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Gradual Drought Under Field Conditions Influences the Glutathione Metabolism, Redox Balance and Energy Supply in Spring Wheat

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Abstract

Glutathione (GSH) metabolism, redox balance and energy supply in spring wheat (*Triticum aestivum* L.) during gradual drought stress under field conditions were investigated. Although levels of total and reduced GSH were decreased, the ratio of GSH/GSSG (glutathione disulfide) was markedly increased by drought. Levels of GSH biosynthetic precursors, cysteine (Cys) and γ-glutamylcysteine (γ-GC), and the activities of their biosynthetic enzymes, γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GSHS) were also significantly increased in stressed plants. Glutathione reductase (GR) activity, which is responsible for the conversion of GSSG to GSH, was also increased under this field stress. However, two other important enzymes in GSH metabolism, glutathione peroxidase (GP) and glutathione S-transferase (GST), showed decreased activity in the droughted plants. These results suggest that the higher ratio of GSH/GSSG, the rate of GSH biosynthesis and the capacity of its redox cycling rather than GSH accumulation might be essential for drought resistance of plants. Activities of the two key Calvin-cycle enzymes possessing exposed sulfhydryl groups, $NADP^+$-dependent glyceraldehydes-3-phosphate dehydrogenase (G3PD) and fructose-1,6-bisphosphatase (FBPase) were not affected by drought stress, whereas, activity of the key enzyme in the pentose-phosphate pathway (PPP), 6-phosphogluconate dehydrogenase (6-PGD), increased in the droughted plants. The ratios of $NADPH/NADP^+$, $NADH/NAD^+$ and ATP/ADP increased in the droughted plants, indicating that an up-regulation of the reduced redox state and the energy supply in the plant cells might be an important physiological strategy for plants responding to drought stress. A simple correlation between the high ratio of GSH/GSSG, the rate of GSH biosynthesis and the redox cycle and the high reduction states of redox status in the plant cells was also observed under field drought.

Key words: Energy supply; Gradual field drought; GSH metabolism; Redox balance; Spring wheat (*Triticum aestivum* L.)

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Introduction

Drought results in a water deficit in plant tissues, which, in turn, can lead to an imbalance in the redox poise of plant cells, and thus inducing oxidative stress in plants (Noctor and Foyer 1998a; Smirnoff 1998). To counteract the toxicity of active oxygen species (AOS), a highly efficient antioxidative defense system, including both non-enzymatic and enzymatic constituents, are present in plant cells (Noctor and Foyer 1998a; Polle 2001).

Glutathione (GSH) is a cellular protectant and is also the major reservoir of nonprotein reduced sulfur in plants (May and others 1998; Noctor and others 1998; Foyer and others 2001). Owing to its redox-active thiol group, GSH is associated with stress resistance, and is an important antioxidant in the cellular milieu, responsible for maintenance of antioxidative machinery of the cells under stress. GSH is oxidized by various free radicals and oxidants to glutathione disulfide (GSSG), while glutathione reductase (GR, EC 1.6.4.2) uses NADPH to reduce GSSG to GSH (Noctor and others 1998). GSH directly reduces most active oxygen species and also scavenges hydrogen peroxide via glutathione peroxidase (GP, EC 1.11.1.9), which is involved in the detoxification of lipid peroxides rather than hydrogen peroxide *per se* (Noctor and others 1998; Nagalakshmi and Prasad 2001). Glutathione S-transferase (GST, EC 2.5.1.18) is another GSH-dependent detoxifying enzyme in plants, which catalyzes the conjugation of GSH with potentially dangerous xenobiotics such as herbicides (Noctor and others 1998).

GSH is synthesized from glutamate, cysteine and glycine in two ATP-dependent reactions catalyzed by γ-glutamyl cysteine synthetase (γ-GCS; EC 6.3.2.2) and glutathione synthetase (GSHS; 6.3.2.3) (Noctor and others 1998). It has been shown that GSH synthesis is regulated by cysteine (Cys) availability, feedback inhibition of γ-GCS by GSH, transcriptional control of γ-GCS and translational regulation of γ-GCS by the ratio of reduced to oxidized glutathione (Foyer and others 2001). In some cases, increases in the levels of GSH during stress responses have been correlated with an increase of γ-GCS activity (May and others 1998).

