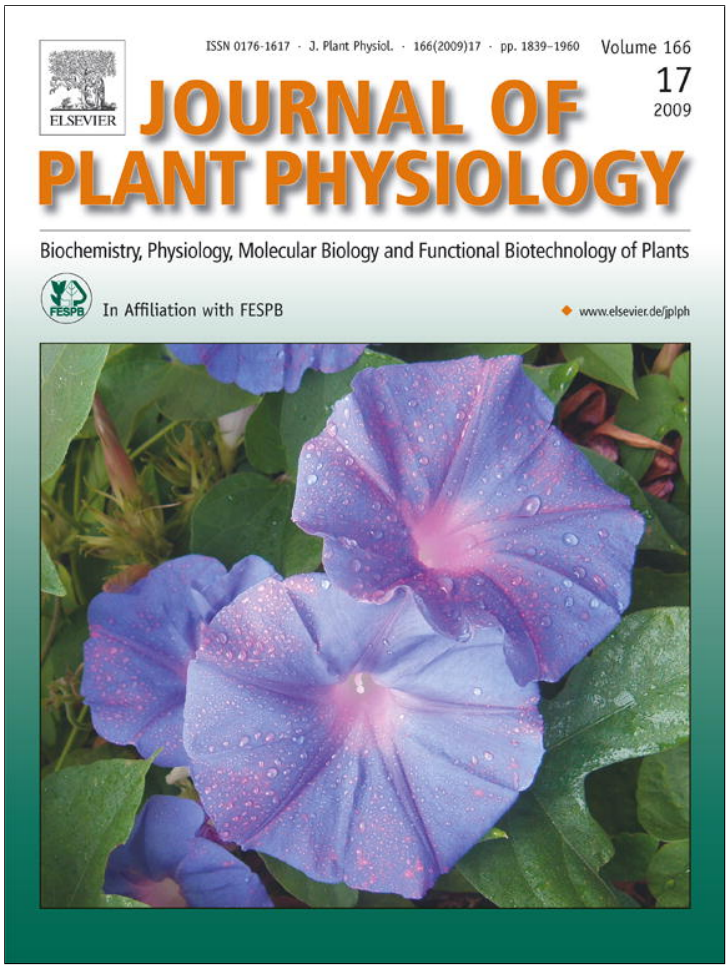

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Glutathione transferase activity and expression patterns during grain filling in flag leaves of wheat genotypes differing in drought tolerance: Response to water deficit

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Summary

Total glutathione S-transferase (GST, EC 2.5.1.18) and glutathione peroxidase (GPOX) activity were measured spectrophotometrically in *Triticum aestivum* cv. MV Emese and cv. Plainsman (drought tolerant) and cv. GK Élet and Cappelle Desprez (drought-sensitive) flag leaves under control and drought stress conditions during the grain-filling period, in order to reveal possible roles of different GST classes in the senescence of flag leaves. Six wheat *GST*s, members of 3 GST classes, were selected and their regulation by drought and senescence was investigated. High GPOX activity (EC 1.11.1.9) was observed in well-watered controls of the drought-tolerant Plainsman cultivar. At the same time, *TaGSTU1B* and *TaGSTF6* sequences, investigated by real-time PCR, showed high-expression levels that increased with time, indicating that the gene products of these genes may play important roles in monocarpic senescence of wheat. Expression of these genes was also induced by drought stress in all of the four investigated cultivars, but extremely high transcript amounts were detected in cv. Plainsman. Our data indicate genotypic variations of wheat GSTs. Expression levels and early induction of two senescence-associated GSTs under drought during grain filling in flag leaves correlated with high yield stability.

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GPOX, glutathione peroxidase; GST, glutathione S-transferase; AOS, activated oxygen species; RWC, relative water content; DHAR, dehydroascorbate reductase; DPA, days post-anthesis.

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Introduction

Higher plants have developed a wide range of defense systems to survive stress caused by pathogens and constantly changing weather and other environmental conditions (Cushman and Bohnert, 2000; Zhu, 2002; Wang et al., 2003). One of the most potentially damaging stress factors is drought, which often leads to an imbalance between antioxidant defense and the amount of activated oxygen species (AOS). AOS are necessary for inter- and intracellular signaling (Breusegem et al., 2001), but can cause damage at various levels of the organization at high concentrations (Asada, 1999). To protect against the toxicity of AOS, aerobic organisms are equipped with an array of defense mechanisms, including one based on the glutathione S-transferases (GSTs).

Plant GST genes encode 25–29 kDa proteins, which form heterodimers, homodimers or monomers. GSTs play important roles in protection against cytotoxic endogenous and xenobiotic compounds (Marrs, 1996; Dixon et al., 1998, 2002a). In addition to detoxification by conjugating a glutathione tripeptide to a wide range of xenobiotics, GST isoenzymes have a function in hormone transport and maintaining homeostasis, including the cellular response to auxins (Bilang et al., 1993), cytokinins (Gonneau et al., 1998) and ethylene (Zhou and Goldsbrough, 1993). Some GST isoforms show glutathione peroxidase (GPOX) activity, suggesting that their main function could be the reduction of toxic lipid peroxidation products and the maintenance of membrane integrity under, for example, osmotic stress (Dixon et al., 2003). The protective role of GSTs against different stressors has been shown in several plant species (Marrs, 1996; Edwards et al., 2000; Basantani and Srivastava, 2007), and transgenic tobacco plants overproducing a GST gene with GPOX activity exhibited significant oxidative stress tolerance (Roxas et al., 2000). GSTs catalyze alternative GSH-dependent biotransformation reactions such as the conversion of maleylacetoacetate to fumarylacetoacetate or reduction of dehydroascorbate (Dixon et al., 2002b), and also have a role in the metabolism of secondary products such as anthocyanins and cinnamic acid (Alfenito et al., 1998). These differing functions coincide with the high diversity of the protein and nucleotide sequences. Plant GSTs fall into eight classes, seven soluble: phi, tau, theta, zeta, dehydroascorbate reductase (DHAR), lambda and tetrachlorohydroquinone dehalogenase, and one membrane bound (microsomal) class of GSTs (GSTF, GSTU, GSTT, GSTZ, GSTDHAR, GSTTCHQD, GSTM, respectively; Edwards and Dixon, 2005).

