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

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Received 3 August 2012; Revised 30 December 2012; Accepted 3 January 2013

Abstract

A wheat stay-green mutant, \textit{tasg1}, was previously generated via mutation breeding of HS2, a common wheat cultivar (\textit{Triticum aestivum} L.). Compared with wild-type (WT) plants, \textit{tasg1} exhibited delayed senescence indicated by the slower degradation of chlorophyll. In this study, the stability of proteins in thylakoid membranes was evaluated in \textit{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 \textit{tasg1} plants could maintain higher Hill activity, actual efficiency ($\Phi_{PSII}$), maximal photochemical efficiency of PSII ($F_{v}/F_{m}$), and Ca\textsuperscript{2+}-ATPase and Mg\textsuperscript{2+}-ATPase activities than the WT plants under drought stress. Furthermore, the abundance of some polypeptides in thylakoid membranes of \textit{tasg1} was greater than that in the WT under drought stress. Expression levels of \textit{TaLhcb4} and \textit{TaLhcb6} were higher in \textit{tasg1} compared with the WT. Under drought stress, the accumulation of superoxide radical (O\textsubscript{2}\textsuperscript{-}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was lower in \textit{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 \textit{tasg1} compared with the WT, and the higher antioxidant competence of \textit{tasg1} may play an important role in the enhanced drought tolerance of \textit{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 \textit{et al}., 2007; Zhao \textit{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 \textit{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 \textit{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 (*Pn*) 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 (O2·−) and hydrogen peroxide (H2O2) 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 (ΦPSII) and the maximal photochemical efficiency of PSII (*Fv/Fm*) 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 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 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 specific primers. The specific primers for genes encoding LHCI (TaLhca1, TaLhca2, and TaLhca3) and LHCCI (TaLhcb1, TaLhcb4, and TaLhcb6) subunits were designed based on published wheat expressed sequence tags (ESTs). TaLhca1, F (5'-CACGTGGCCGACCATCTGGT-3') and R (5'-CAGCCGGCCGTCTTACAGT-3'); TaLhca2, F (5'-CCC AACCGGAAAGACC-3') and R (5'-CCGACGACAGGGGAAAGCAT-3'); TaLhca3, F (5'-CCTACACGCTACGATTCCTC-3') and R (5'-CGCACTCGCTAGTTTCC-3'); TaLhcb1, F (5'-GG AGAACACACAATACACC-3') and R (5'-CCCCATTTAGGTGCGATTTCC-3'); TaLhcb4, F (5'-AAAGGCCGAGGAGGAAAC-3') and R (5'-CCACCCGACATTAAGGGA-3'); and TaLhcb6, F (5'-TACCGCGACTCTACGGTCA-3') and R (5'-CCCAAAAGTGCTACGGACA-3').

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-glycero-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.

$H_{2}O_{2}$ 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 chlorhydate (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 ($S_{1}$) (25 °C). A test tube was filled with leaf discs and distilled water, the the mixture was cooked (100 °C) for 30 min and then reduced to room temperature (25 °C) to determine the final electric conductance ($S_{2}$). The relative electric conductivity (REC) was evaluated as: REC = $S_{1} × 100/S_{2}$.

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 \textit{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 \textit{tasg1} compared with the WT (Fig. 1A–C). Meanwhile, the areas of the flag leaves of \textit{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 \textit{tasg1} and WT plants

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

Fig. 1. Naturally senescent WT (left) and \textit{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 \textit{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 \textit{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 (ΦPSII) and the maximal photochemical efficiency of PSII (Fv/Fm) 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 ΦPSII and Fv/Fm of PSII photochemistry was observed between *tasg1* and the WT in the early stage of drought stress. In the later stage of drought stress...
stress, $FV/Fm$ was decreased, but not significantly, while $\Phi_{PSII}$ decreased significantly. However, $\Phi_{PSII}$ 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).
Protein stability and antioxidant competence contribute to drought stress resistance in \textit{tasg1}.

