

RESEARCH PAPER

Enhanced stability of thylakoid membrane proteins and antioxidant competence contribute to drought stress resistance in the *tasg1* wheat stay-green mutant

Fengxia Tian¹, Jiangfeng Gong¹, Jin Zhang², Meng Zhang¹, Guokun Wang¹, Aixiu Li¹ and Wei Wang^{1,*}

¹ State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Tai'an, Shandong, 271018, PR China

² State Key Laboratory of Tree Genetics and Breeding, Research Institute of Forestry, Chinese Academy of Forestry, Beijing, 100091, PR China

*To whom correspondence should be addressed. E-mail: wangw@sdau.edu.cn

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Abstract

A wheat stay-green mutant, *tasg1*, was previously generated via mutation breeding of HS2, a common wheat cultivar (*Triticum aestivum* L.). Compared with wild-type (WT) plants, *tasg1* exhibited delayed senescence indicated by the slower degradation of chlorophyll. In this study, the stability of proteins in thylakoid membranes was evaluated in *tasg1* under drought stress compared with WT plants in the field as well as in seedlings in the laboratory. Drought stress was imposed by controlling irrigation and sheltering the plants from rain in the field, and by polyethylene glycol (PEG)-6000 in the laboratory. The results indicated that *tasg1* plants could maintain higher Hill activity, actual efficiency (Φ_{PSII}), maximal photochemical efficiency of PSII (F_v/F_m), and Ca^{2+} -ATPase and Mg^{2+} -ATPase activities than the WT plants under drought stress. Furthermore, the abundance of some polypeptides in thylakoid membranes of *tasg1* was greater than that in the WT under drought stress. Expression levels of *TaLhcb4* and *TaLhcb6* were higher in *tasg1* compared with the WT. Under drought stress, the accumulation of superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) was lower in *tasg1* compared with the WT not only at the senescence stage but also at the seedling stages. These results suggest greater functional stability of thylakoid membrane proteins in *tasg1* compared with the WT, and the higher antioxidant competence of *tasg1* may play an important role in the enhanced drought tolerance of *tasg1*.

Key words: Antioxidant competence, drought stress, stability, stay-green mutant, thylakoid membrane proteins, wheat.

Introduction

Drought stress is a serious environmental factor that can severely limit the growth, performance, and productivity of wheat, one of the most important food crops worldwide (Chaves and Oliveira, 2004). The photosynthetic activity of the chloroplast is one of the most sensitive physiological processes to drought stress, which damages the thylakoid membrane, disturbs its functions, and ultimately decreases photosynthesis and crop yield (Shah and Paulsen, 2003; Huseynova *et al.*, 2007; Zhao *et al.*, 2007). Thus, preservation of the photosynthetic apparatus is an important strategy for enhancing photosynthetic activity under drought stress.

Leaf senescence in plants is an internally programmed degeneration process, during which the photosynthetic apparatus is gradually disorganized, leading to death. The most prominent visible change in leaf senescence is associated with chlorophyll degradation and a progressive decline in photosynthetic capability (Matile *et al.*, 1996, 1999). Stay-green or non-yellowing mutations in various plant species have been reported to maintain leaf greenness longer than their wild-type (WT) counterpart during senescence (Thomas and Smart, 1993; Spano *et al.*, 2003; Hörtensteiner, 2009). Some 'functional stay-green' mutants can photosynthesize

for longer and may therefore be expected to give a higher yield (Thomas and Howarth, 2000; Yoo *et al.*, 2007; Zheng *et al.*, 2009). It has been estimated that a delay in the onset of senescence in *Lolium temulentum* by just 2 d increases the amount of carbon fixed by the plant by 11% (Thomas and Howarth, 2000). In contrast, so-called ‘non-functional stay-green’ mutants, for example type C, remain green as a result of lesions in chlorophyll catabolism but lack photosynthetic competence (Thomas and Howarth, 2000).

With type C mutants, the pathways involved in chlorophyll degradation have been well established in recent years (Sato *et al.*, 2009; Schelbert *et al.*, 2009). Two types of chlorophyll are found in higher plants, Chl *a* and Chl *b*. Chl *a* is a component of all chlorophyll–protein complexes, while Chl *b* is contained only in PSI-associated light-harvesting complex I (LHCI) and PSII-associated LHCII. LHCI and LHCII are localized in thylakoid membranes and function in energy harvest and transfer. LHCII is mainly localized in grana, the stacking region of the thylakoid membranes, and is thought to play an important role in the formation and maintenance of grana stacks due to intermolecular forces (Allen and Forsberg, 2001; Standfuss *et al.*, 2005). The apoproteins of LHCI and LHCII are encoded by the *Lhca* and *Lhcb* gene families, respectively. *Lhca1–Lhca4* genes encode the polypeptides of LHCI associated with PSI. *Lhcb1*, *Lhcb2*, and *Lhcb3* genes encode the polypeptides of trimeric LHCII. *Lhcb4*, *Lhcb5*, and *Lhcb6* proteins (often called CP29, CP26, and CP24, respectively) are suggested to be monomeric proteins that are present as one copy per PSII unit. The expression of *Lhca* and *Lhcb* and the stability of LHCI and LHCII are of great importance for keeping the photosynthetic activity at a high level (Standfuss *et al.*, 2005; Sato *et al.*, 2009).

In a previous study, a functional stay-green wheat mutant *tasg1* with improved drought tolerance was generated. The net photosynthetic rate (P_n) of *tasg1* was consistently higher than that of the WT during water senescence induced by drought stress (Tian *et al.*, 2012). In the present experiments, the stability and activity of proteins in thylakoid membranes involved in photosynthesis, as well as the expression of select LHC genes, were detected. The accumulated levels of superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) were found to be lower in *tasg1* compared with the WT under drought stress. These data contribute to the better understanding of stay-green mechanisms for the improvement of drought resistance in wheat cultivars.

Materials and methods

Plant materials

The wheat stay-green mutant, *tasg1*, was previously generated via mutation breeding by applying the ethyl methane sulphonate (EMS) mutagen to HS2, a common wheat cultivar (*Triticum aestivum* L.), as the WT. All the experiments were conducted in two sets: one, in the field and the other in the laboratory.

Field experiments

Wheat plants were grown in the fields of Shandong Agricultural University, China, during the growing seasons October–June 2011.

Six 4 m² interspersed plots were established via random block design in the field, with three replicate plots for each genotype. Wheat seeds were hand-sown in eight lines, with rows spaced 25 cm apart. Seeds were planted at ~1–2 cm depth and 5 cm spacing. Conventional agricultural management was maintained during growth and development of the seedlings.

Drought stress treatment was imposed by controlling irrigation and sheltering the plants from rain, with conventional normal water conditions for controls.

Physiological parameters were measured using intact flag leaves of both *tasg1* and WT plants on 20 May 2011 (~14 d after flowering) and on 4 June 2011 (~6 d before harvest), ~10 d (the early stage of drought stress) and 25 d (the later stage of drought stress) after the drought stress was imposed, respectively.

Laboratory experiments

For the hydroponic seedlings, seeds of both the WT and *tasg1* were germinated on filter paper moistened with water for 24 h at 25 ± 1 °C after being sterilized with 0.2% sodium hypochlorite. The seeds were then placed in a well-ordered fashion on a nylon gauze sheet at the appropriate density and cultured in trays (25 cm × 18 cm × 5 cm) containing Hoagland solution. These trays were placed in a growth chamber at 25 ± 1 °C under a 12 h light (300 μmol m⁻² s⁻¹)–dark cycle and a relative humidity of 70%. After complete unfolding, second leaves were harvested for senescence-inducing treatments as described below. Osmotic stress was induced using 20% polyethylene glycol (PEG)-6000 solution (–1.45 MPa) with water as a control. The solution was changed every 12 h.