GSTs comprise approximately 2% of the soluble protein in wheat seedlings (Pascal and Scalla, 1999). High GST activity is a common characteristic of the most widespread *Triticum* cultivars, and several studies have revealed a high correlation between their GST activity and stress tolerance (Bartoli et al., 1999). Recently, the results of several transcriptome analyses revealed that GSTs may play an important role in senescence (Buchanan-Wollaston et al., 2005; Gregersen and Holm, 2007), and Kunieda et al. (2005) identified a special, senescence-induced GST in barley leaves.

Senescence has attracted widespread attention, particularly in monocarpic plants such as cereals, especially during the grain-filling period. The duration and rate of grain filling determine the final grain weight, a key component of the total yield. Water stress during grain filling usually induces early senescence and shortens the grain-filling period, but increases remobilization of assimilates from the leaves to the grains (Plaut et al., 2004).

Using wheat cultivars with different drought susceptibility, pre- and post-anthesis water deficit induced early senescence in both the resistant and the sensitive wheat lines. Based on higher numbers of grains per ear and a better total grain yield, the Plainsman and MV Emese cultivars can be regarded as drought-resistant, Cappelle Desprez and GK Élet as drought-sensitive wheat cultivars (Guóth et al., 2009). In this study, the changes in GST activity and expression patterns in the flag leaves of wheat cultivars with different drought resistance were investigated under drought stress during the grain-filling period. Our aim was to define the roles of different types of GSTs in defense under drought stress conditions during grain filling.

Materials and methods

Plant material

Our experiments were carried out on four wheat genotypes: *Triticum aestivum* L. cv. MV Emese, a drought-resistant Hungarian cultivar, *Triticum aestivum* cv. GK Élet a drought-sensitive Hungarian cultivar, *Triticum aestivum* cv. Plainsman a drought-resistant American cultivar and the drought-sensitive French Cappelle Desprez. The breeding pedigree analysis showed a closer genetic relationship between the two Hungarian cultivars: similar ancestors were found, and the difference in drought susceptibility was clearly established. No data were found regarding the common origin of

Plainsman and Cappelle Desprez. Plants were grown in plastic pots (3 plants per pot) containing a mixture of soil (Terra, Hungary) and sand (1:1, v/v) under 300 μmol m^{-2} s^{-1} light intensity provided by OSRAM HQL 400 W/R lamps, 12 h/12 h day/night illumination, at 25 °C/20 °C day/night temperature, at 55–60% air humidity. Drought stress was induced by reducing the water supply 4 days before the booting stage (7–8 days before anthesis). The plants were irrigated every 2nd day to 60% total soil water capacity for control plants and 25% for stressed plants. The experiments were carried out in two seasons. Samples were taken at anthesis and 4, 9, and 12 days post-anthesis (DPA) from the whole flag leaves of 6–7 plants.

Relative water content (RWC)

To determine the RWC, penultimate leaves were weighed immediately to obtain fresh weight (FW), then floated on distilled water for 24 h and weighed again for turgid weight (TW). Leaves were then dried at 80 °C for 24 h for dry weight (DW) measurements. The RWC was calculated according to the following formula: RWC (%) = 100(FW−DW)/(TW−DW).

Chlorophylls *a*, *b* and carotenoid content

Samples from tissue mixture of total flag leaves were homogenized in ice-cold 100% (v/v %) acetone (1.5 mL for 250 mg sample), and extracted for 24 h. Samples were centrifuged at 5000*g* for 15 min at 4 °C. The pellet was extracted again with 80% (v/v %) acetone (1.5 mL for 250 mg sample) for 24 h. The supernatants were collected after centrifugation (5000*g*, 15 min, 4 °C). The pigment composition was measured by a double-beam spectrophotometer according to Lichtenthaler and Wellburn (1983). This method implies measurement of absorbed light in plant extract at 470, 646.8 and 663.2 nm.

Malondialdehyde (MDA) content determination

MDA formation was assayed by using a thiobarbituric acid method (Ederli et al., 1997). 100 mg leaf tissue was homogenized with 1 mL 0.1% trichloroacetic acid (TCA); to avoid further lipid peroxidation 100 μL 4% butylhydroxytoluene (BHT) was added to the extract. After centrifugation at 12,000*g* for 20 min, 250 μL of supernatant was mixed with 1 mL 0.5% thiobarbituric acid in 20% TCA and the mixture was incubated in boiling water for 30 min. The absorbance was measured at 532 nm and adjusted for non-specific absorbance at 600 nm. MDA concentration was estimated by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Enzyme assays

Tissue homogenization and extraction steps were carried out at 4 °C. Crude protein extracts were prepared by homogenizing 0.2–0.5 g of flag leaf tissues in 2 mL of extraction buffer (0.1 M phosphate buffer pH 7.0, containing 1 mmol L^{-1} phenylmethylsulfonyl fluoride and 1% polyvinyl-polypirrolidone). The homogenate was then centrifuged at 10,000*g* for 15 min, and the supernatant was decanted.

Glutathione transferase (EC 2.5.1.18) activity was determined spectrophotometrically by using an artificial substrate, 1-chloro-2,4-dinitrobenzene (CDNB) according to Habig et al. (1974). Reactions were initiated by the addition of CDNB, and the increase in A_{340} was determined. One unit (U) is the amount of enzyme producing 1 μmol conjugated product in 1 min, $\varepsilon_{340} = 9.6$ mmol L^{-1} cm^{-1}. The enzyme activity was expressed in terms of specific activity (U g^{-1} FW). FW was used for calculation because in this developmental stage the protein content is greatly affected by the amount of RUBISCO, which is remobilized more rapidly from leaves than the other proteins during senescence (Pécsváradi et al., personal communication). GPOX (EC 1.11.1.9) activity was measured using the method of Awasthi et al. (1975) with cumene hydroperoxide as a substrate, as described in Csiszár et al. (2004). The reaction mixture contained 4 mmol L^{-1} GSH, 0.2 mmol L^{-1} NADPH, 0.05 U glutathione reductase (GR, Type II from wheat, Sigma), 100 μL enzyme extract, and 0.5 mmol L^{-1} substrate in phosphate buffer (0.1 mol L^{-1}, pH 7.0) in a total volume of 1 mL. The decrease of NADPH was followed by measuring the absorbance at 340 nm; 1 U was equaled to converted NADPH μmol min^{-1}. The non-specific absorbance decrease was corrected for by using additional measurements without substrate, $\varepsilon_{340} = 6.22$ mmol L^{-1} cm^{-1}.

