Original article

Redox markers for drought-induced nodule senescence, a process occuring after drought-induced senescence of the lowest leaves in soybean (Glycine max)

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Running title: Drought stress responses in soybeans

Abstract

Background and Aims Water is an increasingly scarce resource that limits crop productivity in many parts of the world, and the frequency and severity of drought are predicted to increase as a result of climate change. Improving tolerance to drought stress is therefore important for maximizing future crop yields. The aim of this study was to compare the effects of drought on soybean (Glycine max) leaves and nodules in order to define phenotypic markers and changes in cellular redox state that characterize the stress response in different organs, and to characterize the relationships between leaf and nodule senescence during drought.

Methods Leaf and crown nodule metabolite pools were measured together with leaf and soil water contents, and leaf chlorophyll, total protein contents and chlorophyll *a* fluorescence

quenching parameters in nodulated soybeans that were grown under either well-watered conditions or deprived of water for up to 21 days.

Key Results Ureides, ascorbate, protein, chlorophyll and the ratios of variable chlorophyll a fluorescence (F_v') to maximal chlorophyll a fluorescence (F_m') fell to levels below detection in the oldest leaves after 21 days of drought. While these drought-induced responses were not observed in the youngest leaf ranks, the F_v'/F_m' ratios, pyridine nucleotide levels and the reduction state of the ascorbate pool were lower in all leaf ranks after 21 days of drought. In contrast to leaves, total nodule protein, pyridine nucleotides, ureides, ascorbate, and glutathione contents increased as a result of the drought treatment. However, the nodule ascorbate pool was significantly less reduced as a result of drought. Higher levels of transcripts encoding two peroxiredoxins were detected in nodules exposed to drought stress but senescence-associated transcripts and other mRNAs encoding redox-related proteins were similar under both conditions.

Conclusions: While the physiological impact of the drought was perceived throughout the shoot, stress-induced senescence occurred only in the oldest leaf ranks. At this stage, a number of drought-induced changes in the nodule metabolites were observed but no markers of senescence could be detected. We conclude that stress-induced senescence in the lowest leaf ranks precedes nodule senescence, suggesting that leaves of low photosynthetic capacity are sacrificed in favour of nodule nitrogen metabolism.

Keywords: ascorbic acid, cysteine proteases, drought, nodules, peroxiredoxin, redox regulation, soybean; senescence, ureides

INTRODUCTION

Water is an increasingly scarce resource that limits crop productivity in many parts of the world. Drought is a major limitation to crop growth and the productivity of current agriculture and is one of the most important environmental threats to food security worldwide **(**Cutforth *et al.*, 2007). The frequency and severity of drought are predicted to increase as a result of climate change together with increases in the land areas experiencing drought (Jury and Vaux, 2007). While plant responses to drought are increasingly well understood (Claeys and Inzé, 2013) gaining a working knowledge of the genetic basis of drought tolerance remains more elusive. The acquisition of drought tolerance probably involves molecular, cellular, physiological and developmental adjustments (Yamaguchi-Shinozaki and Shinozaki, 2006; Manavalan *et al.*, 2009). Drought resistant cultivars are considered to produce higher yields than sensitive cultivars under conditions of water limitation (Fenta *et al.,* 2012, 2014). Plants employ different strategies to avoid or tolerate drought. These are: (i) drought escape, e.g. reproduction before soil water becomes limiting in late-season drought; (ii) dehydration tolerance, the ability of plant tissues to survive and recover from dehydration, and (iii) dehydration avoidance, where plants use adaptive mechanisms to delay loss of turgor and the onset of dehydration. Plants can avoid the negative impacts of drought through reduced transpiration, osmotic adjustment or improved capture of water from the soil (Chaves and Oliveira 2004; Blum, 2005). Rapid canopy development, and the timing and sensitivity of reproductive development in relation to seasonal droughts are considered to be important traits underpinning drought tolerance (Blum, 2005). In the first instance, drought is perceived by the root system from which signals are transmitted to the leaves, activating "water saving" strategies, including stomatal closure to control water loss by transpiration (Pastori and Foyer, 2002; Flexas *et al.,* 2006; Cruz de Carvalho, 2008). Drought is also considered to increase the accumulation of reactive oxygen species (ROS; Tuteja, 2007, Iturbe-Ormaetxe *et al.,* 1998)

leading to oxidative signalling that accompanies a network of hormonal responses (Noctor *et al.,* 2014).

Soybean is the most important grain legume crop worldwide. It is used for human and animal consumption. As a legume, soybean is able to establish a symbiotic union with nitrogen-fixing soil bacteria from *Rhizobium* genus that are housed with root nodules (Van Heerden *et al.,* 2007). Symbiotic nitrogen fixation provides an important nitrogen source for agriculture underpinning sustainable agriculture. As in all legumes, exposure to drought leads to an inhibition of nodule nitrogen fixation and a breakdown of symbiosis that limits yields in many parts of the world (Ladrera *et al.,* 2007). It is therefore not surprising that the yield of the soybean crop is decreased by drought, particularly when it occurs during flowering and early pod expansion (Pedersen *et al.*, 2005), times when the nodules are already subject to a developmentally-regulated senescence program. Drought is therefore considered to be the greatest threat to soybean production and profitability worldwide (Neves-Borges *et al.,* 2012). For example, in 2008-2009 drought-induced losses in total soybean production in Brazil, the second largest soybean producer, were estimated to be almost 11 million tons (Franchini *et al.*, 2009). Hence, the delivery of high-yielding drought-tolerant varieties through marker-assisted selection programs is essential to farmers. While functional genomics tools have allowed the dissection of the molecular mechanisms that define the drought stress response in soybean, there has been relatively little advance in shoot and root phenotyping to assist molecular genetics approaches (Fenta *et al.,* 2012, 2014). Currently, traits such as rooting depth (root mass at depth), water use efficiency, nitrogen fixation and leaf wilting are important in the evaluation of soybean germplasm for drought tolerance.

Legumes are able to establish a symbiotic relationship with bacteria from the genus *Rhyzobium,* which are able to fix atmospheric nitrogen (N_2) into organic nitrogenous compounds. In tropical legumes such as soybeans the primary products of symbiotic nitrogen fixation are ureides, such as allantoin and allantoate. Ureides, which are derived from urea, are less soluble than amides, which are the major products of symbiotic nitrogen fixation in temperate legumes. In some studies, ureides have been shown to accumulate in the nodules exposed to drought (Sinclair and Serraj, 1995; Serraj *et al.,* 1999b). However, the drought-induced accumulation of ureides has not always been observed (Serraj *et al.,* 1999; Serraj *et al.,* 1999b; King and Purcell, 2005; Todd *et al.,* 2006, Ladrera *et al.,* 2007). Moreover, no correlation was found between ureide accumulation and the stress-induced inhibition of nitrogen fixation (Ladrera *et al.,* 2007).

The effects of drought on soybean nodules are well documented (Evans *et al.,* 1999; Purcell *et al.,* 2000; Streeter, 2003) as are the effects on leaves or leaves and roots (Liu *et al.,* 2005; Stolf-Moreira *et al.,* 2010). There is a strong association between root and nodule parameters and shoot biomass in drought-tolerant soybeans under both glass-house and field conditions (Fenta *et al.,* 2012, 2014). This finding led us to consider whether there was a relationship between leaf and nodule senescence under drought because this topic is poorly documented in the literature. The timing of the senescence genetic programs in leaves and nodules appears to be considered as something like a "chicken and egg" scenario in the overall plant stress response. This study was therefore undertaken to define the phenotypic markers of the drought response in soybean. Moreover, the relative progression of drought-induced changes in metabolism was measured in the different leaf ranks and in crown nodules in order to resolve the sequence of events with regard to the timing of senescence in different organs.

The molecular and metabolic responses of the reduction/oxidation (redox) systems of leaves and nodules to drought in relation were measured in relation to organ senescence.

MATERIALS AND METHODS

Plant material and growing conditions

Seeds of *Glycine max* (L.) Merr. cv. Williams 82 were inoculated with 0.5 grams per seed of a cell powder of *Bradyrhizobium japonicum* strain WB74-1 (Soygro bio-fertilized Limited, South Africa) and placed one seed per pot (18 cm height and 22 cm diameter) filled up until the rim with vermiculite fine grade (0.5-2 mm). Seedlings were watered twice per week with nitrogen free Hoagland's nutrient solution and demineralised water was added the remaining days to keep optimum conditions.

