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Down-regulation of catalase activity contributes to senescence induction in wheat leaves exposed to shading stress

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Abstract

In shaded wheat (*Triticum aestivum* L.) leaves, the suppression of blue radiation (BR) triggers senescence. This phenomenon is correlated to an increase in oxidative stress symptoms and a decrease of catalase (CAT) activity, among other traits. Previous data suggest that the radiation signal transduction pathway may involve changes in Ca^{2+} and H_2O_2 homeostasis. For better a understanding of the interaction among the spectral composition of radiation, Ca^{2+} availability, and the antioxidant metabolism in the regulation of shade-induced senescence, detached wheat leaves were placed in a growth chamber and exposed to either blue (B, high BR transmittance) and/or green (G, very low BR transmittance) *Lee*® filters in the absence or presence of 0.8 mM verapamil (a Ca^{2+} channels blocker), 4.0 mM EGTA (a Ca^{2+}chelator), or 8.0 mM 3-amino-1,2,4-triazole (a CAT inhibitor). At defined time points, the leaf samples were analyzed for changes in chlorophyll content, specific activities of CAT, ascorbate peroxidase (APX), and guaiacol peroxidase (POX), CAT isozymes, and gene expression of *CAT1, CAT2*, and two senescence markers (*TaSAG1* and *TaSAG3*). BR transmittance decreased the chlorophyll degradation rate and *SAG* genes expression either in leaves continuously exposed under the B filter, as well as in leaves previously exposed under the G filter. The effect of BR was associated with the maintenance of a high CAT (but not APX and POX) activity, and it was suppressed either in the presence of 3-AT or when Ca^{2+} availability was decreased. BR altered the CAT activity both at the transcriptional and at the post-transcriptional level. Nevertheless, different responses of CAT isozymes and CAT genes expression profiles to specific treatment combinations indicate that they differed in their regulatory pathways.

Additional key words: antioxidatnts, blue radiation, calcium, gene expression, *Triticum aestivum*

Introduction

Senescence is a physiological process during which leaves experience major changes in their morphology and metabolism (Gepstein 2004, Keskitalo *et al*. 2005, Lim *et al.* 2007, Love *et al.* 2008). Even though it is a genetically controlled process, many exogenous factors can prompt it. Leaves beneath a dense canopy experience a marked reduction of the photon flux density (PFD) as well as of the red (660 nm) and blue (400 - 450 nm) wavelengths due to chlorophyll and other pigments absorption, and it has been shown that changes in either PFD or spectral composition can induce leaf senescence in an independent way (reviewed in Causin and Barneix 2007). Although a decrease in the red to far red ratio was shown to trigger leaf senescence in different dicotyledoneous species (Guiamét *et al.* 1989, Rouseaux *et al.* 1996, 1997, Yang *et al.* 2012), we have recently

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Abbreviations: APX - ascorbate peroxidase; 3-AT - 3-amino-1,2,4-triazole; BR - blue radiation; CAT - catalase; EGTA - ethylene-glycol-bis-(β-amino-ethylether)-N,N,N',N'-tetraacetic acid; PFD - photon flux density; POX - guaiacol peroxidase; PVPP - polyvinylpolypyrrolidone.
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demonstrated that in leaves of wheat, chlorophyll and soluble protein degradation rates under shade conditions are only slightly affected by changes in the red to far red ratio, but they significantly increase when blue wavelengths are suppressed (Causin *et al.* 2006, Causin and Barneix 2007, Causin *et al.* 2009, see also Fig. 1 Suppl.). Among other factors, the development of senescence symptoms is correlated with changes in the content of H_2O_2 and other indicators of oxidative stress, or with a decrease of catalase (CAT, EC 1.11.1.6) activity. These results led us to hypothesize that in wheat leaves, blue radiation (BR) suppression would act as a stress signal that initiates senescence, and that oxidative processes are an important component of this phenomenon. Even though the pathway of the BR signal perception and transduction remains to be elucidated, previous work suggested that changes in Ca^{2+}availability associated to BR signaling pathways are involved in the regulation of this process (Causin *et al.* 2006).

