Improved drought resistance in a wheat stay-green mutant *tasg1* under field conditions

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Abstract

We investigated the drought resistance of a wheat (*Triticum aestivum* L.) stay-green mutant *tasg1* and its wild-type (WT) in field experiments conducted for two years. Drought stress was imposed by controlling irrigation and sheltering the plants from rain. Compared with the WT, *tasg1* exhibited a distinct delayed senescence under both normal and drought stress conditions, as indicated by slower degradation of chlorophyll and decrease in net photosynthetic rate than in WT. At the same time, *tasg1* mutants maintained more integrated chloroplasts and thylakoid ultrastructure than did WT plants under drought stress. Lower malondialdehyde content and higher antioxidative enzyme activities in *tasg1,* compared to WT, may be involved in the stay-green phenotype and drought resistance of *tasg1*.

Additional key words: antioxidative defense system, chlorophyll degradation, chloroplast and thylakoid ultrastructures, drought stress, photosynthesis.

Introduction

Drought stress restricts crop growth and productivity (Bahieldina *et al.* 2005). Climate models predict that drought will become more frequent as a long-term consequence of global warming (Salinger 2005, Cook *et al.* 2007), emphasizing the urgent need to develop adaptive strategies in crop breeding for a changing environment.

The stay-green trait is characterized by a plant's ability to tolerate post-flowering drought stress, and has been found in many crop plants. Stay-green plants are further divided into functional stay-green and non-functional stay-green mutants, depending on whether retention of green color is coupled with retention (functional stay-greens) or loss (non-functional stay-greens) of photosynthetic activity (Thomas and Howarth 2000).

The possibility of controlling senescence to increase drought resistance was confirmed by experiments in which drought-induced production of cytokinins, known to delay senescence, improved plant survival and yield (Rivero *et al.* 2007). In sorghum, delayed leaf senescence has been linked to higher grain yield, particularly in environments in which available water during grain filling is inadequate to support potential transpiration (Borrell e*t al.* 1999, 2000a,b). Borrell *et al.* (2000b) also reported that stay-green sorghum hybrids produced 47 % more post-anthesis biomass than their counterparts under terminal moisture deficit conditions. Four green-retaining wheat lines had higher yields than their parents under drought (Spano *et al.* 2003).

In this paper we showed that a wheat stay-green mutant, *tasg1,* exhibits distinctly delayed senescence and evaluated physiological mechanisms of its drought resistance under field conditions.

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; c_i - intercellular CO_2 concentration; DAA - days after anthesis; DS - drought stress; E - transpiration rate; g_s - stomatal conductance; MDA - malondialdehyde; OA - osmotic adjustment; P_N - net photosynthetic rate; POD - peroxidase; RWC - relative water content; SOD - superoxide dismutase.
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Materials and methods

A wheat stay-green mutant, named *tasg1*, was generated in our laboratory by application of ethyl methane sulphonate EMS) to wheat (*Triticum aestivum* L.) cultivar HS2 (WT). Plants were grown in the fields of Shandong Agricultural University, China, during the growing seasons (October to June) of 2008 - 2009 and 2009 - 2010. Six 5 m^2 interspersed plots were established *via* random block design in the field, with three replicate plots for each genotype. Wheat kernels were hand-sown in 10 lines, with rows spaced 25 cm apart. Wheat kernels were planted at about 1 - 2 cm depth and 5 cm spacing. Conventional agricultural management was maintained during growth and development of the seedlings. Drought stress (DS) was imposed by controlling irrigation and sheltering the plants from rain, together with conventional water conditions for controls (CK). Soil water content was measured gravimetrically after 25-d DS (on 1 June, 2009 and 5 June, 2010) and a statistically significant difference between DS and CK was achieved.

The measurements of physiological parameters were made on intact flag leaves from 26 April to 2 June 2009, and 29 April to 6 June 2010. The April dates were about 8 d before flowering and the June dates about 2 d before harvest.

The measurements of gas exchange parameters, including net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), and intercellular CO_2 concentration (c_i), were made on intact flag leaves, using a portable photosynthesis system (*CIRAS-2*, *PP Systems*, Norfolk, UK) between 9:00 and 11:00, at photon flux density (PFD) of 1 200 μmol m^{-2} s^{-1}. The measurements lasted approximately 10 min, during which no significant changes in the environment were observed. Six replicate measurements were conducted (with six flag leaves from different wheat plants used for each genotype). After determining photosynthetic parameters, we harvested the leaves and used for determination of biochemical parameters in the laboratory.