Screening of databases, phylogenetic analyses

Wheat GST sequences were identified using an *in silico* approach. Screening for wheat GSTs was initially performed on the TIGR (The Institute for Genomic Research) wheat database using published plant GST sequences from DDBJ/EMBL/GenBank sequence database (http://compbio.dfci.harvard.edu/tgi). A minimum cut-off E value ($\leq$e−20)

was applied to select significant matches. To discriminate between duplicated genes, a threshold of at least 95% nucleotide sequence identity was used. Nucleotide sequences of known wheat GST genes and tentative consensus sequences (TCs) were aligned using the CLUSTALW program (Thompson et al., 1994). According to the conserved sequences used for classification of GST proteins (Dixon et al., 2002a), and using genes that were already assigned to GST classes, we could identify six classes of wheat GSTs (Dixon et al., 2002b). A family tree was constructed from approximately 300 amino acid long sequences and drawn with Phylodendron D.G. Gilbert version 0.8d.

RNA purification, expression analyses with real-time RT-PCR

RNA was extracted from flag leaf samples harvested at different developmental stages (anthesis, 4, 9, 12 DPA) according to Chomczynski and Sacchi (1987). DNase digestions were applied (Fermentas). First strand cDNA was synthesized using MMLV reverse transcriptase (Fermentas). Primers were designed using Primer express and Primer 3 software. Primers were synthesized in the Nucleic Acid Synthesis Laboratory, Biological Research Center (Szeged, Hungary). Primer pairs are shown in Table 1. The expression rate of GST genes was monitored by quantitative real-time PCR (BioRad, MJ Research) using SYBR green probes (Applied Biosystems; Karsai et al., 2002). Each reaction was repeated at least three times. QRT-PCR was initiated with denaturation at 95 °C for 10 min followed by 41 cycles of denaturation at 95 °C for 15 s and annealing extension at 60 °C for 1 min. Data analysis was performed using Opticon monitor software. To determine the specificity of the reaction, a melting curve analysis of the product was performed immediately after the final PCR cycle by increasing the temperature from 55 to 90 °C (0.2 °C s^{-1}). Data were normalized using the wheat elongation factor α subunit (EF-1) and a gene with unknown function (NP-1) as high and low controls, respectively (Jukanti et al., 2006). These two internal standards showed constant expression levels in senescing leaf tissues during the grain-filling period (Jukanti et al., 2006).

Analysis and statistics

RWC, pigments MDA and enzyme assays

For all measurements, the means $\pm$ SD were calculated from the data of at least three separate samples. Differences between treatment means were determined by Duncan's multiple range test. Columns denoted by the same letters did not differ significantly at a probability level of $P<0.05$. In some cases, differences between treatment means were determined using the Student's *t*-test.

Real-time RT-PCR

Data were calculated using $2^{(-\Delta\Delta Ct)}$ formula (Livak and Schmittgen, 2001): the additive effect of concentration, gene and replicate was minimized by subtracting the Ct number of the target gene from that of the average of the two reference genes, which yielded ΔCt. This value was subtracted from all other

Table 1. Gene-specific primers used for QRT-PCR.

Primer	Description	Sequence (5′–3′)
NP_1 F	Gene with unknown function	ccaagacgaagcagaacaga
NP_1 R		acacatccaacgcaagagaa
EF_1 F	Elongation factor-1 subunit	aacttcacctcccaggtcat
EF_1 R		gtcaccagctcagcaaactt
AJ441055 F	GSTF6	caagaagccgtgatttgcta
AJ441055 R		gcgacaccaacaagaaaaga
AY064481 F	GST19E50	agcagcaaccaagggaaaaat
AY064481 R		cgccacgttcgtcgacatg
X56004 F	GSTA2	ttcgagtgcatcatcattcc
X56004 R		ccttcaccttggggtactca
AJ414698 F	GSTU1B	cggagggaaggaacaaataa
AJ414698 R		cactgactgacccaaccaac
AJ414699 F	GSTU1C	ggtagttgtttggttttgttagtgtga
AJ414699 R		gcaggtggcaacacttgaca
AF002211 F	GSTZ	atgagagccttgaggtggtt
AF002211 R		cacacatctcccaaatggac

The first two primer pairs were utilised to amplify low- and high-expression standards, while primer pairs 3–8 amplify wheat GST genes (Table 3).F: forward primer; R: reverse primer.

ΔCt values, which yield the ΔΔCt (Yuan and Stewart 2005). There were differences between expression levels of *GST*s of the control samples on 0 DPA (initial control) of different cultivars. To demonstrate these differences, the lowest initial control sample's transcript amounts of all cultivars were taken as an arbitrary unit.

Results

Changes in RWC, chlorophyll and carotenoid contents, and GST and GPOX activity

One thousand grain mass of the investigated genotypes under drought stress is a good parameter of the drought stress tolerance of wheat cultivars. Data presented in Figure 1 show that yield of cv. Cappelle Desprez decreased to a greater extent under drought stress, while Plainsman had very high yield stability. Drought stress, to which the plants were exposed from the booting stage till the 12th day post-anthesis, had a different impact on the water content of the leaves of different wheat cultivars. There were no significant differences in the leaves of tolerant Plainsman either in the control or in the water-stressed plants during this period, while the leaves of drought-sensitive GK Élet showed some decrease in RWC even in controls, and the drought stress significantly decreased RWC values even at anthesis in the two sensitive cultivars (Table 2).

The changes in pigment content show that there was no serious senescence until the 12 DPA.

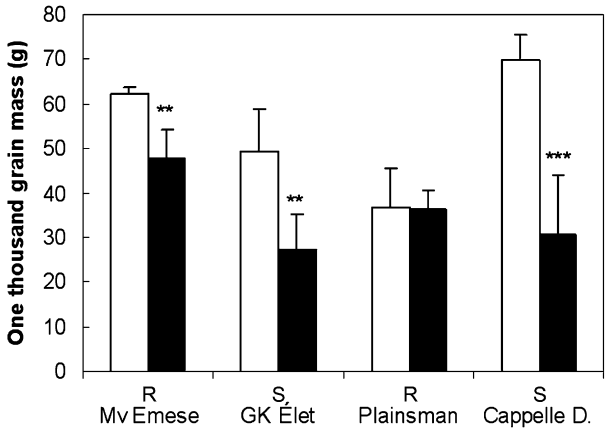


Figure 1. One thousand grain mass of MV Emese, GK Élet, Plainsman, Cappelle Desprez cultivars during the early grain-filling period (□ well-watered conditions, ■ drought stress). R and S for resistant and sensitive genotypes, respectively. Statistical differences compared with controls are indicated by $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, Student *t*-test.

