog (1997) and Edwards et al. (2000). The encoded proteins contain the triplet of amino acid residues His-Lys-Lys, which are conserved only in the tau class GST sequences, and are located at position 53 to 55 (numbering of amino acid residues is according to the *TtGSTU1* sequence throughout the text, unless stated otherwise). The conserved triplet of His-Asn-Gly in the tau class is also conserved in these three GSTs at position 61 to 63 (Fig. 2A). *TtGSTU3* is a unique GST, however, in that it appears to be a tau class GST protein based on its deduced amino acid sequence, but its gene sequence does not contain a single intron, which is characteristic of tau class GST genes.

The hydropathy profiles of the *TtGSTU1* and *TtGSTU2* proteins, as determined by the method of Kyte and Doolittle (1982), are almost identical. Both proteins contain a strong hydrophilic region around amino acids 110 to 130 (Fig. 2B, only *TtGSTU1* is shown). This hydrophilic region is located where exon 2 of each gene starts (at amino acid 109 in *TtGSTU1* and *TtGSTU2*; Fig. 2A). The function of this hydrophilic region is not known, but may play an important role in substrate binding and/or substrate specificity. Alternatively, this region may play a role in determining the intracellular localization of the GST proteins. This region in *TtGSTU1* and *TtGSTU2* is very similar in sequence to the same region of the mouse (*Mus musculus*) mGSTA4-4 protein (amino acid residues 110–120), which also contains several charged residues and was shown to be important for a proposed electrostatic interaction with the plasma membrane of mouse liver hepatocytes (Singh et al., 2002). Hydropathy analysis of the *TtGSTU3* protein showed minor differences relative to *TtGSTU1*, although slightly less hydrophilic character was noted around amino acids 110 to 130 (Fig. 2A).

**Repeat Elements Are Dispersed in the Intergenic Regions of the 14-kb Contiguous Sequence of BAC 1**

Two open reading frames (encoding the proteins *TtGSTU1* and *TtGSTU2*) were found within the completely sequenced 14-kb interval from BAC 1. A BLAST search with this 14-kb interval identified sequences with similarity to several repeat elements from barley, maize, and the diploid wheat *Triticum monococcum*. The 14-kb interval contains about 2.6 kb of sequence upstream of the TATA box of *TtGSTU2* (Fig. 1) until the *Eco*RI site (and start of the 14-kb contiguous sequence) is reached. In this region, about 1 kb of sequence (bp 1–1,039 of accession no. AY013753) was highly homologous to the long terminal repeat (LTR) of several retrotransposons, including the *Angela*-type retrotransposons from *T. monococcum* (Wicker et al., 2001). This 1-kb portion also shares 82% nucleotide identity with a portion of

**A**

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TtGSTU1  MA-GGDDLKLLGAWPSPFVTRVKLALALKGLSYEDVEEDLYKKSELLLKSNPVHK  54
TtGSTU2  MA-GGDDLKLLGAWPSPFVTRVKLALALKGLSYEDVEEDLYKKSELLLKSNPVHK  54
TtGSTU3  MAEGGDELKLLGSWSSPFVTRAKLALAIKGLSYENVDEDVHNKSDLLLGSNPVHK  55

                                                         *
TtGSTU1  KIPVLIHNGAPVCESMIILQYIDEVFASTGPSLLPADPYERAIARFWVAYVDDKL  109
TtGSTU2  KIPVLIHNGAPVCESMIIVQYIDEVFAGTGPSLLPADPYERAVARFWVPYVDDKL  109
TtGSTU3  KIPVLIHNGVPVCESMIIVQYIDEAFAGTGPPILPADPHERAVARFWAAYVDDKL  110

TtGSTU1  VAPWRQWLRGKTEEEKSEGKKQAFAAVGVLEGALRECSKGGGFFGGDGVGLVDVA  164
TtGSTU2  VAPWRQWLRGKTEEEKSEGKKQAFAAVEVLEGALRECSKGGGFFGGDSVGLVDVA  164
TtGSTU3  VSQWTNSSRSKSR-AGAEAMTEMLVAVEALEGALRECSKGKGFFGGDGVGLVDIA  164

TtGSTU1  LGGVLSWMKVTEALSGDKIFDAAKTPLLAAWVERFIELDAAKAALPDVGRLLEFA  219
TtGSTU2  LGGVLSWMKVTEVLSGDKIFDAAKTPLLAAWVERFSGLDAAMAALPDVGRLLEFA  219
TtGSTU3  LGSMHSWLMAIEAMSGAKVFDPARTPLLAAWMERFGSLDAARAVLPDVGRVWELA  219

TtGSTU1  KAREAAAAASK------------------------------  230
TtGSTU2  KAREAAAAASN------------------------------  230
TtGSTU3  KIKHGQACRSFNFKQQVASRHGGMTYVCVCVNCEVEWPVFTF  261
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**Figure 2.** Comparison of tau class GST proteins from *T. tauschii*. A, Alignment of the deduced amino acid sequences of *Tt*GSTU1, *Tt*GSTU2, and *Tt*GSTU3. Identical amino acids and conservatively substituted residues are shaded with light and dark gray, respectively. The amino acid residue where exon 2 of *Tt*GSTU1 and *Tt*GSTU2 starts is indicated by an asterisk above the sequences. GenBank accession numbers are as follows: *Tt*GSTU1 and *Tt*GSTU2 (AY013753), and *Tt*GSTU3 (AY013754). B, Hydropathy profile of the *Tt*GSTU1 protein, as determined by the method of Kyte and Doolittle (1982) using a window of 10 amino acid residues.

**B**



the LTR of the *BARE-1 copia*-like retroelement from barley (Manninen and Schulman, 1993).

Approximately 8 kb of sequence (bp 3,889–11, 859 of accession no. AY013753) is located between the *TtGSTU2* and *TtGSTU1* genes (Fig. 1). Within this 8-kb intergenic sequence, there are three regions that show homology to the LTRs of several retrotransposons from cereal species. Sequences from position 5,261 to 5,890 are similar to the LTRs of *Angela*-type retrotransposons (Wicker et al., 2001), and also shares about 83% identity with the barley *BARE-1 copia*-like retroelement over a 170-bp region (Manninen and Schulman, 1993). Position 8,062 to 8,451 was also similar to the LTRs of *Angela*-type retrotransposons (Wicker et al., 2001) and also shares 86% identity with the *BARE-1 copia*-like retroelement in barley over a 130-bp region (Manninen and Schulman, 1993). Position 8,775 to 9,124 shares about 90% nucleotide identity with LTRs of the *BARE-1*-like retrotransposons *Angela-2*, *Angela-3*, and *Angela-4* (Wicker et al., 2001).