The pigments were extracted from 0.05 g of fresh leaves in 10 cm^3 of 80 % aqueous acetone. Chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) contents were determined with a *Hitachi U-2001* (Japan) spectrophotometer at 663.2, 646.8, and 470.0 nm. Pigment contents were calculated according to Lichtenthaler (1987).

In the middle of the leaves, near a vein, small pieces (1 mm^2) were cut and fixed in 2 % glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.0) for 12 h at 4 °C. The segments were washed and post-fixed in 1 % osmium tetroxide for 4 h in the same buffer, and then dehydrated in a graded series of ethanol solutions and embedded in *Epon-812*. Ultrathin sections cut on a *Reichert Ultratome* (*OM-U3, Reichert-Jung*, Vienna, Austria) were stained with uranyl acetate and lead citrate and examined under transmission electron microscope (*JEOL-1200EX*, Tokyo, Japan).

The relative water content (RWC) of the wheat flag leaves was determined according to Ma *et al.* (2006). Leaf water potential (Ψw) was measured with a HR-33 T dew point microvoltmeter (*Wescor*, Logan, UT, USA) after equilibration in the chamber for 2.5 h. Osmotic potential and osmotic adjustment (OA) were determined with a vapor pressure osmometer (*Vapro Osmometer 5520, Wescor*). For the measurement of osmotic potential at full pressure potential (ψ_s^{100}), tissues were rehydrated with deionized water for 6 - 8 h at 4 °C in the dark. OA was calculated as the CK-DS difference in ψ_s^{100} (ψ_{sc}^{100} - ψ_{ss}^{100}).

The soluble protein content was measured following the method of Bensadoun and Weinstein (1976), with bovine serum albumin (BSA) as a standard. Total soluble sugar content was analyzed with anthrone reagent with a *Bausch and Lomb* spectrophotometer (Yemm and Willis 1954).

Antioxidant enzymes were extracted by grinding 1 g of fresh flag leaves with 10 cm^3 extraction buffer (0.05 M phosphate, pH 7.8, containing 0.1 mM ethylene diamine tetracetic acid (EDTA) and 1 %, (m/v) polyvinyl-pyrrolidone (PVP), in an ice bath and centrifuging at 12 000 g for 20 min. Enzyme activities were measured in the supernatant.

Enzyme activities were determined according to the following methods: superoxide dismutase (SOD; EC 1.15.1.1) and ascorbate peroxidase (APX; EC1.11.1.11) following Cakmak and Marschner 1992, catalase (CAT; EC 1.11.1.6) according to Durner and Klessig 1996 and peroxidase (POD; EC 1.11.1.7) according to Scebba *et al.* 2001. Enzyme assays were carried out using a UV-visible spectrophotometer (*Shimadzu UV-1601*) at 25 °C.

The assay for O_2^- was performed as described by Sui *et al.* (2007). Fresh leaves (0.5 g) were thoroughly ground in an ice bath in 5 cm^3 of grinding medium containing 0.05 M phosphate buffer (pH 7.8). The homogenate was centrifuged at 5 000 *g* for 10 min at 4 °C. The supernatant with phosphate buffer (pH 7.8) and 0.5 cm^3 of 10 mM hydroxyl-ammonium chloride was incubated at 25 °C for 20 min; 1 cm^3 of 17 mM *p*-aminobenzene sulfonic acid and 1 cm^3 of 7 mM α-naphthylamine were then added, and the mixture was incubated at 25 °C for 20 min. Finally, 1 cm^3 of ethyl ether was added to the mixture, which was centrifuged at 1 500 *g* for 5 min. The water phase was used for determination of absorbance at 530 nm. The malon-dialdehyde (MDA) level was assayed according to Quan *et al.* (2004).

All experiments were repeated at least three times. Statistical analysis was conducted using *Data Processing System* (*DPS*; Zhejiang University, China). Differences among wheat lines or treatments were compared using Duncan's multiple range tests at 0.05 probability levels.