The plants were kept in a glass house with 16 h day length and 30/25°C day/night temperature, with a light intensity of 350 µmol m^{-2} s^{-1}.

Drought treatments

The drought experiment was performed on plants that had been grown for five weeks. At this stage the cotyledons has senesced and the plants had two unifoliolate lower leaves (L) and five trifoliolate (TF) leaves. A total of 30 plants were used in each experiment. Control plants received nutrients and water as in the first five weeks of growth. For the drought treatments, plants were deprived of both water and nutrients for 21 days.

Three plants were sampled from each of the control and drought-treated plants at the times indicated on the figures and tables. Crown nodules were harvested and leaf discs were collected from each leaf rank on the plant. These were weighed and immediately frozen in

liquid N_2 until analysis. The roots and a soil core (10 cm length, 2.5 cm diameter) were taken from each pot in an area close to the plant. Each sample was weighed and then placed in an oven at 80°C until a constant weight was reached, for the measurement of the dry weight values. The root and soil water contents were calculated from the fresh and dry weight measurements.

Protein content

The content of protein was determined following the method described by Bradford (1976).

Pigment content

Chlorophylls *a* and *b* and total carotenes were extracted in 95% ethanol by homogenising frozen leaf discs in a mortar that had been pre-cooled using liquid N_2. The extracts were centrifuged at 12,000 x *g* for 10 min at 4°C. The absorbance of the supernatant solutions was then measured at 648.6 and 664.2 nm. The pigments were then quantified as described by Lichtenthaler (1987).

Chlorophyll *a* fluorescence

The quantum efficiency of photosystem II (PSII) reaction centres was determined by the ratios of variable chlorophyll a fluorescence (Fv') to maximal chlorophyll a fluorescence (Fm') in light-adapted leaves using a portable fluorometer, Fluorpen fp100 (Photon Systems Instruments, Brno, The Czech Republic) using the equipment settings for quantum yield parameter.

Ascorbic acid, glutathione and pyridine nucleotides

Metabolites were measured in extracts from frozen plant material that had been homogenized in a pre-cooled mortar at liquid N_2 temperatures. Frozen 1 M perchloric acid was then added and the mixtures were homogenised until they had thawed. After centrifugation at 14000 *g* for 10 min (at 4°C), the pellets were discarded and the supernatants were used for assay of ascorbate and glutathione contents. Total ascorbate and the ratio between reduced ascorbate and its oxidized form, dehydroascorbate, were determined in extracts of leaf discs and nodules as described by Foyer *et al.,* (1983). Reduced glutathione (GSH) and homoglutathione (hGSH) were measured together with glutathione disulphide (GSSG) and homoglutathione disulphide (hGSSG) in extracts of leaf discs and nodules as described by Noctor and Foyer (1998). The spectrophometric technique used in these studies does not to discriminate between glutathione and homoglutathione. In these experiments, GSH was used to produce a standard curve and a correction factor of 2.6 was applied as described by Klapheck (1988) and has been used in other similar studies (Pérez-Chaca et al., 2014).

The reduced and oxidized forms of pyridine nucleotides were extracted from leaf discs and nodules and assayed as described by Foyer *et al.,* (2008). Oxidized pyridine nucleotides (NAD^+ and $NADP^+$) were extracted with acid as described for ascorbate and glutathione. Reduced pyridine nucleotides (NADH and NADPH) were extracted in 0.2 M NaOH and supernatant fractions were neutralized with 0.2 N HCl. Pyridine nucleotides were assayed using the phenazine methosulfate-catalyzed reduction of dichlorophenolindophenol in the presence of ethanol and alcohol dehydrogenase (for NAD^+ and NADH) or glucose 6-phosphate (G6P) and G6P dehydrogenase (for $NADP^+$ and NADPH) as described by Queval and Noctor (2007).

Ureide contents

Ureide contents were determined in extracts of leaf discs and nodules according to the method of Young and Conway (1942). Leaf discs and nodules were ground in a mortar that had been precooled to liquid N_2 temperatures and homogenised with frozen 0.2 M NaOH until the samples had thawed. Samples then were boiled for 20 min. After cooling, NaOH was added to the diluted extracts to ensure the conversion of allantoin to allantonic acid. The samples were then boiled to hydrolize allantonic acid before phenylhydrazine and potassium ferricyanide solutions are added and colour change measured at 525 nm. Calibration curves were performed using allantoin as the standard.

RNA extraction, cDNA synthesis, and qRT-PCR analysis

Real-time qPCR was performed as described previously (Pellny et al., 2009). Frozen nodule material was ground with a mortar and pestle in liquid nitrogen and about 100 mg fresh material was used for mRNA extraction using RNEasy Plant Minikit (Qiagen, France) according to the manufacturer's instructions. RNA reverse transcription and quantitative PCR were performed on an Eppendorf Realplex2 real-time PCR system by one-step RT-PCR using Quantifast SYBR Green RTPCR Kit (Qiagen) following the manufacturer's instructions. The data was analysed using the $2-\Delta\Delta CT$ method (Livak and Schmittgen, 2001). Relative expression was normalized against the constitutively expressed β-actine or soybean elongation factor. Melting curves were performed for each sequence in order to confirm the identity of amplification products. Accession numbers and sequences used for forward and reverse primers used are shown in Table 1.

Table 1. Primer sequences for amplification of different vacuolar, papain-like cysteine proteases and redox-associated proteins.

Primers for γECS, hGS, GR MDAAR and DHAR were designed based on NCBI reference sequences.

Phytozome ID	Forward primer (5' to 3')	Reverse primer (3' to 5')
Glyma18g52780 (Actin 1)	TGTTCCCTGGTATTGCTGAC	AAGGTGCTAAGAGATGCCAAG
Glyma02g44460 (ElF 1-β)	GTGGTACGATGCTGTCTCTC	CCACTGAATCTTACCCCTTGAG
γECS (XM_006586262.1)	CTCAAACAGGGGAAGCAGAG	CACTTTGGCTGGAAACCAAT
hGS (NM_001250121.1)	AATTCTGGTCGTGGTTCAGG	TGAAATTGCTTGTCCATCCA
MDAR (XM_006599044.1)	GTGGTGGGAATTGGAATACG	TGCTTTGACTGGAAATGCTG
DHAR (NM_001250000.1)	GAGATTGCTTTGGGGCATTA	TTCCACTTTAGGACGCCAAC
Glyma06g41610 (Tx1)	CAGTGGATAAAATTGTGGGTGC	GGCAACTGTTCAATGTGTTCG
Glyma07g09240 (Prx1)	TGAAAGGAAAGGGTGTGGAC	GAGGGCATTGGTGTATTTGG
Glyma09g32540 (Prx2)	ACGTTCCTGGCTTCATTGAG	TTCTCTGGGAATGTTTTGGC
Glyma12g13920(Grx1)	TTCCTTATAGGTCATGGCAACC	GTGTGGGCTAATTGTGAAGTG
Glyma16g07870 (Grx2)	ACCTATTGCCCTTTCTGC	TCCGTCCACTCAACTAATGC
Glyma17g14680 (VPE1)	CTACGGAAACTACAGGCATC	GTTCTCCGTCGTCACATTAT
Glyma05g04230 (VPE2)	CACCATCCCTTGTAAATTGT	GGGGTTTCAGTGCATAATAA
Glyma14g10620 (VPE3)	GGTCGTGGATGTTGCTGAGG	ATCTGCTTGATGCCTGTAGTTTCC
Glyma04g04400 (CP1)	GATCTTTAATGGCCACGATCCTCAT	CAGCACCTTGAAAGGGGTAATCCT
Glyma17g05670 (CP2)	GCTTGTCACTGCTCATTTTCGC	TTTTCCGGTGTAGGGATATGC
Glyma10g35100 (CP3)	GAGGCCATGCCCTCATGT	TCACCTCTCTCCCCAGTGTAGG
Glyma14g40670 (CP4)	ATATGGAGCGTGTGACTCGG	GTAATATCCATTCTCTCCCCAGCTC
Glyma04g03090 (CP5)	AAGCTGTGGTGCATGTTGGG	AGTGGCGCTTGTCTTTGCAG

Data analysis

The data presented are the means ± standard error (SE) of three independent plants per treatment and per time point. For comparisons and statistical analysis a t-test was performed for each level and time point between control and drought plants, and significance levels are indicated with asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

RESULTS

Water content in soybean tissues during drought treatment

Nodulated soybean plants were grown to the fifth trifoliate leaf stage, as illustrated in Fig. 1A. At this stage, the plants had lost their cotyledons and had two unifoliolate leaflets (L) and five trifoliolate leaves (TF). Batches of plants were then subjected to drought by depriving the plants of water for 21 days. In comparison to well-watered controls, the water contents of roots (Fig. 1B) and the vermiculite planting medium (Fig. 1B,C) decreased from day 9 onwards in plants experiencing drought (Fig. 1B, C). In contrast, leaf water contents were similar to the well-watered controls in all but the lowest leaf ranks of the plants subjected to drought. The lowest leaf rank, L, contained little water after 21 days of drought (Figure 1I). Similarly, TF1 had significant lower water contents than control plants after 21 days of drought (Fig. 1D). Visual inspection showed that the L leaves were dry and yellow, and the TF1 leaves were flaccid and yellow (Fig. 1 J).