However, chlorophyll *a*+*b* content decreased in MV Emese, both in control and stressed plants on the last sampling days. There was also a slight decrease in the Cappelle Desprez cultivar in control conditions, and it decreased due to stress even from 0 DPA. The carotenoid content changed similarly in Cappelle Desprez on these sampling days, but no significant differences were detectable in the other cases. In the drought-tolerant cultivars, MDA contents of flag leaf tissues increased stepwise until the end of the experiment, both in the control and the drought-stressed plants. The drought-sensitive cultivars displayed less marked changes. The MDA level was enhanced significantly only at 12 DPA due to drought stress in GK Élet, and moderately elevated MDA contents were generally detected in drought-stressed Cappelle Desprez samples compared with the control samples.

The highest levels of extractable GST (Figure 2) and GPOX (Figure 3) activity were found in the Plainsman cultivar. In control plants, the activity of both transferase and peroxidase was enhanced after anthesis; transferase exhibited a slower change in cv. MV Emese and GK Élet. The induction of GST activity following drought stress was detected earlier in resistant cultivars than in sensitive ones. In Cappelle Desprez, no significant changes were detectable, and in GK Élet, GST activity increased only on 12 DPA in the drought-stressed plants (Figure 2).

In control conditions, the extractable GPOX activity increased significantly in every investigated cultivar. In MV Emese, Plainsman and Cappelle Desprez, drought stress further increased GPOX activity at the first sampling day at anthesis. The GPOX activity of the sensitive GK Élet cultivar showed significant decline due to stress (Figure 3).

These results indicate the possible involvement of increased GST and GPOX activity in maintaining normal metabolism during the initiation of natural senescence of flag leaves and successful acclimatization under stress in some wheat genotypes.

Clustering of GST coding sequences

24 members of the GST gene family were identified from GenBank/DDBJ/EMBL databases (Table 3). 18 wheat *GST* genes (700–1500 bp cDNAs) were selected for homology searching of tentative consensus sequences (TC) in the TIGR database with high homology to known *GST*s. This screening led to the identification of 98 putative GST sequences. A homology-based tree was created

Table 2. The effects of soil drought on the relative water content (RWC %); chlorophylls *a*, *b* and carotenoid content of wheat plants during grain-filling period.

	DPA	RWC (%)		Chlorophyll *a*+*b* ($\mu g\,g^{-1}$ DW)		Carotenoids ($\mu g\,g^{-1}$ DW)		MDA ($nmol\,g^{-1}$ FW)	
		Well-watered	Drought-stressed	Well-watered	Drought-stressed	Well-watered	Drought-stressed	Well-watered	Drought-stressed
MV Emese, R	0	88.78±9.6 ab	80.67±1.4 b	21.07±0.5 a	22.39±1.8 a	1201.02±124.2 a	1290.12±129.5 a	42.23±3.4 c	40.89±5.2 c
	4	95.99±0.7 a	89.50±1.4 ab	22.15±1.7 a	21.96±4.7 a	1260.08±59.4 a	1242.77 ±33.8 a	50.45±2.1 ab	52.88±0.3 a
	9	94.40±1.2 a	86.49±1.5 ab	21.14±1.7 a	17.55±1.3 b	1185.67±264.7 a	1010.25±219.4 a	44.53±1.1 bc	56.89±7.0 a
	12	87.49±7.9 ab	79.66±3.1 b	17.37±1.4 b	17.03±1.8 b	1077.14±116.1 a	1008.08±102.5 a	53.27±0.8 a	56.16±4.4 a
GK Élet, S	0	92.74±1.2 ab	82.86±1.2 cd	19.09±0.8 a	17.96±0.4 ab	1064.79±58.1 a	1027.45±22.0 a	44.24±3.2 bc	50.60±6.7 b
	4	92.52±2.2 ab	75.35±7.6 de	16.02±1.7 bc	16.30±3.7 bc	907.01±84.5 a	962.14±220.5 a	46.20±1.9 bc	47.72±4.0 bc
	9	93.94±0.6 a	70.69±2.7 e	17.79±1.6 ab	14.38±1.5 c	998.92±74.2 a	899.77±141.2 a	43.22±1.1 bc	49.43±1.5 b
	12	84.90±3.8 bc	75.66±5.2 de	18.90±2.0 a	16.96±0.6 ab	1092.30±44.0 a	989.27±48.0 a	41.39±3.9 c	65.67±5.6 a
Plainsman, R	0	92.68±2.4 a	94.05±1.0 a	21.25±0.2 a	19.71±2.9 ab	1178.58±9.3 a	1060.19±167.2 a	39.47±3.6 de	41.80±0.9 cde
	4	94.91±0.7 a	93.89±0.2 a	22.32±2.1 a	21.26±0.9 a	1224.93±124.25 a	1170.76±35.2 a	38.29±2.3 e	44.38±3.1 bcde
	9	95.35±0.9 a	95.19±1.0 a	19.67±0.8 ab	17.41±3.2 b	1071.94±32.5 a	976.22±163.8 a	49.97±4.6 b	47.04±0.7 bcd
	12	94.25±0.3 a	94.16±0.5 a	22.83±0.9 a	19.48±2.9 ab	1260.89±34.5 a	1088.60±52.2 a	48.49±7.5 bc	59.55±5.9 a
Cappelle, Desprez, S	0	92.50±0.9 a	77.46±3.0 d	20.31±1.3 a	16.44±1.1 bc	1077.76±75.7 a	945.88±70.8 bc	47.01±2.9 bc	55.88±5.5 a
	4	89.23±1.7 ab	80.87±1.1 cd	15.81±0.4 c	14.94±1.4 cd	849.22±17.2 cd	865.06±78.8 cd	44.12±4.1 c	53.03±2.9 ab
	9	92.88±1.2 a	86.34±4.0 abc	16.04±1.0 bc	15.65±0.9 c	928.40±58.8 bc	921.87±52.8 bc	46.56±1.1 bc	41.35±3.1 c
	12	91.70±2.4 a	82.36±7.5 bcd	18.35±0.4 ab	13.67±1.1 d	1012.91±26.4 ab	791.75±54.8 d	42.32±2.2 c	48.94±7.3 abc

DPA: days post-anthesis. R and S for resistant and sensitive genotypes, respectively. Means denoted by the same letters were not significantly different ($p<0.05$, Duncan test).