Results

In well watered CK plants, no significant difference between *tasg1* and WT was observed in plant development or phenology before the flag leaves appeared, except that the flag leaf of WT plant emerged on about 25 April, while on *tasg1* mutant one or two days later. The flowering date of *tasg1* was also delayed by one or two days compared to WT. The stay-green phenotype of *tasg1* was expressed at the beginning of anthesis, and was especially apparent during natural senescence. DS accelerated plant senescence in both wheat genotypes, but this was always delayed in *tasg1* compared to WT. Meanwhile, the area of the flag leaves of *tasg1* at the grain filling stage was 28 and 23 % larger than in WT under CK and DS, respectively (data not shown).

No obvious difference was found in Chl *a* content between *tasg1* and WT at the initial phase of senescence (data not shown). However, from the 22nd to the 30th day after anthesis (DAA), Chl *a* content in *tasg1* was always higher than in WT, especially under DS. Similar differences were observed in Chl *b* content (Table 1).

Net photosynthetic rate (P_N) was higher in *tasg1,*

Table 1. Chlorophyll *a* content, chlorophyll *b* content, net photosynthetic rate, transpiration rate, stomatal conductance, intercellular CO_2 concentration, superoxide production, malondialdehyde contents, superoxide dismutase, peroxidase, catalase and ascorbate peroxidase actiities in flag leaves of field-grown *tasg1* and WT wheat plants under CK and DS conditions. Means ± SE, n = 3. DAA - days after anthesis. Data from year 2009, those from 2010 were similar.

Parameters	DAA	CK WT	CK *tasg1*	DS WT	DS *tasg1*
Chl *a* content	22	2.68 ± 0.09	2.77 ± 0.08	2.41 ± 0.03	2.43 ± 0.10
[mg g^{-1}(f.m.)]	26	2.02 ± 0.12	2.29 ± 0.14	1.70 ± 0.30	2.05 ± 0.10
	30	1.25 ± 0.35	1.95 ± 0.18	0.89 ± 0.14	1.72 ± 0.13
Chl *b* content	22	1.12 ± 0.03	1.13 ± 0.13	1.04 ± 0.03	0.97 ± 0.02
[mg g^{-1}(f.m.)]	26	0.84 ± 0.05	0.98 ± 0.06	0.73 ± 0.06	0.87 ± 0.11
	30	0.50 ± 0.02	0.76 ± 0.10	0.40 ± 0.02	0.69 ± 0.02
P_N	22	4.43 ± 0.05	4.20 ± 0.10	5.00 ± 0.40	4.13 ± 0.20
[µmol m^{-2} s^{-1}]	26	4.01 ± 0.30	3.84 ± 0.30	4.20 ± 0.05	4.34 ± 0.12
	30	2.53 ± 0.30	3.66 ± 0.30	2.03 ± 0.32	3.29 ± 0.40
E	22	4.43 ± 0.50	4.20 ± 0.10	5.00 ± 0.40	4.13 ± 0.20
[mmol m^{-2} s^{-1}]	26	4.00 ± 0.30	3.84 ± 0.30	4.20 ± 0.50	4.34 ± 0.12
	30	2.53 ± 0.30	3.66 ± 0.30	2.03 ± 0.32	3.29 ± 0.40
g_s	22	226.00 ± 6.00	214.00 ± 10.0	206.00 ± 13.0	230.50 ± 12.0
[mmol m^{-2} s^{-1}]	26	190.00 ± 7.00	200.00 ± 12.0	167.00 ± 15.0	163.00 ± 7.00
	30	54.00 ± 2.00	118.00 ± 9.00	14.00 ± 2.00	88.00 ± 6.00
c_i	22	249.17 ± 9.00	230.29 ± 8.00	239.40 ± 12.0	227.60 ± 6.00
[µmol mol^{-1}]	26	241.83 ± 6.00	214.00 ± 10.0	240.33 ± 13.0	236.50 ± 12.0
	30	290.50 ± 2.00	233.67 ± 9.00	317.00 ± 12.0	255.00 ± 6.00
O_2^- production rate	22	2.14 ± 0.08	1.15 ± 0.09	1.88 ± 0.16	1.08 ± 0.04
[µmol mg^{-1}(protein) min^{-1}]	26	1.61 ± 0.05	0.82 ± 0.11	2.01 ± 0.22	0.78 ± 0.18
	30	2.93 ± 0.06	1.55 ± 0.05	4.64 ± 0.20	2.46 ± 0.24
MDA content	22	14.11 ± 0.80	13.12 ± 0.60	14.52 ± 0.90	13.86 ± 0.60
[µmol g^{-1}(f.m.)]	26	17.40 ± 0.75	15.84 ± 0.80	17.57 ± 1.02	18.00 ± 0.80
	30	18.48 ± 1.20	16.83 ± 0.70	23.18 ± 0.80	21.12 ± 0.50
SOD activity	22	37.50 ± 1.24	38.60 ± 0.12	31.75 ± 1.86	35.70 ± 1.80
[U mg^{-1}(protein)]	26	25.20 ± 1.50	37.09 ± 2.70	24.00 ± 1.81	33.09 ± 1.37
	30	17.10 ± 0.34	25.20 ± 0.06	10.60 ± 0.65	19.80 ± 0.78
POD activity	22	189.00 ± 4.50	181.00 ± 9.00	177.00 ± 2.60	217.78 ± 7.80
[U mg^{-1}(protein)]	26	138.00 ± 3.30	188.00 ± 7.00	157.00 ± 5.30	177.00 ± 6.30
	30	92.00 ± 4.50	136.00 ± 6.00	35.00 ± 6.30	120.00 ± 5.40
CAT activity	22	169.00 ± 10.2	190.00 ± 10.0	150.49 ± 3.40	170.00 ± 12.0
[U mg^{-1}(protein)]	26	143.11 ± 23.0	172.00 ± 7.99	129.72 ± 13.0	160.77 ± 13.0
	30	83.00 ± 10.3	117.12 ± 13.5	58.00 ± 11.0	111.00 ± 7.53
APX activity	22	70.00 ± 6.71	80.40 ± 2.27	68.00 ± 3.73	72.00 ± 8.85
[U mg^{-1}(protein)]	26	45.00 ± 4.53	59.10 ± 1.88	29.00 ± 3.06	35.50 ± 10.2
	30	25.30 ± 9.11	40.80 ± 7.97	3.70 ± 0.99	14.90 ± 6.28

