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Antioxidative defence under drought stress in a wheat stay-green mutant

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Abstract

A wheat stay-green mutant, named *tasg1*, was generated using the mutagen ethyl methane sulphonate applied to wheat (*Triticum aestivum* L.) cv. HS2. A drought stress was imposed by controlling irrigation and sheltering plants from rain. The antioxidant defence was characterized in the flag leaves of the *tasg1* and wild-type (WT). Compared with WT, *tasg1* had higher reduced ascorbate/oxidized ascorbate ratio, reduced glutathione/oxidized glutathione ratio, and antioxidant enzyme activities during senescence under both normal and drought stress conditions. The *DHAR* gene expression remained higher in *tasg1* than in WT during the drought stress and *tasg1* had a higher antioxidant defence competence which may contribute towards the delayed leaf senescence. The different transcriptional responses of some wheat senescence-associated genes to the drought stress between *tasg1* and WT were observed. These results suggest that the competent antioxidative capacity might play an important role in the enhanced drought tolerance in *tasg1*.

Additional key words: ascorbate-glutathione cycle, reactive oxygen species, senescence-associated gene, *Triticum aestivum*.

Introduction

Drought is the primary limitation to wheat production worldwide (Møller *et al.* 2007) and disturbs almost all plant functions (Yamaguchi-Shinozaki *et al.* 2002). Mutants that retain greenness during senescence are collectively called stay-green mutants and different types (A, B, C, D, and E) have been defined (Thomas and Howarth 2000). They are further divided into functional stay-green and non-functional stay-green mutants depending on whether retention of green colour is coupled with retention (functional stay-greens) or loss (non-functional stay-greens) of photosynthetic activity (Thomas and Howarth 2000). The first two classes (type A and B) are functionally stay-green, and the remaining three are non-functional. An increased drought resistance in some stay-green mutants was confirmed. Rivero *et al.* (2007) reported that the suppression of drought-induced leaf senescence by the production of cytokinins in transgenic tobacco plants results in outstanding drought tolerance and improved plant survival and yield. Borrell *et al.* (2000) also reported that stay-green sorghum hybrids produced 47 % more post-anthesis biomass than their counterparts under terminal moisture deficit conditions. But the mechanism underlying the increased drought resistance in some stay-green mutants remains unknown.

We generated a new wheat mutant, *tasg1*, with delayed leaf senescence using ethyl methane sulphonate (EMS) as mutagen. *Tasg1* is a functional stay-green mutant with drought resistance characteristics according to our previous studies (Tian *et al.* 2012, Hui *et al.* 2012). In the present work, the antioxidant defence mechanism and the expression of senescence-associated genes were analyzed. These data will be helpful for better understanding the stay-green mechanism and for improving the drought resistance of wheat cultivars.

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbate; Chl - chlorophyll; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; DS - drought stress; EDTA - ethylenediamine tetraacetic acid; EMS - ethyl methane sulphonate; GR - glutathione reductase; GSH - glutathione; GSSG - oxidized glutathione; H_2O_2 - hydrogen peroxide; MDHAR - monodehydroascorbate reductase; $O_2^{\bullet-}$ - superoxide radical; R-OOH - hydroperoxides; ROS - reactive oxygen species; SAG - senescence-associated gene.
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Materials and methods

A wheat stay-green mutant, named *tasg1*, was generated in our laboratory using the mutagen EMS applied to a wheat (*Triticum aestivum* L.) cultivar HS2 which is further considered as wild-type (WT). Plants were grown in the field of the Shandong Agricultural University, China, during the growing seasons (October to June) of 2011 and 2012. Six 4 m^2 interspersed plots were established *via* random block design in the field, with three replicate plots for each genotype. Conventional agricultural management was maintained during growth and development of the seedlings. A drought stress (DS) was imposed by controlling irrigation and sheltering the plants from rain from May 1st to May 15th (flowering stage); plants under normal water conditions served as controls (CK). We harvested the flag leaves to determine physiological and biochemical parameters every five days after the drought stress was imposed, and we immediately submersed them in liquid nitrogen and stored at -80 °C till used.