Plants possess both enzymatic and non-enzymatic systems to counteract oxidative stress and among them, the CAT activity plays a central role in stress defense (Willekens *et al.* 1997). Plant CATs are heme-containing enzymes usually present in multiple isoforms. In Angiosperms studied so far, CATs are encoded by a small family of genes showing complex spatial and temporal patterns of expression (Luna *et al.* 2004, Smykowski *et al.* 2010, Mhamdi *et al.* 2012). The active enzyme is believed to be a homo- or hetero-tetramer made up of four subunits of about 60 kDa each. An adequate CAT activity is essential for the removal of H_2O_2 produced by photorespiration (Noctor *et al.* 2000), but also appears to be critical for maintaining the redox balance in the cell and for the prevention of oxidative damage under different stress conditions (Willekens *et al.* 1997, Orendi *et al.* 2001, Procházková *et al.* 2001, Luna *et al*. 2004, Vandenabeele *et al.* 2004, Azpillicueta *et al.* 2008, Pena *et al.* 2011). The presence of a Ca^{2+}/calmodulin binding motif as well as an antioxidant responsive element (ARE) in the promoter of plant *CAT* genes probably underlies this important protective role (Polidoros and Scandalios 1999, Yang and Poovaiah 2002). In wheat, two CAT isoforms account for most of the enzyme activity present in the leaves, and two genes (*CAT1* and *CAT2*) have been demonstrated to encode CAT subunits (Luna *et al.* 2004, Yang *et al.* 2006). In wheat leaves, the expression of both genes is modulated by PFD, and the *CAT1* expression shows characteristics of circadian control (Luna *et al.* 2004). CATs are mostly PFD-sensitive. At moderate to high PFD, the prosthetic heme sensitizes their inactivation by BR in the presence of O_2 (Shang and Feierabend 1999). However, in rye leaves, BR was shown to be most effective in activating *CAT1* mRNA expression (Schmidt *et al.* 2006), which indicates that changes in both PFD and spectral composition may exert complex effects on the activity of CAT isozymes. To our knowledge, no information is available on how changes in spectral composition affect the activity and gene expression of CAT isoforms during shade induced senescence.

In the present work we studied the effect of changes in PFD and spectral composition on CAT activity and *CAT* genes expression profiles in wheat leaves exposed to a shading stress and analyzed the role of Ca^{2+} ions as well as CAT suppression in the regulation of the senescence rate.

Materials and methods

Plant growth and experiments: Wheat (*Triticum aestivum* L., cv. INTA Oasis) caryopses were surface sterilized in 10 % (v/v) H_2O_2 for 12 min, germinated on wet tissue paper, after 24 h transplanted to plastic pots containing a mixture of sand : *Agrolite* : *Vermiculite* (2:1:1), and grown in a greenhouse at a natural (12 - 13 h depending on the time of the year) photoperiod. Pots were periodically irrigated with tap water and, after a week, fertilized as indicated in Causin *et al*. (2006). To study the effect of radiation spectral quality on leaf senescence under shading conditions, four fully expanded leaf blades (usually the third ones) of 26-d-old plants were excised and incubated for different times in plastic boxes (3 - 4 independent replicates per treatment conditions) whose lids were covered with *Lee Filters* (Andover, UK), green *#089* (which simulates the transmittance spectrum of a green leaf) or blue *#075*, hereafter referred as treatment G or B, respectively. These filters were chosen because, even though they differed in the percent transmission of blue wavelengths, they decreased the photosynthetically active photon flux density (PPFD) to a similar extent and supplied a similar proportion of red (R = 660 nm) to far-red (FR = 730 nm) radiation (Causin *et al.* 2006, Fig. 1 Suppl.). Where indicated, boxes without added filters were used as non-shaded controls (Ctr). The incubations were performed in a chamber supplied with an equal number of white (*Sylvania F27W T8/LD/54*) and blue + red radiation (*Sylvania Grolux F30W T12*) fluorescent tubes (an 18-h photoperiod), at day/night temperatures of 26 ± 2/17 ± 2 ºC, and a relative humidity of 60/70 %. Average values for PFD were 192 ± 3 (non-shaded Ctr), 38 ± 2 (treatment G), and 37 ± 3 (treatment B) μmol m^{-2} s^{-1}. The measurements of PFD were performed with a *LI-190* quantum sensor (*LI-COR*, Lincoln, NE, USA) attached to a *CAVA-RAD* data logger (Cavadevices, Argentine). Depending on the experiment, the excised leaves were incubated on distilled water or on aqueous solutions containing either 4.0 mM ethyleneglycol-bis-(β-amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA; an

apoplastic Ca^{2+}chelator), 0.8 mM verapamil (a Ca^{2+} channels blocker), or 8.0 mM 3-amino-1,2,4 triazole (3-AT; a CAT inhibitor) which were added from appropriate stock solutions at specified time intervals. The solutions were supplied with 5 mm^3 dm^{-3} *Tween 20* to increase tissue contact with the medium. Leaves were sampled at different time intervals, briefly rinsed in distilled water, blotted dry and immediately ground with liquid N_2. Tissue samples were kept at -70 ºC for the further analysis. When indicated, the same procedure was performed with samples of leaves detached prior to beginning radiation treatments (T_0). All the experiments were repeated at least twice. Whenever possible, data from different experiments were averaged, otherwise data from one representative experiment are presented.

Chlorophyll content and enzyme activities: Chlorophyll *a+b* content was measured either in N,N-dimethylformamide extracts (10 mg of leaf fresh mass per 4 cm^3) according to Porra (2002) or with a *SPAD Chlorophyll Meter 502* (*Konica Minolta*, Tokyo, Japan), depending on the experiment. On average, eight measurements per leaf blade and three to five leaves were measured per treatment.