In the laboratory, and the three observed polypeptides of \( \sim 28 \), 38, and 50 kDa in \textit{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 \textit{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 \textit{TaLhca1}, \textit{TaLhca2}, and \textit{TaLhca3}, were down-regulated gradually during drought stress in both the WT and \textit{tasg1} (Fig. 5). The mRNA levels of all three \textit{TaLhca} genes tested were reduced to the minimum at 48 h after water stress treatment. For example, \textit{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 \textit{tasg1}. Meanwhile, \textit{TaLhca2}, at the last tested time point of 48 h, still remained at a higher level in \textit{tasg1} than in the WT.

Expression levels of genes involved in LHCII, namely \textit{TaLhcb1}, \textit{TaLhcb4}, and \textit{TaLhcb6}, were not consistent with those involved in LHCI (Fig. 5). The expression levels of \textit{TaLhcb4} and \textit{TaLhcb6} were higher in \textit{tasg1} compared with the WT, especially at the last tested time point (48 h). The degradation of \textit{TaLhcb4} and \textit{TaLhcb6} was inhibited in \textit{tasg1}, decreasing slowly as the period of drought stress was extended. Even at the last tested time point (48 h), the transcript levels of \textit{TaLhcb4} and \textit{TaLhcb6} did not decrease significantly and still remained high. The expression level of \textit{TaLhcb4} was significantly different between the WT and \textit{tasg1} in the early stage during drought stress, while that of \textit{TaLhcb6} was largely different between the two plant types in the later stage during drought stress. However, the expression levels of \textit{TaLhcb1} were similar between the WT and \textit{tasg1} over the course of drought stress treatment.

To analyse pigment-binding proteins further, the abundance of \textit{TaLhca2}, \textit{TaLhcb4}, and \textit{TaLhcb6} was detect 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 \textit{tasg1}, especially at the last tested time point (48 h) according to Fig. 5. Immunoblot analysis (Fig. 6) revealed that the accumulation of \textit{TaLhca2}, \textit{TaLhcb4}, and \textit{TaLhcb6} was decreased in both the WT and \textit{tasg1} at 48 h after treatment under drought stress. There was no significant difference in accumulation of \textit{TaLhca2} between the WT and \textit{tasg1}. However, the contents of \textit{TaLhcb4} and \textit{TaLhcb6} were higher in \textit{tasg1} than in the WT under drought stress. The protein accumulation of \textit{TaLhcb6} was 2.15-fold higher in \textit{tasg1} than in the WT at 48 h after treatment under drought stress. The mRNA accumulation of \textit{TaLhcb6} was 1.97-fold higher in \textit{tasg1} compared with the WT (Fig. 5). These observations suggest that \textit{TaLhcb4} and \textit{TaLhcb6} could be maintained at a higher level in \textit{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 \textit{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 \textit{tasg1} and the WT. However, the amount of carbonylation was significantly higher in \textit{tasg1} than in the WT at 48 h after treatment under drought stress.

![Fig. 5.](https://example.com/fig5.png) 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 \textit{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.
protein carbonylation products accumulated in the WT was greater than that in \textit{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).

\(\text{O}_2^-\) accumulation was detected by monitoring the precipitation of purple formazan when reacting NBT with \(\text{O}_2^-\). As shown in Fig. 8A, under normal conditions and in the early stage of drought stress, little NBT precipitation was detected in \textit{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 \textit{tasg1}, but, compared with the WT, the accumulation of \(\text{O}_2^-\) was less than in \textit{tasg1}. DAB staining also showed less accumulation of \(\text{H}_2\text{O}_2\) in \textit{tasg1} than in the WT (Fig. 8C, D).

Effect of drought stress on relative electrical conductivity

At 10 d under drought, the REC of the WT was higher than that of \textit{tasg1} under normal water conditions. Drought stress increased the REC in both wheat varieties, but it was lower in \textit{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 \textit{tasg1} was consistently lower over the course of the experiment (Fig. 9). The less severe membrane damage in \textit{tasg1} compared with the WT was consistent with the greater antioxidant competence of \textit{tasg1} (Figs 7–9).