Water content of the soil was measured after 10 d of DS and statistically significant differences between DS and CK were achieved. The soil water content in 0 - 20 cm and 20 - 40 cm depths was 6.32 and 7.58 % in CK, and 4.88 and 7.13 % in DS, respectively.

Total leaf H_2O_2 content was measured according to the method described by Sui *et al.* (2007) with slight modifications. Leaf samples (0.2 g) were homogenized with 3 cm^3 of a 50 mM phosphate buffer (pH 6.8). The homogenate was centrifuged at 6 000 *g* for 25 min. The supernatant was mixed with 1 cm^3 of 0.1 % (m/v) titanium sulfate in 20 % (m/v) H_2SO_4, and the mixture was then centrifuged at 6 000 *g* for 15 min. The absorbance change at 410 nm was monitored using a UV-visible spectrophotometer (*Shimadzu UV-1601*, NSW, Australia). H_2O_2 content was calculated according to the standard curve plotted with known concentrations of H_2O_2. Hydroperoxides (R-OOH) were assayed using ferric-xylenol orange as described by Gay and Gebicki (2000).

Leaf discs (1 g) were homogenized in 5 cm^3 of 10 % (m/v) trichloroacetic acid containing 1.5 mM ethylene-diaminetetraacetic acid (EDTA). After centrifugation at 14 000 *g* for 15 min, the supernatant was diluted 1:50 with 5 % (m/v) Na_2HPO_4 (pH 7.5) for neutralization. Total ascorbate (AsA) content was determined according to the method of Arakawa *et al.* (1981). Dehydro-ascorbate (DHA) content was calculated as the difference between total AsA and reduced AsA. Glutathione (GSH) metabolite pool was measured using the enzymatic recycling assays according to the method of Anderson *et al.* (1992). All values are reported as GSH equivalents according to the standard curve plotted with known concentrations of GSH.

Antioxidant enzymes were extracted by grinding 0.5 g of fresh flag leaves with 5 cm^3 of an extraction buffer consisting of 0.05 M phosphate, pH 7.8, 0.1 mM EDTA, and 1 % (m/v) polyvinylpyrrolidone (PVP) in an ice bath and then centrifuged at 12 000 *g* and 4 °C for 20 min. The supernatant was used for the following enzyme assays. Glutathione reductase (GR; EC 1.6.4.2) activity was assayed according to Schaedle and Bassham (1997). Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was measured according to Hossain and Asada (1984). The rate of reaction was corrected for the non-enzymatic reduction of dehydroascorbate by reduced glutathione. Monodehydroascorbate reductase (MDHAR; EC1.6.5.4) activity was measured according to Arrigoni *et al.* (1981). The absorbance change of 0.1 in 1 min was defined as one unit of GR, DHAR, and MDHAR activities.

For laboratory experiments, seeds of both WT and *tasg1* germinated at 25 ± 1 °C for 24 h on filter paper moistened with water after being sterilized with 0.2 % (m/v) sodium hypochlorite. The seeds were then placed in an orderly fashion on a nylon gauze sheet at an appropriate density and cultured in trays (25 cm × 18 cm × 5 cm) containing a Hoagland solution. These trays were placed in a growth chamber at a temperature of 25 ± 1 °C, a 12-h photoperiod, an irradiance of 300 μmol m^{-2} s^{-1}, and a relative humidity of 70 %. After complete unfolding, second leaves were harvested for senescence-inducing treatments as described below. An osmotic stress was induced using a 20 % (m/v) polyethylene glycol (PEG) 6000 solution (the osmotic potential of -1.45 MPa). The solution was changed every 12 h.

Total RNA was extracted from the control and PEG-treated wheat leaves with a *Trizol* reagent (*TaKaRa*, Shiga, Japan) according to the manufacturer's protocol and then was treated with *DNaseI* (RNase-free, *Promega*, Madison, USA). The total RNA was subjected to the first-strand cDNA synthesis with a *RevertAid* first strand cDNA synthesis kit (*Fermentas*, USA) according to the manufacturer's protocol. The cDNA was diluted to 0.1 cm^3 with sterile water. Real-time PCR was performed using the first-strand cDNA generated above, and selected primers (listed in Table 1) were described previously (Baek and Skinner 2003) or designed using the *Primer Premier 5.0* software. Each primer was composed of about 20 nucleotides with melting temperatures around 60 °C. A primer set for each antioxidant enzyme was designed to produce an amplicon ranging from about 90 to 110 nucleotides. The *tubulin* cDNA was used as reference. *Tubulin* has been accepted widely as housekeeping gene in growing plants (Coker and Davies 2003). PCR was carried out in a 0.02 cm^3 reaction containing a 1 × *SYBR Green* PCR master mix (*Tiangen*, Beijing, China), 500 nM forward and reverse primers, and 0.002 cm^3 of cDNA template. The quantitative analysis was performed using the *Bio Rad CFX Manager* (Hercules, USA) system with PCR conditions of 40 cycles at 94 ºC for 20 s, 61 ºC for 30 s,