It has been found that in plants a number of redox-based sensory systems underly the mechanisms by which plants monitor quantitative and qualitative changes in the environment and organize their responses to stress (May and others 1998). GSH/GSSG, ascorbate (ASC)/dehydroascorbate (DHA), and NADPH/NADP are three interdependent redox couples present in plant cells and their relationships have been shown in the ascorbate-glutathione cycle (May and others 1998; Noctor and Foyer 1998a; Asada 1999). Recently, it has been found that, as a cellular sensor, GSH may act to ensure maintenance of the cellular NADPH pool under stress. This points to the strategies that plants have adopted to ensure a supply of NADPH for stress defenses (May and others 1998). However, there is a lack of information concerning how the pools of GSH and NADPH function in plants when they are subjected to environmental stress such as drought.

To evaluate the metabolism of GSH and its functions in redox and energy balances under drought stress, the activities of key enzymes of GSH biosynthesis, γ-GCS and GSHS, and enzymes involved in degradation and regeneration of GSH such as GST, GP and GR were investigated in the leaves of spring wheat. The ratios of NADH/NAD, NADPH/NADP and ATP/ADP, and the activities of some enzymes correlated with the redox and energy balances of cells in the plant were also investigated.

Materials and Methods

Plant Materials and Growth Conditions

Plants of spring wheat (*Triticum aestivum* L.) cv. 8139 were grown under irrigated and water stress condition. Seeds were provided by the Institute of Agriculture, Dingxi County, Gansu, P. R. China. Wheat seeds were sown on March 9, 2001 and the seedlings emerged on March 23. Before sowing, fields in the arid region (located in Lanzhou City, Gansu Province, in the northwest of China) were irrigated. Drought treatment was applied by withholding irrigation throughout the plant development and movable rainout shelters were used to shield the plants from rain after sowing. The controls were continuously maintained under optimal irrigation by irrigating regularly by hand. At regular intervals, the soil moisture content was monitored and after about a month the soil moisture contents between the droughted field and the control field began to show obvious differences (Chen and other 2003a). The final fully expanded leaves used for measurements were harvested on May 5 and 15, respectively, and samples were collected at 9 AM and immediately frozen in liquid N_2. For determination of the pools of glutathione, the fresh leaves were collected on May 6 and 17, respectively, in 2002, after a similar drought-treatment process as described above.

Leaf Water Status

The leaf water potential was measured with a pressure chamber. The dry matter of leaves was determined after drying for 72 h at 80°C, and the relative water content (RWC) was evaluated using the following equation; $RWC = (FW - DW)/(SW - DW) \cdot 100\%$, where *FW* is fresh weight, *DW* is dry weight, and *SW* is saturated weight in water (Iturbe-Ormaetxe and others 1998).

Determinations of Reduced Thiol Levels

Leaf thiols were extracted and determined as their monobromobimane (MBB)-derivatized products as described by Creissen and others (1999). MBB-derivatized thiols were separated by C_{18} reversed-phase HPLC and detected using an on-line fluorometer according to the method of Newton and others (1981). Identification and quantification of thiol compounds were performed by comparison of retention times of known amounts of standard compounds derivatized with MBB.

Estimation of the Pools of Glutathione

Fresh leaves were homogenized with a mortar and pestle at temperatures between 0 and approximately 4°C in 5% sulfosalicylic acid (w/v), centrifuged at 15,000*g* for 20 min, and the supernatant was used for total and GSSG determinations by the 5,5′-dithio-bis-(2-nitrobenzoic acid)/GR recycling procedure, as described by Nagalakshmi and Prasad (2001). A standard curve prepared by using GSH and GSSG was used in the calculation of the amounts of total glutathione, reduced GSH and GSSG.