An 868-bp sequence flanks the 3′ end of the *TtGSTU1* mRNA until the *Kpn*I restriction site (and end of the 14-kb contiguous sequence) is reached. A BLAST search with this sequence showed that a region of about 660 bp (toward the 3′ end) shares 95% nucleotide identity with intron 10 of the *T. tauschii* starch synthase I gene (Li et al., 1999), which may indicate the presence of a repeat family that is present throughout different parts of the wheat ge-
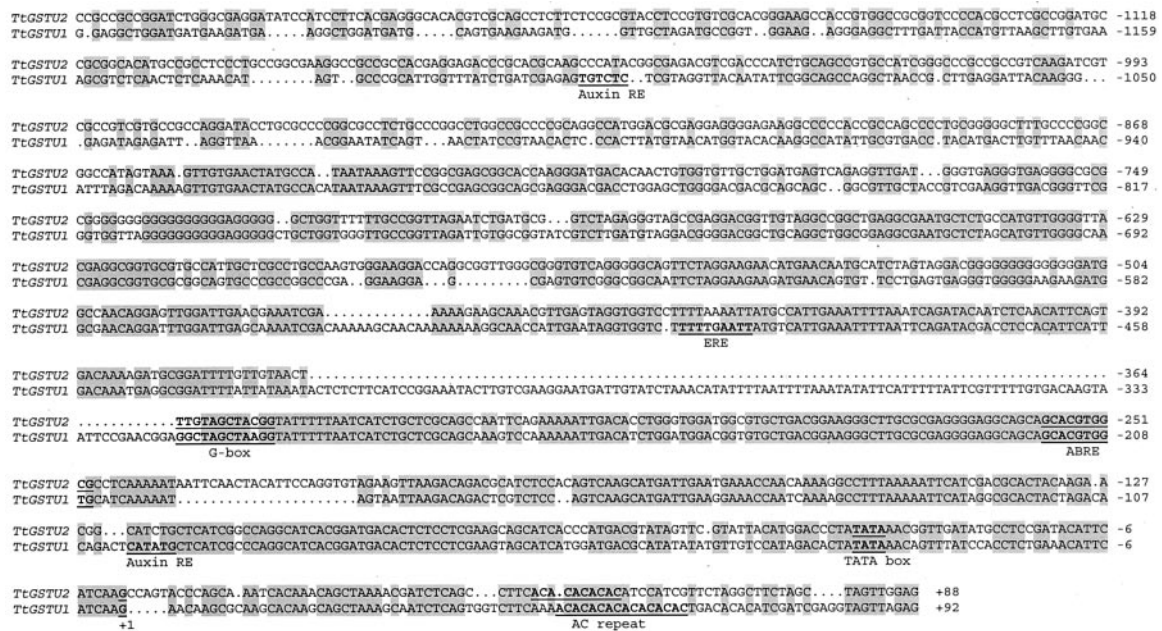
nome. This region also shares homology with the intron of the maize ACCase gene (accession no. U90128), which was noted to contain a number of retroelements, such as *colonist-1* and *colonist-2*.

## 5′-Flanking Regions of *TtGSTU1* and *TtGSTU2*

In comparison with the 5′-UTR present in the *TtGSTU1* cDNA, the start site for transcription initiation was set approximately 90 bp upstream of the MET start codon for both GST genes (Fig. 3). The major distinguishing feature of the 5′-UTRs in the two genes is an "AC" dinucleotide simple sequence repeat, present just upstream of the translational start site. The *TtGSTU1* genomic sequence contains eight copies of the AC repeat, whereas the *TtGSTU2* gene has five AC repeats (Fig. 3). An appropriately placed TATA box can be easily recognized 36 bp 5′ to the transcription start site in both genes. Comparison of alignments of the nucleotide sequences of the promoters of *TtGSTU1* and *TtGSTU2* revealed that the two genes are very similar for about 800 nucleotides upstream of the TATA boxes, except for two large gaps that were noted: a 22-bp gap in the *TtGSTU1* promoter at position −195, and a large gap in the *TtGSTU2* promoter at position −363 (Fig. 3).

Preliminary analysis of the UTRs and promoter regions using a plant transcription factor homology database (Higo et al., 1999) identified several potential cis-acting regulatory elements. Sequences similar

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TtGSTU2 CCGCCGCCGGATCTGGGCGAGGATATCCATCCTTCACGAGGGCACACGTCGCAGCCTCTTCTCCGCGTACCTCCGTGTCGCACGGGAAGCCACCGTGGCCGCGGTCCCCACGCCTCGCCGGATGC -1118
TtGSTU1 G.GAGGCTGGATGATGAAGATGA.....AGGCTGGATGATG.....CAGTGAAGAAGATG......GTTGCTAGATGCCGGT..GGAAG..AGGGAGGCTTTGATTACCATGTTAAGCTTGTGAA -1159

TtGSTU2 CGCGGCACATGCCGCCTCCCTGCCGGCGAAGGCCGCCGCCACGAGGAGACCCGCACGCAAGCCCATACGGCGAGACGTCGACCCATCTGCAGCCGTGCCATCGGGCCCGCCGCCGTCAAGATCGT -993
TtGSTU1 AGCGTCTCAACTCTCAAACAT........AGT..GCCCGCATTGGTTTATCTGATCGGAGTGTCTC..TCGTAGGTTACAATATTCGGCAGCCAGGCTAACCG.CTTGAGGATTACAAGGG... -1050
                                                           Auxin RE

TtGSTU2 CGCCGTCGTGCCGCCAGGATACCTGCGCCCGGCGCCTCTGCCCGGCCTGGCCGCCCGGCAGGCCATGGACGCGAGGAGGGGAGAAGGCCCCCACCGCCAGCCCCTGCGGGGGCTTTGCCCCGGC -868
TtGSTU1 .GAGATAGAGATT..AGGTTAA.......ACGGAAATATCAGT...AACTATCCGTAACACTC.CCACTTATGTAACATGGTACACAAGGCCATATTGCGTGACC.TACATGACTTGTTTAACAAC -940

TtGSTU2 GGCCATAGTAAA.GTTGTGAACTATGCCA..TAATAAAGTTCCGGCGAGCGGCACCAAGGGATGACACAACTGTGGTGTTGCTGGATGATCAGAGGTTGAT...GGGTGAGGGTGAGGGGCGCG -749
TtGSTU1 ATTTAGACAAAAAGTTGTGAACTATGCCACATAATAAAGTTTCGCCGAGCGGCAGCGAGGGACGACCTGGAGCTGGGGACGACGCAGCAGC..GGCGTTGCTACCGTCGAAGGTTGACGGGTTCG -817