and 68 ºC for 35 s. The absence of primer-dimer formation was examined in single and no-primer controls. Each sample was examined in triplicate using relative quantification analysis. This method normalizes the expression of the specific gene versus the control reference with the formula $2^{-\Delta\Delta C_T}$, where $\Delta C_T = C_T$ of specific gene - C_T of reference gene, and $\Delta\Delta C_T = \Delta C_T$ - an arbitrary constant. The threshold cycle value is defined as the PCR cycle number that crosses an arbitrarily placed threshold line.

Six genes related to senescence were selected to examine their different expression in *tasg1* and WT. First, total RNA from wheat leaves that were grown under normal conditions and drought stress treatments for 0, 5, 10, and 15 d was isolated according to the manufacturer's protocol (*Trizol*, *TaKaRa*, Shiga, Japan), and subsequently used for the reverse transcription polymerase chain reaction (RT-PCR). Then, total RNA was treated with DNaseI (RNase-free; *Promega*) to remove genomic DNA, and the reverse transcription was performed using the primer oligo $(dT)_{18}$ and Moloney murine leukemia virus (M-MLV) reverse transcriptase (*Promega*) at 42 °C for 60 min. Subsequently, the PCR reaction with equal aliquots of cDNA samples was performed using special primers.

The amplification of the *tubulin* gene (using primers *tubulin F*: 5′-ACCGCCAGCTCTTCCACCCT-3′ and *tubulin R*: 5′-TCACTGGGGCATAGGAGGAA-3′) exhibiting a constitutive expression was used as positive control, from which a linear relationship between the amount of RNA used for amplification and the amount of the cDNA fragment amplified as well as the quality of both extracted RNA and RT-PCR reactions were determined. The amplification conditions were as follows: 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. The cycle was repeated 28 times. RT-PCR products were separated on a 1.0 % (m/v) agarose gel. The experiments were independently repeated three times under the identical conditions.

All experiments were repeated at least three times. Statistical analysis was conducted using the data processing system of the Zhejiang University, Zhejiang, China. Differences between means among the wheat lines or treatments were compared using the Duncan's multiple range tests at a 0.05 probability level.

Table 1. Primer sequences. The annealing temperature for each of the primer couple and the size of the amplicons are also indicated (the *tubulin* cDNA was used [a] - for *DHAR* expression and [b] - for *SAG* expressions).

Gene	Forward primer sequence 5′-3′	Reverse primer sequence 5′-3′	[°C]	Size [bp]
DHAR	TCAAGCCAGATTTAGTCACC	TGGAAGCGTGGAGCGATTTG		20
Tubulin[a]	ATCTGTGCCTTGACCGTATCAGG	GACATCAACATTCAGAGCACCATC		23
TaSAG1	TCAAGCCAGATTTAGTCACC	TGGAAGCGTGGAGCGATTTG	56	616
TaSAG3	TGTTCTTGACGACGATGGTG	TGAGCACTAAGCGCAGCA	52	213
TaSAG4	CCTCACCAGCCTCAAGTTCC	GTCTTCTCCGTCCTGTCAGT	54	223
TaSAG6	TCGTCCTGGTCATGCTCGCT	TCGTCCTGGTCATGCTCGCT	54	454
TaSAG7	CAAGCGCCCCTACACCGTCC	TGGTACTGCTGGGCGAAGAA	52	321
TaSAG9	ACAAGTTCAACCCCGTCAAG	CCATCAGCTTCATCAGACCC	56	439
Tubulin[b]	ACCGCCAGCTCTTCCACCCT	TCACTGGGGCATAGGAGGAA	58	579