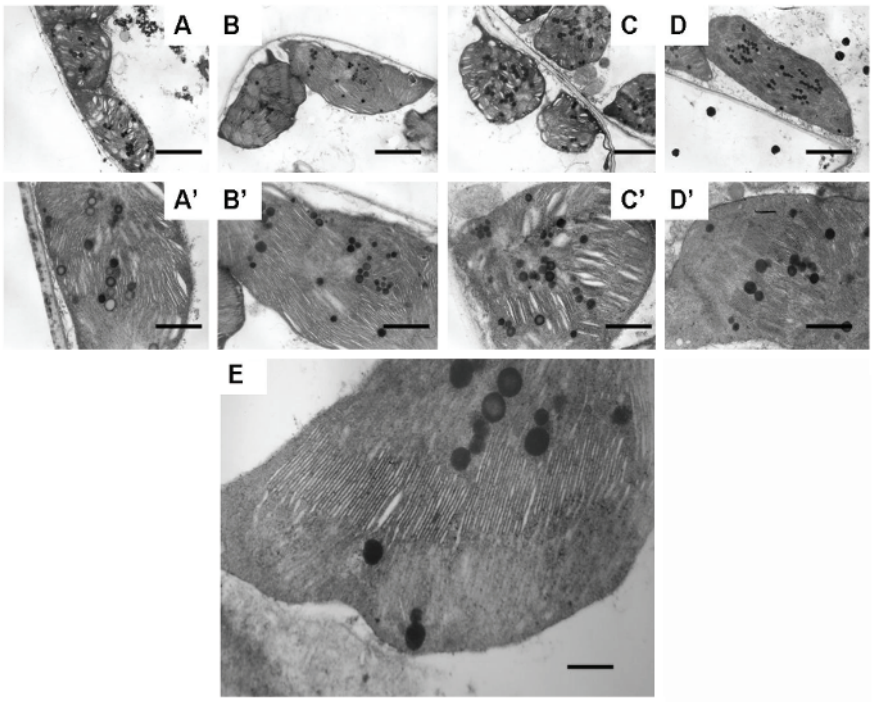

Fig. 1. Chloroplast ultrastructure in flag leaves of *tasg1* (*B,D*) and WT (*A,C*) under CK (*A,B*) and DS (C,*D*) (at 5th DAA, *bar*s = 200 nm). Thylakoid ultrastructure in flag leaves of *tasg1* (*B′,D′*) and WT (*A′ C′*) under CK (*A′,B′*) and DS (*C′,D′*) (at 15th DAA, *bars* = 400 nm). Chloroplast grana stacks fused in flag leaves of *tasg1* when subjected to DS at 15th DAA (*E*) (*bar* = 200 nm). Photographs obtained in 2009.