Determining the water content of soil

Water conditions were assessed by measuring the water content of the soil at 10 d and 25 d after drought stress treatments were imposed. A statistically significant difference in soil water content between drought treatment plots and normal water control plots was achieved (Table 1).

Determining the Hill activity of the chloroplast

Chloroplast isolation and Hill activity assays were determined following the procedure of Zhao *et al.* (2007), which was modified from Ye and Qian (1985). The chlorophyll content of the chloroplast suspension was measured according to Arnon (1949).

Chlorophyll *a* fluorescence analysis

The actual PSII efficiency under irradiance (Φ_{PSII}) and the maximal photochemical efficiency of PSII (F_v/F_m) were measured with a portable pulse-modulated fluorometer FMS-2 (Hansatech Instruments Ltd, King's Lynn, UK). For quenching analyses, the leaves were illuminated with actinic light intensities of 800 μmol m⁻² s⁻¹ for 16 min (which was found to be sufficient for the induction of steady-state light conditions) and subsequently kept for a further 10 min in the dark.

Table 1. Soil water content (%) in the field under both drought stress (DS) and normal water (CK) conditions. Values are mean ± SE ($n=3$). The letters next to the values demonstrate significant difference at 0.05 thresholds.

Date	0–20 cm depth		20–40 cm depth	
	DS	CK	DS	CK
20 May 2011	4.88 ± 0.20 b	6.32 ± 0.20 a	7.13 ± 0.03 b	7.58 ± 0.14 a
4 June 2011	4.78 ± 0.22 b	6.43 ± 0.29 a	7.08 ± 0.12 b	7.55 ± 0.19 a

Determinations of thylakoid membrane Ca^{2+} -ATPase and Mg^{2+} -ATPase activities

Ca^{2+} -ATPase activity and Mg^{2+} -ATPase activity were determined following the procedure of Wang *et al.* (2010), which was modified from Huang (1985).

Detection of thylakoid polypeptides

Thylakoid membranes were prepared according to the method of Zhang *et al.* (1999). Fresh leaves (1 g) were homogenized in an ice-cold 5 ml solution of HMSN buffer containing 0.4 M sucrose, 0.01 M NaCl, 5 mM MgCl_2 , and 0.01 M HEPES (pH 7.8) in a tissue homogenizer. The homogenate was filtered through four layers of gauze and centrifuged at 5000 g at 4 °C for 10 min. The supernatants and most of the loose pellets were discarded. The remaining chloroplast deposit was suspended in 5 ml of HMSN buffer and centrifuged at 5000 g at 4 °C for 10 min. The supernatants and most of the loose pellets were discarded. The remaining chloroplast deposit suspended in 1 ml of HMSN buffer was used to examine the thylakoid polypeptides.

Chlorophyll contents in thylakoid suspension were estimated using 80% acetone following the equation of Lichtenthaler (1987).

Thylakoid polypeptides were separated by urea and SDS-PAGE according to Parida *et al.* (2003) with minor modification to the 15% separating gel and were detected after staining and de-staining as described in Parida *et al.* (2003). In total, 20 µg of chlorophyll was loaded per lane.

Semi-quantitative RT-PCR of pigment-binding protein-related gene expression

Six genes related to pigment-binding proteins were selected to examine the different expression in *tasg1* and the WT. First, total RNA from wheat leaves, which were grown under normal conditions and drought stress treatments for 0, 12, 24, 36, and 48 h, was isolated according to the manufacturer's protocol (Trizol, Takara, China), and subsequently used for reverse transcription-polymerase chain reaction (RT-PCR). Then, total RNA was treated with DNase I (RNase-free; Promega USA) to remove genomic DNA, and reverse transcription was performed using the primer oligo(dT)₁₈ and Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega) at 42 °C for 60 min. Subsequently, a PCR with equal aliquots of cDNA samples was performed using special primers. The specific primers for genes encoding LHCI (*TaLhca1*, *TaLhca2*, and *TaLhca3*) and LHCII (*TaLhcb1*, *TaLhcb4*, and *TaLhcb6*) subunits were designed based on published wheat expressed sequence tags (ESTs): *TaLhca1*, F (5'-CAACCTGCCGACCATCCTG-3') and R (5'-CAGCCGCCGTTCTTGAT-3'); *TaLhca2*, F (5'-CCCCAACCGCAAGAACC-3') and R (5'-CCGACGAAGGCGAGCAT-3'); *TaLhca3*, F (5'-CCTCACCAGCCTCAAGTTCC-3') and R (5'-CGCACGCTCACGTTTCC-3'); *TaLhcb1*, F (5'-GGAGAACACACAATACACC-3') and R (5'-CCCATTATGTGTGCAGTTC-3'); *TaLhcb4*: F (5'-AAAGGCCGAGGAGGACAA-3') and R (5'-CCACCGACCACTTAAGAGG-3'); and *TaLhcb6*, F (5'-TCAGCGACCTACCGTCA-3') and R (5'-CCCAAAGAAGTCACGGACA-3').

Amplification of the *T. aestivum tubulin* gene (using primers *TubulinF*, 5'-ACCGCCAGCTCTTCCACCCT-3' and *TubulinR*, 5'-TCACTGGGGCATAGGAGGAA-3') exhibiting constitutive expression was used as a positive control, from which a linear relationship between the amount of RNA used for amplification and the amount of cDNA fragment amplified, as well as the quality of both extracted RNA and RT-PCRs were determined.

The amplification conditions were as follows: 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C. The cycle was repeated 28 times. The RT-PCR products were separated on a 1.0% (m/v) agarose gel. The experiments were independently repeated three times under identical conditions.

Detection of pigment-binding proteins by immunoblotting

Total protein was extracted from wheat leaves. Protein content was determined by the dye binding assay according to Bradford (1976). Proteins were separated by SDS-PAGE on a 12% gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Saint-Quentin, France). Proteins were routinely detected with the Lhca2, Lhcb4, and Lhcb6 antibody (Sigma), and with the Rubisco large subunit as control.

Detection of protein carbonylation by immunoblotting

Total protein was extracted from wheat leaves (Dalle-Donna *et al.*, 2002; Qiu *et al.*, 2008). Proteins were separated by SDS-PAGE following the procedures of Zhang *et al.* (2012), which were modified from Dalle-Donna *et al.* (2002). Protein carbonylation was routinely detected with the anti-dinitrophenyl (DNP) antibody (Sigma).

Staining detection of O_2^- accumulation

O_2^- accumulation was visualized with nitroblue tetrazolium (NBT). Detached leaves were infiltrated with 6 mM NBT. Chlorophyll was removed from the leaves prior to imaging by infiltrating them with lacto-glycerol-ethanol (1:1:4, v/v/v) and boiling in water for 5 min. The location of formazan deposits was visualized by subtracting background (non-formazan) pixels from the leaf image.

H_2O_2 staining with diaminobenzidine

Detached leaves were infiltrated with 6–8 ml of diaminobenzidine (DAB) solution (1 mg ml⁻¹ DAB, Sigma pH 3.8) with NaOH according to the method of Giacomelli *et al.* (2007) with slight modification. Leaves were then left in the solution overnight in the dark. The following day, the solution containing the leaves was brought to the boil for a few minutes, and then incubated at room temperature for 3 h under mild agitation. The leaves were then cleared with chlorhydrate (10 ml of water added to 25 g of chlorhydrate).