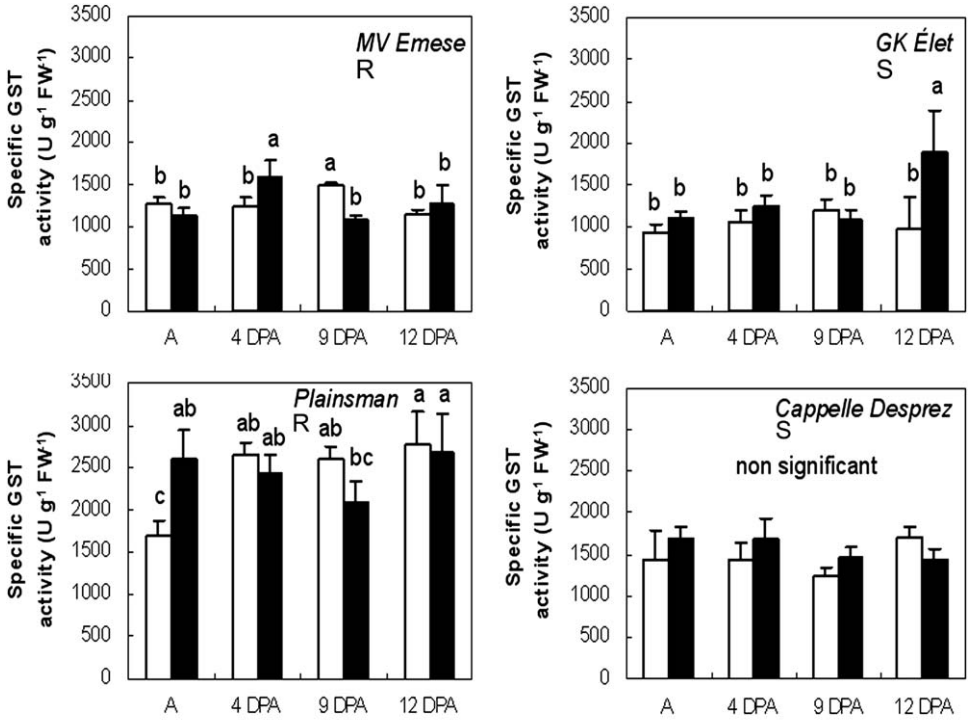


Figure 2. Specific GST activity (U g^{-1} FW) in MV Emese, GK Élet, Plainsman, Cappelle Desprez cultivars during the early grain-filling period (□ well-watered conditions, ■ drought stress). R and S for resistant and sensitive genotypes, respectively. Means denoted by the same letters were not significantly different ($P<0.05$, Duncan test).

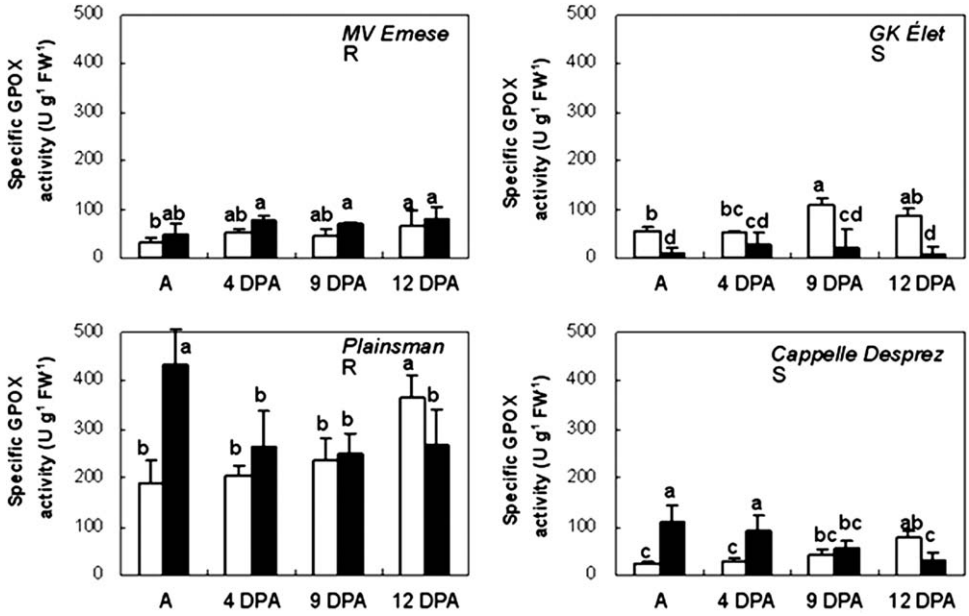


Figure 3. Specific GPOX activity (U g^{-1} FW) in MV Emese, GK Élet cultivar, Plainsman, Cappelle Desprez during the early grain-filling period (□ well-watered conditions, ■ drought stress). R and S for resistant and sensitive genotypes, respectively. Means denoted by different letters indicate a significant difference ($P<0.05$, Duncan test).

after selecting approximately 900 nucleic acid long sequence regions with the highest similarity (Figure 4). Based on conserved sequences used for classification of GST proteins and the genes already assigned to GST classes, we could identify six classes of wheat *GST*s (Dixon et al., 2002a,b).

The phi (GSTF) and tau (GSTU) class *GST*s are the most heterologous classes, containing 38 and 26

Table 3. Wheat GST genes with their GenBank accession numbers.

Accession number	Length (bp)	Class	Name	Molecule type	Submitted by
X56012	2178	phi	*TaGSTA1*	Genomic DNA	Dudler et al. (1991)
X56004	3196	phi	*TaGSTA2*	Genomic DNA	Mauch et al. (1991)
AF387085	911	phi	–	mRNA	Zhu and Ma (unpublished)
AF184059	914	phi	–	mRNA	Goetzberger et al. (2000)
AY064481	965	phi	*19e50*	mRNA	Theodoulou et al. (2003)
AJ441055	904	phi	*TaGSTF6b*	mRNA	Cummins et al. (2003)
AJ440796	927	phi	*TaGSTF1*	mRNA	Cummins et al. (2003)
AJ440795	897	phi	*TaGSTF6*	mRNA	Cummins et al. (2003)
AJ440794	866	phi	*TaGSTF5*	mRNA	Cummins et al. (2003)
AJ440793	721	phi	*TaGSTF4*	mRNA	Cummins et al. (2003)
AJ440792	930	phi	*TaGSTF3*	mRNA	Cummins et al. (2003)
AJ440791	865	phi	*TaGSTF2*	mRNA	Cummins et al. (2003)
AJ414701	1043	tau	*TaGSTU3*	mRNA	Thom et al. (2002)
AJ414700	926	tau	*TaGSTU2*	mRNA	Thom et al. (2002)
AJ414699	1008	tau	*TaGSTU1C*	mRNA	Thom et al. (2002)
AJ414698	1051	tau	*TaGSTU1B*	mRNA	Thom et al. (2002)
AJ414697	1085	tau	*TaGSTU1A*	mRNA	Thom et al. (2002)
AF479764	1018	tau	*TaGSTU4 (28e45)*	mRNA	Theodoulou et al. (2003)
AF002211	945	zeta	*TaGSTZ1*	mRNA	Subramaniam et al. (1999)
AF109714	2947	zeta	*TaGSTZ1*	Genomic DNA	Subramaniam et al. (1999)
AY377972	384	zeta	–	Genomic DNA	Ghaffari unpublished
AY064480	901	theta	*Cla47*	mRNA	Theodoulou et al. (2003)
Y17386	1031	lambda	*TaGSTl1*	mRNA	Theodoulou et al. (2003)
AY074784	956	dhar	*TaGSTDHAR*	mRNA	Chen et al. (2003)