Catalase, ascorbate peroxidase (APX, EC 1.11.1.11), and guaiacol peroxidase (POX, EC 1.11.1.7) activities were assayed in crude homogenates obtained by grinding a 0.1 g leaf sample with 1.2 cm^3 of an extraction buffer [50 mM potassium phosphate, pH 7.6, containing 1.0 mM ethylenediaminetetraacetic acid (disodium salt), 1.0 mM ascorbic acid, 1.0 mM dithiotreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.15 % (v/v) *Triton X100*] in the presence of 20 - 25 mg polyvinylpolypyrrolidone. The homogenates were centrifuged (18 000 *g*, 25 min), the supernatants collected and centrifuged for a second time (19 500 *g*, 10 min) prior to enzyme assays. All procedures were performed at 4 ºC. Reaction buffers were either 50 mM potassium phosphate, pH 7.0 (for CAT and APX assays) or 150 mM potassium phosphate, pH 6.1 (for POX assay). CAT activity was measured according to Aebi (1984). For calculations, the slope of the initial (20 s) decrease in absorbance due to H_2O_2 consumption was taken into account. One unit was the amount of enzyme that decomposed 1 µmol of H_2O_2 per min in 1 cm^3 of reaction mixture at pH 7.0 and 25 ºC. APX and POX activities were assayed following the procedures described in Procházková *et al*. (2001). CAT isozymes were separated on 9 % (m/v) non-denaturating polyacrylamide gels (15 µg of protein from crude extracts per lane), at 4 ºC (3 h, 150 V). After electrophoresis, CAT isoforms were visualized according to Woodbury *et al*. (1971). Densitometric analysis of zymograms was performed on scanned images of the gels using the *Image J* free software (*Image J 1.47v*. *NIH*, Bethesda, USA) and their content was expressed as relative units. Protein content in the crude extracts was determined with the method of Bradford (1976).

Analysis of *CAT1* and *CAT2* expression by RT-PCR: Total RNA was isolated from 100 mg of frozen, powdered leaves by the addition of 1 cm^3 of a *TRIzol*® reagent according to the manufacturer´s protocol (*Molecular Research Center Inc.,* Cincinnati, USA). RNA content was determined using *NanoDrop 2000* (*Thermo Scientific*, Whaltham, USA). RNA (1 µg) was treated with *DNase I* (*Promega*, Madison, USA) and then converted to cDNA with *oligo-dT 15* (*Invitrogen,* Carlsbad, USA) using *RevertAid TM M-MuLV* reverse transcriptase (*Invitrogen*). The primers and accession numbers (GeneBank ID) used were: wheat *CAT1* (E16461) F 5'-ACTACGACGGGCTCATG-3' and R 5'-GCCCTGAAGCAGATTTCT-3', wheat *CAT2* (X94352), F 5'-CCTTAATCAGCAGGGATG-3' and R 5'-AGATAGAACACGCGGAG -3'. PCR conditions were: 5 mm^3 of cDNA diluted 1/40 per reaction, and annealing at 95 ºC for 3 min, 35 cycles of 95 ºC for 30 s, 52 ºC for 30 s, 72 ºC for 40 s, and a final extension at 72 ºC for 10 min. PCR reactions were performed using a programmable *Thermocycler T 18* (*Ivema*, Buenos Aires, Argentina). The PCR-amplified products were fractionated by electrophoresis on a 1.5 % (m/v) agarose gel and stained with ethidium bromide. Fragments of wheat *actin* and *tubulin* genes were amplified as internal standards. The primers and accession numbers used were: *actin* (AB181991) F 5'-GGATCGGTGGCTCTATTT TG-3' and R 5'-TGTACCCCTTATTCCTCTGAGG-3', *tubulin* (*Tubb5*, U76896) F 5'-TTCCTGCACTGG TACACGGG-3' and R 5'-AGCCATCAGAAATAGCC CCG-3'. Gels were photographed with a digital imaging system (*FOTODYNE*, Hartland, USA) and analyzed with a *Gel-Pro* analyzer (*Exon-Intron*, Loganville, USA). Each expression profile presented is representative of at least three biological replicates.