Relative electric conductivity

Six leaf discs (0.8 cm in diameter) were put into 10 ml of distilled water and a vacuum was applied for 30 min, and then surged for 3 h to measure the initial electric conductance (S1) (25 °C). A test tube was filled with leaf discs and distilled water, the mixture was cooked (100 °C) for 30 min and then reduced to room temperature (25 °C) to determine the final electric conductance (S2). The relative electric conductivity (REC) was evaluated as: $\text{REC} = \text{S1} \times 100/\text{S2}$.

Statistical analysis

The statistical significance of differences in measured parameters was tested by using the procedures of DPS (Zhejiang University, China). All pairwise comparisons were analysed using Duncan's test. Differences between the means among wheat lines or treatments were compared using Duncan's multiple range tests at 0.05 probability levels.

Results

Stay-green phenotype of *tasg1* in the field

Under normal field conditions, no significant difference between *tasg1* and the WT was observed in plant development or phenotype before the flag leaves appeared. However, flag leaves of the WT generally emerged on 28 April, while those of *tasg1* did not appear until 1 d or 2 d later. The flowering date of *tasg1* was also delayed by 1 d or 2 d compared with

the WT. The stay-green phenotype of *tasg1* was expressed at the beginning of anthesis and was especially apparent with late natural senescence. Drought stress accelerated the plant senescence in both wheat varieties, but it was delayed in *tasg1* compared with the WT (Fig. 1A–C). Meanwhile, the areas of the flag leaves of *tasg1* were 28% and 23% larger than those of WT leaves under normal water and drought stress conditions, respectively, at the grain filling stage (Fig. 1D).

Differential effects of drought stress on activity and abundance of the photosynthesis protein complexes in the thylakoid membrane of tasg1 and WT plants

In a previous study, photosynthetic activity was found to be higher in *tasg1* than in the WT, and the duration of the high photosynthesis period was longer in *tasg1* than in the WT under drought conditions (Tian *et al.*, 2012). In the

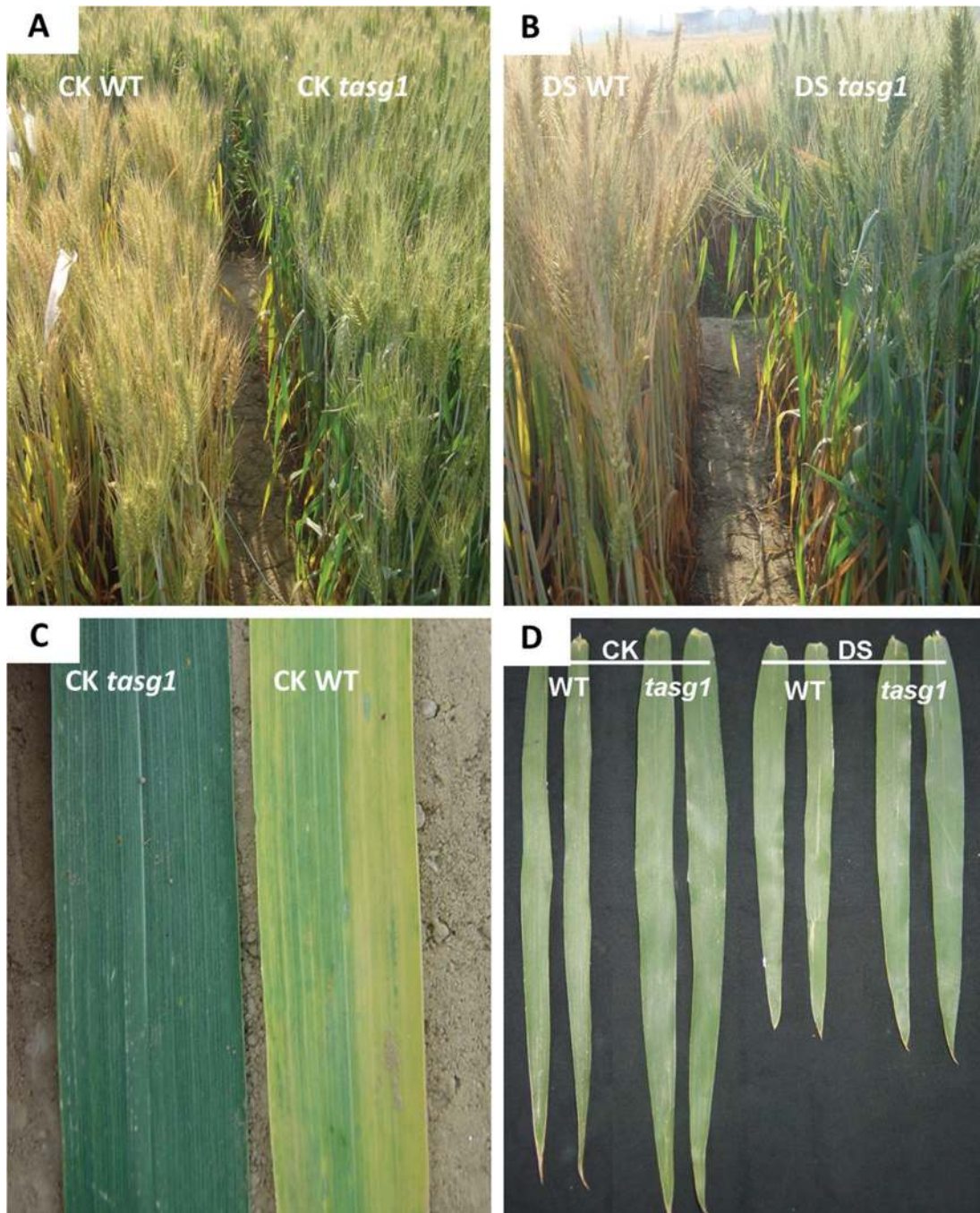


Fig. 1. Naturally senescent WT (left) and *tasg1* (right) plants were maintained with normal water (A) or induced with drought stress (B) at the late filling stage in the field. (C) Naturally senescent flag leaves of WT (right) and *tasg1* (left) plants at the late filling stage in the field. The images in A–C were obtained on 2 June 2011. (D) Flag leaves of WT and *tasg1* plants under normal water and drought stress conditions at the filling stage in the field. Photos in D were taken on 25 May 2011.

current study, the protein activity and components involved in photosynthesis were detected in the thylakoid membrane of the flag leaves. The results are shown in Figs 2–5 and are described below.

Hill reaction activity

As shown in Fig. 2, under normal water conditions, there was no obvious difference in the Hill reaction activity between the WT and *tasg1* at 10 d under drought. The Hill reaction activity was reduced by 31.4% and 47.9% in leaves of *tasg1* and WT plants, respectively, under normal water conditions due to senescence. Meanwhile, the Hill reaction activity was reduced by 44.1% and 51.1% in leaves of *tasg1* and WT plants, respectively, at 25 d under drought. These results suggest that the Hill reaction activity was less affected by drought stress in *tasg1* than in the WT.