sequences. The zeta (GSTZ), theta (GSTT), lambda (GSTL) and DHAR (GSTDHAR) *GST*s are represented by 8, 11, 7 and 8 TCs, respectively.

For analysis of *GST* expression during the early grain-filling period in the four wheat cultivars, 6 GST coding sequences likely associated with senescence and stress tolerance were selected. Kunieda et al. (2005) previously identified and characterized a *S*enescence-*I*nduced GST (SIGST) in the flag leaves of barley. They suggested that the sequence of this type of GST contains highly conservative sequences in higher plants, and they found that this sequence was similar to the three alleles of *TaGSTU1* gene. Thom et al. (2002) reported that, in the investigated wheat tau group GSTs, TaGSTU1 showed the highest activity during treatment with a model stress metabolite, crotonaldehyde. In an earlier independent experiment, *TaGSTU1B*, *TaGSTU1C*, *TaGST19E50* and *TaGSTZ* showed up-regulation, among several GST coding sequences, due to osmotic and/or drought stress in two drought stress-tolerant wheat cultivars (Secenji et al., unpublished). These sequences are grouped into three classes: *GSTU1B* and *GSTU1C* belong to the tau, *GSTZ* to the zeta and *GST19E50* belongs to the phi class. The phi class GSTs have broad substrate specificity; they are also involved in the response to phytohormones, to oxidative stress caused by salt or temperature stress, phytopathogens or herbicides (Cummins et al., 1997; Roxas et al., 2000). We chose two other phi class sequences: *TaGSTF6*, whose encoded protein was characterized by Cummins et al. (2003), which possessed high conjugating activity against stress metabolite analogues, and *TaGSTA2*, which showed highly significant similarity to a pathogen-induced GST (Dudler et al., 1991). *TaGSTA2*, a genomic DNA sequence shows complete homology to TC248571.

Expression patterns of selected wheat GST sequences

Among the control samples, the highest transcript amounts were detected in Plainsman (Figure 5). *TaGSTU1B* and *TaGSTF6* showed time-dependent induction in the controls of GK Élet and Plainsman cultivars. Among tau class GSTs, *TaGSTU1B* showed greater induction under stress than *TaGSTU1C* in all cultivars except Cappelle Desprez. Among phi class sequences, the transcript amounts of *TaGSTF6* and *TaGST19E50* were increased by stress in Plainsman, MV Emese and Cappelle Desprez, and, to a lesser extent, in GK Élet. Transcript levels of *TaGST19E50* were increased by stress in all four cultivars, but the elevation in Cappelle Desprez was highest. The supposed pathogen-inducible *TaGSTA2* was down-regulated due to aging on 4, 9 and 12 DPA compared