Photosynthesis and leaf chlorophyll contents

The ratio of light-adapted variable chlorophyll *a* fluorescence (Fv') to maximal chlorophyll *a* fluorescence (Fm') was used as a measure of the photosynthetic capacity. Decreases in the Fv'/Fm' ratio indicate that fewer open reaction centres are available in photosystem II to undertake photochemistry. Despite the observation that the water contents of all but the

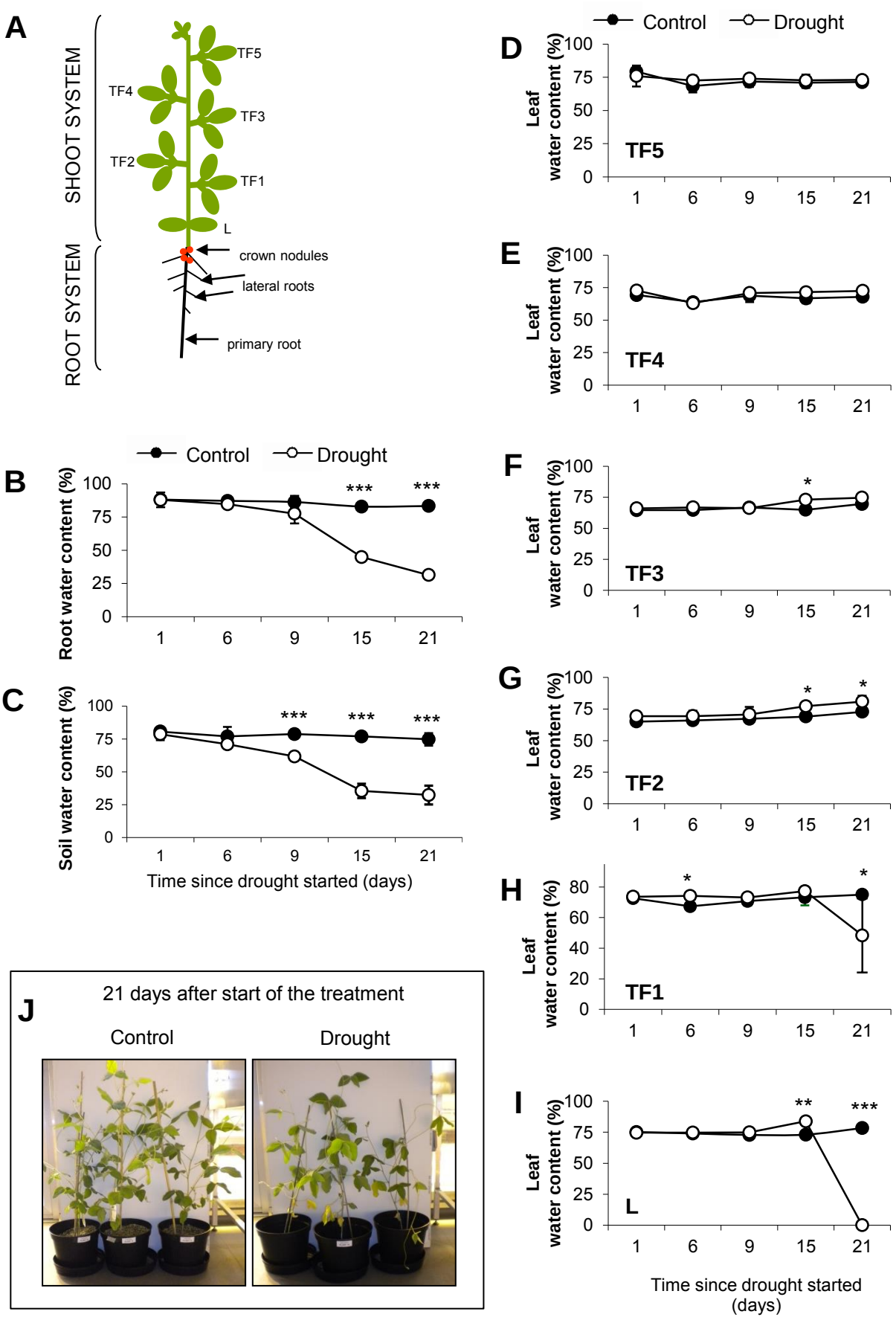

Figure 1. The effects of drought on leaf and soil water contents. A) Schematic representation of a soybean plant indicating the different leaves ranks at the time of the experiment (L = unifoliolate leaves, TF= trifoliolate leaves); B-I) root water content (B), soil water content (C), the water content of leaves at different ranks on the stem (D-I) and the phenotype of the plants after 21 days of treatment. (J). Data are mean ± SE of 3 different samples. Statistical differences for each time point between the control and drought are denoted by asterisk (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