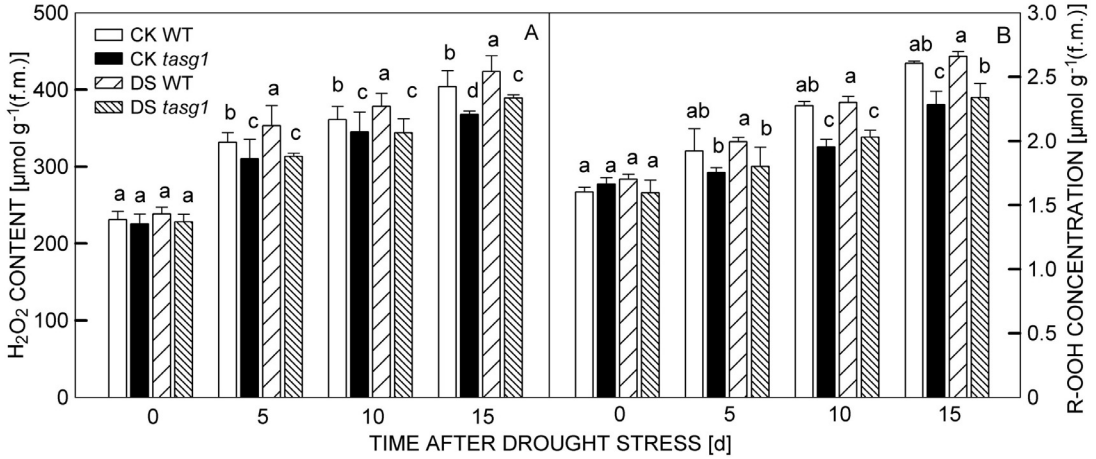

Fig. 1. Effects of drought stress on the H_2O_2 content (*A*) and R-OOH content (*B*) in flag leaves of WT and *tasg1* plants. Means ± SD (n = 5). Different letters indicate significant differences at a 0.05 level at the same time point.

Results

Reactive oxygen species (ROS) production in plants was monitored by measuring the content of H_2O_2 and hydroperoxides (R-OOH). There was no obvious difference in the H_2O_2 content between WT and *tasg1* under