Extractions and Assays of the Antioxidant Enzymes

Leaf tissue samples (approximately 0.5 g) were ground to a powder with liquid N_2 and homogenized in 3 ml of ice-cold extraction buffer (50 mM potassium phosphate buffer, pH 7.8, 5 mM ASC, 5 mM DTT, 100 mM NaCl, 5 mM EDTA, and 2% PVP-40). The extracts were centrifuged at 15,000*g* for 15 min, and the supernatants were used for the enzyme assays. All the above operations were performed at 0 to 4°C (Biemelt and others 1998). Superoxide dismutase (SOD) was assayed by the nitroblue tetrazolium (NBT) method modified from that described by Dhindsa and others (1980). One unit of SOD was defined as that being contained in the volume of extract that caused a 50% inhibition of the SOD-inhibitable fraction of the NBT reduction. Peroxide dismutase (POD) and catalase (CAT) activities were measured according to the method of Cakmak and others (1993). Ascorbate peroxidase (APX) was assayed as described by Asada (1984).

Extractions and Assays of the Enzymes Associated with Metabolism of Glutathione

Leaf tissue samples (approximately 1.0 g) were ground to a powder with liquid N_2 and homogenized in 4 ml of ice-cold extraction buffer: γ-GCS and GSHS with buffer A (100 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 5.0% PVP-40); GST, GP, and GR with buffer B (100 mM potassium phosphate buffer, pH 7.0, 1.0 mM EDTA, and 4.0% PVP-40). The extracts were centrifuged at 15,000*g* for 20 min, and the supernatants were used for the enzyme assays. All the above operations were performed at 0 to 4°C. The γ-GCS assay was carried out according to the method of Creissen and others (1999). The GSHS assay was carried out according to Hell and Bergmann (1988). GST and GR activities were measured as described by Sudhakar and others (2001), and GP activity was determined by the method of Hartikainen and others (2000).

Extractions and Assays of G3PDH and FBPase

Leaf tissue samples were ground to a powder with liquid N_2 and homogenized in ice-cold extraction buffer containing 100 mM Tris-HCl, pH 8.1, 1 mM Na_2 EDTA, 10 mM $MgCl_2$, and 4% (w/v) PVP-40. The extracts were centrifuged at 15,000*g* for 20 min, and the supernatants were used for the enzyme assays. To avoid oxidation of the sulfhydryl groups, the solution was depleted in oxygen under vacuum and all extractions were carried out at nitrogen atmosphere. Activities of glyceraldehydes-3-phosphate dehydrogenase (G3PD) and fructose-1,6-biphosphatase (FBPase) were assayed as described by Loggini and others (1999). Initial activity was assayed immediately after homogenization. Total activity was assayed on aliquots of enzyme extract incubated for 20 min with 20 mM 1,4-dithiothreitol (DTT).

Extraction and Assay of 6-Phosphogluconate Dehydrogenase (6-PGD)

Leaf tissue samples were ground to a powder with liquid N_2 and homogenized in extraction buffer containing 100 mM Tris-HCl, pH 7.5, 10 mM H_3BO_4, 30 mM DTT, 15% (w/v) glycerol (Bailey-Serres and

Table 1. Water Conditions and Antioxidant Enzyme Activities of Spring Wheat Leaves during Field Drought.

Parameters	C_1	D_1	C_2	D_2
Water potential (-MPa)	1.26 ± 0.09[a]	1.36 ± 0.06[b]	1.16 ± 0.11[a]	1.43 ± 0.04[c]
Relative water content (RWC, %)	90.5 ± 0.3[a]	85.2 ± 0.5[b]	89.8 ± 0.6[a]	83.2 ± 0.7[c]
Dry weight/fresh weight	0.173 ± 0.002[a]	0.186 ± 0.003[b]	0.202 ± 0.002[c]	0.215 ± 0.007[d]
SOD (U mg^{-1} protein min^{-1})	5.2 ± 0.1[a]	11.7 ± 0.7[b]	6.6 ± 0.4[c]	9.9 ± 0.4[d]
POX (°mol tetraguaiacol formation mg^{-1} protein min^{-1})	1.68 ± 0.08[a]	2.01 ± 0.19[b]	1.53 ± 0.05[c]	2.74 ± 0.41[d]
CAT (°mol H_2O_2 oxidized mg^{-1} protein min^{-1})	0.17 ± 0.09[a]	0.21 ± 0.09[b]	0.15 ± 0.02[a]	0.26 ± 0.08[c]
APX (°mol ascorbate oxidized mg^{-1} protein min^{-1})	0.78 ± 0.05[a]	1.31 ± 0.11[b]	0.49 ± 0.12[c]	I.52 ± 0.09[d]

The leaves were harvested around 9 AM on May 5 and 15, 2001, respectively. Results are the means ± SD of 10 repetitions for water potential and 3 to 4 repetitions for relative water content (RWC) and ratio of dry weight/fresh weight. For antioxidant enzymes, the values are means ± SD of three independent experiments with 3 to 4 replicates. One-way analysis of variance was used for comparisons between the means. Values in each row followed by different letters are significantly different at $P \leq 0.05$. C_1, control on May 5; D_1, drought-stressed on May 5; C_2, control on May 15; D_2, drought-stressed on May 15.

