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METHYLENE BLUE SENSITIVITY 1 (MBS1) is required for acclimation of Arabidopsis to singlet oxygen and acts downstream of β -cyclocitral

Authors

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Abstract

Singlet oxygen ($^1\text{O}_2$) signalling in plants is essential to trigger both acclimatory mechanisms and programmed cell death under high light stress. However, because of its chemical features, $^1\text{O}_2$ requires mediators, and the players involved in this pathway are largely unknown. The β -carotene oxidation product, β -cyclocitral, is one such mediator. Produced in the chloroplast, β -cyclocitral induces changes in nuclear gene expression leading to photoacclimation. Recently, the METHYLENE BLUE SENSITIVITY protein MBS has been identified as a key player in $^1\text{O}_2$ signalling leading to tolerance to high light. Here, we provide evidence that MBS1 is essential for acclimation to $^1\text{O}_2$ and cross-talks with β -cyclocitral to mediate transfer of the $^1\text{O}_2$ signal to the nucleus, leading to photoacclimation. The presented results position MBS1 downstream of β -cyclocitral in $^1\text{O}_2$ signalling and suggest an additional role for MBS1 in the regulation of plant growth and development under chronic $^1\text{O}_2$ production.

Introduction

Reactive oxygen species (ROS) are produced in plants as inevitable by-products of oxygenic metabolism. At high doses, these molecules are generally highly toxic, causing oxidation of biomolecules such as lipids and proteins. As a result, several efficient antioxidant mechanisms have been put in place to prevent their accumulation in plants (Apel & Hirt [2004](#); op den Camp et al. [2003](#); Rastogi et al. [2014](#)). Previously, the general notion on the effect of ROS was their cytotoxicity. However, in recent years, ROS have been implicated in several beneficial developmental, stress response and biotic interaction processes in plants such as root gravitropism, cell expansion, systemic acquired resistance, stomatal closure, hypersensitive defence reactions, stress acclimation and retrograde signalling (Apel & Hirt [2004](#); Carol & Dolan [2006](#); Foreman et al. [2003](#); Gechev et al. [2006](#); Pogson et al. [2008](#); Ramel et al. [2013a](#); Baxter et al. [2014](#)). These processes make use of the signalling properties of ROS.

Signalling by ROS was previously attributed mainly to H_2O_2 , as evidenced by transcriptional regulation of H_2O_2 -specific genes detected via microarray hybridization (Gechev et al. [2005](#); Levine et al. [1994](#); Vanderauwera et al. [2005](#)). This was probably because of the high spatial mobility of H_2O_2 which can travel a reasonable distance away from its site of production and across membranes through specialized aquaporins called peroxoporphins (Bienert et al. [2006](#); Henzler & Steudle [2000](#)). More recently, signalling properties have been discovered also for other ROS, and specific marker genes for different ROS have been identified (Demidchik et al. [2003](#); op den Camp et al. [2003](#); Ramel et al. [2013a](#)).

Singlet oxygen ($^1\text{O}_2$) is a peculiar non-radical ROS. In plants, it is produced in the reaction centre of photosystem II (PSII) mainly by the process of energy transfer from triplet excited state chlorophyll to ground state oxygen, as a means to de-excite the chlorophylls (Krieger-Liszka & Trebst, [2006](#); Triantaphylides & Havaux, [2009](#)). Furthermore, photooxidative damage to cells has been ascribed mainly to $^1\text{O}_2$ in photosynthetic organisms exposed to high light stress (Triantaphylides et al. [2008](#); Gonzalez-Perez et al. [2011](#)). As a highly reactive molecule, $^1\text{O}_2$ oxidizes biomolecules, leading to direct toxicity in plants (Apel & Hirt [2004](#); Triantaphylides et al. [2008](#)). However, low doses of this molecule serve a signalling role, leading to photoacclimation or to programmed cell death in plants (Triantaphylides & Havaux [2009](#)). The production of $^1\text{O}_2$ has been associated with the rapid induction of a specific set of nuclear encoded genes, as illustrated in the conditional *flu* mutant (op den Camp et al. [2003](#)) and in the *chlorina1* (*ch1*) mutant of *Arabidopsis thaliana* (Ramel et al. [2013a](#)). The discovery of the *flu* and *ch1* mutants (Espineda et al. [1999](#); Meskauskiene et

al. [2001](#); Ramel et al. [2013a](#)) which overproduce $^1\text{O}_2$ from the photosensitizer protochlorophyllide and from over-excitation of PSII because of lack of PSII light-harvesting complexes, respectively, have paved the way for numerous studies on the signalling functions of this ROS.