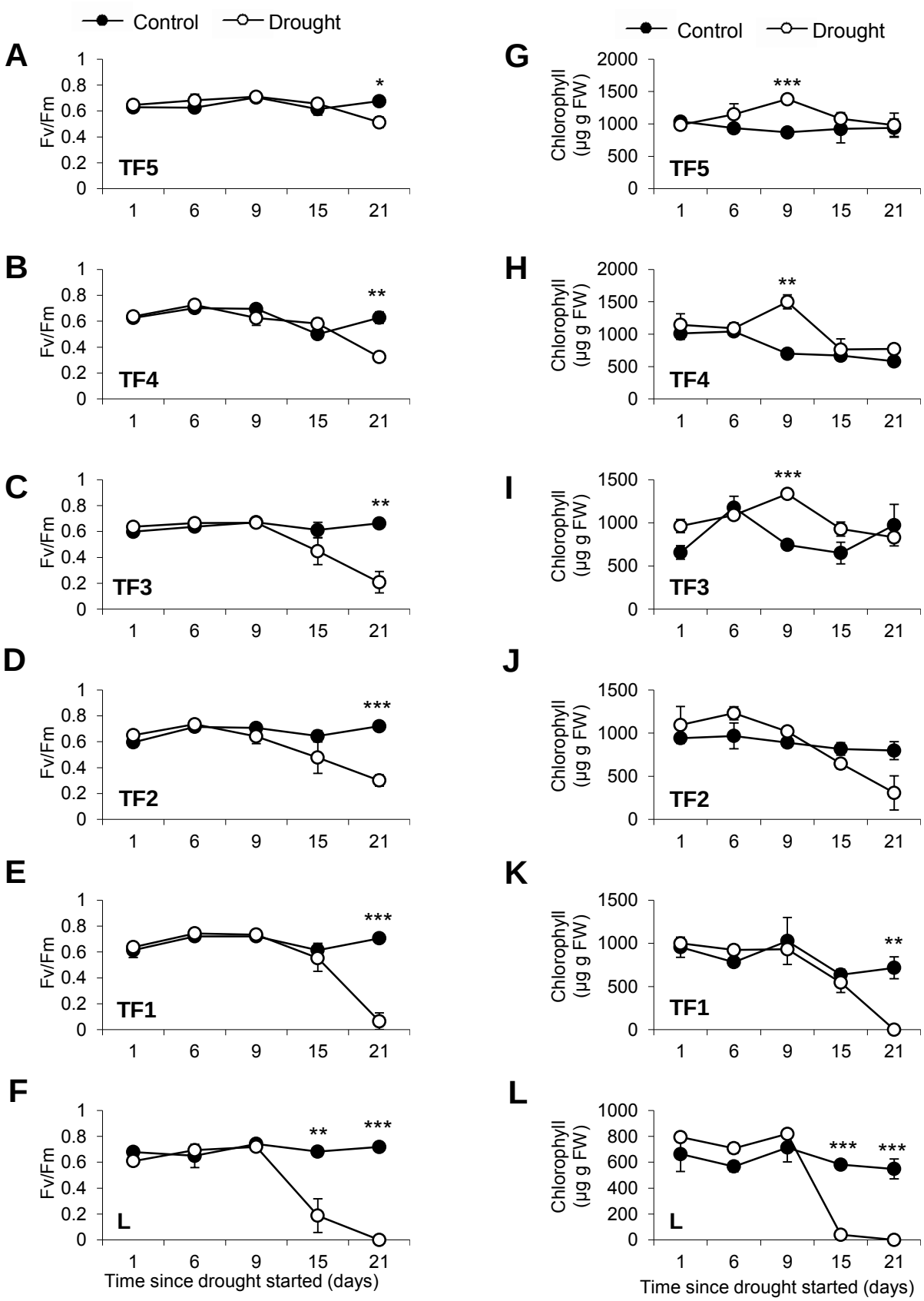

Figure 2. The effects of drought on photosystem II efficiency (Fv'/Fm') in light adapted leaves (A-F) and chlorophyll levels (G-L) in the different leaf ranks . Data are means ± SE of 3 different samples. Statistical differences for each time point between the control and drought are denoted by asterisk (* p<0.05, ** p<0.01, *** p<0.001).

lowest leaf ranks were similar in the plants experiencing drought to well-watered controls, the physiological impact of the imposed stress was observed by the decreased Fv'/Fm' ratios in all leaf ranks after 21 days of drought treatment (Fig. 2A-F). However, in contrast to other leaves, the Fv'/Fm' ratios of the TF1 leaf ranks were no longer measurable at day 21 indicating that complete inhibition of photosynthesis (Fig. 2E). Leaf rank L showed significant decreases in Fv'/Fm' ratios from day 15 of the drought treatment. , The Fv'/Fm' ratios were below the levels of detection day 21 as the leaves had already lost chlorophyll (Fig. 2G-L). The levels of chlorophyll were similar in youngest leaf ranks (TF3, TF4 and TF5) under both well-watered and drought conditions (Fig. 2A-D).

Leaf protein and ureide contents

Leaf protein contents were significantly decreased from day 15 in the TF1 and L leaf ranks (Fig. 3E,F). In contrast, protein levels were similar in the youngest leaf ranks (TF2-TF5) of plants experiencing drought stress and well-watered controls (Fig. 3A-D). A similar trend was observed with regard to the ureide contents of the youngest leaf ranks (Fig. 3H-K). However, the TF1 and L leaves accumulated ureides under drought stress relative to well-watered controls (Fig. 3M)

Leaf ascorbate, glutathione and pyridine nucleotide contents

The levels of total ascorbate (Fig. 4E) were similar in the youngest leaf ranks of well-watered controls and drought stressed plants (TF3-TF5; Fig. 4A-C). In contrast, the lower leaf ranks tended to accumulate ascorbate in response to drought (Fig 4.D-F). However, the reduction state of the ascorbate pool expressed as the percentage of pool that was present as the reduced form was decreased in all the leaf ranks after 21 days of drought (Fig. 4G-L).

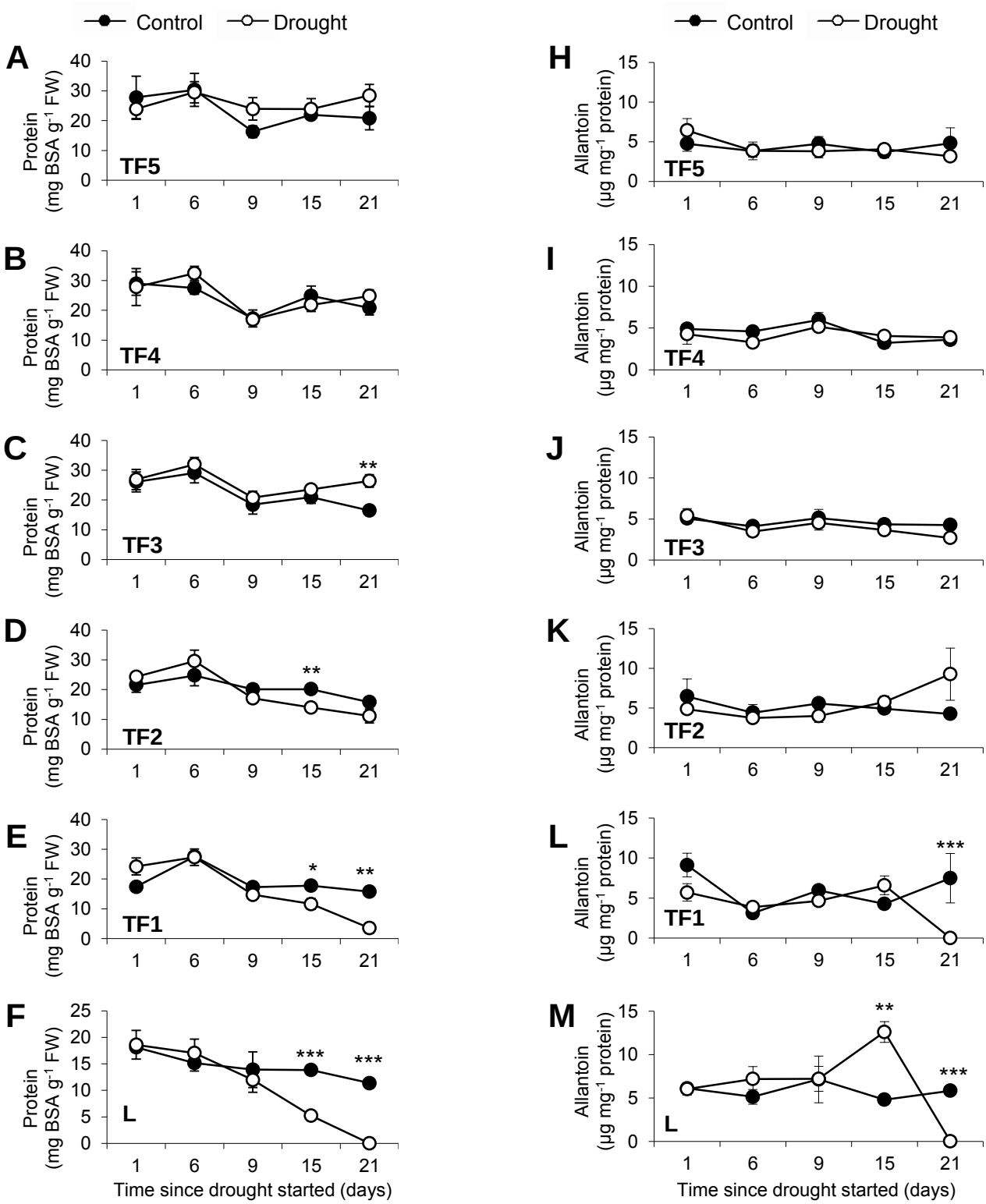

Figure 3. The effects of drought on the protein (A-G) and ureide (H-N) contents of leaves at different leaf ranks. Data are the mean ± SE of 3 different samples. Statistical differences for each time point between the control and drought are denoted by asterisk (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