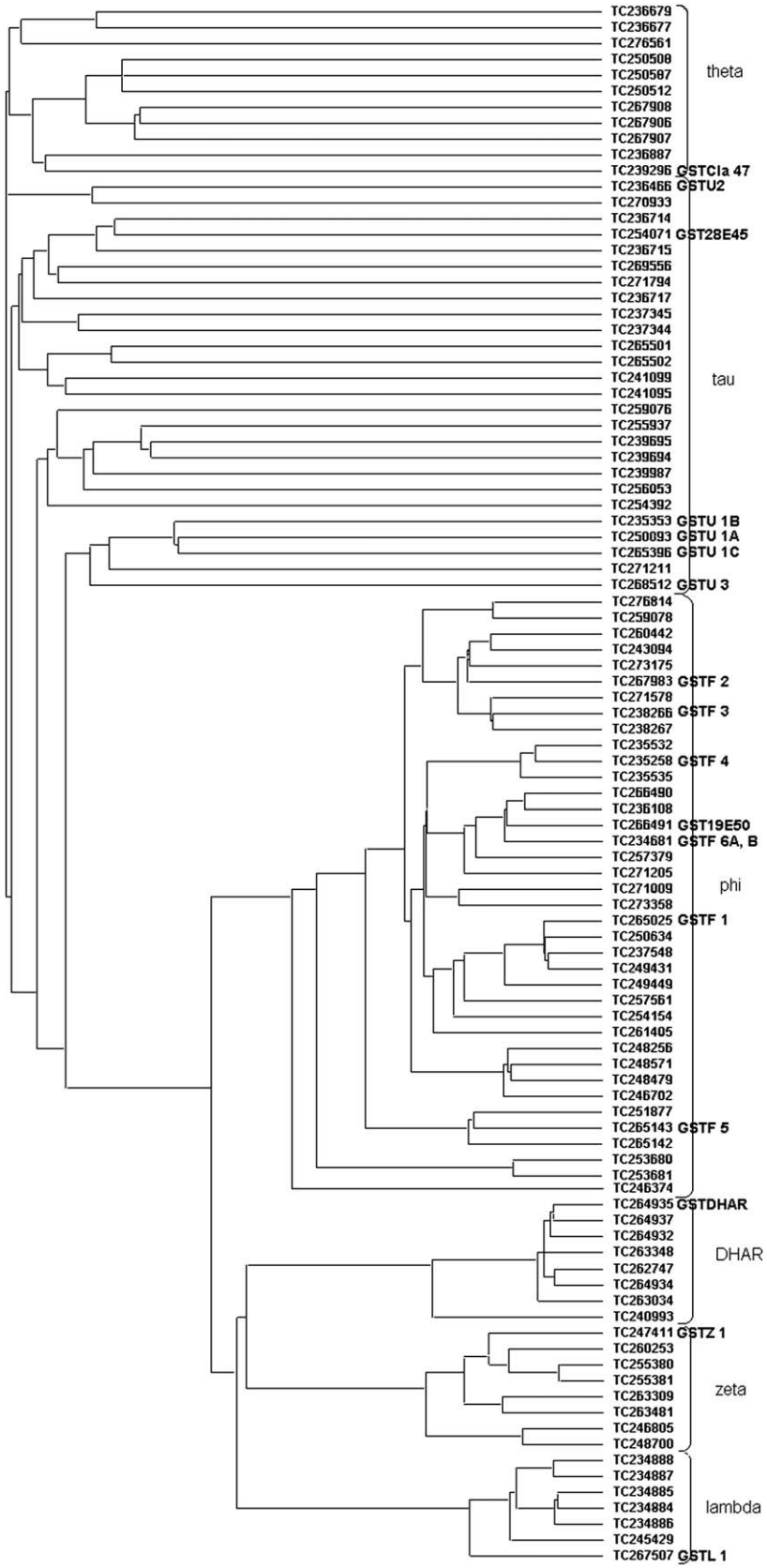


Figure 4. Phylogenetic tree and classes of GSTs, based on the coding sequences of wheat GSTs. Tentative consensus sequences and the corresponding GST genes with highly significant similarity are shown.

with the day of anthesis in three cultivars (GK Emese, Plainsman and Cappelle Desprez). *TaGSTZ* was the least affected by stress and was mostly down-regulated on the last 2 sampling days (Figure 5).

Discussion

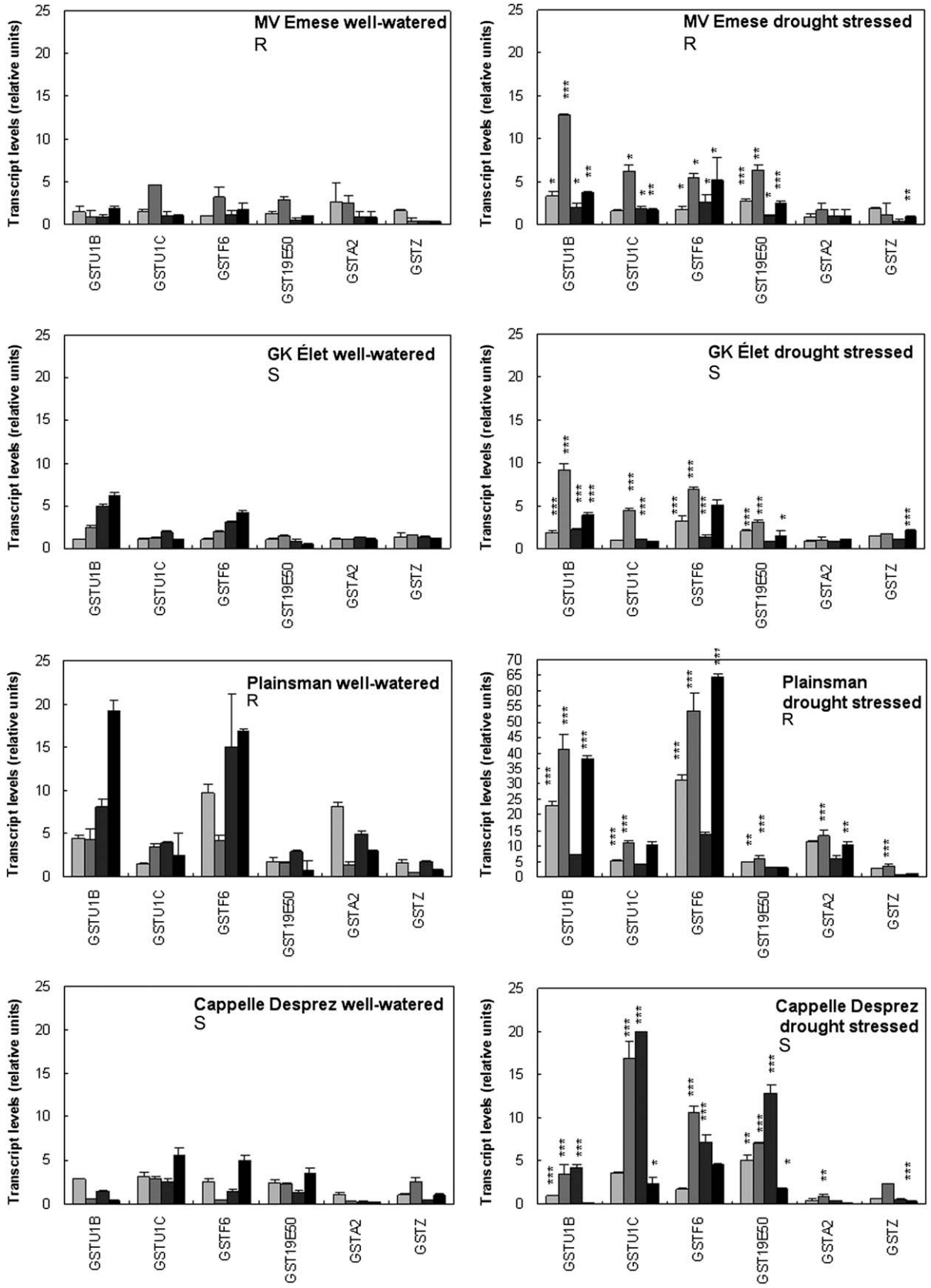
The timing of flag leaf senescence is an important factor in grain filling and yield, both under stress and optimal conditions. In our experiments, wheat flag leaves were sampled at four points, beginning at anthesis and continuing until the 12th day post-anthesis (12 DPA), covering the premilk stage and the beginning of the medium milk stage. Senescence involves dynamic intracellular changes and a great number of gene products playing important roles in the regulation of this process. During senescence, maintaining protection against oxidative stress is important to protecting the cells from premature death, and delayed senescence mutants have been found to have increased tolerance to oxidative stress (Woo et al., 2004).

Several parameters are able to predict leaf senescence, including an increase of MDA and the decrease of chlorophyll content (Yang et al., 2001). According to our results, the MDA content showed a small increase in the drought-resistant MV Emese and Plainsman, both in control and drought-stressed plants from 4 DPA, before the chlorophyll degradation occurred. More significant changes in some symptoms of senescence (chlorophyll degradation, changes in P_N, Φ_{PSII}, qP, and NPQ) appeared only after 12 DPA (Guóth et al 2009).