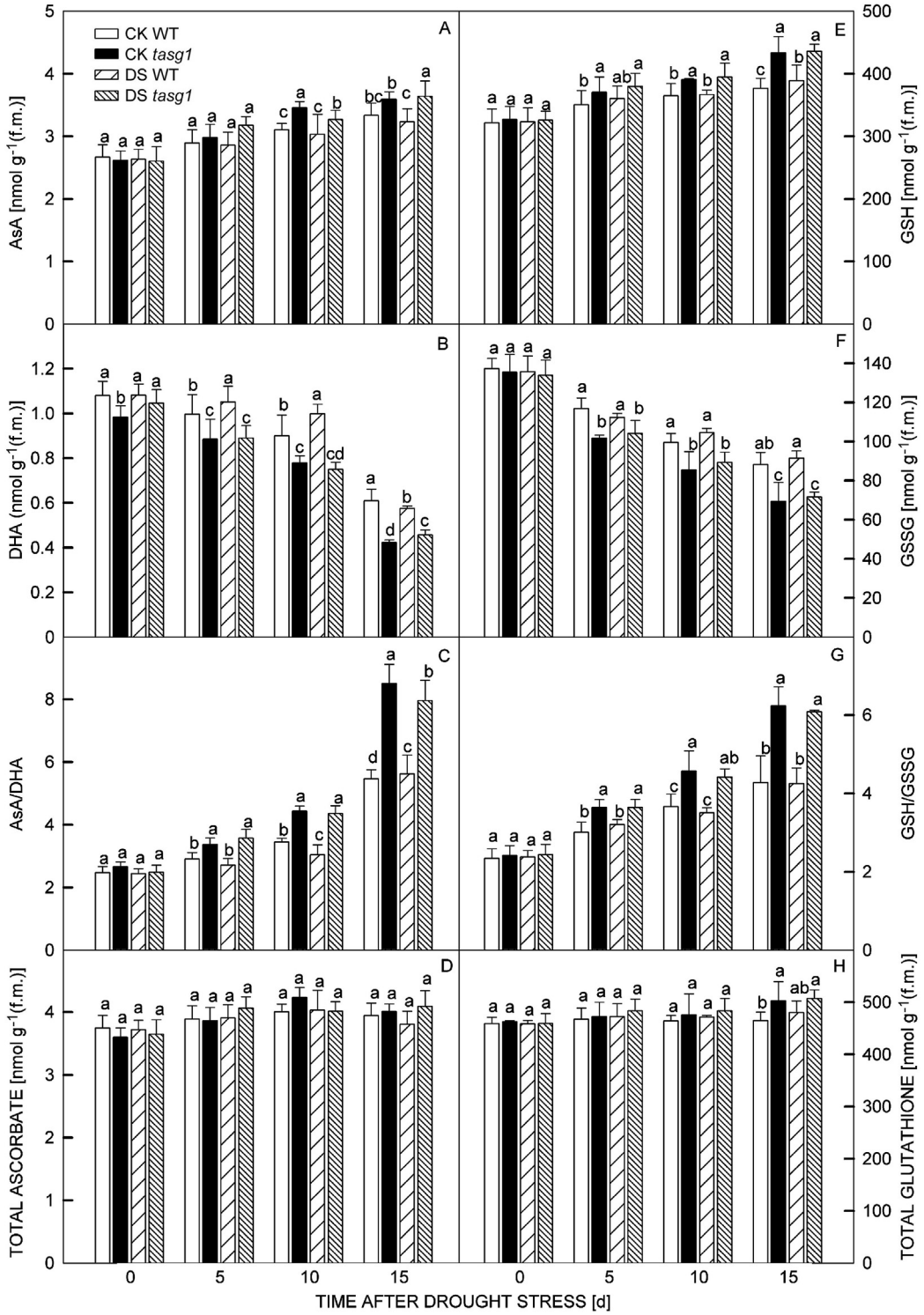

Fig. 2. Effects of drought stress on the ascorbate-glutathione cycle in flag leaves of WT and *tasg1* plants: *A* - AsA, *B* - DHA, *C* - AsA/DHA ratio, *D* - total ascorbate (AsA+DHA), *E* - GSH, *F* - GSSG, *G* - GSH/GSSG ratio, and *H* - total glutathione (GSH+GSSG). Means ± SD ($n = 3$). Different letters indicate significant differences at a 0.05 level at the same time point.

CK. DS resulted in a significant increase in H_2O_2 content in both the wheat genotypes, but the increase was greater in WT than in *tasg1*. After 15 d under CK, the H_2O_2 content increased by 75.2 and 63.0 % in leaves of the *tasg1* and WT plants, respectively, due to senescence. On the other hand, the H_2O_2 content increased by 77.6 and 70.6 % in leaves of the *tasg1* and WT plants, respectively, under DS (Fig. 1*A*). The trend in changes of R-OOH content was in accordance with that of H_2O_2 (Fig. 1*B*). No obvious difference was found between *tasg1* and WT at the initial phase of DS. However, in the late stage of DS, the R-OOH content in *tasg1* was higher than in WT.

As concern the ascorbate cycle, there was no significant difference in the content of AsA, DHA, and AsA+DHA, and the AsA/DHA ratio between the WT and *tasg1* plants under CK (Fig. 2*A-D*). DS decreased the content of DHA, but increased the content of AsA and the AsA/DHA ratio more in *tasg1* than in WT.

In the glutathione cycle, DS remarkably decreased the content of oxidized glutathione (GSSG) in both WT and *tasg1*, and the decrease in *tasg1* was more serious than in WT (Fig. 2*F*). Furthermore, DS induced a greater increase in GSH content and GSH/GSSG ratio in *tasg1* than in WT (Fig. 2*E,G*). However, there was no considerable change in GSH+GSSG under DS as compared with CK in both *tasg1* and WT (Fig. 2*H*).