Analysis of *TaSAG* genes expression by real-time quantitative (RT-qPCR): Two leaf senescence-associated gene homologs were used as developmental markers (Kajimura *et al.* 2010). Reverse transcription reactions were performed using 2 µg of purified total RNA (see above) and using *oligo-dT* according to the *M-MLV* reverse transcriptase protocol (*Life Technologies,* Carlsbad, USA). RT-qPCR reactions were performed using an *Eppendorf Master cycler Realplex* machine. The primers and accession numbers used were: *TaSAG1* (CJ637025) F 5'-GTTGCCATTGAAGCGTTG-3' and R 5'-CACTCC TGTCCGAATATAGC-3', *TaSAG3* (CJ683824) F 5'-TATACAGCAGTAGATTCCAAGAG-3' and R 5'-CACGCCATAGAAGAACACC-3'. The reaction mixture contained 5 mm^3 of cDNA diluted 1/30, 20 µM specific primers, 0.75 U of *Taq* DNA polymerase (*Life Technologies*), 3 mM $MgCl_2$, 10 mM dNTP, 0.025 mm^3 of *SYBR Green* (*Roche*, Mannheim, Germany) in a final volume of 25 mm^3. Wheat *β-tubulin* (*Tubb2,* U76745) was used as internal standard with the following primers sequences: F 5'-CAATGTCAAGTCCAGCG

TCT-3' and R 5'-AGGTCGTTCATGTTGCTCTC-3'. PCR conditions were: 95 ºC for 2 min, 40 cycles of 95 ºC for 15 s, 68 ºC for 35 s, 95 ºC for 15 s, 60 ºC for 15 s, and 95 ºC for 15 s. We completed each run with a melting curve to confirm the specificity of amplification.

Statistical analyses: Within each experiment, data from each sampling were analyzed separately with conventional one-way or two-way *ANOVA* (*Statistica*, *Stat Soft Inc.*, Tulsa, USA), depending on the experimental design. The model included radiation treatment and, where appropriate, pharmacological treatments as fixed factors. When necessary, data were log transformed to meet *ANOVA* assumptions. Post-hoc comparisons for significant main effects or interaction terms were performed using the Tukey HSD test. A given effect was assumed significant at $P < 0.05$, unless otherwise stated.

Results

A high BR transmission in the shading treatments consistently decreased the senescence rate of excised leaves, although the efficiency was higher in treatments with a longer exposure to the B filter (Fig. 1*A*, compare treatments B, GB_{30}, and GB_{54}). In all cases, the senescence retardant effect of BR was accompanied by the maintenance of a higher CAT activity than in the treatment G (Fig. 1*B*).

To further analyze the role of CAT activity as well as the influence of Ca^{2+} ions on this process, an experiment was conducted where leaves firstly exposed under the G filter for 52 h were then exposed under the filter B in the absence (treatment GB) or presence of the CAT inhibitor 3-AT (treatment GB_{3AT}), the Ca^{2+} chelator EGTA (treatment GB_{EGTA}), or the Ca^{2+} channel blocker verapamil (treatment GB_{VP}). Leaves continuously exposed to filter B or G were used as controls. The senescence retardant effect of BR was consistently suppressed when leaves were exposed to GB_{AT}, GB_{EGTA}, or GB_{VP} (Fig. 2*A*). The analysis of CAT activity confirmed that BR is an important cue for its stimulation (compare treatments B, G, and GB in Fig. 2*B*). As for chlorophyll degradation, the presence of either 3-AT or EGTA (but not verapamil) markedly inhibited the CAT stimulatory effect of BR (Fig. 2*B*). The fact that only a minor effect was exerted by verapamil (Fig. 2*B*, the treatment GB_{vp}) suggests that this Ca^{2+} channel blocker could alter the senescence rate by interfering with BR-regulated processes other than CAT activity. The activities of APX and POX increased with time in all the experimental conditions (Fig. 2*C*,*D*). Nevertheless, as opposed to CAT, the activitiesy of these peroxidases were either not stimulated (POX), or rather inhibited (APX) by BR. Moreover, after 80 h, the activity of APX and, to a lesser extent, POX was up-regulated in those conditions where the senescence rate decreased with respect to the treatment B (Fig. 2*C*,*D*, the treatments G, GB_{3AT}, GB_{EGTA}, and GB_{VP}).

The expressions of *TaSAG1* and *TaSAG3* slightly increased with time in the treatment B, but they were significantly higher than at T_0 in leaves exposed under the G filter for 52 and 80 h (Fig. 3). At 80 h, the expressions of both genes were down-regulated to almost initial levels in the treatment GB indicating that BR not only prevented chlorophyll degradation, but acted as overall senescence retardant signal when supplied to the shade stressed leaves. As for chlorophyll degradation, the effect of BR on *TaSAG* genes expression was also consistently suppressed when either the Ca^{2+} availability or CAT activity was negatively affected (Fig. 3, the treatments GB_{3AT}, GB_{EGTA}, and GB_{VP}).