PSII photochemistry

The actual PSII efficiency under irradiance (Φ_{PSII}) and the maximal photochemical efficiency of PSII (F_v/F_m) are commonly used as sensitive indicators of plant photosynthetic performance responding to stress conditions (Maxwell and Johnson, 2000), and both of these parameters represent a measure of the functional status of PSII. As shown in Fig. 3A and B, no significant difference in Φ_{PSII} and F_v/F_m of PSII

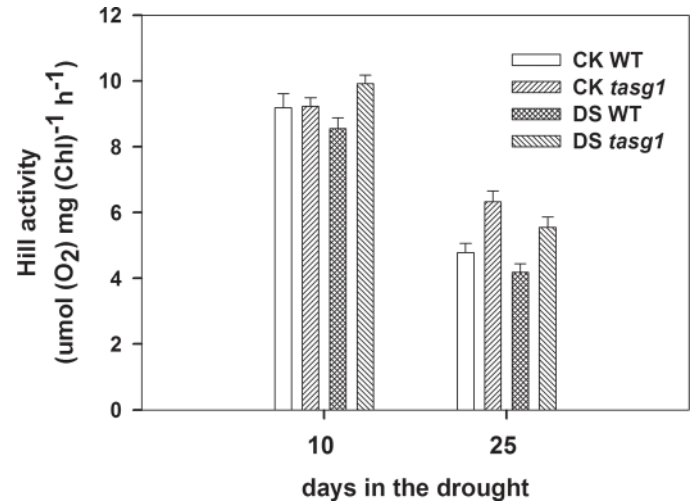


Fig. 2. Hill activity of chloroplasts in flag leaves of WT and *tasg1* plants in the early and later stages of drought stress in the field. Each bar is the mean \pm SD of four replications. Bars with the same letter were not significantly different at $P < 0.05$.

photochemistry was observed between *tasg1* and the WT in the early stage of drought stress. In the later stage of drought

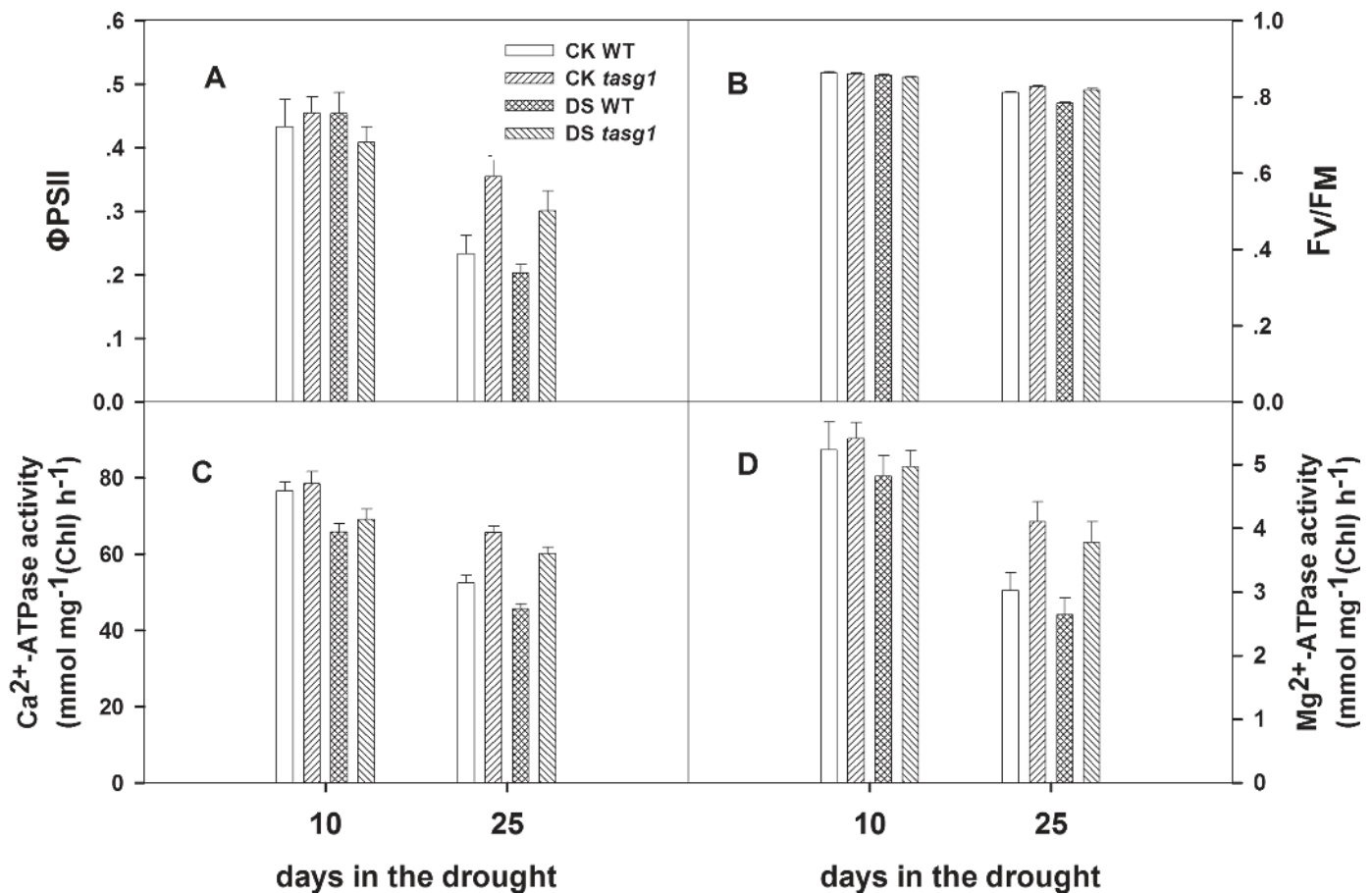


Fig. 3. F_v/F_m (A), Φ_{PSII} (B), and the activities of Ca²⁺-ATPase (C) and Mg²⁺-ATPase (D) in the thylakoid membrane in WT and *tasg1* flag leaves in the early and later stages of drought stress. Each bar is the mean \pm SD of four replications. Bars with the same letter were not significantly different at $P < 0.05$.

stress, F_v/F_m was decreased, but not significantly, while Φ_{PSII} decreased significantly. However, Φ_{PSII} in leaves of *tasg1* was 52.1% and 47.9% higher than in the WT under normal and drought stress conditions, respectively. These findings suggest that the PSII complex in *tasg1* could better withstand drought stress than the WT.

As shown in Fig. 3C and D, under normal water conditions and at 10 d under drought, differences in the activities of Ca^{2+} -ATPase and Mg^{2+} -ATPase between *tasg1* and the WT were not significant, but they were significantly higher in *tasg1* than in the WT at 25 d under drought. Drought stress decreased the activities of Ca^{2+} -ATPase and Mg^{2+} -ATPase in both *tasg1* and WT plants, but both activities were consistently higher in *tasg1* than in the WT during drought stress, with significant differences observed at 25 d under drought.

These results suggest greater stability of ATPases in *tasg1* compared with the WT under drought stress.

From the results shown above (Figs 2, 3), it can be deduced that the activity of thylakoid membrane proteins, including PSII and ATPase, were better maintained in *tasg1* than in the WT under drought stress.

Polypeptide abundance in thylakoid membrane

Next, the changes in protein abundance in the thylakoid membrane under drought stress were investigated. In flag leaves under field conditions, the level of the 28 kDa polypeptide in *tasg1* was higher than that in the WT under both normal and drought stress conditions on both days of the drought, 10 d and 25 d (Fig. 4A). The polypeptides were similarly detected in WT and *tasg1* wheat seedlings at the second-leaf stage in