To date, only few components of the $^1\text{O}_2$ chloroplast-to-nucleus retrograde signalling pathway(s) have been identified. Being a highly reactive molecule with a short life span lower than 0.5–1 μs in plant cells (Bisby et al. [1999](#); Li et al. [2012](#); Telfer [2014](#)), it is most likely that $^1\text{O}_2$ reacts with and oxidizes molecules in the vicinity of its production. Thus, chloroplast-to-nucleus retrograde signalling is believed to proceed by means of mediators (Farmer & Davoine [2007](#); Triantaphylides & Havaux [2009](#)). Recently, it has been demonstrated that the β -carotene oxidation products, β -cyclocitral (β -cc) and dihydroactinidiolide, function as $^1\text{O}_2$ intermediate signalling molecules, causing changes in $^1\text{O}_2$ -specific nuclear gene expression, ultimately leading to photoacclimation (Ramel et al. [2012](#); Shumbe et al. [2014](#)). Jasmonic acid is also an important messenger, mediating the process of cell death in *A. thaliana* exposed to high light stress (Ramel et al. [2013b](#)). In addition, a few protein factors have also been identified as mediators of $^1\text{O}_2$ signalling, including the EXECUTER and topoisomerase VI proteins identified in the *Arabidopsis flu* mutant (Kim et al. [2012](#); Lee et al. [2007](#); Simkova et al. [2012](#)), SINGLET OXYGEN RESISTANT 1 (SOR1), a member of the bZIP group of transcription factor identified in the green alga *Chlamydomonas reinhardtii* (Fischer et al. [2012](#)) and the METHYLENE BLUE SENSITIVITY 1 protein (MBS1) identified in both *C. reinhardtii* and *A. thaliana* (Shao et al. [2013](#)).

MBS1 is a small zinc finger protein located in the cytosol and in the nucleus under normal growth conditions, but concentrated in specific stress granules and processing bodies upon oxidative stress. It was first identified in a genetic screen for *C. reinhardtii* mutants defective in response to $^1\text{O}_2$ generated by the photosensitizer methylene blue (Shao et al. [2013](#)). The absence of MBS1 expression in the *mbs1* mutant and in the double mutant *mbs1/RNAi-mbs2* in *A. thaliana* (which possesses two highly similar MBS genes) led to increased susceptibility to high light stress (Shao et al. [2013](#)), a condition known to produce predominantly $^1\text{O}_2$ (Krieger-Liszka & Trebst, [2006](#); Triantaphylides et al. [2008](#)). Furthermore, the insertional knockout mutant *mbs1* also showed de-regulation of the expression of $^1\text{O}_2$ -specific markers, indicating a role of the MBS1 protein in mediation of $^1\text{O}_2$ signalling upstream of the induction of antioxidant defence mechanisms (Shao et al., [2013](#)). In an attempt to gain deeper insights into the mechanisms of signalling by $^1\text{O}_2$, here we have pinpointed MBS1 as a novel downstream intermediate in the β -cc signalling pathway, which ultimately regulates the process of photoprotection in *A. thaliana*. Unlike in wild-type (WT) plants where β -cc causes induction of many $^1\text{O}_2$ -responsive genes and greater tolerance to photooxidative stress (Ramel et al. [2012](#)), we show that the absence of MBS1 in the *mbs1* mutant led to de-regulation of $^1\text{O}_2$ -inducible genes after β -cc treatment. The corresponding β -cc-induced tolerance to high light stress was unachieved in the mutant, therefore indicating a crucial role of MBS1 in β -cc-mediated $^1\text{O}_2$ signalling.