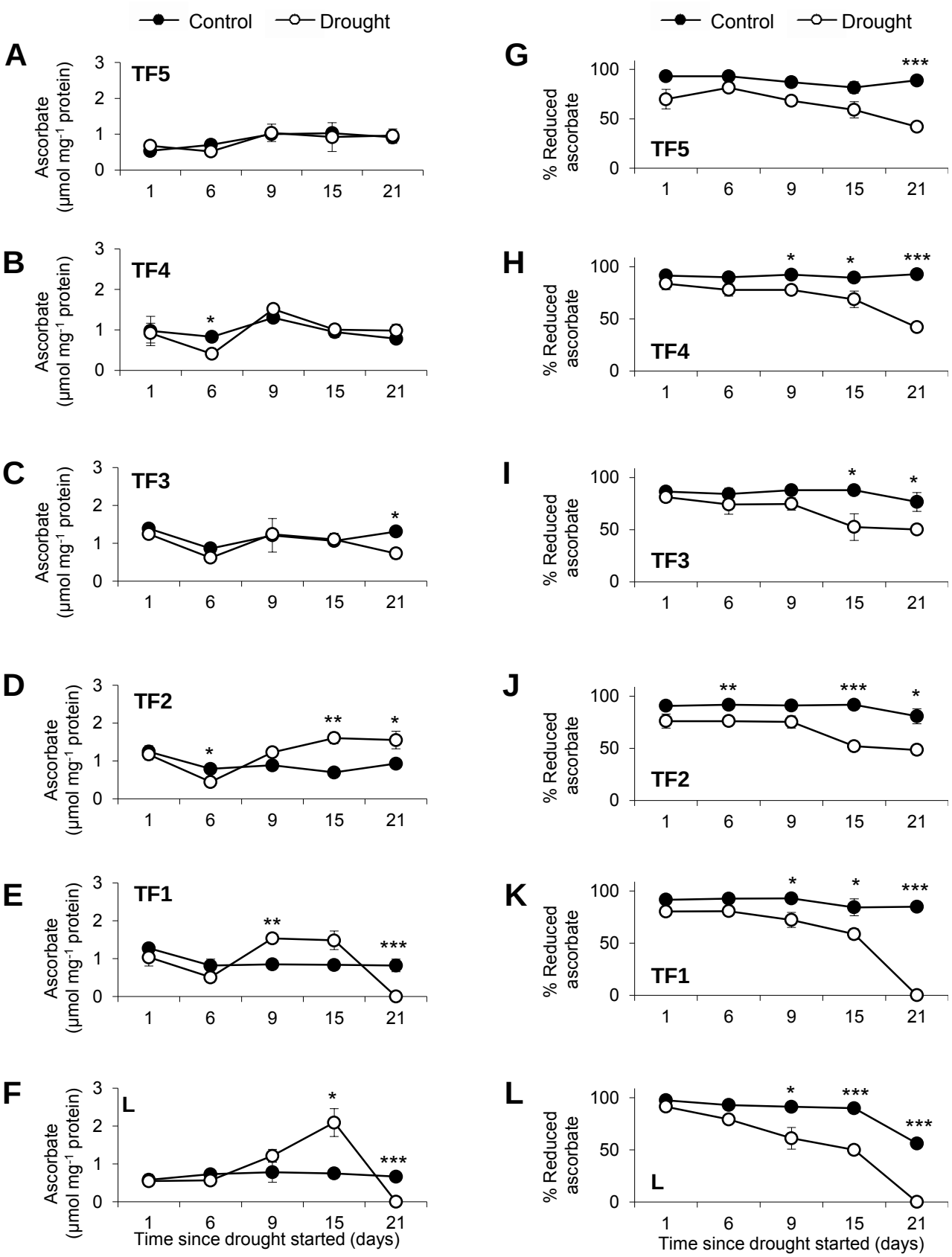

Figure 4. The effects of drought on the total (reduced plus oxidised) ascorbate levels (A-F) and on the percentage present in the reduced form (G-M) of the leaves at different leaf ranks. Data are the means ± SE of 3 different samples. Statistical differences for each time point between the control and drought are denoted by asterisk (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Table 2. The levels of pyridine nucleotides and percentage of the reduced forms in the leaves and nodules of well-watered plants and on plants deprived of water for 1 and 21 days. The plant leaf level codes are the same as in Figure 1.

Data are the mean ± SE. Significant differences of each drought time point compared with the well-watered plants are indicated with asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Plant leaf level	NAD + NADH (nmol mg^{-1} protein)			% NADH		
	Well-watered	**Drought (day 1)**	**Drought (day 21)**	**Well-watered**	**Drought (day 1)**	**Drought (day 21)**
TF5	1.87 ± 0.33	1.80 ± 0.13	1.05 ± 0.14	20.8 ± 0.9	24.1 ± 5.5	26.3 ± 7.8
TF4	1.71 ± 0.43	2.46 ± 0.48	1.25 ± 0.09	24.0 ± 2.6	19.2 ± 2.9	21.9 ± 1.4
TF3	1.42 ± 0.25	1.99 ± 0.14	0.97 ± 0.02	20.0 ± 1.0	23.8 ± 2.5	23.4 ± 4.6
TF2	1.42 ± 0.30	0.98 ± 0.23	1.98 ± 0.27	23.4 ± 1.8	31.5 ± 5.6	27.7 ± 1.9
TF1	1.77 ± 0.17	1.89 ± 0.16	0.00 ± 0.00 ***	20.0 ± 1.2	19.3 ± 3.7	0.0 ± 0.0 ***
L	1.76 ± 0.27	2.15 ± 0.19	0.00 ± 0.00 ***	24.5 ± 1.6	20.2 ± 2.2	0.0 ±0.0 ***
nodule	6.85 ± 0.41	7.01 ± 0.66	5.34 ± 0.70	23.8 ± 0.6	25.3 ± 3.0	21.8 ± 0.2 *
	NADP + NADPH (nmol mg^{-1} protein)			**% NADPH**		
	Well-watered	**Drought (day 1)**	**Drought (day 21)**	**Well-watered**	**Drought (day 1)**	**Drought (day 21)**
TF5	1.69 ± 0.22	1.84 ± 0.29	1.09 ± 0.07	44.6 ± 5.8	40.8 ± 12.5	58.3 ± 6.6
TF4	1.33 ± 0.07	1.23 ± 0.04	1.15 ± 0.16	44.3 ± 5.2	21.5 ± 3.2	50.8 ± 8.7
TF3	1.18 ± 0.17	1.48 ± 0.22	0.99 ± 0.16	44.4 ± 6.1	28.2 ± 1.9	50.6 ± 8.1
TF2	1.48 ± 0.12	1.40 ± 0.17	1.64 ± 0.37	42.5 ± 4.5	29.6 ± 4.3	56.3 ± 4.8
TF1	1.57 ± 0.16	1.35 ± 0.14	0.00 ± 0.00 ***	35.1 ± 4.6	30.2 ± 4.3	0.0 ±0.0 ***
L	1.26 ± 0.17	1.55 ± 0.16	0.00 ± 0.00 ***	37.7 ± 3.3	27.4 ± 5.9	0.0 ± 0.0 ***
nodule	1.04 ± 0.05	0.99 ± 0.07	1.58 ± 0.14**	30.5 ± 3.4	33.6 ± 6.0	32.8 ± 4.6