Nguyen 1992), after centrifugation for 20 min at 12,000*g* the supernatant was used for the assays of enzyme activity as described by Bailey-Serres and Nguyen (1992).

Total protein content was determined using a coomassie dye with bovine serum albumin as the standard (Bradford 1976).

Determination of Pyridine Nucleotides

Extraction of NAD^+(H) and $NADP^+$(H) was carried out according to the method of Sijmons and others (1984). Pyridine nucleotide concentrations were determined with enzymatic cycling as described by Slater and Sawyer (1962), using alcohol dehydrogenase (EC 1.1.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) for NAD^+(H) and $NADP^+$(H), respectively. These enzymes were added to the assay mixtures after 10 min equilibration. The decrease in absorbance at 600 nm of the final electron acceptor in the cycling assay, 2,6-dichlorophenol-indophenol, was measured at 30°C with a spectrophotometer. A standard curve prepared using standard pyridine nucleotides was used for calculating the amounts of NADH, NADPH, NAD^+ and $NADP^+$, respectively.

Determination of ATP and ADP Levels

ATP and ADP were assayed with the method described by Brown and Wray (1968). ATP and ADP concentrations were determined with enzymic cycling using glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and hexokinase (EC 2.7.1.1) for ATP and lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.1.40) for ADP, respectively.

Results

Drought induced a slight decrease in the leaf water potential and relative water content (RWC), and an increase in the ratio of leaf dry weight to fresh weight (Table 1) in the spring wheat plants. With the increase in drought period the leaf water potential and RWC decreased at a faster rate. In addition, antioxidant enzyme activities, including SOD, POX, CAT and APX, all significantly increased at two sampling stages in the field drought treatment (Table 1).

Drought caused a 26.9 and 50.0% decrease in reduced GSH at both the 1st and 2nd stage of sampling, respectively. However, levels of the precursors of GSH biosynthesis, Cys and γ-GC, were markedly increased by drought, reaching levels 181 to 200% and 142 to 181% of their control values, respectively (Table 2). The pools of glutathione were measured by the 5,5′-dithio-bis-(2-nitrobenzoic acid)/GR recycling procedure. The results also showed a decrease in glutathione content by drought (Table 2). Although levels of total and reduced GSH were decreased, the ratios of GSH/GSSG were increased at both stages of sampling, indicating that the redox status of glutathione was changed to a more reduced state by drought (Table 2). Field drought significantly increased activities of γ-GCS and GSHS, two key enzymes in the glutathione biosynthesis, by 57 and 76 % and 35 and 113%, respectively at the two stages (Table 3). It should be noticed that the activity of γ-GCS was considerably high than that of GSHS in both control and droughted plants. In addition, decreased activities of GP and GST were observed under field drought; the decrease continued with the duration of stress. In contrast, GR activity increased under drought conditions at the two stages (Table 3).

Table 2. Levels of Reduced Thiols (°mol g^{-1} DW) and the Pools of Glutathione (°mol g^{-1} DW) in Leaves of Spring Wheat under Field Drought.

	C_1	D_1	C_2	D_2
Results obtained by HPLC				
Cys (°mol g^{-1} DW)	0.72 ± 0.08[a]	1.54 ± 0.14[b]	0.66 ± 0.05[a]	1.19 ± 0.13[c]
γ-GC (°mol g^{-1} DW)	0.18 ± 0.05[a]	0.33 ± 0.02[b]	0.20 ± 0.03[a]	0.29 ± 0.03[c]
GSH (°mol g^{-1} DW)	9.3 ± 0.9[a]	6.8 ± 0.7[b]	9.4 ± 0.1[a]	4.7 ± 1.5[c]
Results from DTNB/GR recycling procedure				
Total glutathione (°mol g^{-1} DW)	13.0 + 0.8[a]	8.2 + 0.4[b]	13.9 + 1.0[a]	7.3 + 0.5[c]
GSH (°mol g^{-1} DW)	11.8 + 1.1[a]	7.5 + 0.6[b]	12.6 + 0.0.9[a]	6.8 + 0.8[c]
GSSG (°mol g^{-1} DW)	1.22 + 0.07[a]	0.62 + 0.05[b]	1.31 + 0.18[a]	0.43 + 0.06[c]
GSH/GSSG	9.7 + 0.8[a]	12.1 + 0.7[b]	9.3 + 0.6[a]	15.5 + 0.9[c]