No obvious difference was found in the glutathione reductase (GR) activity between *tasg1* and WT at the initial phase of DS. However, the GR activity in *tasg1* was always higher than in WT under late DS (Fig. 3*A*). DS induced a significant decrease in the activity of DHAR in both WT and *tasg1*. But the decrease in WT was more serious than in *tasg1* (Fig. 3*B*). The activity of DHAR was 26.1 and 21.5 % higher in *tasg1* than in WT under CK and DS, respectively. When DS was applied, the MDHAR activity decreased considerably in WT. The MDHAR activity in *tasg1* also decreased but much less than in WT (Fig. 3*C*).

The *DHAR* gene was selected to determine the different transcriptional responses to the drought stress

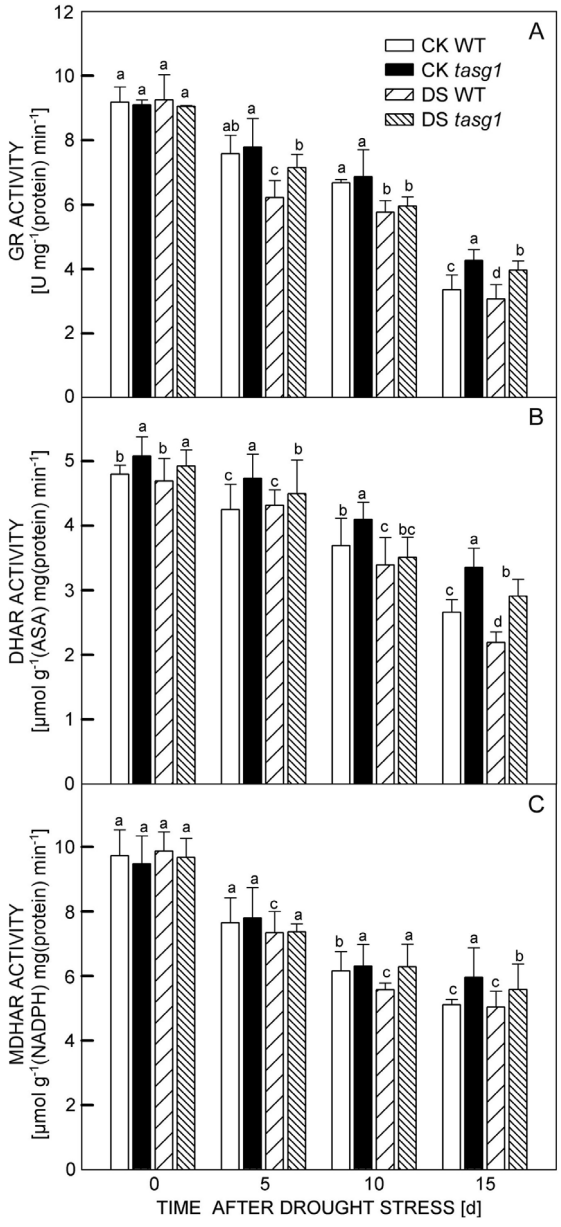

Fig. 3. Effects of drought stress on the antioxidant enzyme activities in flag leaves of WT and *tasg1*: *A* - GR, *B* - DHAR, and *C* - MDHAR. Means ± SD (n = 3). Different letters indicate significant differences at a 0.05 level at the same time point.

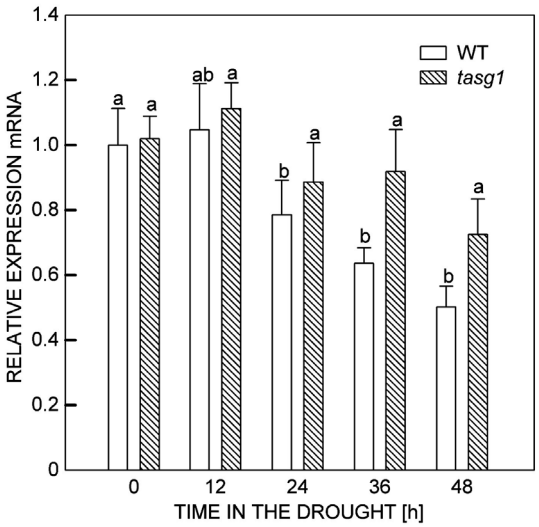

Fig. 4. Changes in the *DHAR* mRNA expression in response to a drought stress measured using real-time RT-PCR. The drought stress was induced using a 20 % PEG-6000 solution for 0, 12, 24, 36, and 48 h with water as control. The expression data correspond to means of triplicates, normalized to *α-tubulin*, and using a control sample from 0 h as calibrator. The expression of data is in arbitrary units ± SD. Different letters indicate significant differences at a 0.05 level at the same time point.

between *tasg1* and WT (Table 1). As revealed by RT-PCR, the expression of the *DHAR* gene was similar in WT and *tasg1* before the PEG treatment (Fig. 4). After 12 h, the expression increased in both the wheat genotypes. However, the expression then decreased continuously and the decrease was greater in WT than in *tasg1*.