Table 2. Water potential (Ψw), osmotic potential at full turgor (Ψs^{100}), osmotic adjustment (OA), relative water content (RWC), soluble sugar content and soluble protein content in flag leaves of *tasg1* and WT under CK and DS at 15th DAA (30 May 2010). Means ± SE, *n* = 3. Different letters indicate significant differences at a 0.05 level.

Treatments	Ψw [MPa]	Ψs^{100} [MPa]	OA [MPa]	RWC [%]	Soluble sugar [mg g^{-1} (f.m.)]	Soluble protein [mg g^{-1} (f.m.)]
CK WT	-0.61 ± 0.03a	-1.28 ± 0.02a		93.30 ± 0.90a	23.8 ± 1.80a	2.09 ± 0.135a
DS WT	-1.03 ± 0.03b	-1.44 ± 0.01a	-0.06 ± 0.014a	81.12 ± 0.55b	24.4 ± 1.21a	2.25 ± 0.945b
CK *tasg1*	-0.62 ± 0.02a	-1.27 ± 0.02b		94.01 ± 1.35a	26.5 ± 1.24b	2.39 ± 1.061b
DS *tasg1*	-0.95 ± 0.01c	-1.31 ± 0.03c	-0.04 ± 0.009b	89.32 ± 0.50c	28.6 ± 0.89c	2.66 ± 0.743c

compared to WT, at 30 DAA, which was consistent with the differences in Chl content. Somewhat similar differences were observed in E and g_s, but c_i was significantly lower in *tasg1* than WT. This may be related to the higher photosynthetic activity in *tasg1*.

O_2^- production and MDA content were lower in *tasg1* than WT. The activities of several antioxidant enzymes, including SOD, POD, CAT and APX were mostly suppressed by DS, but the activities of these enzymes were always higher in *tasg1* than in WT.

In CK, chloroplasts were arranged regularly along the cell wall, but their shape was slightly different in the two genotypes. Chloroplasts were approximately roundish

Table 3. Kernel yields of field-grown *tasg1* and WT in 2010. Values are mean ± SE (*n* = 3). Different letters indicate significant differences at a 0.05 level.

Treatments	Number of kernels [$spike^{-1}$]	Number of spikes [m^{-2}]	Kernel mass [mg]	Yield [m^{-2}]
CK WT	39.4±1.8a	300.1±4.1a	51.7±0.7a	0.609±0.030a
DS WT	35.0±2.0c	268.2±3.5ab	50.6±1.0b	0.475±0.038b
CK *tasg1*	50.6±1.4b	302.0±4.65a	43.8±0.4a	0.667±0.044a
DS *tasg1*	44.0±2.0d	272.0±4.0a	42.5±1.0b	0.509±0.033b

in WT (Fig. 1*A*), but prolonged in *tasg1* Fig. 1*B*). After DS, some damage to the chloroplast envelope was found in WT (Fig. 1*C*), accompanied with the shift of the organelles from the cell wall to the center of the cell. Compared to WT chloroplasts, *tasg1* chloroplasts showed less damage induced by DS (Fig. 1*D*). Under CK, the thylakoid lamellae were closely arranged and assembled to form the grana in the WT (Fig. 1*A′*). Lamellae were more closely arranged in *tasg1* (Fig. 1*B′*). DS resulted in swollen and loosely scattered thylakoid lamellae in WT (Fig. 1*C′*), but these changes were not obvious in *tasg1* (Fig. 1*D′*).

There were no obvious differences in water potential (Ψ_w), osmotic potential (Ψ_s), or RWC between WT and *tasg1* under sufficient water supply. DS resulted in a significant decrease in Ψ_w, Ψ_s, and RWC in both wheat genotypes, but the decrease was greater in WT than in *tasg1*. The higher Ψ_s in *tasg1* may be related to the greater OA of *tasg1* under DS, which in turn may be related to the accumulation of soluble sugars and proteins.