Discussion

\textit{Improved functions of protein complexes in the thylakoid membrane in \textit{tasg1} facilitate photosynthesis and drought resistance}

In a previous study, \textit{tasg1} was shown to retain photosynthetic competence, consistent with its delayed progression
Protein stability and antioxidant competence contribute to drought stress resistance in \textit{tasg1}

of chlorophyll degradation compared with WT plants at 30 d after anthesis. Therefore, \textit{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, \textit{tasg1} exhibited markedly delayed senescence with retention of leaf colour, and the stay-green phenotype of \textit{tasg1} was also maintained under drought stress (Fig. 1), suggesting improved drought resistance in \textit{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 \( P_{\text{m}} \) of \textit{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 \textit{tasg1} and how \textit{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 \textit{tasg1} under drought stress in this study, the Hill reaction activity, \( \Phi_{\text{PSII}} \) and \( F_{\text{v}}/F_{\text{m}} \) 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 LHCl and LHCI 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 
\( \text{tasg1} \) and WT plants by drought stress, but it remained at a higher level in 
\( \text{tasg1} \). 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 
\( \text{tasg1} \) 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 \( \Phi_{\text{PSII}} \) 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 \( \Phi_{\text{PSII}} \) and \( F_{v}/F_{m} \) of PSII photochemistry were observed between 
\( \text{tasg1} \) and the WT, while drought stress significantly decreased \( \Phi_{\text{PSII}} \) (Fig. 3A) and \( F_{v}/F_{m} \) (Fig. 3B). However, compared with the WT, those decreases in 
\( \text{tasg1} \) were attenuated under drought stress, indicating that the PSII complex in 
\( \text{tasg1} \) could better withstand photoinduced inactivation than the WT under drought stress. Furthermore, drought stress significantly (\( P < 0.05 \)) decreased the activities of \( \text{Ca}^{2+}\text{-ATPase} \) and \( \text{Mg}^{2+}\text{-ATPase} \) in the thylakoid membrane, but the decreases in the WT were more drastic than those in 
\( \text{tasg1} \) (Fig. 3C, D), suggesting the better drought tolerance of ATPase in 
\( \text{tasg1} \) 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 
\( \text{tasg1} \) 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, 
\( \text{tasg1} \) 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 
\( \text{tasg1} \) mutant are protected from drought stress-induced damage (Tian et al., 2012). Under drought stress conditions in 
\( \text{tasg1} \), 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 LHClI 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 LHClI proteins, TaLhcb4 and TaLhcb6, were found to be higher in 
\( \text{tasg1} \) 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 
\( \text{tasg1} \) 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 
\( \text{tasg1} \) than in the WT at the last tested time point of 48 h (Fig. 5). However, the WT and 
\( \text{tasg1} \) 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 
\( \text{tasg1} \) 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-Calderentey 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 
\( \text{tasg1} \) need to be studied further. In a previous study (Tian et al., 2012), 
\( \text{tasg1} \) 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 LHClI stability (Bellemare et al., 1982; Paulsen et al., 1993; Horn and Paulsen, 2004), which may contribute to the maintenance of grana stacks in 
\( \text{tasg1} \).

Enhanced antioxidant activities in 
\( \text{tasg1} \) 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 $O_2^-$ 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 antioxidative 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 reliably maintain thylakoid membrane polypeptides at high levels, which could also contribute to protection from cell membrane damage in tasg1 (Tian et al., 2012).

**Acknowledgements**

This work was supported by National Natural Science Foundation of China (No. 30671259).

**References**

Allen JF, Forsberg J. 2001. Molecular recognition in thylakoid structure and function. *Trends in Plant Science* 6, 317–326.

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* 55, 373–399.

Arnon DI. 1949. Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* 24, 1–15.

Bellemare G, Bartlett SG, Chua NH. 1982. Biosynthesis of chlorophyll a/b-binding polypeptides in wild type and the chlorina-f2 mutant of barley. *Journal of Biological Chemistry* 257, 7762–7767.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* 72, 248–254.

Chaves MM, Oliveira MM. 2004. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. *Journal of Experimental Botany* 55, 2365–2384.

Cohen E, Bieschke J, Percivalle RM, Kelly JW, Dillin A. 2006. Opposing activities protect against age-onset proteotoxicity. *Science* 313, 1604–1610.