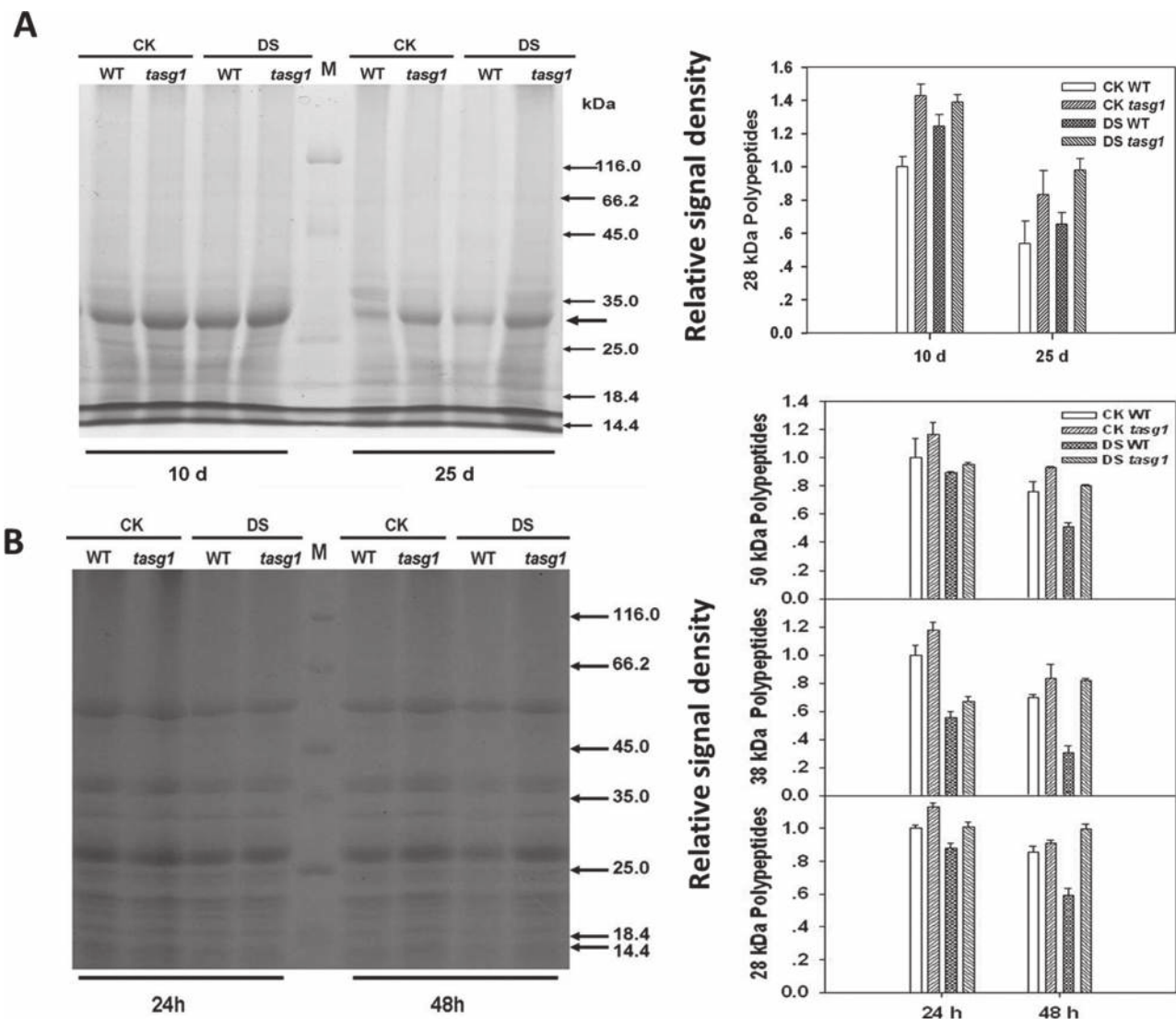


Fig. 4. Effects of drought stress on thylakoid polypeptides in WT and *tasg1* flag leaves in the field (A) and in the leaves of WT and *tasg1* seedlings (B). M represents the molecular mass marker. Lanes to the left of M represent the WT and *tasg1* under normal conditions (CK) and in the early stage under drought stress (DS) by treatment with 20% PEG-6000 solution for 24 h. Lanes to the right of M represent the WT and *tasg1* under normal conditions and in the later stage under drought stress by treatment with 20% PEG-6000 solution for 48 h. The graphs on the right show the relative density of signals in the corresponding samples of the SDS-PAGE in the left panels. Quantitative analysis was performed using the Tanon GIS system (Tanon, Shanghai China).

the laboratory, and the three observed polypeptides of ~28, 38, and 50 kDa in *tasg1* were consistently higher than those in the WT after treatment with 20% PEG-6000 solution with water as control for 24 h and 48 h (Fig. 4B).

Transcriptional levels of genes involved in LHCI and LHCII
Six genes encoding pigment-binding proteins were selected to determine the different transcriptional responses to drought stress between *tasg1* and the WT. RNA was obtained from wheat seedlings in which PEG-6000 was used to simulate water stress. As revealed by semi-quantitative RT-PCR, the expression of genes encoding pigment-binding proteins showed distinct responses to drought stress, and the expression patterns varied for different genes (Fig. 5).

Expression levels of genes involved in LHCI, namely *TaLhca1*, *TaLhca2*, and *TaLhca3*, were down-regulated gradually during drought stress in both the WT and *tasg1* (Fig. 5). The mRNA levels of all three *TaLhca* genes tested were reduced to the minimum at 48 h after water stress treatment. For example, *TaLhca3* was the most sensitive gene to drought stress, and it was significantly repressed by 48 h of drought stress in both the WT and *tasg1*. Meanwhile, *TaLhca2*, at the last tested time point of 48 h, still remained at a higher level in *tasg1* than in the WT.

Expression levels of genes involved in LHCII, namely *TaLhcb1*, *TaLhcb4*, and *TaLhcb6*, were not consistent with those involved in LHCI (Fig. 5). The expression levels of *TaLhcb4* and *TaLhcb6* were higher in *tasg1* compared with the WT, especially at the last tested time point (48 h). The degradation of *TaLhcb4* and *TaLhcb6* was inhibited in *tasg1*, decreasing slowly as the period of drought stress was extended. Even at the last tested time point (48 h), the transcript levels of *TaLhcb4* and *TaLhcb6* did not decrease significantly and still remained high. The expression level

of *TaLhcb4* was significantly different between the WT and *tasg1* in the early stage during drought stress, while that of *TaLhcb6* was largely different between the two plant types in the later stage during drought stress. However, the expression levels of *TaLhcb1* were similar between the WT and *tasg1* over the course of drought stress treatment.

To analyse pigment-binding proteins further, the abundance of *TaLhca2*, *TaLhcb4*, and *TaLhcb6* was detected by western blotting with the Rubisco large subunit as a control. The expression levels of these three genes were different to a certain extent between the WT and *tasg1*, especially at the last tested time point (48 h) according to Fig. 5. Immunoblot analysis (Fig. 6) revealed that the accumulation of *TaLhca2*, *TaLhcb4*, and *TaLhcb6* was decreased in both the WT and *tasg1* at 48 h after treatment under drought stress. There was no significant difference in accumulation of *TaLhca2* between the WT and *tasg1*. However, the contents of *TaLhcb4* and *TaLhcb6* were higher in *tasg1* than in the WT under drought stress. The protein accumulation of *TaLhcb6* was 2.15-fold higher in *tasg1* than in the WT at 48 h after treatment under drought stress, while the mRNA accumulation of *TaLhcb6* was 1.97-fold higher in *tasg1* compared with the WT (Fig. 5). These observations suggest that *TaLhcb4* and *TaLhcb6* could be maintained at a higher level in *tasg1* than in the WT under drought stress.