In order to better understand the role of radiation spectral composition in the regulation of CAT activity, we performed CAT zymograms, and analyzed *CAT1* and *CAT2* expression profiles. Native PAGE of crude extracts revealed the presence of two CAT isoforms, although

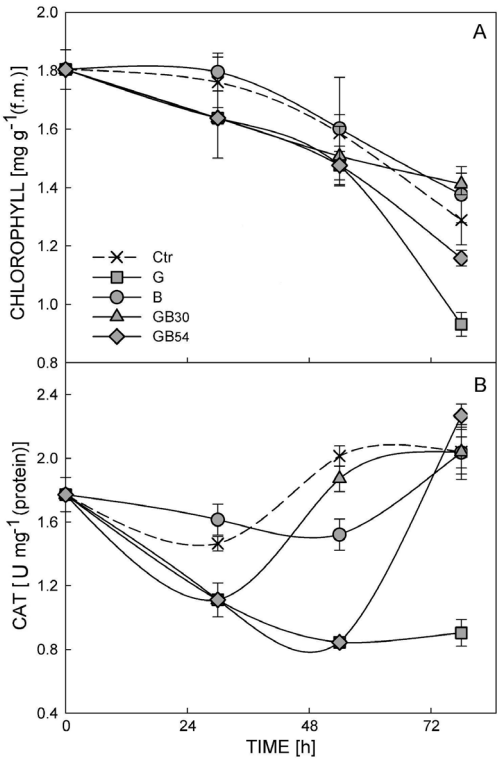

Fig. 1. The chlorophyll content (*A*) and CAT specific activity (*B*) in excised wheat leaves floated on distilled water and exposed to white radiation (control, Ctr) or shaded under the green (G) or blue (B) filter. A set of leaves from the treatment G were changed to the B after either 30 h (treatment GB_{30}) or 54 h (treatment GB_{54}). Data are means ± SD ($n = 4$).

CAT2 was the prominent enzyme (Fig. 4). Even though the activity of both the isoforms declined when BR was suppressed, or in the presence of either 3-AT or EGTA, the densitometric measurements indicate that the minor isoform, CAT1, was more sensitive than CAT2 (Fig. 4). As for the total CAT activity, verapamil did not

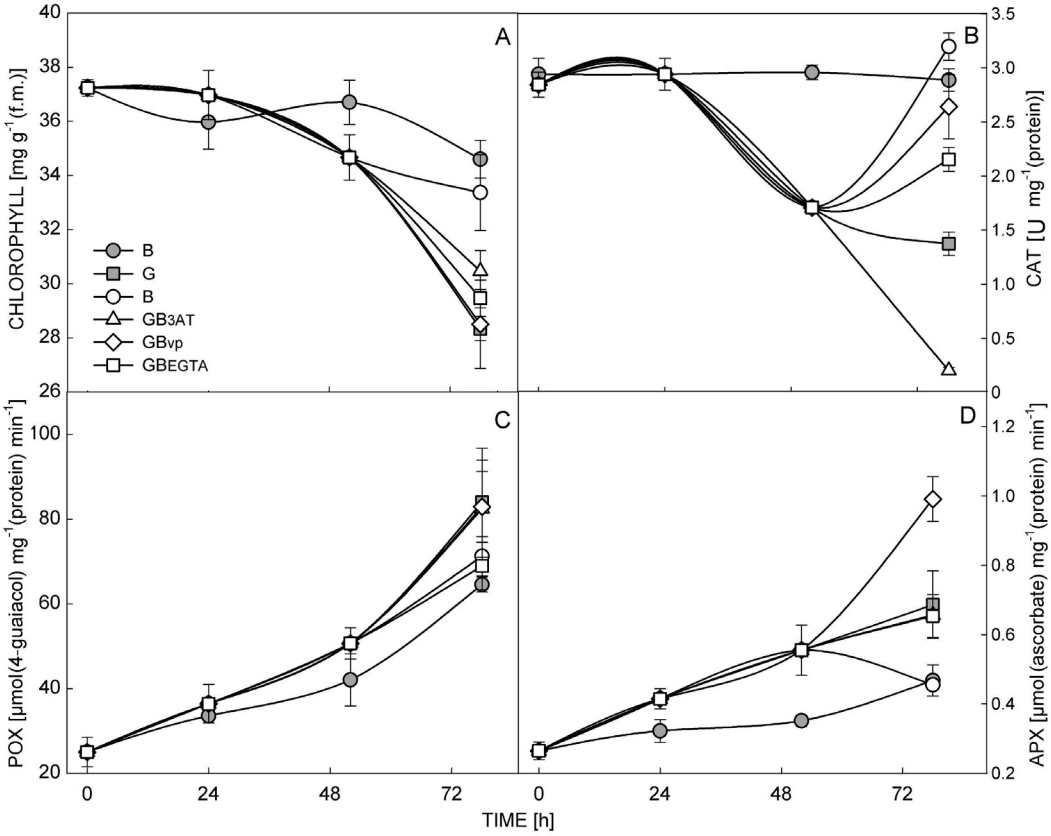

Fig. 2. The chlorophyll content (*A*), CAT specific activity (*B*), POX specific activity (*C*), and APX specific activity (*D*), in excised wheat leaves exposed under the G filter and after 52 h under the B filter in the absence (treatment GB) or presence of 8.0 mM 3-AT (treatment GB_{3AT}), 4.0 mM EGTA (treatment GB_{EGTA}), or 0.8 mM verapamil (treatment GB_{VP}). Leaves continuously exposed to the filter B or G without pharmacological treatments were used as controls. Means ± SD (n = 4).