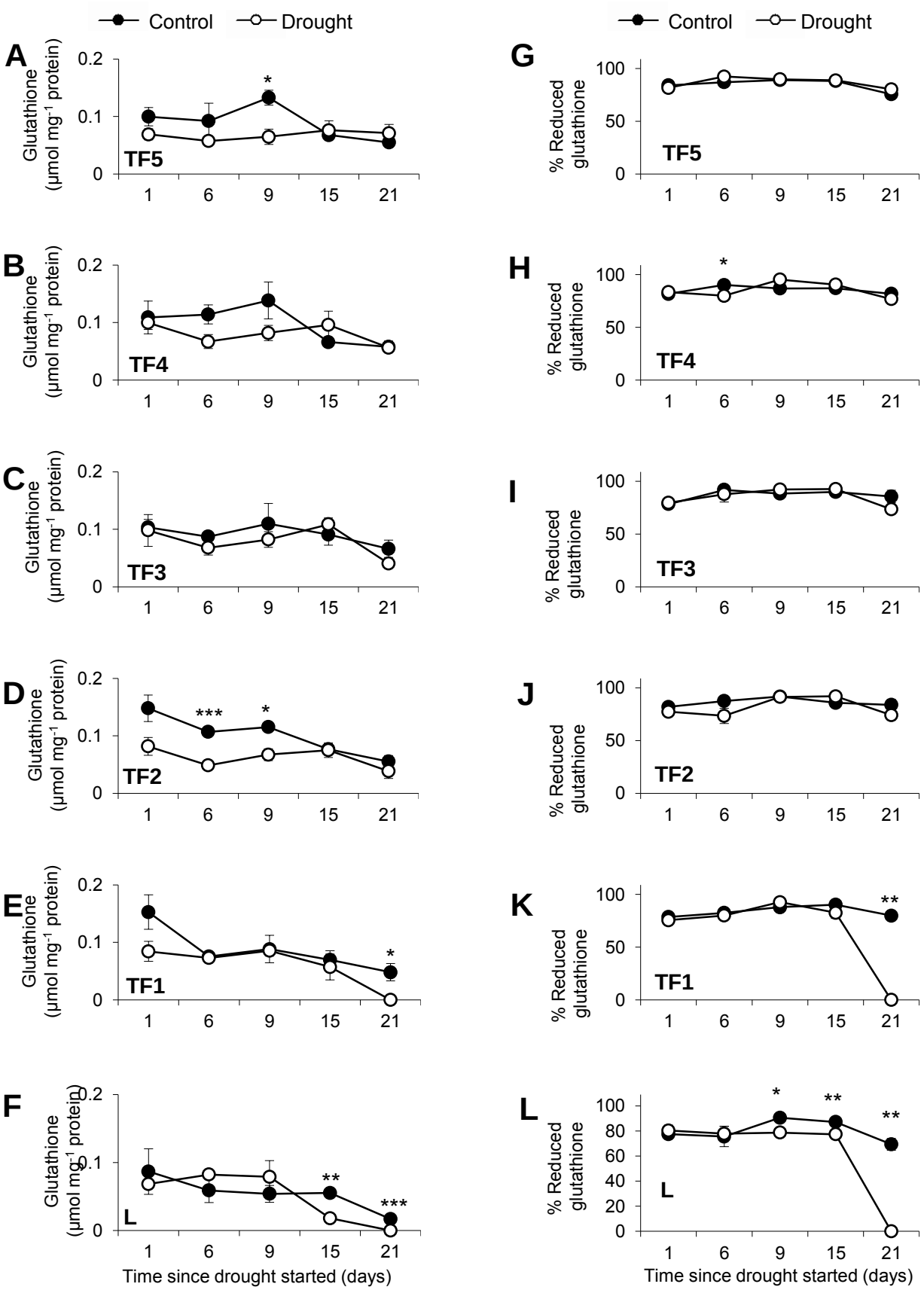

Figure 5. The effects of drought on the total glutathione and homoglutathione (GSH and hGSH plus GSSG and hGSSG) pool (A-F) and percentage of reduced form (G-M) in the leaves at different leaf ranks. Data are the mean s± SE of 3 different samples. Statistical differences for each time point between the control and drought are denoted by asterisk (* p<0.05, ** p<0.01, *** p<0.001).

The levels of total glutathione (GSH plus GSSG) and homoglutathione (hGSH plus hGSSG) tended to decrease with leaf development, a feature that was observed in all but the youngest leaf ranks (Fig. 5). Drought had little effect on the abundance of glutathione (GSH plus GSSG) and homoglutathione (hGSH plus hGSSG) in all but the lowest leaf ranks, which had significantly lower levels of these antioxidants after 21 days of the drought stress treatment relative to well-watered controls (Fig. 5F). Moreover, the reduction state of the glutathione pool expressed as the percentage of pool that was present as GSH plus hGSH was lower in the TF1 leaf ranks relative to well watered controls after 21 days of drought (Fig. 5K).

The levels of NAD and NADH were significantly decreased in the youngest leaf ranks (TF2, TF3, TF4 and TF5) of plants exposed to drought stress compared to well-watered controls (Table 2). In contrast levels of NADP and NADPH were similar in the youngest leaf ranks of drought-treated plants and well-watered controls (Table 2).

The effects of drought on nodule parameters

Crown nodules harvested throughout the 21 days of the drought stress treatment were not greatly visually different from the crown nodules on the well-watered controls (Fig. 6A), except that they were slightly less pink in the central zone, where developmental senescence is known to be initiated (Puppo *et al.,* 2005). In contrast to leaves, nodule protein (Fig. 6B) ureide (Fig. 6C) contents increased as a result of drought treatment. While the reduction state of the nodule ascorbate pool was significantly lower than in well-watered controls even on the first days of the stress treatment (Fig. 6E), the reduction state of the nodule glutathione pool (Fig. 6G) was similar in the nodules of well-watered controls and plants experiencing drought. While pyridine nucleotides were not detectable in the TF1 leaf ranks after 21 days of

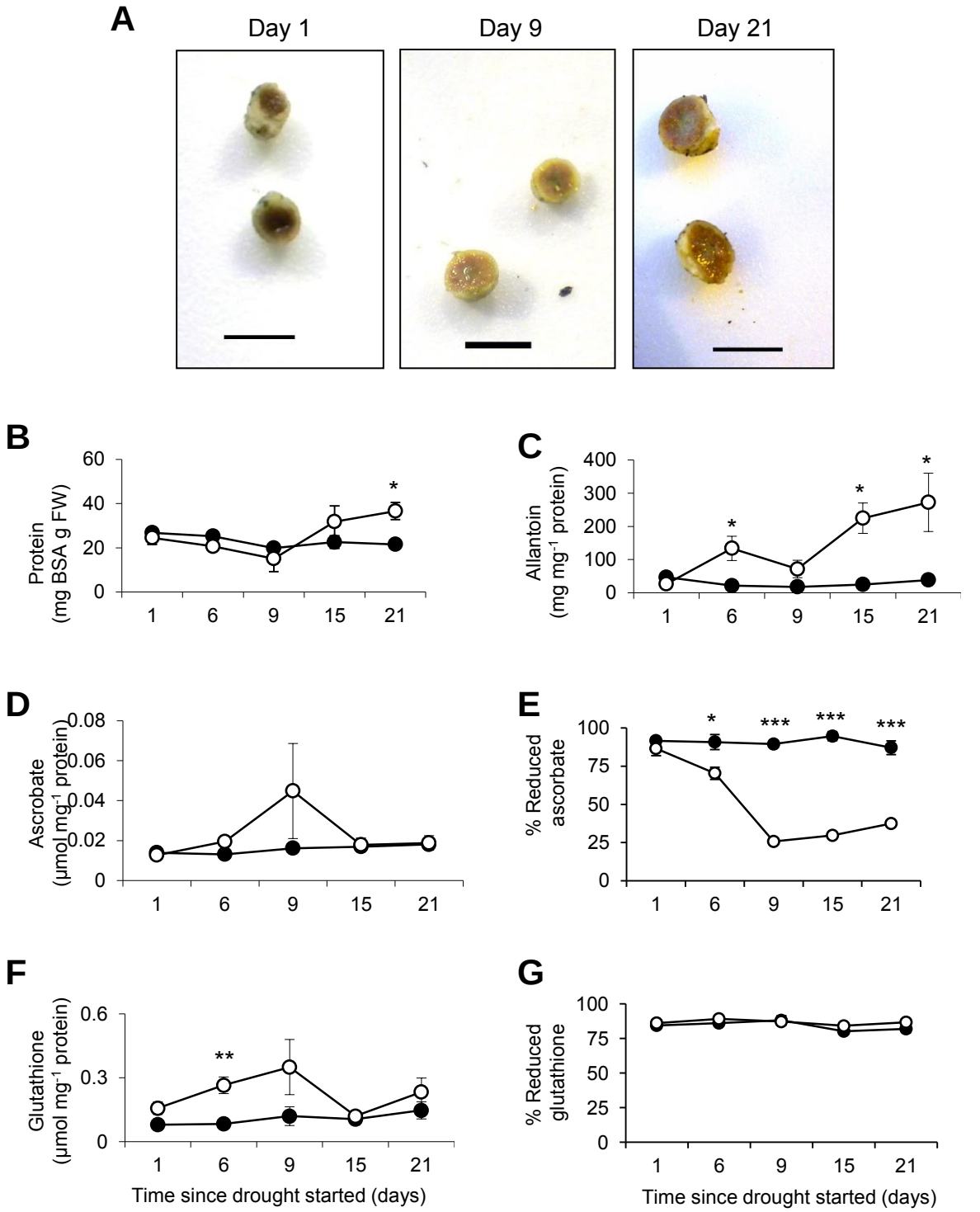

Figure 6. The effects of drought on the nodule phenotype (A) 1, 9 and 21 days after the onset of drought treatment, (B) protein content, (C) ureide content, measured as allantoin, (D) total ascorbate, (E) the percentage of reduced ascorbate, (F) total glutathione plus homoglutathione content, (G) percentage of reduced glutathione plus homoglutathione. In B-G data are the means ± SE of 3 independent nodule samples. Statistical differences for each time point between the control and drought are denoted by asterisk (* $p<0.05$). Scale bar = 0.5 cm