Effects of drought stress on antioxidant activity

To study the mechanisms underlying differences in senescence and drought resistance, the antioxidant activities in *tasg1* and the WT were compared, and the results are shown in Figs 7–9. First, protein carbonylation was detected by immunoblotting (Fig. 7). Drought stress increased protein carbonylation in the leaves of *tasg1* and the WT. However, the amount of

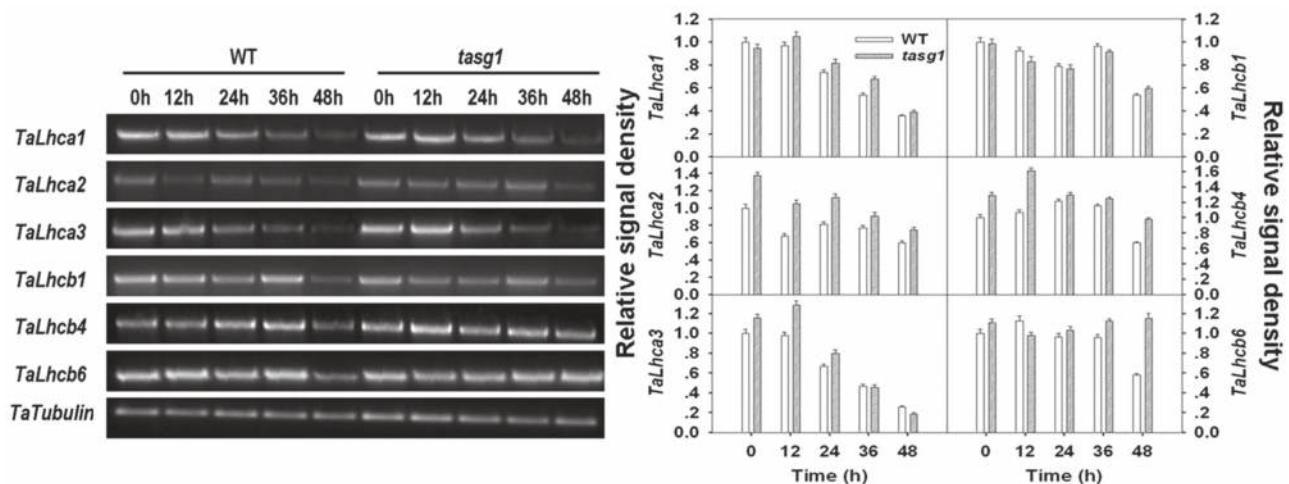


Fig. 5. Expression of LHCI and LHCII genes in response to drought stress in wheat. Total RNA from leaves of wheat grown under normal and drought stress conditions for 0, 12, 24, 36, and 48 h was isolated and reverse transcribed. RT-PCR products amplified using specific primers were separated on a 1.0% (m/v) agarose gel. The constitutively expressed *T. aestivum tubulin* gene was amplified as a positive control. Three independent experiments were performed under identical conditions. The right panel shows the corresponding relative densities of the RT-PCR signals in the left panel. Quantitative analysis was performed using the Tanon GIS system.

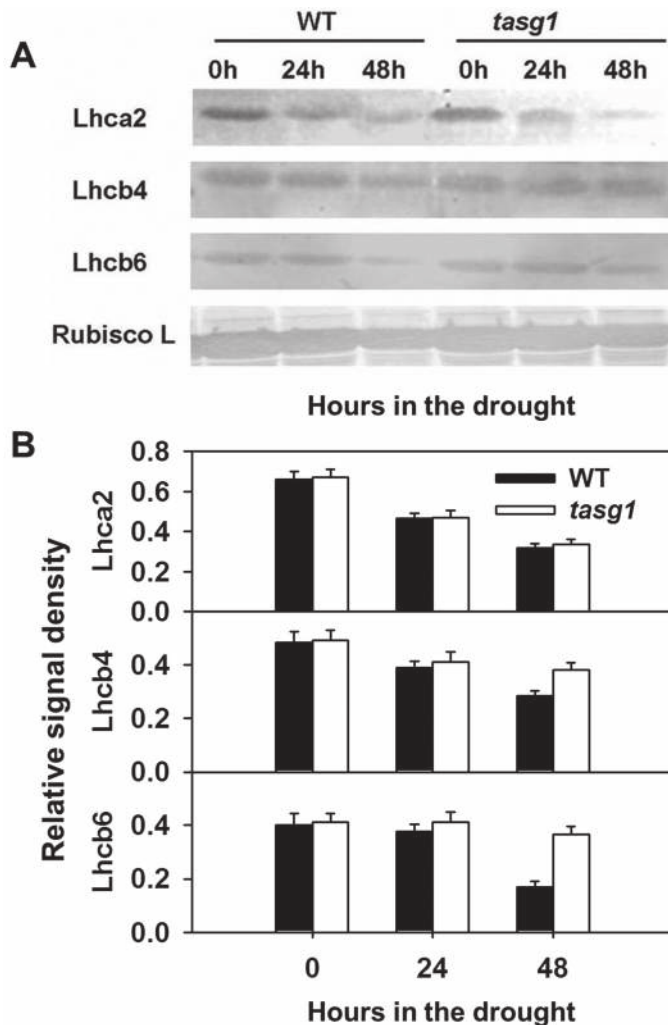


Fig. 6. Immunoblot analysis of degradation of photosynthesis-related proteins (Lhca2, Lhcb4, and Lhcb6) during induced drought stress in the WT and *tasg1* by 20% PEG for 0, 24, and 48 h. Rubisco L, the Rubisco large subunit. The lower panel shows the corresponding relative densities of the western blotting signals in the upper panel. The amounts of band signal by densitometry and normalized to that of the loading control (Rubisco L). Quantitative analysis was performed using the Tanon GIS system.

protein carbonylation products accumulated in the WT was greater than that in *tasg1* under both normal and drought stress conditions. Meanwhile, in the control experiment using PVDF membranes treated with HCl only without 2, 4-dinitrophenylhydrazine (DNPH), no protein carbonylation was found (data not shown).

$O_2^{\cdot-}$ accumulation was detected by monitoring the precipitation of purple formazan when reacting NBT with $O_2^{\cdot-}$. As shown in Fig. 8A, under normal conditions and in the early stage of drought stress, little NBT precipitation was detected in *tasg1*, but obvious NBT staining was observed in the WT. In the later stage of drought stress (Fig. 8B), NBT precipitation was significantly increased in both the WT and *tasg1*, but, compared with the WT, the accumulation of $O_2^{\cdot-}$ was less than in *tasg1*. DAB staining also showed less accumulation of H_2O_2 in *tasg1* than in the WT (Fig. 8C, D).

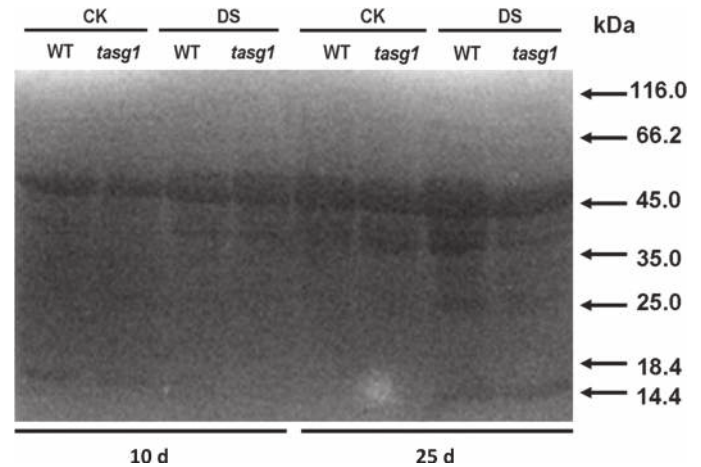


Fig. 7. Protein carbonylation levels were detected with anti-DNP antibodies in WT and *tasg1* flag leaves in the early and later stages of drought stress in the field. Protein gel blot analysis of protein carbonylation following derivatization of protein carbonyls with DNPH in HCl.