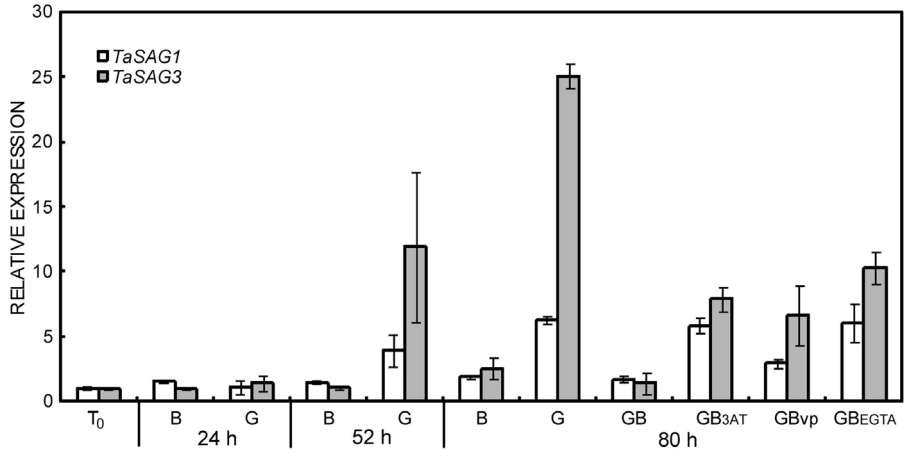

Fig. 3. Relative abundances of *TaSAG1* and *TaSAG3* transcripts at 0, 24, 52, and 80 h in excised wheat leaves exposed under the G filter and after 52 h under the B filter in the absence (treatment GB), or presence of 8.0 mM 3-AT (treatment GB_{3AT}), 4.0 mM EGTA (treatment GB_{EGTA}), or 0.8 mM verapamil (treatment GB_{VP}). Leaves continuously exposed to the filter B or G without pharmacological treatments were used as controls. Data were normalized considering the expression at T_0 = 1. Means ± SD (n = 3).

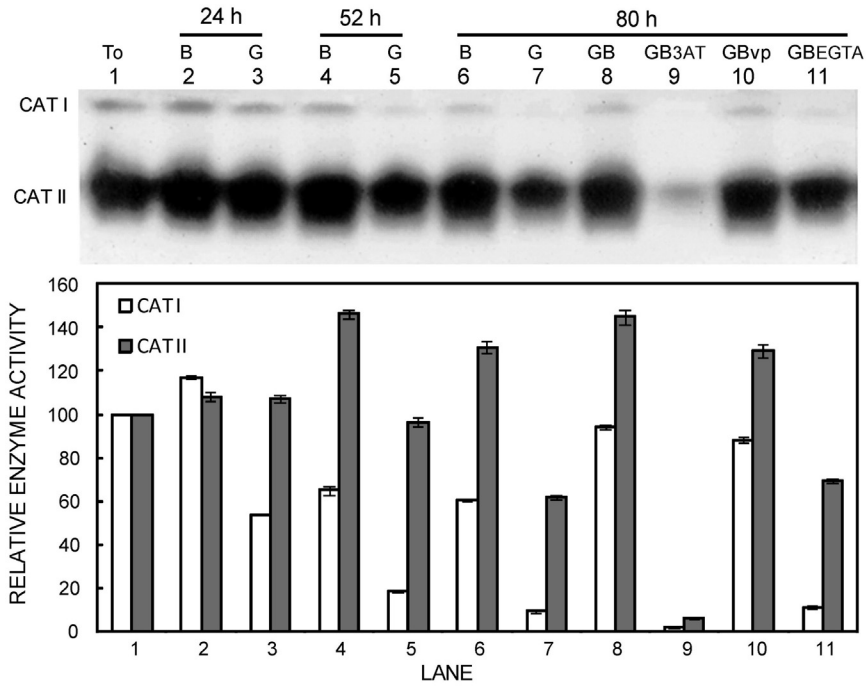

Fig. 4. Activities of CAT isoforms at 0, 24, 52, and 80 h in excised wheat leaves exposed under the G filter and after 52 h under the B filter in the absence (treatment GB), or presence of 8.0 mM 3-AT (treatment GB_{3AT}), 4.0 mM EGTA (treatment GB_{EGTA}), or 0.8 mM verapamil (treatment GB_{VP}). Leaves continuously exposed to the filter B or G without pharmacological treatments were used as controls. Values from the densitometric analysis represent means ± SD (n = 3). Data were normalized considering density units at T_0 = 100 %. Note that the picture was inverted ("negative image") to better visualize CAT I bands.