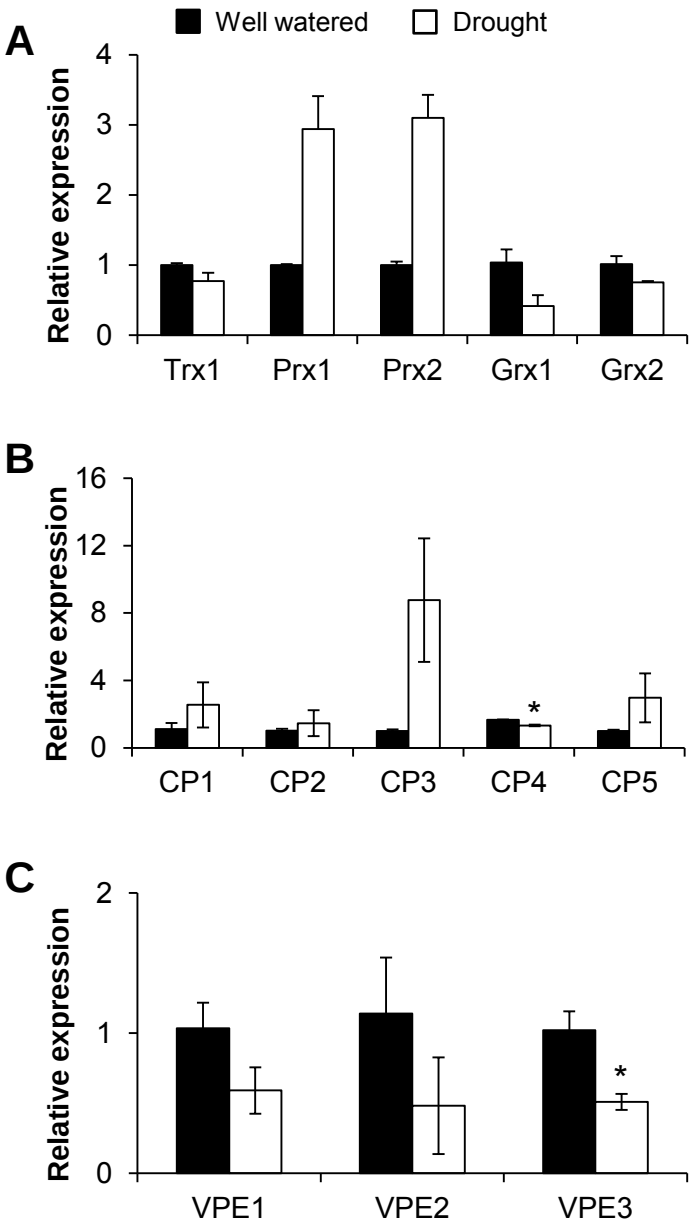

Figure 7. The effects of drought on the abundance of selected transcript in nodules. (A) Thioredoxin (TRX1, Glyma06g41610), peroxiredoxins (Prx1 (Glyma07g09240) and Prx2 (Glyma09g32540) and glutaredoxins (Grx1 (Glyma12g13920) and Grx2 (Glyma16g07870)), (B) Cysteine proteases (CP1 to CP5, Glyma04g04400, Glyma17g05670, Glyma10g35100, Glyma14g40670 and Glyma04g03090 respectively). (C) Vacuolar processing enzymes (VPE1 to VPE3, Glyma17g14680, Glyma05g04230 and Glyma14g10620 respectively). Data are the means ± SE of 3 different samples of nodules harvested from either well watered or drought-treated plants at day 15 of the experiment Statistical differences for each time point between the control and drought are denoted by asterisk (* $p<0.05$).

drought, nodule pyridine nucleotide levels were either similar to well-watered controls or even increased (Table 2).

To explore the effects of drought further, we selected a number of nodule-expressed sequences encoding different proteins associated with redox regulation and senescence (cysteine proteases, CP, and vacuolar processing enzymes, VPE). The abundance of transcripts encoding a thioredoxin (TRX1, Glyma06g41610) and two glutaredoxins (Grx1, Glyma12g13920, and Grx2, Glyma16g07870) were similar in the nodules from well-watered and drought stressed plants. The abundance of transcripts encoding two peroxiredoxins (Prx1, Glyma07g09240, and Prx2, Glyma09g32540) however was higher in the plants exposed to drought for 15 days than the well-watered controls (Fig. 7A). Of the selected transcripts encoding CPs (Fig. 7B) and VPEs (Fig. 7C) only CP4 (Glyma14g40670) and VPE3 (Glyma14g10620) were significantly reduced changed as a result of drought treatment (Fig. 7B,C). Although values were not significant, CP3 transcripts tended to increase, possibly indicating the onset of nodule senescence (Fig. 7B). Of the transcripts encoding nodule antioxidant enzymes that were selected for further analysis, only transcripts encoding dehydroascorbate reductase (DHAR) were significantly changed as a result of drought treatment (Fig. 8). DHAR transcripts were decreased in the nodules of plants exposed to drought for 9 and 21 days compared to those of one day of drought (Fig. 8).

DISCUSSION

The negative impact of drought on symbiotic N_2 fixation in soybean root nodules is well documented (Sinclair and Serraj, 1995; King and Purcell, 2005; Marino *et al.,* 2007). Several mechanisms could account for the stress-induced inhibition of nitrogenase activity including

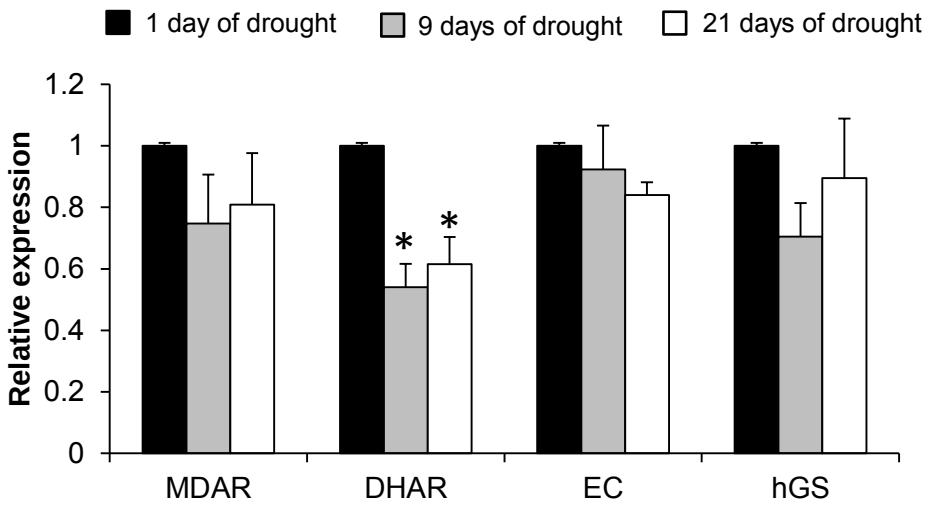

Figure 8. The effects of drought on the abundance of selected transcripts encoding antioxidant enzymes. Samples of nodules were harvested from either well watered or drought-treated plants on days 1, 9 and 21 days of the experiment. Data are the means ± SE of 3 different samples. Statistical differences for each time point between the control and drought are denoted by asterisk (* $p<0.05$).

(1) carbohydrate depletion, (2) changes in the oxygen diffusion barrier, and (3) feedback regulation by ureide accumulation (Serraj *et al.,* 1999, Van Heerden *et al.,* 2008). Drought-induced decreases in nitrogenase activity may result from changes in carbon (Gordon *et al.,* 1999) and/or nitrogen metabolism (Ladrera *et al.,* 2007). While we have not measured nitrogenase activity in the present study, the data presented in Fig. 6C support the evidence from earlier studies (Vadez *et al.,* 2000, Ladrera *et al.,* 2007) showing that ureides accumulate in the nodules of plants experiencing drought stress. Therefore, if nitrogenase activity was inhibited as a result of the imposition of drought, the extent of inhibition experienced under these conditions was not sufficient to prevent ureide accumulation in the nodules. Legume species that produce ureides are known to be more sensitive to drought than those that produce and transport amides because of lower ureide solubility. It is possible that ureide accumulation in the nodules causes a negative feedback inhibition of symbiotic N_2 fixation (Sinclair and Serraj, 1995). However, the drought-induced increases in nodule ureides measured in the present study were accompanied by higher levels of nodule protein, pyridine nucleotides, ascorbate and glutathione (Fig. 6). The accumulation of these low molecular weight antioxidants suggests that the nodule response to the imposition of drought, is to shore-up the defences that perturbations in cellular redox state.