TtGSTU2 CGGGGGGGGGGGGGGGGGAGGGGG..GCTGGTTTTTTGCCGGTTAGAATCTGATGCG...GTCTAGAGGGTAGCCGAGGACGGTTGTAGGCCGGCTGAGGCGAATGCTCTGCCATGTTGGGGTTA -629
TtGSTU1 GGTGGTTAGGGGGGGGGGAGGGGGCTGCTGGTGGGTTGCCGGTTAGATTGTGGCGGTATCGTCTTGATGTAGGACGGGGACGGCTGCAGGCTGGCGGAGGCGAATGCTCTAGCATGTTGGGGCAA -692

TtGSTU2 CGAGGCGGTGCGTGCCATTGCTCGCCTGCCAAGTGGGAAGGACCAGGCGTTGGGCGGTGTCAGGGGGCAGTTCTAGGAAGAACATGAACAATGCATCTAGTAGGACGGGGGGGGGGGGGATGA -504
TtGSTU1 CGAGGCGGTGCGCGGCAGTGCCCGCCGGCCCGA..GGAAGGA...G.........CGAGTGTCGGGCGGCAATTCTAGGAAGAAGATGAACAGTGT.TCCTGAGTGAGGGTGGGGGAAGAAGATG -582

TtGSTU2 GCCAACAGGAGTTGGATTGAACGAAATCGA.............AAAAGAAGCAAACGTTGAGTAGGTGGTCCTTTTAAAATTATGCCATTGAAATTTTAAATCAGATACAATCTCAACATTCAGT -392
TtGSTU1 GCGAACAGGATTTGGATTGAGCAAATCGACAAAAAGCAACAAAAAAAAGGCAACCATTGAATAGGTGGTC.TTTTTGAATTATGTCATTGAAATTTTAATTCAGATACGACCTCCACATTCATT -458
                                                                            ERE

TtGSTU2 GACAAAAGATGCGGATTTTGTTGTAACT...................... -364
TtGSTU1 GACAAATGAGGCGGATTTTATTATAAATACTCTCTTCATCCGGAAATACTTGTCGAAGGAATGATTGTATCTAAACATATTTTAATTTTAAATATATTCATTTTTATTCGTTTTTGTGACAAGTA -333

TtGSTU2 ..........TTGTAGCTACGGTATTTTTAATCATCTGCTCGCAGCCAATTCAGAAAAATTGACACCTGGGTGGATGGCGTGCTGACGGAAGGGCTTGCGCGAGGGGGAGGCAGCAGCACGTGG -251
TtGSTU1 ATTCCGAACGGAGGCTAGCTAAGGTATTTTTAATCATCTGCTCGCAGCAAAGTCCAAAAAATTGACATCTGGATGGACGGTGTGCTGACGGAAGGGCTTGCGCGAGGGGGAGGCAGCAGCACGTGG -208
               G-box                                                                                            ABRE

TtGSTU2 CGCCTCAAAAATAATTCAACTACATTCCAGGTGTAGAAGTTAAGACGACGCATCTCCACAGTCAAGCATGATTGAATGAAACCAACAAAAGGCCTTTAAAAATTCATCGACGCACTACAAGA.A -127
TtGSTU1 TGCATCAAAAAT..................AGTAATTAAGACAGACTCGTCTCC.AGTCAAGCATGATTGAAGGAAACCAATCAAAAGGCCTTTAAAAATTCATAGGCGCACTACTAGACA -107

TtGSTU2 CGG...CATCTGCTCATCGGCCAGGCATCACGGATGACACTCTCCTCGAAGCAGCATCACCCATGACGTATAGTTC.GTATTACATGGACCCTATATAAACGGTTGATATGCCTCCGATACATTC -6
TtGSTU1 CAGACTCATATGCTCATCGCCCAGGCATCACGGATGACACTCTCTCGAAGTAGCATCATGGATGACGCATATATATGTTGTCCATAGACACTATAAAACAGTTTATCCACCTCTGAAACATTC -6
             Auxin RE                                                                           TATA box

TtGSTU2 ATCAAGCCAGTACCCAGCA.AATCACAAACAGCTAAAACGATCTCAGC...CTTCACA.CACACACATCCATCGTTCTAGGCTTCTAGC....TAGTTGGAG +88
TtGSTU1 ATCAAG.....AACAAGCGCAAGCACAAGCAGCTAAAGCAATCTCAGTGGTCTTCAAAACACACACACACACACTGACACACATCGATCGAGGTAGTTAGAG +92
             +1                                                   AC repeat
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**Figure 3.** Comparison of the 5'-flanking sequences of the two tandem GST genes, *TtGSTU1* and *TtGSTU2*, isolated from the BAC 1 clone. Numbering is determined from the putative transcription start site by comparison of the 5'-UTR sequence of the *TtGSTU1* gene with the *TtGSTU1* cDNA. Potential transcriptional regulatory elements identified by homology searches are underlined in bold and labeled accordingly.

to the TATA box of many eukaryotic promoters were found at position −36 of both *TtGSTU1* and *TtGSTU2*. The sequence RYACGTGGYR (R = A/G and Y = C/T), which was identified as an ABA-responsive element (ABRE) in Arabidopsis (Iwasaki et al., 1995), was found in both *TtGSTU1* and *TtGSTU2* promoters at −230 and −249, respectively (Fig. 3). An ethylene-responsive enhancer element AWTTCAAA (W = A/T) identified in the carnation *GSTI* gene (Itzhaki et al., 1994) and a fruit-ripening gene (Montgomery et al., 1993) was located in the minus strand of the *TtGSTU1* promoter at −525 (Fig. 3). The sequences of TGTCTC (Ulmasov et al., 1995a) and CATATG (Xu et al., 1997), which were related to auxin-responsive expression, were found in *TtGSTU1* at −1,118 and −119 (Fig. 3). The auxin-responsive element TGTCTC was also found in the minus strand of *TtGSTU2* at −2,078 (not shown). A G box-like sequence (Menkens et al., 1995) containing the palindromic hexamer TAGCTA was found at position −308 in the *TtGSTU1* gene and position −351 in the *TtGSTU2* gene (Fig. 3). This same sequence in the *TtGSTU2* gene is actually a palindromic octamer, GTAGCTAC.