Six wheat senescence-associated genes (SAG) in the flag leaf were selected to determine different transcriptional responses to the drought stress between *tasg1* and WT (Table 1). As revealed by semi-quantitative RT-PCR (Fig. 5), the expression of these genes showed distinct responses to the PEG treatment, and the expression patterns varied for different genes. *TaSAG1*, *TaSAG6*, and *TaSAG9* were the most sensitive genes to the drought stress, their expressions were up-regulated immediately after the PEG treatment in both WT and *tasg1* (Fig. 5). The expressions of *TaSAG1*, *TaSAG6*, and *TaSAG9* were higher in WT compared to *tasg1* for various periods after the PEG treatment. On the other hand, the expression of *TaSAG1*, *TaSAG6*, and *TaSAG9* were firstly inhibited in *tasg1*, but further slowly increased. Even at the last tested time point (48 h), the expressions of *TaSAG1*, *TaSAG6*, and *TaSAG9* did not increase significantly in WT and remained low. *TaSAG3*, *TaSAG4*, and *TaSAG7* showed no significant accumulation in both *tasg1* and WT after the PEG treatment.

Discussion

The efficient removal of ROS is a key factor for plant stress tolerance. ROS are produced in a controlled manner through normal metabolic processes in aerobic organisms (Elstner *et al.* 1994, Gratão *et al.* 2005) as signaling molecules in pathogen defense, programmed cell death, and abiotic stress responses (Desikan *et al.* 2001, Mittler 2002). However, stress conditions can unbalance a steady-state level in ROS production (Foyer and Noctor 2005, Sharma and Dietz 2009). Failure to control ROS accumulation leads to a phenomenon known as oxidative stress (Bartosz 1997, Foyer and Noctor 2000).

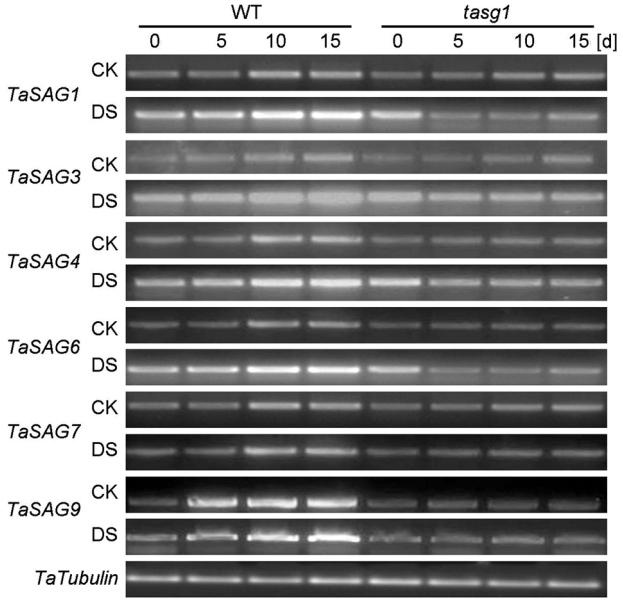

Fig. 5. *SAG* gene expressions in responses to a drought stress in wheat. Total RNA from wheat flag leavesthat were grown under the drought stress for 0, 5, 10, and 15 d was isolated and reverse transcribed. Subsequently, RT-PCR was performed using specific primers, and the RT-PCR products were separated on a 1.0 % (m/v) agarose gel. The tubulin line was as positive control of the RT-PCR reactions with the *tubulin* gene which exhibits a constitutive expression. The experiments were independently repeated three times under the identical conditions.