The yield of *tasg1* was 9.5 % and 7.0 % higher than WT under CK and DS conditions, respectively, but these differences were not significant. The greater number of kernels per wheat spike was the major factor contributing to the higher yield of *tasg1,* although the number of wheat spikes in each plot in *tasg1* also contributed to the increased yield. The size and mass of each kernel was lower in *tasg1* than WT.

Discussion

Plant senescence is an internally programmed degenerative process 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 corresponding WT during senescence (Thomas and Smart 1993, Spano *et al.* 2003, Hörtensteiner 2009). Some stay-green mutants might therefore be expected to give a higher yield (Zheng *et al.* 2009). For example, a delay in the onset of plant senescence in *Lolium temulentum* by just 2 d increased the amount of carbon fixed by 11 % (Thomas and Howarth 2000). Here, we report on the wheat stay-green mutant, *tasg1*. Compared with its WT, *tasg1* exhibited markedly delayed senescence with characteristic retention of leaf colour and delayed chlorophyll loss (Table 1). In accordance with the delayed chlorophyll degradation and retained photosynthetic function, *tasg1* represents a functional stay-green mutant (Thomas and Howarth 2000). Further, a slightly higher yield in *tasg1* than WT was observed. This is consistent with previous reports (Rivero 2007, Hörtensteiner 2009).

In addition, this stay-green phenotype can be more drought resistant (Yoo *et al.* 2007, Hörtensteiner 2009). Xu *et al.* (2000) suggested that greenness was a reliable indicator of leaf senescence and should be useful to sorghum breeders in evaluating progeny to select for drought tolerance.

Water stress damages thylakoid membranes, disturbs their function, and ultimately decreases photosynthesis and crop yield (Shah and Paulsen 2003, Huseynova *et al.* 2007, Zhao *et al.* 2007). 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). The P_N of *tasg1* was always higher than that of WT during water senescence induced by DS. This was related to more stable chloroplast ultrastructure in *tasg1* than in WT, with thylakoid stacking in the grana nearly unchanged under DS (Fig. 1*A*). In some cases, stacks with significantly more lamellae per granum were observed, probably due to the fusion of several grana stacks, as reported earlier (Kusaba *et al.* 2007, Schelbert *et al.* 2009). We also observed fusion of several grana stacks in *tasg1* under DS (Fig. 1*E*). This phenomenon was not observed in *tasg1* grown under CK. By contrast, in WT plants grown under DS, plastoglobules became obvious, grana were largely unstacked, and overall thylakoid membrane density was reduced. Retention of grana stacking might be connected with inhibition of degradation of light-harvesting complex 2 (LHC 2), which was also reported at the very late stage of leaf senescence in *nyc1* mutant (Kusaba *et al.* 2007).

Under DS, the rate of CO_2 fixation in the chloroplast is often insufficient to consume all absorbed light energy, which leads to the formation of ROS (Foyer and Noctor 2005, Zimmermann and Zentgraf 2005). During senescence, many antioxidant enzymes show reduced activity (Prochazkova *et al.* 2001), which also contributes to enhanced ROS accumulation. Therefore, higher antioxidant competence is useful in delaying senescence. Zavaleta-Mancera *et al.* (2007) found that cytokinin could preserve chloroplast integrity during dark-senescence by promoting CAT and APX activities and reducing the content of H_2O_2 in wheat leaves. Some maize stay-green mutants also have higher antioxidant capacity (Prochazkova *et al.* 2001). In our experiments, the accumulation of O_2^- increased at late stages of senescence, leading to an increase of MDA. DS also increased O_2^- accumulation and MDA contents, but they were always lower in *tasg1* than in WT. This may be related to increased activities of SOD, POD, CAT and APX in *tasg1* as compared to WT. These results are consistent with results reported previously (Prochazkova

et al. 2001, Srivalli and Khanna-Chopra 2004, Hu *et al.* 2010). These results suggest that more efficient antioxidative system may be involved in the stay-green phenotype and drought resistance of *tasg1*.

In conclusion, our results demonstrate that the stay-green mutant *tasg1* has higher drought resistance than WT under field conditions. *Tasg1* maintains chloroplast and thylakoid ultrastructure better than WT, contributing to improved P_N and yield under drought stress. Improved water balance and effective antioxidative system may also be involved in the drought resistance of *tasg1*.

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