## Expression of GST Genes in *T. tauschii* following Treatment with Safeners and Hormones

RNA gel-blot analysis showed that constitutive GST expression was detected in roots of *T. tauschii*, but expression in shoots was low or not detectable (Fig. 4). GST expression was highly induced by the safeners cloquintocet-mexyl and fluxofenim in *T. tauschii* shoots and roots. Induction of GST expression by these safeners in wheat shoots is consistent with previous results (Riechers et al., 1998). Because potential ABA-, ethylene-, and auxin-responsive regulatory elements were identified (by homology searches) in the promoters of the GST genes (Fig. 3),
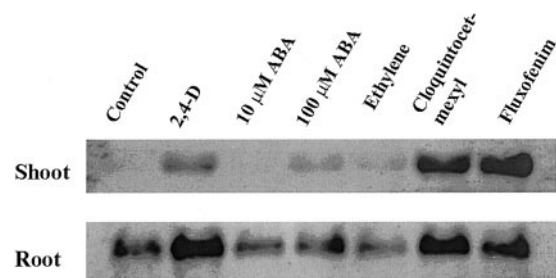


**Figure 4.** RNA gel-blot analysis of GST expression in *T. tauschii* after treatment with herbicide safeners and plant hormones. Total RNA (10 $\mu$g lane$^{-1}$) was analyzed, and the blot was probed with the digoxigenin (DIG)-labeled *TtGSTU1* cDNA coding region from *T. tauschii*. Etiolated seedlings were treated for 48 h with aqueous solutions containing 100 $\mu$M 2,4-dichlorophenoxyacetic acid (2,4-D), 10 $\mu$M or 100 $\mu$M ABA, 20 $\mu$g mL$^{-1}$ 2-chloroethyl-phosphonic acid for ethylene treatment, and 10 $\mu$M of the safeners cloquintocet-mexyl and fluxofenim. All treatment solutions were applied as vermiculite drenches to the seedlings 72 h after transferring pots to room temperature; the seedlings were exposed to the treatments for 48 h, then shoots and roots were harvested (5-d total growth period). Uniform loading was verified by comparing RNA intensities after ethidium bromide staining and by hybridizing the blots with a wheat actin probe (data not shown).

we also examined expression in response to these plant hormones. The synthetic auxin herbicide 2,4-D highly induced GST expression in roots and also induced expression in shoots, although to lower levels than the safeners in shoot tissue. ABA at a relatively high concentration (100 versus 10 $\mu$M) slightly increased GST expression in *T. tauschii* shoots (Fig. 4). Ethylene had no effect on GST expression in *T. tauschii* shoots or roots (Fig. 4). The fact that both safeners caused the greatest increase in GST expression, relative to the plant hormones, suggests that safeners may be tapping into a different regulatory pathway for induction of GST expression, or that the signal for induction may be stronger and/or longer lasting for a safener relative to the plant hormones examined.

## Differential Expression of *TtGSTU1* and *TtGSTU2* in *T. tauschii*

Due to the high degree of similarity between the two GST genes at the nucleic acid level, we used semiquantitative reverse transcriptase (RT)-PCR (Kinoshita et al., 1992) to detect individual expression patterns of the two tightly linked GST genes in roots and shoots of *T. tauschii*. Primers were designed (Table I) that would selectively amplify only one of the two tandemly duplicated GST genes. Constitutive GST expression in both roots and shoots was contributed mainly by *TtGSTU1*, and this gene also seems to be the most highly induced and expressed in safener-treated seedlings (Fig. 5). *TtGSTU1* was also induced by 100 $\mu$M ABA and 2,4-D in shoots (Fig. 5). *TtGSTU2* expression was not detectable in control shoots, but was weakly expressed in control roots. However, *TtGSTU2* expression could be detected in control shoots when amplifying for 35 cycles (data not shown), compared with the 25 cycles shown in Figure 5. *TtGSTU2* expression was induced by safeners, 100 $\mu$M ABA, and 2,4-D in both roots and shoots, although the level of expression was always less than that of *TtGSTU1* (Fig. 5). Ethylene had little effect on *TtGSTU1* and *TtGSTU2* expression in either shoots or roots (Fig. 5).

We could not detect the expression of *TtGSTU3* by RT-PCR in either untreated *T. tauschii* seedlings or treated seedlings (data not shown), which suggests that *TtGSTU3* may be transcriptionally inactive (a pseudogene), or has an expression pattern entirely
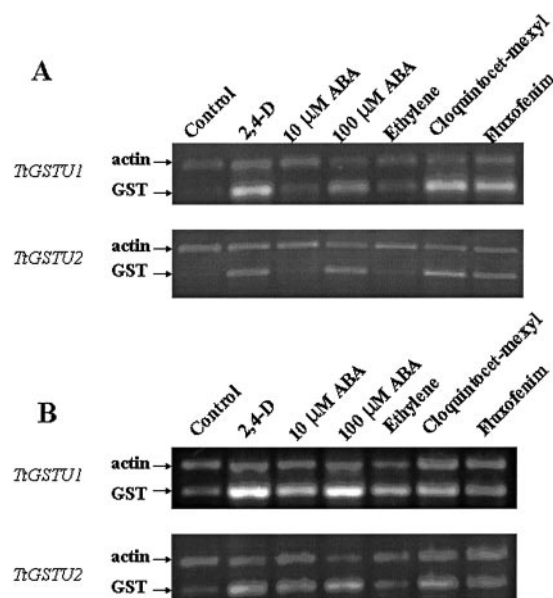


**Figure 5.** Semiquantitative RT-PCR analysis of individual GST gene expression in *T. tauschii*. Expression was analyzed in shoots (A) and roots (B) of *T. tauschii*. Seedling growth conditions and treatments, and total RNA samples were the same as those used for RNA gel-blot analysis shown in Figure 4. Total RNA (5 $\mu$g) was used to synthesize first strand cDNA, and a fraction (1/20) of the first strand cDNA was used as template for PCR amplification of individual gene transcripts. Ethidium bromide-stained RT-PCR products were separated in 1.2% (w/v) agarose gels and analyzed with 1D image analysis software (Eastman-Kodak, Rochester, NY). The wheat actin gene was used as a constitutively expressed control gene and loading control.

different from *TtGSTU1* and *TtGSTU2*. This might include expression in other tissues or organs, or possibly under different stress conditions.
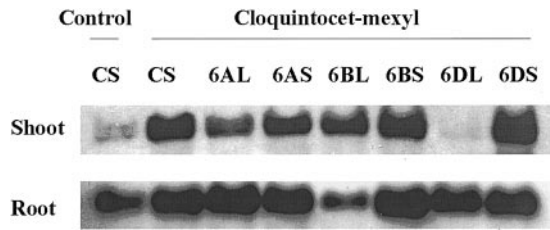
## GST Expression in Cultivated, Hexaploid Bread Wheat and Other *Triticum* Spp.