The zeta class GST has been shown to accumulate in carnation petals during senescence (Itzhaki et al., 1994). The protein of *Arabidopsis* GSTZ1 plays a significant role in phenylalanine and tyrosine degradation (Dixon et al., 2000). The maleylacetoacetate isomerase (MAAI) function suggests that GSTZ proteins are involved in the re-utilization and translocation of nitrogen metabolites. In our experiments, expression of the zeta class GSTZ1 was slightly higher in the Plainsman, GK Élet and MV Emese cultivars. Although there were some time-dependent differences in GSTZ1 expression levels in flag leaves in different cultivars, the level of this transcript was relatively constant until the medium milk stage both in control and stress conditions. These results suggest that zeta class GSTs are less involved either in flag leaf senescence during the early grain-filling period or in its drought stress response mechanisms.

Transcriptome analysis of flag leaves of wheat during senescence revealed 5 up-regulated genes encoding GSTs and a cytosolic GPOX enzyme (Gregersen and Holm 2007). One of these GST genes belongs to the zeta class (TC248700), two of them (TC248404 and TC234681 also known as *TaGST19E50*) to the phi class, and TC265396 showed highly significant similarity to *TaGSTU1 A/B/C*. Kunieda et al. (2005) found that GST enzyme activity increased along with progressing senescence, and that GSTs transcriptional regulation is controlled by signal transduction linked to oxidative stress. The participation of tau class GSTs in the broad network of catalytic and regulatory functions involved in oxidative stress response has been shown in tomato (Kilili et al., 2004), parsley (Loyall et al., 2000) and rice (Soranzo et al., 2004). Xu et al. (2002) found that *TtGSTU1* and *TtGSTU2* of *Triticum tauschii* were up-regulated by 100 μM ABA treatment in both shoot and root tissues, and it was confirmed that the promoter region of *TtGSTUs* contained ABA-, ethylene-, and auxin-responsive elements. Both of these findings indicate a potential role in stress responses.

In our experiments, estimation of *GST* expression patterns showed differences in expressed genes in the flag leaves of the investigated cultivars during the early grain-filling period. The expression patterns of *GSTU1B* and *1C* differed in the four cultivars under control conditions, and the marked increase of *GSTU1B* transcript levels was found only in the drought-resistant cv. Plainsman. The drought-sensitive GK Élet also exhibited a significant induction, but lower transcript levels. The transcript amounts of *TaGSTU1B* and *TaGSTF6* were strongly up-regulated by drought stress on the day of anthesis and on most of the sampling days in the drought-tolerant Plainsman cultivar, a lesser increase was found in the expression rate of *GSTU1B* in MV Emese and GK Élet. According to our results, one of the drought-resistant cultivars (Plainsman) accumulated the highest levels of GSTU1B, GSTF6 and GSTA2 transcripts in response to drought treatment, whereas one of the drought-sensitive cultivars, Cappelle Desprez accumulated the highest levels of GSTU1C and GST19E50 transcripts (Figure 5). This indicates that the investigated GST sequences undergo different types of regulation in the four cultivars and even *TaGSTU1* (similar to barley senescence-induced GST) displayed different expression in the wheat lines. The different responses found in selected GST transcripts appear to depend on genotypes rather than their drought susceptibility. The elevation of *TaGSTU1B* and *TaGSTF6* expression levels before the appearance of visible symptoms of senescence indicates that these genes may play a role in the regular progression of senescence. Interestingly, these sequences were strongly induced under drought



stress, especially in the drought-resistant Plainsman cultivar, and to a lesser extent, in cv. MV Emese. The products of these genes are presumably involved in a strong detoxification and/or they can promote the mobilization or prevent the degradation of macromolecules, which facilitate the grain filling under drought stress.

The GST sequences were chosen on the basis of their possible roles in stress response and senescence. It is primarily the plant-specific phi and tau class GSTs that mediate glutathione transferase activity towards a diverse range of xenobiotics (Edwards and Dixon, 2005). Diversity in the substrate specificity and GST and GPOX activity of some members of phi and tau class GSTs in wheat were investigated by Thom et al. (2002) and Cummins et al. (2003). In our experiments, some similarities were found in the tendencies between the extractable GST activity and the expression levels of the chosen genes. The drought-resistant Plainsman showed the highest GST and GPOX activity in both drought-stressed and well-watered samples. The highly significant increase on the day of anthesis, in correlation with changes in expression patterns, confirms the rapid reaction of the Plainsman to water deficit. This genotype exhibited great yield stability under drought, which may occur in connection with an extremely strong detoxification capacity in tissues. The possibility that GST participates in a successful grain filling in wheat under water deficit requires further investigation. In the sensitive cultivar GK Élet, the decreased GPOX activity and the largely unaffected GST activity up to the last sampling day show that the activation of the defense mechanisms was not sufficient to maximize grain filling under water stress. The GST activity data and expression levels indicate that the faster reaction and the more intense defense against the toxic stress metabolites had an important role in the successful mobilization of storage reserve material in the Plainsman cultivar. MV Emese and GK Élet both showed a moderate rise in the expression of GSTs, but the effects of drought on the extractable GST and GPOX activity differed between the two cultivars, and GPOX activity remained very low in GK Élet cultivar. Some similar tendencies were found between the GST activity and expression level of some sequences, but no complete correspondence can be established because GST isoenzymes can display different activity towards the artificial substrate CDNB (which was used to establish the GST activity in our experiment).

In summary, our results on the wheat GST transcript measurements in flag leaves indicate the major roles of some GST isoenzymes both in monocarpic senescence and drought stress responses during the grain-filling period. Differences were observed among the genotypes, and the early GST activation detected in drought-tolerant wheat lines suggests the involvement of GSTs in the protection of optimal cell metabolism. *TaGSTU1B* and *TaGSTF6* can be important for regular progression of flag leaf senescence. In correlation with the enzyme activity, these gene products presumably indicated strong detoxification, which could facilitate the grain filling under drought stress.

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Figure 5. Transcript levels of phi, tau and zeta class *TaGST*s in GK Élet, MV Emese, Plainsman and Cappelle Desprez cultivars during the early grain-filling period (□ anthesis, ■ 4 DPA; ■ 9 DPA, ■ 12 DPA). R and S for resistant and sensitive genotypes, respectively. Vertical bars represent standard deviations. The lowest relative transcript level in the control samples on the day of anthesis (initial control) was equal to one for each gene. Data were normalized using the wheat elongation factor α subunit (EF-1) and a gene with unknown function (NP-1) as high and low controls, respectively (Jukanti et al., 2006). Statistical differences compared with controls are indicated by $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

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