Dalle-donne I, Rossi R, Giustarini D, Gagliano N, Simplicio PD, Colombo R, Miliani A. 2002. Methionine oxidation as a major cause of the functional impairment of oxidized actin. *Free Radical Biology and Medicine* 32, 927–937.

Dubey RS. 2005. Photosynthesis in plants under stressful conditions. In: Pessarakli M, ed. *Handbook of photosynthesis*, 2nd edn. New York: CRC Press, Taylor and Francis Group, 717–737.

Foyer CH, Noctor G. 2005. Redox homeostasis and antioxidative signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell* 17, 1866–1875.

Giacomelli L, Masi A, Ripoll DR, Lee MJ, van Wijk KJ. 2007. *Arabidopsis thaliana* deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low. *Plant Molecular Biology* 65, 627–644.

Gillet B, Beely A, Peltier G, Rey P. 1998. Molecular characterization of CDSP 34, a chloroplastic protein induced by water deficit in *Solanum tuberosum* L. plants, and regulation of CDSP 34 expression by ABA and light illumination. *The Plant Journal* 16, 257–262.

Horn R, Paulsen H. 2004. Early steps in the assembly of light harvesting chlorophyll a/b complex. *Journal of Biological Chemistry* 279, 44400–44406.

Hörtenersteiner S. 2009. Stay-green regulates chlorophyll and chlorophyll-binding protein degradation during senescence. *Trends in Plant Science* 14, 155–162.

Huang, ZH. 1985. *Techniques of plant physiological experiment*. Shanghai: Shanghai Scientific & Technical Publishers, 111–115.

Hui Z, Tian FX, Wang GK, Wang GP, Wang W. 2012. The antioxidative defense system is involved in the delayed senescence in a wheat mutant tasg1. *Plant Cell Reports* 31, 1073–1084.

Huseynova IM, Suleymanov SY, Aliyev JA. 2007. Structural functional state of thylakoid membranes of wheat genotypes under water stress. *Biochimica et Biophysica Acta* 1767, 869–875.

Kusaba M, Ito H, Morita R, et al. 2007. Rice NON-YELLOW COLORING1 is involved in light-harvesting complex II and grana degradation during leaf senescence. *The Plant Cell* 19, 1362–1375.

Lichtenenthaler HK. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods in Enzymology *350–382*.

Matile P, Hörtenersteiner S, Thomas H, Kraütler B. 1996. Chlorophyll degradation in senescent leaves. *Plant Physiology* 112, 1403–1409.

Matile P, Hörtenersteiner S, Thomas H. 1999. Chlorophyll degradation. *Annual Review of Plant Physiology and Plant Molecular Biology* 50, 67–95.

Maxwell K, Johnson GN. 2000. Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany* 51, 659–668.

Noctor G, Foyer CH. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Biology* 49, 249–279.
Oksman-Caldentey KM, Saito K. 2005 Integrating genomics and metabolomics for engineering plant metabolic pathways. Current Opinion in Biotechnology 16, 174–179.

Parida AK, Das AB, Mittra B. 2003. Effects of NaCl stress on the structure, pigment complex composition, and photosynthetic activity of mangrove Bruguiera parviflora chloroplasts. Photosynthetica 41, 191–200.

Park SY, Yu JW, Park JS, et al. 2007. The senescence-induced stay green protein regulates chlorophyll degradation. The Plant Cell 19, 1649–1664.

Paulsen H, Finkenzeller B, Kühlein N. 1993. Pigments induce folding of light-harvesting chlorophyll a/b-bonding protein. European Journal of Biochemistry 215, 809–816.

Poppek D, Grune T. 2006. Proteasomal defense of oxidative protein modifications. Antioxidants and Redox Signaling 8, 173–184.

Qiu QS, Huber JL, Booker FL, Jain V, Leakey ADB, Fiscus EL, Yau PM, Ort DR, Huber SC. 2008. Increased protein carbonylation in leaves of Arabidopsis and soybean in response to elevated [CO2]. Antioxidants and Redox Signaling 173–184.