It is now well established that glutathione and homoglutathione play important roles in the nodulation process and in drought tolerance **(**Matamoros *et al.,* 1999, Frendo *et al.,* 2005). While we were not able to measure the homoglutathione pool in these studies, the redox state of the glutathione pool was similar in the nodules of well-watered plants and those that were experiencing drought stress. The drought response measured in the present study showed that the nodule appears to be rich in metabolites, antioxidants and protein. Drought-induced structural changes in the oxygen diffusion barrier have been reported to alter nodule

permeability to O_2 lowering in symbiosome O_2 concentrations (O_i) leading to inhibition nitrogenase activity indirectly because of lower nodule respiratory activity (Serraj and Sinclair, 1996). Conversely, stress-induced changes to nodule O_2 homeostasis can also lead to an increase in O_i and the fractional oxygenation of leghemoglobin (Kuzma *et al.,* 1995). Low symbiosome O_i would favour decreased ROS production and high levels of reduction of the nodule ascorbate and glutathione/homogluthaione pools. The data presented here show that the nodule ascorbate pool was significantly less reduced in drought-stressed plants compared to well-watered controls. This finding might suggest that nodule ROS production was increased rather than decreased and hence nodule O_i was increased following the imposition of drought stress.

After 21 days of drought, the stress had an impact on photosynthesis (as measured by the Fv'/Fm' ratio) and cellular redox state (as indicated by the enhanced oxidation of the ascorbate pool) the leaves at all ranks on the stem, the negative impacts of drought being most severe in the TF1 and L leaf ranks. The imposition of drought stress leads to a genome-wide preprograming of gene expression regulation in leaves (Molina, *et al.,* 2008). However, the effects of drought on the nodule transcriptome are much less well characterised (Afonso-Grunz *et al.,* 2014). A similar sequence of events has been suggested to occur in leaf and nodule senescence programs in which catabolic nutrients are recycled, followed by organ degeneration (Van de Velde *et al.,* 2006). The most abundantly expressed genes in the senescent zones of the *Medicago truncatula* nodule were cysteine proteases (CP) that were highly homologous to SAG12, a well characterised marker of leaf senescence (Van de Velde *et al.,* 2006). CPs are synthesized in legume nodules during senescence (Asp *et al.,* 2004). In order to determine whether the drought stress treatment used in these studies triggered nodule senescence, we selected five CP sequences from the soybean database (www.soybase.org),

which we have designated CP1 to CP5 (Glyma04g04400, Glyma17g05670, Glyma10g35100, Glyma14g40670 and Glyma04g03090, respectively) and three sequences encoding vacuolar processing enzymes which we have designated VPE1 to VPE3 (Glyma17g14680, Glyma05g04230 and Glyma14g10620, respectively). We selected these proteases because the levels of the transcripts encoding these enzymes were changed during nodule senescence as measured by RNAseq analysis (data not shown; van Wyk, unpublished results). The abundance of transcripts encoding cysteine proteases Glyma04g04400, Glyma17g05670, Glyma10g35100 was decreased during the process of developmental nodule senescence during nodule senescence while Glyma14g40670 and Glyma04g03090 and the mRNAs encoding the VPEs measured here was increased as determined in our RNAseq analysis (van Wyk, unpublished results). Similarly, the genes related to redox processes that were measured in this study were selected because their transcripts were changed in abundance by more than 50-fold as a result of developmental nodule senescence in our RNAseq analysis (van Wyk, unpublished results).

VPEs are involved in protein re-mobilisation during leaf senescence and seed-set (Muntz and Shutov, 2002). None of the selected transcripts encoding CPs or VPEs (Fig. 7B, C) were increased in abundance in the nodules as a result of drought treatment. This finding suggests that there had been no substantial triggering of the nodule senescence program at this stage of the drought stress treatment. We also selected sequences from the soybean database that encode proteins involved in redox-related processes in the nodules (Figs. 7, 8.) to determine whether the drought-induced enhanced oxidation of the nodule ascorbate pool was accompanied by changes in the levels of redox-related transcripts. While the abundance of very few redox-related transcripts was changed in response to drought as has been noted earlier in leaves (Noctor *et al.,* 2014), the abundance of transcripts encoding two

peroxiredoxins which we have called Prx1 (Glyma07g09240) and Prx2, (Glyma09g32540) were significantly higher in the plants exposed to drought than in the well-watered controls (Fig. 7A). Prx1 has homology to PrxIIC, whose expression is induced by oxidants such as hydrogen peroxide and lipid peroxides and also by the addition of ascorbate (Horling *et al.,* 2003). Prx2 (also called TPX1 in the database) encodes a thioredoxin-dependent peroxidase of unknown function. These findings may support the view that oxidative stress in nodules is caused by enhanced ROS formation rather than a decrease in the antioxidant defences (Evans *et al.,* 1999).

Leaf senescence is a highly regulated catabolic process in which leaf constituents are remobilised and transported to other plant organs. The progression of leaf senescence in different ranks on the stem is influenced by the availability of light and hence photosynthetic carbon metabolism that underpins the fitness of plants, particularly when they are grown within in dense canopies (Brunel-Muguet *et al.,* 2013). Senescence begins with the lowest leaves on the shoot. The light gradient or partial shading of lower leaves is considered to regulate the order of the senescence process in order to allow remobilization of resources toward leaves that are exposed to higher light and hence have higher photosynthetic capacities (Boonman *et al.,* 2006). The partial shading of leaves in dense canopies is thought to lower transpiration rates and hence of cytokinin delivery to lower leaves resulting in lower photosynthetic activities that in some way targets the leaves for early senescence (Boonman *et al.,* 2006). The program of drought-induced senescence observed in this study clearly targets the lowest leaves on the shoot, which had lost all their protein, chlorophyll and photosynthetic capacity after 21 days of water deprivation. The data presented here clearly demonstrate that the senescence program is complete in the lower leaves before any molecular or metabolic markers (such as loss of protein) are observed in the crown nodules. This suggests that

senescence of the lower leaves precedes that of the nodules and younger leaves. This finding is important in understanding the stress response because it appears that symbiotic N_2 fixation and ureide production are maintained in a similar manner to the younger more photosynthetically-competent leaves by re-allocation of carbon resources from the lower less leaves.

The data presented here show that soybean plants were not deprived of nitrogen as a result of drought because the products of symbiotic nitrogen fixation were abundant in the leaves even after 21 days of exposure to this stress. While we cannot rule out a deficiency in other nutrients such as phosphate under the conditions used in this study, we consider that the experimental design reflects the natural situation that occurs when plants are deprived of water. Changes in sub-sets of transcript profiles can be useful indicators of deficiencies in different nutrients. We have performed an RNAseq analysis in similar studies on drought in soybean and we have not found any transcript changes that would indicate major deficiencies in essential nutrients. We have not included phenotypic parameters for soybean growth in response to drought in this study because we have previously extensively characterised the effects of drought on a range of shoot and root parameters in different soybean cultivars grown under glasshouse or field conditions (Fenta *et al.,* 2012; 2014). Such studies have demonstrated the importance of maintaining shoot growth, photosynthesis and symbiotic nitrogen fixation in drought tolerance in soybean (Fenta *et al.,* 2012; 2014).

CONCLUSION

While plant responses to drought are complex and need to be analysed at system-levels using genomics and physiological approaches, it is also important to understand the order and sequence of events that underpin survival strategies. The concept that the nodules are the first target of drought stress is well established in the literature but the data presented here

demonstrate that this is not the case. Rather, the soya plants balance the provision of nitrogen and carbon resources and the lower leaves are sacrificed prior to the nodules. Preserving the nodules rather than leaves of low photosynthetic capacity clearly has a physiological advantage in stress situations. The next step is therefore to characterise the genes that expressed in the nodule in order to preserve symbiotic N_2 fixation at the early stages of drought. Here we have identified transcripts encoding two thioredoxin-dependent peroxidases as being important in the drought-induced response program. While little is known about the functions of thioredoxin-dependent peroxidases, many are thought to have signalling as well as defensive functions. These proteins are therefore potential new targets for further study in relation to signalling and defence functions at the crucial early stages of the nodule response to drought. This new knowledge adds to the body of information required for the improvement of soybeans that is required to sustain the wide range of soya applications in the food and animal feed industries.

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