In addition to examining GST expression in *T. tauschii*, we also investigated GST expression in cultivated, hexaploid bread wheat (ABD genomes). Homoeologous GST genes were previously mapped to the short arms of group 6 chromosomes in cultivated, hexaploid bread wheat via Southern hybridization (Riechers et al., 1998), using the *T. tauschii* TtGSTU1 cDNA to probe ditelosomic, aneuploid wheat lines of group 6 chromosomes that are missing either the

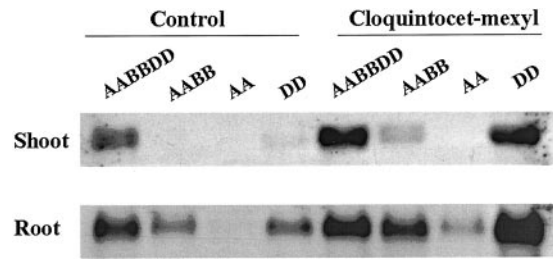| Table I. *Primers for RT-PCR* | | |
|---|---|---|
| Name | Sequence | PCR Product Size |
| | | *bp* |
| 5'Cons | 5'-aagggcctgagctacgag-3' | – |
| 3'*TtGSTU1* | 5'-tgctggcggctcacttg-3' | 622 |
| 3'*TtGSTU2* | 5'-gtgtgctggctcagttag-3' | 622 |
| 3'*TtGSTU3* | 5'-gcatcaagcgagccgaaac-3' | 527 |
| Actin forward | 5'-ctggactcacaccttctacaacgagctccgtgt-3' | – |
| Actin reverse | 5'-atccagacactgtacttcctt-3' | 765 |

**Figure 6.** RNA gel-blot analysis of GST expression in chromosome group 6 ditelosomic, aneuploid wheat lines. GST expression was analyzed in aneuploid lines derived from cultivated, hexaploid bread wheat cv Chinese Spring, in both roots and shoots. Treatments included the control (unsafened) or 10 $\mu$M of the safener cloquintocet-mexyl for 48 h as described in Figure 4. Total RNA (10 $\mu$g lane$^{-1}$) was analyzed, and the blot was probed with the DIG-labeled *TtGSTU1* cDNA coding region from *T. tauschii*. L, Long arm only (missing the short arm); S, short arm only (missing the long arm); CS, cultivated, hexaploid bread wheat cv Chinese Spring base genotype.



**Figure 7.** RNA gel-blot analysis of wheat genome contribution to GST expression. Four wheat species were examined, each differing in genome composition and/or ploidy level. Wheat seedlings were either unsafened (control) or treated with 10 $\mu$M of the safener cloquintocet-mexyl for 48 h as described in Figure 4. Total RNA (10 $\mu$g per lane) was analyzed, and the blot was probed with the DIG-labeled *TtGSTU1* cDNA coding region from *T. tauschii*. AABBDD, Cultivated, hexaploid bread wheat; AABB, *T. turgidum* subsp. *durum*; AA, *T. monococcum*; DD, *T. tauschii*.

short or long arm of chromosome 6A, 6B, or 6D. RNA gel-blot analyses of these same lines were conducted, again using the *TtGSTU1* cDNA as a probe. It is clear that in safener (cloquintocet-mexyl)-treated wheat shoots, removal of the tandem GST genes in the chromosome 6DL line (missing the short arm only) almost completely eliminated GST expression (Fig. 6). However, removal of the homoeologous GST genes in the chromosome 6AL and 6BL lines (missing the short arms of 6A and 6B, respectively) had relatively minor effects on GST expression levels, even though the 6A or 6B GST alleles were no longer present.

GST expression in cultivated, hexaploid bread wheat roots showed a different pattern when individual group 6 chromosome arms were removed (Fig. 6). When the short arms of chromosomes 6A and 6D were removed (in lines 6AL and 6DL), there was no detectable difference in the level of GST expression in safener-treated roots. However, when the short arm of chromosome 6B was removed (in the 6BL line), there was a significant decrease in GST gene expression. This suggests that in safener-treated cultivated, hexaploid bread wheat roots, the GST allele(s) on chromosome 6BS is a major contributor to GST expression; in contrast, the GST alleles on chromosome 6DS are most important in safener-treated cultivated, hexaploid bread wheat shoots and the GST alleles on chromosomes 6AS and 6BS appear to be minor contributors in shoots.

This difference in genome contribution to GST gene expression in roots and shoots is also noted in Figure 7, where several *Triticum* spp. were analyzed for their response to the safener cloquintocet-mexyl in roots and shoots. *Triticum* spp. were chosen that differ in genome constitution (diploids A only or D only, and tetraploid AB) and were compared with cultivated, hexaploid bread wheat (ABD genomes). GSTs were highly induced in the shoots of cultivated, hexaploid bread wheat (AABBDD) and *T. tauschii* (DD), al-

though there was also a relatively high constitutive level of GST mRNA in cultivated, hexaploid bread wheat cv Chinese Spring (AABBDD; Fig. 7). In contrast, there was no induction of GST expression in the shoots of *T. monococcum* (AA) and relatively minor induction in *Triticum turgidum* subsp. *durum* (AABB) shoots (Fig. 7). However, the safener increased GST expression in the roots of all four wheat species, although the lowest induction occurred in *T. monococcum* roots (Fig. 7). These results confirm our previous observation that GST genes in the D genome of wheat are contributing the vast majority of safener-induced expression in shoots; however, in roots, it appears that GST genes in both the B and D genomes are the major contributors to GST expression (Figs. 6 and 7).

Cultivated, hexaploid bread wheat cv Chinese Spring was used in all of our expression studies because it is the base genotype from which the ditelosomic aneuploids were derived (Sears, 1954). There was a relatively high level of constitutive GST expression in shoots of cv Chinese Spring, compared with *T. turgidum* subsp. *durum*, *T. monococcum*, and *T. tauschii*, yet it also showed safener induction (Figs. 6 and 7). To further investigate this finding, three other varieties of cultivated, hexaploid bread wheat (CITR 9038, Ernie, and P25R57) were used to compare and
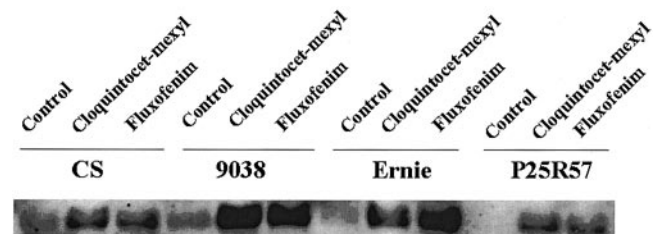


**Figure 8.** Cultivated, hexaploid bread wheat varietal response to safeners and differential GST expression. Wheat seedlings were either unsafened (control) or treated with 10 $\mu$M of the safener cloquintocet-mexyl or fluxofenim for 48 h as described in Figure 4. CS, Chinese Spring.