Reinders J, Sickmann A. 2007. Modificomics: posttranslational modifications beyond protein phosphorylation and glycosylation. Biomolecular Engineering 24, 169–177.

Sato Y, Morita R, Katsuma S, Nishimura M, Tanaka A, Kusaba M. 2009. Two short-chain dehydrogenase/reductases, NON-YELLOW COLORING1 and NYC1-LIKE, are required for chlorophyll b and light-harvesting complex II degradation during senescence in rice. The Plant Journal 57, 120–131.

Sato Y, Morita R, Nishimura M, Yamaguchi H, Kusaba M. 2007. Mendel’s green cotyledon gene encodes a positive regulator of the chlorophyll degrading pathway. Proceedings of the National Academy of Sciences, USA 104, 14169–14174.

Schelbert S, Aubry S, Burla B, Agne B, Kessler F, Krupinska K, Hörtenstein, S. 2009. Phophytin phosphoribide hydrolase (pheophytinase) is involved in chlorophyll breakdown during leaf senescence in Arabidopsis. The Plant Cell 21, 767–785.

Shah NH, Paulsen GM. 2003. Interaction of drought and high temperature on photosynthesis and grain-filling of wheat. Plant and Soil 257, 219–226.

Spano G, Di Fonzo N, Perrotta C, Platani C, Ronga G, Lawlor DW, Napier JA, Shewry PR. 2003. Physiological characterization of ‘stay green’ mutants in durum wheat. Journal of Experimental Botany 54, 1415–1420.

Standfuss J, van Scheltinga, ACT, Lamborghini M, Kühlbrandt W. 2005. Mechanisms of photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5 Å resolution. EMBO Journal 24, 919–928.

Swiatek M, Kuras R, Sokolenko A, et al. 2001. The chloroplast gene ycf9 encodes a photosystem II (PSII) core subunit, PsbZ, that participates in PSII supramolecular architecture. The Plant Cell 13, 1347–1367.

Thomas H, Howarth CJ. 2000. Five ways to stay green. Journal of Experimental Botany 51, 329–337.

Thomas H, Smart CM. 1993. Crops that stay green. Annals of Applied Biology 123, 193–223.

Tian FX, Gong JF, Wang GP, Wang GK, Fan ZY, Wang W. 2012. Improved drought resistance in a wheat stay-green mutant tasg1 under field conditions. Biologia Plantarum 56, 509–515.

Wang GP, Zhang XY, Li F, Luo Y, Wang W. 2010. Overaccumulation of glycine betaine enhances tolerance to drought and heat stress in wheat leaves in the protection of photosynthesis. Photosynthetica 48, 117–126.

Ye JY, Qian YQ. 1985. Detection of Hill reaction with spectrophotometer. In: Xue YL, Xia ZA, eds. Plant physiology experiment handbook. Shanghai: Shanghai Science and Technology Press, 104–107.

Yoo SC, Cho SH, Zhang H, Paik HC, Lee CH, Li J, Yoo JH, Lee BW, Koh HJ, Seo HS, Paek NC. 2007. Quantitative trait loci associated with functional stay-green SNU-SG1 in rice. Molecules and Cells 24, 83–94.

Zhang J, Guo QF, Feng YN, Li F, Gong JF, Fan ZY, Wang W. 2012. Manipulation of monoubiquitin improves salt tolerance in transgenic tobacco. Plant Biology 14, 315–324.

Zhang LX, Paakkarinen V, van Wijk KJ, Aro EM. 1999. Cotranslational assembly of the D1 protein into photosystem II. Journal of Biological Chemistry 274, 16062–16067.

Zhao XX, Ma QQ, Liang C, Fang Y, Wang YQ, Wang W. 2007. Effect of glycinebetaine on function of thylakoid membranes in wheat flag leaves under drought stress. Biologia Plantarum 51, 584–588.

Zheng HJ, Wu AZ, Zheng CC, Wang YF, Cai R, Shen XF, Xu RR, Liu P, Kong LJ, Dong ST. 2009. QTL mapping of maize (Zea mays) stay-green traits and their relationship to yield. Plant Breeding 128, 54–62.