Levels of reduced thiols were determined by HPLC in 2001. The values are means ± SD of two independent experiments with 3 replicates. C_1, control on May 5; D_1, drought-stressed on May 5; C_2, control on May 15; D_2, drought-stressed on May 15. The pools of glutathione were measured by the 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB)/GR recycling procedure in 2002. The values are means ± SD of three independent experiments with 3 to 6 replicates. C_1, control on May 6; D_1, drought-stressed on May 6; C_2, control on May 17; D_2, drought-stressed on May 17. One-way analysis of variance was used for comparisons between the means. The values in each row followed by different letters are significantly different at $P \leq 0.05$.

Table 3. Activities of Enzymes Associated with Metabolism of Glutathione in Leaves of Spring Wheat under Field Drought.

	C_1	D_1	C_2	D_2
γ-GCS (nmol mg^{-1} protein min^{-1})	3.26 ± 0.13[a]	5.12 ± 0.33[b]	2.83 ± 0.36[a]	4.97 ± 0.63[b]
GSHS (nmol mg^{-1} protein min^{-1})	0.20 ± 0.02[a]	0.27 ± 0.03[b]	0.15 ± 0.02[c]	0.32 ± 0.05[b]
GP (nmol GSH mg^{-1} protein min^{-1})	23.1 ± 2.3[a]	13.7 ± 0.5[b]	20.0 ± 2.8[a]	8.9 ± 1.9[c]
GST (nmol CDNB conjugated mg^{-1} protein min^{-1})	49.4 ± 5.1[a]	42.0 ± 2.3[b]	54.3 ± 2.6[a]	43.6 ± 2.5[b]
GR (nmol NADPH oxidized mg^{-1} protein min^{-1})	40.1 ± 5.3[a]	53.7 ± 5.0[b]	45.6 ± 3.5[a]	63.2 ± 4.3[c]

The values are means ± SD of three independent experiments with 3 replicates for γ-GCS and GSHS and 4 to 6 replicates for GP, GST and GR. One-way analysis of variance was used for comparisons between the means. Values in each row followed by different letters are significantly different at $P \leq 0.05$. C_1 control on May 5; D_1, drought-stressed on May 5; C_2, control on May 15; D_2, drought-stressed on May 15.

Activities of FBPase and G3PD were not affected by field drought (Table 4). Although FBPase and G3PD activities were almost similar in stressed spring wheat plants, the activation state (percentage of initial activity on total activity) seemed higher in G3PD than in FBPase. However, field drought increased the activity of 6-PGD by 40 and 21% at two samplings (Table 4).

Contents of NADH and NAD^+ in spring wheat slightly increased whereas NADPH and $NADP^+$ were markedly decreased by drought conditions (Table 5). Although the NAD(H) pool showed more oxidation compared to the NADP(H) pool in spring wheat, where ratios of NADH/NAD^+ were much lower (less than 0.2) than ratios of NADPH/$NADP^+$ (in excess of 1.3), both ratios were significantly increased by field drought (Table 5).

Levels of ATP increased markedly in stressed plants, and with duration of drought. However, levels of ADP increased relatively less, and only on May 15 were the levels significantly higher in droughted plants. In contrast, ratios of ATP/ADP significantly increased at the two sampling stages, and in the stressed plants reached 159 and 208% of the controls, respectively (Table 5).