contrast with cv Chinese Spring. These four wheat varieties differed in both basal level of GST expression and safener induction in shoots in response to the two safeners cloquintocet-mexyl and fluxofenim (Fig. 8). The varieties Chinese Spring and CITR 9038 showed the highest basal levels of GST expression, whereas there was no detectable basal GST expression in P25R57. GST expression was highly induced by both safeners in CITR 9038, which is consistent with the large increases in GST enzyme activity (in response to both safeners) reported previously for this wheat line (Riechers et al., 1996b). The variety Ernie showed a higher level of induction of GST expression after treatment with both safeners relative to the variety P25R57 (Fig. 8). These varying ranges of constitutive and safener-induced GST mRNA levels among wheat varieties and genetic lines are consistent with the broad range of constitutive and safener-increased GST activity levels reported previously (Riechers et al., 1996b), and provide further evidence that there is a great amount of genetic diversity present for GST expression levels in wheat germplasm.

Although it was not determined which genome(s) is contributing the constitutive expression observed in cultivated, hexaploid bread wheat cv Chinese Spring shoots (Figs. 6 and 7), the variable constitutive and safener-induced expression levels observed in cultivated, hexaploid bread wheat varieties (Fig. 8) suggest genome complexities indicative of further evolution and diversification of homoeologous GST gene family members after their duplication by polyploidization of the A, B, and D genomes in cultivated, hexaploid bread wheat. The diversification of *TtGSTU1* and/or *TtGSTU2* expression patterns in both *T. tauschii* (by a duplication event) and cultivated, hexaploid bread wheat (by polyploidization) is consistent with a relaxation of purifying selection in duplicate genes of recent origin (Lynch and Conery, 2000; Hofer and Ellis, 2002).

## DISCUSSION

### Genomic Organization

Screening a *T. tauschii* BAC genomic library (average insert size of 119 kb; Moullet et al., 1999) allowed us to isolate the BAC 1 clone (insert size of 150 kb) containing the two tandemly repeated GST genes *TtGSTU1* and *TtGSTU2*, both contained within a 14-kb restriction fragment. Within this 14-kb fragment, there were only two open reading frames, both coding for the two highly homologous tau class GSTs. These two GSTs contain the entire transcription units in two exons interrupted by one intron, with complete conservation of intron position. However, the length and nucleotide sequences of the two genes' introns are different. Similar GST gene clusters have been identified in the carnation genome (Itzhaki and Woodson, 1993) as well as in the Arabi-

dopsis genome (Arabidopsis Genome Initiative, 2000; Edwards et al., 2000). In contrast to *TtGSTU1* and *TtGSTU2*, the two tightly linked carnation GST genes were obtained from a single lambda phage clone, and contained 10 exons and nine introns (Itzhaki and Woodson, 1993), characteristic of zeta class plant GSTs. These two GST genes showed conservation in their intron positions and also the length and nucleotide sequences of the introns (Itzhaki and Woodson, 1993).

The recent findings from carnation, Arabidopsis, and wheat all indicate that GSTs from the same subclass are grouped on chromosomes as tandem duplications (Edwards et al., 2000). Comparisons of *TtGSTU1* and *TtGSTU2* sequences with rice genome sequences from GenBank and the draft recently completed by the Beijing Genomics Institute (http://btn.genomics.org.cn/rice) identified at least two clusters of tandemly duplicated tau class GST genes in rice. One of these clusters contained 20 closely related tau class GST genes on a single BAC clone from rice chromosome 10 (accession no. AC091680). At least 12 of these 20 genes were expressed in leaves, roots, or callus tissue based on their identity with rice GST cDNAs or ESTs. Another tandem duplication of tau class GST genes was found on a rice BAC clone from chromosome 1 (accession no. AP003450). Among these 22 rice tau class GST genes, both *TtGSTU1* and *TtGSTU2* show the highest similarity to the same *OsGSTU4* gene present within the 20-gene cluster on chromosome 10, suggesting that the duplication event leading to *TtGSTU1* and *TtGSTU2* may have occurred after the divergence of the rice and wheat genomes. The high degree of similarity between *TtGSTU1* and *TtGSTU2* in both the coding and 5'-flanking regions (except for a few insertions or deletions in their promoters; Fig. 3) supports the notion that these genes arose due to a recent duplication event. *TtGSTU1* and *TtGSTU2* map to wheat chromosome 6 (Riechers et al., 1998), which is proposed to be syntenic with rice chromosome 2 (Moore et al., 1995; Moore, 2000). Our finding that the rice genes with the highest similarity to *TtGSTU1* and *TtGSTU2* map to chromosome 10 may suggest a lack of microsynteny for these regions (Bennetzen, 2000; Tarchini et al., 2000), or that the true rice orthologs of the wheat GST genes may be present in the estimated 8% of the rice genome not covered by the draft sequence from the Beijing Genomics Institute.

Among cereal crops, rice appears to have a predominance of tau class GST genes, which was also reported for hexaploid wheat (Cummins et al., 1997; Edwards and Dixon, 2000) but in contrast with maize, which was noted to have more phi class GST genes (Edwards and Dixon, 2000). However, these observations in wheat and maize have been based mainly on sequence analysis and immunological characterization of GST enzymes that possess herbicide detoxification activities. A complete sequence

analysis of the entire rice, wheat, and maize genomes would be necessary to confirm this preliminary speculation on the relative abundance of specific GST subclasses in cereal crops.

BLAST searches showed that the intergenic regions of the 14-kb fragment contained sequences similar to the LTRs of retrotransposons, which is in accord with previous reports that showed a large portion of the wheat genome consists of repetitive DNA elements (Wicker et al., 2001). Certain types of retrotransposons are preferentially located near the centromeres of the chromosomes of grass species, such as wheat, barley, sorghum, and maize (Kumar and Bennetzen, 1999). Mapping the chromosomal location of a homologous GST in barley with the *TtGSTU1* cDNA showed that it is located very close to the centromere of chromosome 6HS (Riechers et al., 1998). Based on these results, we postulate that the precise chromosomal location of *TtGSTU1* and *TtGSTU2* in *T. tauschii* and cultivated, hexaploid bread wheat may be the same as in barley (i.e. near the centromere of chromosome 6DS). This would also be consistent with our finding of the retrotransposon-like sequences in the intergenic regions of the BAC 1 clone from *T. tauschii*.