To confirm further differences in antioxidant activities between the two wheat varieties, wheat seedlings at the third-leaf stage were also used to detect accumulation of reactive oxygen species (ROS) accumulation by water stress treatment with 20% PEG-6000 solution or water as a control for 0, 24, and 48 h. The $O_2^{\cdot-}$ accumulation was lower in *tasg1* compared with the WT at all three observed time points, but no obvious difference in H_2O_2 accumulation was observed at this stage (Fig. 8H–J). The results (Fig. 8) collectively show that the accumulation of ROS in *tasg1* was lower than in the WT under both normal and drought stress conditions not only at the senescence stage but also at the seedling stages.

Effect of drought stress on relative electrical conductivity

At 10 d under drought, the REC of the WT was higher than that of *tasg1* under normal water conditions. Drought stress increased the REC in both wheat varieties, but it was lower in *tasg1* than in the WT. At 25 d under drought, leaf senescence increased the REC in both wheat varieties compared with that at 10 d under drought, and drought stress inhibited this increase. However, compared with the WT, the REC in *tasg1* was consistently lower over the course of the experiment (Fig. 9). The less severe membrane damage in *tasg1* compared with the WT was consistent with the greater antioxidant competence of *tasg1* (Figs 7–9).

Discussion

Improved functions of protein complexes in the thylakoid membrane in *tasg1* facilitate photosynthesis and drought resistance

In a previous study, *tasg1* was shown to retain photosynthetic competence, consistent with its delayed progression

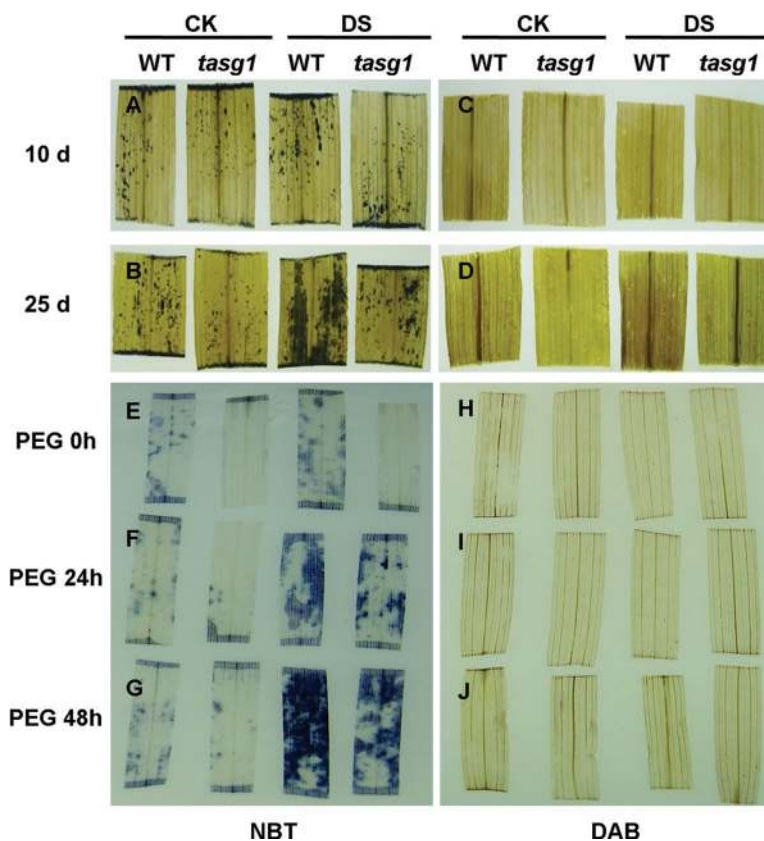


Fig. 8. Changes in $O_2^{\cdot-}$ (A, B) and H_2O_2 (C, D) accumulation in WT and *tasg1* flag leaves. The top row shows samples from plants after drought stress at the earlier stage. The bottom row shows samples from plants after drought stress at the later stage. Changes in $O_2^{\cdot-}$ (E, F, G) and H_2O_2 (H, I, J) accumulation in WT and *tasg1* leaves of third-leaf stage seedlings. Drought stress was induced using 20% PEG-6000 solution with water as a control for 0, 24, and 48 h. Leaves were detached after drought treatment and vacuum infiltrated with NBT or DAB solution. The presence of the purple formazan precipitate indicates the location and content of $O_2^{\cdot-}$ accumulation. The brown polymerization product is the result of H_2O_2 accumulation.

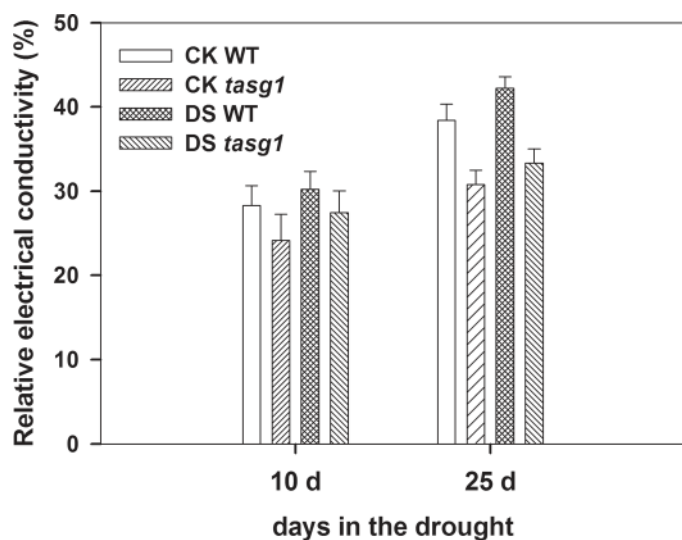


Fig. 9. Effects of water stress on relative electrical conductivity in *tasg1* and the WT. The results are means \pm SD of five independent measurements on each of five plants per experiment.

of chlorophyll degradation compared with WT plants at 30 d after anthesis. Therefore, *tasg1* is a functional stay-green

wheat mutant with the Type B phenotype (in which senescence is initiated on schedule but progresses at a rate lower than the WT) or the combination of Type B and Type A (in which senescence is initiated late but proceeds at a normal rate) (Hui *et al.*, 2012). In this current work, compared with the WT, *tasg1* exhibited markedly delayed senescence with retention of leaf colour, and the stay-green phenotype of *tasg1* was also maintained under drought stress (Fig. 1), suggesting improved drought resistance in *tasg1*.

Photosynthesis in the chloroplast is one of the most stress-sensitive physiological processes. Stress damages the thylakoid membrane, disturbs its functions, and ultimately decreases photosynthesis and crop yield (Shah and Paulsen, 2003; Huseynova *et al.*, 2007; Zhao *et al.*, 2007). In a previous study, the P_n of *tasg1* was found to be greater than that of the WT under drought stress (Tian *et al.*, 2012). However, the functions of the protein complexes in the thylakoid membrane of *tasg1* and how *tasg1* protects the thylakoid membrane from damage under drought stress was still unclear. To determine the stability of the protein complexes in the thylakoid membrane in *tasg1* under drought stress in this study, the Hill reaction activity, Φ_{PSII} and F_v/F_m of PSII photochemistry, ATPase activity, as well as the abundance of polypeptides in the thylakoid membrane were examined. The expression

levels of several genes and proteins involved in LHCI and LHCII were also detected.

The Hill reaction activity is an important index for oxygen-evolving centre (OEC) activity in thylakoid membranes. As can be seen in Fig. 2, in the early stage of drought stress (at 10 d under drought), the Hill reaction activity was reduced in leaves of both *tasg1* and WT plants by drought stress, but it remained at a higher level in *tasg1*. About 2 weeks later (at 25 d under drought), a steep decrease in the Hill reaction activity was observed under both normal and drought stress conditions, suggesting that severe damage to the OEC occurred due to leaf senescence as well as drought stress, but the decrease was smaller in *tasg1* compared with the WT.