Discussion

In the field, plants are subjected to a more gradual stress because water availability in the soil does not change abruptly and, therefore, plant responses might be different under this type of stress condition. Abrupt stress conditions lead to irreversible injuries to membranes (Stewart 1989), whereas a gradual imposition of stress may not lead to significant cellular damage and thus may enable plants to tolerate even more intense stress (Kozlowski and Pallardy 2002). In the field, gradual drought conditions resulted in a slight decrease in leaf RWC and

Table 4. The Initial (I) and Total (T) Activities and Activation State (percentage of initial activity on total activity) of FBPase and G3PD, and Activity of 6-PGD in Leaves of Spring Wheat under Field Drought.

	C_1	D_1	C_2	D_2
6-PGD (nmol $NADP^+$ reduced mg^{-1} protein min^{-1})	61.6 ± 9.7[a]	86.0 ± 11.6[b]	66.7 ± 9.6[a]	80.8 ± 8.6[b]
FBPase I (nmol $NADP^+$ reduced mg^{-1} protein min^{-1})	61.6 ± 11.1[a]	59.8 ± 6.5[a]	53.5 ± 7.4[a]	52.4 ± 6.8[a]
FBPase T (nmol $NADP^+$ reduced mg^{-1} protein min^{-1})	126.5 ± 7.2[a]	108.9 ± 12.0[a,b]	100.9 ± 7.0[b]	113.1 ± 15.5[a]
FBPase I/T (%)	48.7 ± 0.6[a]	54.9 ± 0.4[b]	53.0 ± 0.8[b]	46.3 ± 0.6[a]
G3PD I (nmol NADPH oxidized mg^{-1} protein min^{-1})	67.3 ± 16[a]	87.5 ± 14[a]	175.1 ± 18.2[b]	154.6 ± 17.5[b]
G3PD T (nmol NADPH oxidized mg^{-1} protein min^{-1})	120.7 ± 7.1[a]	130.4 ± 9.4[a]	220.3 ± 26.1[b]	210.9 ± 19.7[b]
G3PD I/T (%)	52.8 ± 3.6[a]	65.0 ± 5.1[b]	78.7 ± 4.6[c]	70.5 ± 2.7[b]

The values are means ± SD of three independent experiments with 4 to 5 replicates. One-way analysis of variance was used for comparisons between the means. Values in each row followed by different letters are significantly different at $P \leq 0.05$. C_1, control on May 5; D_1, drought-stressed on May 5; C_2, control on May 15; D_2, drought-stressed on May 15.

Table 5. Levels of Pyridine Nucleotides (nmol g^{-1} DW) and ATP and ADP (° mol g^{-1} DW) in Leaves of Spring Wheat under Field Drought.

	C_1	D_1	C_2	D_2
NADH (nmol g^{-1} DW)	51.5 ± 8.4[a]	78.3 ± 13.7[b]	54.8 ± 20.3[a]	55.6 ± 12.5[a]
NAD^+ (nmol g^{-1} DW)	468.4 ± 165.2[a]	506.9 ± 108.3[a]	296.1 ± 61.1[b]	307.0 ± 71.7[b]
$NADH/NAD^+$	0.11 ± 0.006[a]	0.15 ± 0.015[b]	0.12 ± 0.009[a]	0.18 ± 0.012[c]
NADPH (nmol g^{-1} DW)	27.1 ± 0.3[a]	22.7 ± 0.4[b]	23.4 ± 0.3[b]	19.5 ± 0.5[c]
$NADP^+$ (nmol g^{-1} DW)	18.9 ± 2.5[a]	14.0 ± 1.4[b]	17.4 ± 0.6[a]	11.4 ± 2.2[c]
NADPH/NADP	1.43 ± 0.08[a]	1.62 ± 0.13[b]	1.34 ± 0.07[a]	1.71 ± 0.10[b]
ATP (° mol g^{-1} DW)	0.76 ± 0.13[a]	1.04 ± 0.14[b]	0.78 ± 0.17[a]	2.03 ± 0.36[c]
ADP (° mol g^{-1} DW)	2.01 ± 0.42[a]	1.75 ± 0.46[a]	1.96 ± 0.16[a]	2.49 ± 0.49[b]
ATP / ADP	0.37 ± 0.025[a]	0.59 ± 0.011[b]	0.39 ± 0.032[a]	0.81 ± 0.056[c]

The values are means ± SD of three independent experiments with 3 to 6 replicates. One-way analysis of variance was used for comparisons between the means. Values in each row followed by different letters are significantly different at $P \leq 0.05$. C_1, control on May 5; D_1, drought-stressed on May 5; C_2, control on May 15; D_2, drought-stressed on May 15.

water potential (Table 1). This was very dissimilar to the changes in plant water status under experimental stresses in which water content and water potential were abruptly decreased, resulting in plants experiencing severe water deficit conditions within a few days (Sairam and others 1998; Smirnoff and Colombe 1998; Ali and others 1999). The antioxidant enzyme activities increased significantly under field drought conditions (Table 1) suggesting that at both sampling stages wheat plants experienced oxidative stress induced by the drought.