## Expression Analyses in *T. tauschii*

Although *TtGSTU1* and *TtGSTU2* have similar gene structures and share very high identities at the amino acid and DNA sequence levels, they display different expression patterns in the roots and shoots of *T. tauschii* seedlings, as well as in response to various chemical inducers. Our RT-PCR results show that *TtGSTU1* is constitutively expressed in both roots and shoots of 5-d-old *T. tauschii* seedlings grown under control (untreated) conditions. Using RNA gel-blot and immunoblot analyses, a similar expression pattern was reported in maize for the phi class *GST-29* gene (*ZmGSTF1*), which was found to be constitutively expressed in a number of maize tissues (Jepson et al., 1994; Holt et al., 1995). Unlike maize *GST-29*, which showed a minimal increase in transcript levels upon herbicide safener treatment (Jepson et al., 1994), *TtGSTU1* was strongly induced by herbicide safeners in shoots. Using RT-PCR and gene-specific primers, *TtGSTU2* transcripts were detected in control roots, but not shoots, of 5-d-old etiolated seedlings. The phi class maize *GST-27* gene (*ZmGSTF2*), like *TtGSTU2*, was constitutively expressed in roots, and no expression was detected in other tissues (Jepson et al., 1994; Holt et al., 1995). Furthermore, herbicide safener treatments caused dramatic increases in the expression of both *TtGSTU1* and *TtGSTU2* (Fig. 5) and the maize *GST-27* gene (Jepson et al., 1994; Holt et al., 1995; Irzyk and Fuerst, 1997). These genes also showed induction in response to treatments with high levels of 2,4-D in maize leaves (Jepson et al., 1994) and *T. tauschii*

shoots (Fig. 4), although the level of induction was always lower than with the safeners in these aerial tissues. Induction of expression in response to ethylene was not detected for the GSTs in *T. tauschii* (Fig. 4) or the maize *GST-27* gene, except at very high concentrations that also led to phytotoxicity (Jepson et al., 1994).

These similar expression profiles imply that the promoters of these inducible wheat and maize GST genes may contain similar safener-responsive regulatory elements. An alignment of the promoters of *TtGSTU1* and *TtGSTU2* with the maize *GST-27* promoter (reported in patent no. WO 93/01294, Bridges et al., 1993; GenBank accession no. A32436) did not reveal any conserved regions, and a search with the plant transcription factor homology database (Higo et al., 1999) only identified several CCAAT box sequences within the first 2 kb of the maize *GST-27* promoter (D. Riechers and S. Moose, unpublished data). Because *TtGSTU1* and *TtGSTU2* are tau class GSTs, and the maize *GST-27* is a phi class GST, there may be different safener-responsive regulatory elements in their promoters. Detailed functional analyses of the promoter sequences for these safener-inducible GSTs in maize and wheat may identify important regulatory elements that govern safener-induced expression.

In comparison with two wheat GSTs reported in the literature, *Tt*GSTU1 shares 30% amino acid sequence identity with the phi class GstA1 protein (Mauch and Dudler, 1993) and 25% amino acid sequence identity with the zeta class TA-GSTZ1 protein (Subramaniam et al., 1999). *GstA1* was further characterized for its expression patterns in wheat, detected at both the mRNA and protein levels (Mauch and Dudler, 1993). *GstA1* was induced in response to challenge by pathogens and a cell-free fungal extract, but not by xenobiotics (Mauch and Dudler, 1993), suggesting a role for this gene in plant defense reactions against pathogen attack. In contrast, both *TtGSTU1* and *TtGSTU2* were highly induced by safener treatment in *T. tauschii*, as well as the synthetic auxin 2,4-D (and the phytohormone ABA to a limited extent). These results suggest that the tau class *Tt*GSTU1 and *Tt*GSTU2 proteins have important roles in xenobiotic metabolism in wheat, and may also have significant yet undefined roles in response to plant stresses.

## Expression Analyses in Cultivated, Hexaploid Bread Wheat and Other *Triticum* Spp.

In addition to examining GST expression in the diploid wheat *T. tauschii*, gene expression was investigated in cultivated, hexaploid bread wheat, other diploid and tetraploid wheat species, as well as in ditelosomic aneuploid wheat lines that are missing individual arms of group 6 chromosomes. GST loci were previously mapped to the short arms of chro-

mosomes 6A, 6B, and 6D in cultivated, hexaploid bread wheat, using the *TtGSTU1* cDNA as a probe (Riechers et al., 1998). This allowed us to use the same probe to detect homoeologous GST transcripts in ditelosomic, aneuploid lines of cultivated, hexaploid bread wheat. The results demonstrated that GST expression in safener-treated wheat shoots was mainly contributed by GSTs from the D genome, whereas GSTs from both the B and D genomes contribute to safener-induced GST expression in wheat roots. The GST gene(s) on chromosome 6AS are not expressed to a significant extent in either control or safener-treated roots or shoots of *T. monococcum*, and also do not appear to be significant contributors to expression in safener-treated cultivated, hexaploid bread wheat seedlings. The most straightforward explanation to describe these expression patterns in wheat shoots is that the tandem GST genes on chromosome 6DS contain safener-responsive element(s) in their promoters or UTRs that are lacking in GST genes on chromosomes 6AS and 6BS. However, our data with ditelosomic, aneuploid wheat lines do not rule out the possibility that a regulatory factor could also be located on chromosome 6DS that controls the response to safeners in wheat shoots.

RNA gel-blot analysis of *Triticum* spp. with different genome constitutions showed that safener treatment dramatically increased GST expression in the shoots of cultivated, hexaploid bread wheat (AABBDD) and *T. tauschii* (DD), but not in *T. monococcum* (AA) or *T. turgidum* subsp. *durum* (AABB). These results are consistent with the results of expression analyses from the ditelosomic, aneuploid wheat lines of group 6 chromosomes, clearly indicating that GST genes in the D genome of wheat contribute most toward safener-induced GST expression in shoots. When assaying for GST enzyme activity in different *Triticum* spp. with a herbicide substrate, much higher GST activities were present in safener-treated shoots of both cultivated, hexaploid bread wheat and *T. tauschii*, relative to *T. turgidum* subsp. *durum* or other wheat species that lack the D genome (Edwards and Cole, 1996; Riechers et al., 1996b). The results of these studies confirm that the D genome is an important source of GST isozymes that are involved in the safener response in hexaploid and diploid wheats containing the D genome (Riechers et al., 1996b, 1997b).