We studied the mechanisms underlying the drought resistance of *tasg1*. First, we compared the ROS accumulation in leaves of *tasg1* with that of WT. When the wheat plants were treated with DS for 15 d, the accumulation of H_2O_2 and R-OOH increased less in *tasg1* than in WT (Fig. 1) suggesting their greater antioxidant competence.

Both enzymatic and non-enzymatic components are involved in the ascorbate-glutathione cycle (Gratão *et al.* 2005). This cycle plays a crucial role in removing ROS and maintaining the cellular redox status in different cell compartments (Noctor and Foyer 1998, Drazkiewicz *et al.* 2003, Singh *et al.* 2006). The capacity of this cycle is dependent on the content and redox status of AsA and GSH. Under environmental stresses, AsA in the chloroplast can alleviate photoinhibition of photosystem (PS) II not only by donating electrons to violaxanthin de-epoxidase to dissipate excess excited energy, but also by providing electrons to ascorbate peroxidase (APX) for detoxification of H_2O_2. AsA regeneration is necessary for the reductive detoxification of H_2O_2 (Hossain *et al.* 1984). In this study, DS resulted in a more significant increase in the content of AsA and the AsA/DHA ratio in *tasg1* than in WT (Fig. 2*A,C*), but the AsA+DHA content increased to the equivalent extent in both WT and *tasg*1 (Fig. 2*D*).

The ascorbate-glutathione cycle uses GSH as electron donor to regenerate AsA from its oxidized form, and is considered as the main pathway of $O_2^{\bullet-}$ and H_2O_2 removal in the chloroplast (Noctor and Foyer 1998). In the present study, we observed a greater increase in GSH and GSH/GSSG in *tasg1* than in WT after DS (Fig. 2*E,G*). Besides, GSSG decreased more seriously in WT (Fig. 2*F*). However, DS did not considerably change the GSH+GSSG content as compared with CK (Fig. 2*H*).

Four enzymes, *i.e.*, APX, GR, DHAR, and MDHAR,

are involved in the removal of H_2O_2 and of $O_2^{\bullet -}$ in this cycle. In our previous study (Tian *et al.* 2012), APX activity was determined from the 22nd to 30th day after anthesis. The APX activity was mostly suppressed by DS, but the activity was higher in *tasg1* than in WT. Here, our results show that DS decreased the activities of GR, DHAR, and MDHAR more in WT than in *tasg1*. Evidently, the enhanced activities of these enzymes concomitant with the enhanced content of AsA and GSH could help to quench ROS in *tasg1*.

We also detected the *DHAR* gene responses to the drought stress between *tasg1* and WT. After the 12 h PEG treatment, the expression of *DHAR* increased in both the wheat genotypes but then decreased. Similarly, the *DHAR* gene expression in the wheat seedlings quickly increased, peaked at 24 h of the drought stress, and then gradually decreased (Kang *et al*. 2013).

Numerous studies have shown that ROS and antioxidant systems are involved in natural senescence (Evans *et al.* 1999, Puppo *et al.* 2005). In the senescence process, many senescence-associated genes are up-regulated (Gregersen and Holm 2007). Among 11 SAGs detected, two genes were found to be up-regulated in the stay-green rice leaves (Jiang *et al.* 2011). In this paper, six wheat SAG genes were identified from wheat EST libraries based on their homology to rice SAGs. Among them, *TaSAG1*, *TaSAG3*, and *TaSAG4* are mainly associated with amino acid metabolism, *TaSAG7* with fatty acid metabolism, *TaSAG9* with sugar metabolism, and *TaSAG6* encodes seed proteins of unknown function. The expression of *TaSAG1*, *TaSAG6*, and *TaSAG9* were higher in WT compared to *tasg1* for the various periods after the PEG treatment (Fig. 5). *TaSAG3*, *TaSAG4*, and *TaSAG7* showed no significant accumulation in both *tasg1* and WT after the PEG treatment.

In conclusion, these results suggest that the ascorbate-glutathione cycle might play a crucial role in the enhanced drought tolerance of *tasg1*. The results in this study combined with our previous observations (Tian *et al.* 2012, Hui *et al.* 2012) suggest that a greater antioxidant competence may contribute to delayed senescence and to drought resistance of *tasg1*. These data are helpful for better understanding the mechanism of drought resistance in stay-green mutants.

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