Glutathione metabolism and the GSH pool are related to the responses of plants to a wide range of artificial stresses (May and others 1998; Noctor and others 1998; Loggini and others 1999; Foyer and others 2001; Nagalakshmi and Prasad 2001). Enzyme activity related to GSH biosynthesis and metabolism can enhance the resistances of plants to various biotic and abiotic stresses. Although GSH levels decreased in the draughted plants, GSH precursor (levels γ-GC and Cys; Table 2) and the activities of biosynthetic enzymes (γ-GCS and GSHS; Table 3) increased significantly suggesting that a higher rate of GSH biosynthesis is involved in the response of wheat plants to drought stress. Other studies have also found decreased levels of GSH under stressed condition (Loggini and others 1999; Nagalakshmi and Prasad 2001). However, the ratio of GSH/GSSG was increased by drought, indicating that a more reduced state of glutathione pools was involved in the response of wheat plants to drought (Table 2). It is generally accepted that γ-GCS is the rate-limiting step in GSH biosynthesis (Noctor and others 1998). The fact that the activity of γ-GCS was significantly higher than that of GSHS in wheat plants (Table 3) indicates that the former might be mainly responsible for GSH production in this plant even though the precise mechanism has not been identified. A similar phenomenon was also found in reed plants (Chen and others 2003b). Because GSH

levels and redox status can regulate expression of genes and activities of the redox sensitive transcription factors and enzymes (Sen 1999; Cnubben and others 2001; Pastori and Foyer 2002), we thought that the decrease in total and reduced GSH but the increase in the GSH biosynthesis rate and GSH/GSSG ratio in wheat under field drought might be an important event. Glutathione might be largely consumed for regulating many important metabolic processes, and therefore, its levels declined under drought stress with the increase in biosynthesis rate. GP and GST are closely associated with plants' stress-resistance. Although GP is involved in the detoxification of lipid peroxides rather than hydrogen peroxide *per se* (Eshdat and others 1997), GST is a multigene family of isoenzymes that detoxifies such endogenously produced electrophiles under heavy metal, salt and oxidative stress conditions by conjugation with GSH (Chiou and Tzeng 2000; Nagalakshmi and Prasad 2001; Sudhakar and others 2001). In the present work, the activities of GP and GST decreased in droughted plants (Table 3), indicating that under gradual stress conditions the two enzymes might not be essential for protecting plants from oxidative damages, and the depletion of GSH might be responsible for the decreases in activities. In contrast, GR, which is responsible for GSH regeneration from GSSG, increased in droughted plants (Table 3). These results suggest that the higher ratio of GSH/GSSH, the rate of GSH biosynthesis and the capacity of its redox cycling, rather than GSH accumulation, might be essential for drought resistance in plants.

High GSH levels, in particular high GSH/GSSG ratios, are associated with low hydrogen peroxide concentrations. In the present study, although the levels of total and reduced GSH were decreased, the ratio of GSH/GSSG was increased by field drought (Table 2). This observation is consistent with a study by Loggini and others (1999). Hydrogen peroxide, even at low concentrations, can inhibit chloroplast sulfhydryl-containing enzymes by readily oxidizing their sulfhydryl groups (Takeda and others 1995; Loggini and others 1999). G3PD and FBPase activities were not significantly affected by drought (Table 4), indicating that the higher rates of GSH biosynthesis and the capacity of redox cycling were essential for protecting sulfhydryl-containing enzymes from oxidative damages.