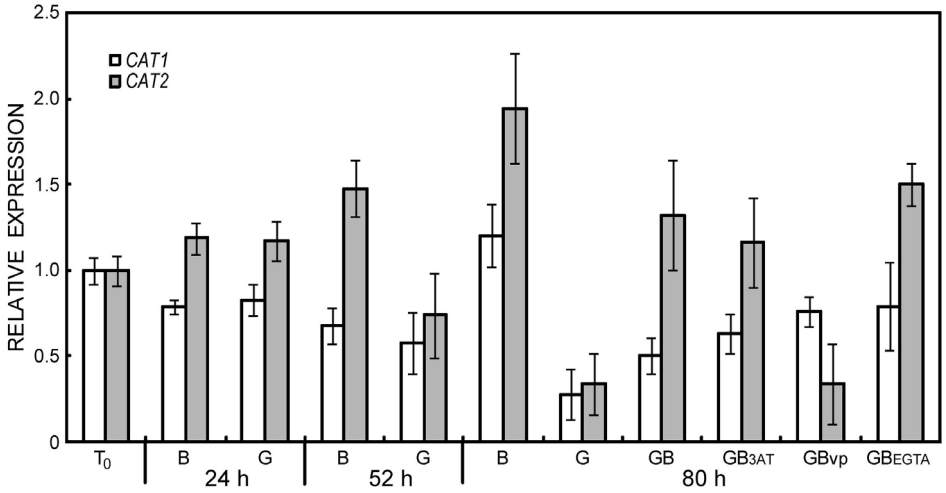

Fig. 5. Relative abundances of *CAT1* and *CAT2* transcripts at 0, 24, 52, and 80 h in excised wheat leaves exposed under the G filter and after 52 h under the B filter in the absence (treatment GB), or presence of 8.0 mM 3-AT (treatment GB_{3AT}), 4.0 mM EGTA (treatment GB_{EGTA}), or 0.8 mM verapamil (treatment GB_{VP}). Leaves continuously exposed to the filter B or G without pharmacological treatments were used as controls. Data were normalized considering the expression levels at T_0 = 1. Means ± SD (n = 3).

significantly suppress the effect of BR on any isoform.

As CAT activity is down-regulated during the night (see Fig. 2 Suppl.), the analysis of *CAT* genes expression was performed on leaves sampled 5 to 6 h after the initiation of the photoperiod. The *CAT1* relative expression decreased with time to a similar extent in both the G- and B-treatments during the first 52 h (Fig. 5). At 80 h, the *CAT1* expression increased to the initial level in leaves exposed to the treatment B, whereas it attained about 30 % of the T_0-expression in leaves shaded under

the filter G (Fig. 5). In leaves under the GB treatment, a partial (statistically significant) recovery of *CAT1* expression was obtained (Fig. 5). Interestingly, this stimulatory effect of BR was independent of the three pharmacological treatments tested. In contrast to *CAT1*, the *CAT2* expression increased with time in leaves continuously exposed to the B filter, whereas it significantly decreased after the 52 and 80 h exposures to the G filter (Fig. 5). As for *CAT1*, the expression of *CAT2* consistently increased at 80 h in leaves from the GB treatment. Although this increment was also independent on the presence of 3-AT or EGTA, verapamil suppressed the stimulatory effect of BR (Fig. 5).

Discussion

Data from the present work support previous evidence indicating that changes in BR transmittance have a central role in the regulation of the senescence rate in wheat leaves exposed to shading (Causin *et al.* 2006, Causin and Barneix 2007). This was confirmed not only by the analysis of chlorophyll degradation rate, but also at the molecular level as indicated by the changes in the expression profiles of two senescence markers (*TaSAG1* and *TaSAG3).*

Under our experimental conditions, the senescence retardant effect of BR was systematically accompanied by the maintenance of a high CAT activity but not by APX or POX activities. Moreover, the effect of BR was suppressed in the presence of 3-AT even when the activities of APX and POX were stimulated. This is consistent with our hypothesis that the increment of senescence symptoms is associated to CAT rather than peroxidases down-regulation. CAT activity has been shown to play a central role in the prevention of cellular damage and the maintenance of the redox balance under different abiotic stresses as well as during senescence (Willekens *et al.* 1997, Corpas *et al.* 2001, Srivalli and Khanna-Chopra, 2001, Yang *et al.* 2006). Although different reports showed that the down-regulation of CAT activity causes serious disorders in leaves exposed to high PFD (*e.g.*, Willekens *et al.* 1997, Vandenabeele *et al.* 2004), our results suggest that a decrease in CAT activity could also play an important role in the increment of senescence rate in the shade stressed leaves.