The reduction in photosynthetic activity under drought stress can be the cause or result of photoinhibition. PSII is believed to play a key role in the response of leaf photosynthesis to environmental perturbations (Swiatek *et al.*, 2001; Dubey, 2005). Both Φ_{PSII} and F_v/F_m represent a measure of the functional status of PSII and have been used as a sensitive indicator of plant photosynthetic performance responding to stress conditions (Maxwell and Johnson, 2000). In this study, under non-stress conditions, no significant differences in Φ_{PSII} and F_v/F_m of PSII photochemistry were observed between *tasg1* and the WT, while drought stress significantly decreased Φ_{PSII} (Fig. 3A) and F_v/F_m (Fig. 3B). However, compared with the WT, those decreases in *tasg1* were attenuated under drought stress, indicating that the PSII complex in *tasg1* could better withstand photoinduced inactivation than the WT under drought stress. Furthermore, drought stress significantly ($P < 0.05$) decreased the activities of Ca^{2+} -ATPase and Mg^{2+} -ATPase in the thylakoid membrane, but the decreases in the WT were more drastic than those in *tasg1* (Fig. 3C, D), suggesting the better drought tolerance of ATPase in *tasg1* than in the WT. All these results were consistent with observations from a previous study (Wang *et al.*, 2010).

The function of the thylakoid membrane is dependent upon its fluidity and integrity, which are affected by its components, such as lipids, membrane proteins, pigments, and ions. Gillet *et al.* (1998) reported that drought induces an increase in the concentration of a 34 kDa thylakoid protein. Similar studies by Huseynova *et al.* (2007) showed that the concentration of a 40.5 kDa thylakoid membrane protein is increased under water stress. It was found here that the abundance of polypeptides from the thylakoid membrane with molecular masses of ~25–35 kDa, especially a 28 kDa polypeptide, was affected by drought stress (Fig. 4A). Meanwhile, the levels of three polypeptides of 28, 38, and 50 kDa were consistently higher in *tasg1* than in WT wheat seedlings at the third-leaf stage when treated with 20% PEG-6000 solution with water as a control for 24 h and 48 h (Fig. 4B). Of these three polypeptides detected in wheat seedlings, only the 28 kDa polypeptide was retained in mature plants (Fig. 4A). This difference suggested that some polypeptides were degraded during leaf development and senescence. Compared with the WT, *tasg1* could maintain the thylakoid membrane protein complexes with better stability against damage by drought stress.

Stay-green mutants are characterized by increased stability of chloroplast membranes and chlorophyll–protein complexes

(Kusaba *et al.*, 2007; Park *et al.*, 2007; Sato *et al.*, 2007). In a previous report, it was also suggested that the chloroplast and thylakoid structures in the *tasg1* mutant are protected from drought stress-induced damage (Tian *et al.*, 2012). Under drought stress conditions in *tasg1*, it had been found that the thylakoid stacks in the grana were nearly unchanged and also a fusion ultrastructure of several grana stacks was observed, which was consistent with other reports (Kusaba *et al.*, 2007; Schelbert *et al.*, 2009). The retention of LHCII is thought to play an important role in the formation and maintenance of grana stacks (Standfuss *et al.*, 2005). In this study, the expression levels of two genes encoding LHCII proteins, *TaLhcb4* and *TaLhcb6*, were found to be higher in *tasg1* compared with the WT, especially at the 48 h time point under drought stress (Fig. 5). Immunoblot analysis (Fig. 6) also revealed that the accumulation of TaLhca2, TaLhcb4, and TaLhcb6 was decreased in both the WT and *tasg1* after 48 h under drought stress. However, some inconsistencies were also found between the mRNA level and protein abundance in the three detected proteins. Expression levels of *TaLhca2* mRNA still remained at a higher level in *tasg1* than in the WT at the last tested time point of 48 h (Fig. 5). However, the WT and *tasg1* have almost the same protein content of TaLhca2 (Fig. 6). At 48 h after drought stress treatment, the mRNA level of *TaLhcb6* was 1.97-fold higher in *tasg1* than in the WT, while protein accumulation of TaLhcb6 was changed 2.15-fold. Previous outcomes indicate that further levels of regulation based on post-transcriptional and post-translational mechanisms are involved in the abiotic stress response (Oksman-Caldentey and Saito, 2005; Reinders and Sickmann, 2007). Beyond protein phosphorylation, other post-translational modifications such as ubiquitination and sumoylation regulate the activation and stability of pre-existing molecules to ensure a prompt response to stress. However, the exact mechanisms of post-transcriptional and post-translational regulatory systems in the WT and *tasg1* need to be studied further. In a previous study (Tian *et al.*, 2012), *tasg1* showed 52.0% and 72.5% higher contents of Chl *b* on the 30th day after anthesis compared with the WT under normal and drought stress conditions, respectively. Chl *b* is known to play an important role in LHCII stability (Bellemare *et al.*, 1982; Paulsen *et al.*, 1993; Horn and Paulsen, 2004), which may contribute to the maintenance of grana stacks in *tasg1*.

Enhanced antioxidant activities in tasg1 may contribute to delayed senescence and drought resistance

Abiotic stresses such as drought, cold, and salinity result in the production and accumulation of ROS, which are highly reactive and toxic to plants, and can lead to senescence and cell death by causing damage to proteins, lipids, DNA, and carbohydrates (Noctor and Foyer, 1998; Apel and Hirt, 2004). Although a variety of enzymatic and non-enzymatic antioxidants can remove the ROS continuously generated by aerobic metabolism and environmental stressors (Foyer and Noctor, 2005), a substantial fraction of cellular proteins is irreversibly damaged (Poppek and Grune, 2006), and the accumulated oxidized proteins can be toxic to plant cells (Cohen *et al.*, 2006).

Derivatization of protein carbonyls with DNPH, followed by immunoblotting with an anti-DNP antibody, is a sensitive and specific method for the detection of oxidatively modified proteins and is generally regarded as an indicator of oxidative stress (Qiu *et al.*, 2008). The lower amounts of accumulated protein carbonylation products observed in *tasg1* compared with the WT under both normal and drought stress conditions (Fig. 7) suggested less oxidative damage to proteins in *tasg1*. The lower levels of electrolyte leakage (Fig. 9) in *tasg1* were also indicative of less cell membrane damage induced by drought stress compared with the WT plant.

Meanwhile, the accumulation of $O_2^{\cdot-}$ and H_2O_2 was lower in *tasg1* than in the WT under normal and drought stress conditions (Fig. 8), which may be related to the higher activity of antioxidative enzymes in *tasg1* (Tian *et al.*, 2012). Furthermore, this lower ROS accumulation in *tasg1* was found not only in the senescence stage (Fig. 8A–D), but also in the seedling stage (Fig. 8E–J), suggesting the native greater antioxidant competence in *tasg1*.

In previous research, *tasg1* accumulated more soluble sugars and proteins under water stress, which could also contribute to protection from cell membrane damage in *tasg1* (Tian *et al.*, 2012).

In summary, the stay-green wheat mutant *tasg1* could stably maintain thylakoid membrane polypeptides at high levels, while its expression of some LHCI- and LHCII-related genes remained steady under drought stress. The improved Hill reaction activity, Φ_{PSII} and F_v/F_m of PSII photochemistry, and ATPase activity resulted in the high photosynthesis activity in *tasg1* compared with the WT plant. Thus, the results in this study combined with previous observations (Hui *et al.*, 2012; Tian *et al.*, 2012) suggest that greater antioxidant competence may contribute to the delayed senescence and drought resistance of *tasg1*.

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