As a rate-limiting enzyme in the PPP, increased 6-PGD activity has been reported under environmental stress conditions such as low pH, salicylic acid treatments, disease, and high soil temperature (Nowotny and others 1998; Chugh and Sawhney 1999; Danson and others 2000; McCue and others 2000; Fukuoka and Enomoto 2001). 6-PGD is one of the major sources of NADPH in the cytoplasm of plant cells. Kuroda and others (1991) reported that the NADPH generated by 6-PGD is used for the detoxification of hydrogen peroxide through the ascorbate-glutathione cycle. Our results showed that 6-PGD activity increased significantly in the droughted plants (Table 4), and the increase corresponded to the elevated ratio of NADPH/$NADP^+$ induced by drought (Table 5). Although the increased activity of 6-PGD did not seem to be directly related to the change in GSH metabolism, the relation between the NADPH supply and GSH metabolism has been studied (May and others 1998). Recent studies have suggested a regulatory role for GSH that may act to ensure maintenance of the cellular NADPH pool under stress conditions. It seems likely that GSH levels are used as a cue in the coordination of mechanisms both for the supply of Cys and the maintenance of the cellular NADPH pool. Coordination of these responses, merely by lowering the GSH pool, provides plants with a simple mechanism to respond defensively to a wide range of stresses through a coordinated up-regulation of the capacity for GSH biosynthesis and its redox cycling (see review of May and others 1998). Our results are consistent with this point of view. With the higher rate of GSH biosynthesis and capacity of its redox cycling, the draughted plants exhibited higher NADPH/$NADP^+$ ratios than the controls (Table 5).

Drought can unbalance the homeostasis of AOS in plant cells and thus subject plants to oxidative stress (Asada 1999; Polle 2001). NADH/NAD and NADPH/NADP are major redox couples in plant cells, which can decide the cellular redox status. In the present work, although the NAD(H) pool maintained a higher oxidation state compared to NADP(H) pool in the spring wheat, both pools tended to produce a more reducted state under gradual drought (Table 5), indicating that the redox status of the plant cells was influenced by drought and a more reduced state in cells might be an advantage for resistance of plants to drought stress. GSH could play an important role in the maintenance of the cellular redox balance (May and others 1998). Our results showed that the increased reduction state of NAD(H) and NADP(H) pools induced by gradual drought corresponded to a high ratio of GSH/GSSG and an increased rate of GSH biosynthesis and capacity for redox cycling under this drought condition, suggesting that a more reduced redox status in plant cells maintained by up-regulation of the ratio of GSH/GSSG, the rate of GSH biosynthesis and the capacity for redox cycling might be an efficient strategy for the adaptation of

the plants to drought stress. Results from an earlier study agreed (Chen and others 2003b).

Drought also caused a change in the energy supply in spring wheat (Table 2). The content of ATP and the ratio of ATP/ADP were markedly increased in the droughted plants, indicating that up-regulation of the energy supply is another important event in the response of spring wheat plants to gradual drought stress along with the reduced redox state in the plant cells. Because the reductive assimilation of inorganic compounds into the cellular matter of plants is mediated by low potential reductants (principally reduced ferredoxin, NADPH, NADH) and nucleoside triphosphates (mainly ATP), and the relative rates of production of the two types of molecules must be closely co-ordinated with their relative rates of consumption (Noctor and Foyer 1998b), similar to ratios of $NADPH/NADP^+$ and $NADH/NAD^+$, up-regulation of the ATP/ADP ratio in droughted plants might also be a physiological strategy of plants responding to drought stress. Although little evidence as to how plants generate more energy under drought stress was directly obtained, this information implies that the higher respiration might be responsible for the up-regulation of the energy supply since the rate of respiration could be enhanced in several plant species by drought (Mudrik and others 2003; Haupt-Herting and others 2001; Huang and Fu 2000).

High or low drought resistance can only be quantified on the basis of contrasting genotypes. In this study, we have only quantified the response of a wheat genotype to gradual field stress. The higher rate of GSH biosynthesis and the capacity for GSH redox cycling, rather than GSH accumulation, might be a more important event in the wheat plant's response to field drought stress. A simple correlation between the high ratio of GSH/GSSG, the rate of GSH biosynthesis and redox cycling and the high reduction redox status was also observed in spring wheat plants responding to field drought. In addition, up-regulation of the energy supply and the ratio of ATP/ADP in droughted plants might be another physiological strategy of plants responding to drought stress.

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