Expression analyses in safener-treated roots from various *Triticum* spp. were also consistent with results found with the ditelosomic, aneuploid wheat lines, although the results were different from those observed in shoots. Safener treatment increased GST transcript levels in the roots of species or lines that contain the B or D genomes. Thus, GST genes in the B and D genomes contribute to safener-induced GST expression in wheat roots, whereas GST genes from the D genome contribute most toward expression in safener-treated shoots. This pattern of genome- and

organ-specific expression of GSTs implies that the genes' promoters or untranslated sequences may contain different transcriptional regulatory elements that control gene expression in wheat roots versus shoots. Interestingly, comparisons of diploid wheats (*T. monococcum* and *T. tauschii*) with cultivated, hexaploid bread wheat indicate that genome contributions to safener-induced GST expression appear to be conserved following the polyploidization of cultivated wheat (Figs. 6 and 7). However, differences in GST expression among cultivated, hexaploid bread wheat varieties show that constitutive and safener-induced GST expression is variable within cultivated, hexaploid wheat (Fig. 8; Riechers et al., 1996b). The recent GST gene duplication event that led to *TtGSTU1* and *TtGSTU2* may have permitted the diversification of gene expression patterns in *T. tauschii* (Lynch and Conery, 2000; Hofer and Ellis, 2002). Further duplication of these genes by the polyploidization events that led to cultivated, hexaploid bread wheat may have allowed for their continued evolution within cultivated wheat (as noted by varietal differences in Fig. 8). Future work will be aimed at characterizing the structure and expression patterns of the *TtGSTU1* and *TtGSTU2* gene homoeologs in *T. monococcum* and cultivated, hexaploid bread wheat. Additional functional studies of these homoeologous genes, which differ in their expression response to herbicide safeners, will help identify the regulatory elements and factors that are important for herbicide safener-induced GST gene expression in wheat.

## MATERIALS AND METHODS

### Screening of a *Triticum tauschii* BAC Library

High-density filters of BAC clones (Moullet et al., 1999) were screened with the coding region of the cDNA encoding the safener-induced *TtGSTU1* (Riechers et al., 1997a), previously isolated from *T. tauschii*. DNA hybridization and washing conditions were as reported by Lagudah et al. (1991). Plasmid DNA from individual BAC clones was isolated using the alkaline lysis method and DNA insert sizes were estimated by pulse field gel electrophoresis according to Moullet et al. (1999).

### DNA Sequencing and Analysis

DNA fragments containing sequences of interest were subcloned into pBluescript SK$^+$ (Stratagene, La Jolla, CA). Sequencing was conducted on both strands and reactions were performed at the sequencing center at the University of Illinois (Urbana) using the Big Dye kit (Perkin-Elmer Applied Biosystems, Foster City, CA) and an ABI Prism 377 (ABI, Sunnyvale, CA). DNA sequence analyses and amino acid alignments were performed using the AlignX tool of Vector NTI Suite V.6 software (InforMax, Inc., Bethesda, MD).

### Plant Material

For RNA extraction and analysis, seeds were planted in plastic pots containing vermiculite. Pots were watered to saturation with deionized water, covered with aluminum foil, and subjected to prechilling at 4°C for 5 d to increase and synchronize seed germination. Pots were then removed from the cold and incubated at room temperature without light for a total of 5 d. For safener and plant hormone treatments, pots were transferred to

room temperature, watered with deionized water, and incubated for 3 d. Then, the pots were watered with 10 $\mu$M cloquintocet-mexyl, 10 $\mu$M fluxofenim, 100 $\mu$M 2,4-D, 10 $\mu$M or 100 $\mu$M ABA, or 20 $\mu$g mL$^{-1}$ 2-chloroethylphosphonic acid (Sigma, St. Louis) for ethylene treatments, and incubated for another 48 h at room temperature. Roots and etiolated shoots were harvested separately, frozen in liquid nitrogen, and stored at $-80°$C until RNA extraction.

## RNA Gel-Blot Analysis

Total RNA was isolated from plant tissues using TRIzol total RNA isolation reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The following manufacturer-recommended modification was used: Equal volumes of a high salt solution (1.2 M sodium citrate and 0.8 M NaCl) and isopropanol were added during the RNA precipitation step to decrease polysaccharide contamination and obtain >90% pure RNA ($A_{260}/A_{280} > 1.8$). RNA was denatured at 55°C in the present of formamide and formaldehyde and separated by electrophoresis in 1.2% (w/v) agarose gels (containing 0.4 M formaldehyde). Equal loading among wells was verified by ethidium bromide staining. RNA was transferred to Nytran N membranes (Schleicher & Schuell, Keene, NH) by capillary blotting in 10× SSC overnight. RNA was fixed to the membrane by cross-linking on an UV Stratalinker (Stratagene). Blots were prehybridized in DIG Easy Hyb buffer (Roche Applied Science, Indianapolis) for 4 h and hybridized to a DIG-labeled TtGSTU1 cDNA coding region probe (Riechers et al., 1997a) overnight at 50°C. The blots were washed at a final stringency in 0.1× SSC and 0.1% (w/v) SDS at 65°C. Blots were developed with CDP-Star chemiluminescent substrate, then exposed to Hyperfilm (Amersham Biosciences, Piscataway, NJ).

## Semiquantitative RT-PCR

Total RNA (5 g) was annealed to an oligo(dT)$_{12-18}$ primer (Invitrogen Life Technologies), then first strand cDNAs were synthesized using Superscript II RT (Invitrogen Life Technologies). The genomic sequences for TtGSTU1, TtGSTU2, and TtGSTU3 were used to design primers for subsequent PCR amplification. Gene-specific primers were designed based on the comparisons of DNA and deduced amino acid sequences of the three GST genes. The forward primer was the same for the three GST genes, and was located at the amino acids 28 to 33 of TtGSTU1 and TtGSTU2, or 29 to 34 of TtGSTU3 (Table I). The reverse primers were designed to be gene specific, and were located near the C-terminal sequences of the open reading frames (Table I). The wheat actin transcript served as an internal, constitutively expressed loading control. The two actin primers used for RT-PCR (Table I) were designed from conserved sequences in the rice (Oryza sativa) actin gene (GenBank accession no. X16280). The reaction mixture contained 1 $\mu$L of first strand cDNA, 0.2 mM dNTPs, 1.0 mM MgCl$_2$, 0.4 $\mu$M each primer, and 1.25 units of Taq polymerase (Invitrogen Life Technologies) in a total volume of 25 $\mu$L. PCR cycling conditions were as follows: an initial denaturation step at 95°C for 10 min, 25 amplification cycles (95°C for 50 s, 65°C for 30 s, and 72°C for 2.5 min), and a final elongation step at 72°C for 10 min. For semiquantitative RT-PCR, linearity for each amplification was confirmed (Kinoshita et al., 1992; Riechers and Timko, 1999). Specificity of amplification for each GST gene was verified by using plasmid controls containing each individual gene fragment under the same PCR conditions as described above (data not shown).

## ACKNOWLEDGMENTS

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