Changes in Ca^{2+} content have been implicated in the regulation of CAT activity (Yang and Poovaiah 2002, Jiang and Zhang 2003). Different environmental signals can trigger rapid and transient increases in cytosolic Ca^{2+}, which in turn may increase the production of $O_2^{\cdot-}$ and H_2O_2, through the stimulation of plasma membrane NADPH-oxidases among other mechanisms (Yang and Poovaiah 2002). Particularly, there is ample evidence that BR may alter Ca^{2+} homeostasis, and that many physiological processes regulated by BR depend either on changes in cytosolic Ca^{2+} and/or on specific Ca^{2+}-binding proteins as secondary messengers (Shinkle and Jones 1988, Elzenga *et al.* 1997, Baum *et al.* 1999, Guo *et al.* 2001, Folta *et al.* 2003, Stoelzle *et al.* 2003, Dodd *et al.* 2010). Some plant CATs have been shown to bind calmodulin which in turn increases their catalytic activity (Yang and Poovaiah 2002). Even though there is no direct evidence that this is also true for wheat CATs, the fact that the stimulatory effect of BR on the CAT activity was suppressed by the presence of EGTA indicates that Ca^{2+} was involved in the signaling process. It has been suggested that the alteration of Ca^{2+} pools by BR may be mediated by the activation of Ca^{2+}-permeable voltage-gated channels (Stoelzle *et al.* 2003, Dodd *et al.* 2010). Verapamil is well known antagonist of plant hyper-polarization-activated Ca^{2+} channels (Demidchik *et al.* 2002, Shang *et al.* 2005), and it was shown to suppress some BR-mediated physiological responses (*e.g.*, Elzenga *et al.* 1997, Shimazaki *et al.* 1997). In our experimental conditions, verapamil exerted only a minor negative effect on either overall as well as CAT-isozymes activities suggesting that (an)other Ca^{2+}channel type(s) might be involved in the control of Ca^{2+} fluxes regulating this enzyme. Nevertheless, the fact that both the chlorophyll degradation rate as well as *SAG* genes expression increased in the presence of verapamil indicates that Ca^{2+} fluxes through voltage-gated channels contributed to the regulation of the overall senescence process by BR. This is in agreement with data reported by Huang *et al.* (1990) and Huang and Kao (1992), who found that failure to maintain the normal transmembrane flux of Ca^{2+}, including through verapamil-sensitive channels, markedly affects dark-induced senescence in detached leaves of certain crops like rice and maize.

Apart from stimulating the CAT specific activity, BR also increased the *CAT1* and *CAT2* expressions. Nevertheless, the control pathways involved seemed to differ between them. In fact, while the *CAT2* expression increased with time in leaves exposed to BR, the *CAT1* expression was particularly stimulated by BR at the later senescent stages. This may in part explain why a high CAT activity could be attained in leaves from the treatment G when they were changed to the B treatment at 52 - 54 h after the excision. On the other hand, although the presence of verapamil partially increased the *CAT1* expression, it suppressed the stimulatory effect exerted by BR on the *CAT2* expression. Interestingly, EGTA did not decrease the expression of both the *CAT* genes in the GB treated leaves, which suggests that, in our experimental conditions, the alteration of apoplastic Ca^{2+} availability by EGTA affected the enzyme activity mainly at the post-transcription level. The existence of differences in the radiation-regulated pathways among

CAT genes has been reported in other plants. For example, Acevedo *et al.* (1996) found that etiolated leaves of barley have greater amounts of *CAT1* mRNA compared with green leaves, whereas mRNA homologous to maize *CAT2* was induced by irradiance suggesting that barley contains radiation-inducible and radiation-repressible CAT genes. In *Arabidopsis thaliana*, mutational disruption of a BR photoreceptor, cryptochrome I, alters the pattern of the dark-expression of *CAT3* mRNA but not *CAT2* mRNA suggesting that in this species, the synthesis of the different CAT subunits are regulated by distinct cues (Zhong *et al.* 1997). Recently, Smykowski *et al*. (2010) identified G-box binding factor 1 (GBF1) as DNA-binding protein of the CAT2 promoter which could be involved in the onset of senescence during bolting time in *A. thaliana* plants, most likely *via* the regulation of intracellular H_2O_2 content. Interestingly, GBF1 belongs to the group G of bZIP transcription factors which have been related to UV and BR signaling in different plant species (Jakoby *et al.* 2002).

In conclusion, changes in BR perception could be among the first cues triggering the signaling pathway regulating senescence rate in wheat leaves exposed to shading. When BR transmission was not suppressed, the development of senescence symptoms was delayed. This effect was in part associated to a stimulation of CAT activity which in turn had an important role in the control of the senescence rate by maintaining the homeostasis of endogenous H_2O_2 content (*e.g.*, Causin *et al.* 2009). The alteration of Ca^{2+} fluxes through verapamil-sensitive and -insensitive channels could be involved in the regulation of CAT isozymes and senescence rate by BR.

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