Materials and Methods

Plant growth and stress treatment

Wild type (WT, ecotype Col 0), *mbs1-1* mutant and MBS1-overexpressing (OE) *A. thaliana* lines (Shao et al. [2013](#)) were grown for 5 weeks in short-day conditions (8 h/16 h, day/night) under a moderate photon flux density (PFD) of $\sim 150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, controlled temperature (20 °C/18 °C, day/night) and a relative air humidity of 65%. Plants of the *ch1* background (single mutant *ch1* and double mutant *ch1 mbs1-1*) were grown at a slightly lower PFD of $\sim 120 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 7–8 weeks (Ramel et al. [2013a](#)). Photooxidative stress was applied by subjecting WT, MBS1 OE and *mbs1* mutant plants to $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ PFD, 7 °C/18 °C temperature day/night respectively, and 380 ppm CO_2 in a growth chamber. High light stress in the *ch1* background was achieved by exposing *ch1* plants to high photon flux density and low temperature ($1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 8 °C). Photoacclimation was induced by exposing *ch1* plants to moderately elevated photon flux density of $450 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 20 °C/18 °C day/night for 2 d (Ramel et al. [2013a](#), with slight modifications). β -cyclocitral (β -cc) and dihydroactinidiolide treatments were performed as previously explained (Ramel et al. [2012](#)). Plants were placed in a transparent airtight plexiglass box, and defined volumes (50, 100 and 200 μL) of pure β -cc or dihydroactinidiolide were applied on cotton balls in the plexiglass boxes. As a control, β -cc and dihydroactinidiolide were replaced with H_2O . The plexiglass boxes were thoroughly sealed and placed in a growth chamber under controlled conditions of light and temperature ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 22 °C) for 4 h. β -cc was obtained from Sigma-Aldrich and dihydroactinidiolide was provided by Chemos.

Root growth

Seeds of the different lines were surface sterilized in 70% EtOH and 0.05% Triton X-100, followed by 100% EtOH. The seeds were sown onto square Petri dishes containing one-half-MS medium supplemented with 0.5 g/l MES-KOH, pH 5.7, 0.8% Plant Agar (Duchefa) and 1% Sucrose, when explicitated, stratified for 2 d at 4 °C in the dark, and placed vertically in a growth chamber under a short daylight period (8 h light/16 h dark) using $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 22 °C.

Quantification of lipid peroxidation and imaging

Lipids were extracted from approximately 0.5 g leaves frozen in liquid nitrogen. The leaves were ground in an equivolume methanol/chloroform solution containing 5 mM Triphenyl Phosphine (PO₃) and 1 mM 2,6-tert-butyl-p-cresol (BHT) ($5 \text{ mL g}^{-1} \text{ FW}$) and citric acid (2.5 mL g^{-1} fresh weight), using an Ultra-Turrax blender. 15-HEDE was added as an internal standard to a final concentration $100 \text{ nmol g}^{-1} \text{ FW}$ and mixed properly. After centrifugation at 700 g and 4 °C for 5 min, the lower organic phase was carefully taken out with the help of a glass syringe and transferred into a 15 mL glass tube. The syringe was rinsed with approximately 2.5 mL chloroform and emptied in the tube containing the upper organic phase. The process was repeated, and the lower layer was again collected and pooled with the first collected fraction. The solvent was evaporated under N₂ gas, at 40 °C. The residues were recovered in 1.25 mL absolute ethanol and 1.25 mL of 3.5 N NaOH and hydrolysed at 80 °C for 30 min. The ethanol was evaporated under N₂ gas at 40 °C for ~10 min. After cooling to room temperature, pH was adjusted to 4–5 with 2.1 mL citric acid. Hydroxy fatty acids were extracted with hexane/ether 50/50 (v/v). The organic phase was analysed by straight phase HPLC-UV, as previously described (Montillet et al. [2004](#)). Hydroxyoctadecatrienoic acid (HOTE) isomers (9-, 12-, 13- and 16-HOTE derived from the oxidation of the main fatty acid, linolenic acid) were quantified based on the 15-HEDE internal standard. ROS-induced HOTES were separated from lipoxygenase (LOX)-induced HOTES using the protocol described in Montillet et al. ([2004](#)).

Lipid peroxidation was also visualized in whole plants by autoluminescence imaging. Stressed plants were dark adapted for 2 h, and the luminescence emitted from the spontaneous decomposition of lipid peroxides was captured by a highly sensitive liquid N₂-cooled charge-coupled device (CCD) camera, as previously described (Birtic et al. [2011](#)). The images were treated using Image J software (NIH, USA)

PSII photochemical activity

Chlorophyll fluorescence from intact leaves was measured with a PAM-2000 fluorometer (Walz), as described previously (Ramel et al. [2012](#)). The maximum quantum yield of PSII was determined by the Fv/Fm ratio, measured in dark-adapted intact leaves. The actual quantum yield of PSII-mediated electron transport ($\Delta F/Fm'$) was measured at different PFDs of white light provided by a Schott KL1500 light source equipped with a light guide. The variable fluorescence Fv was calculated as the difference between maximum fluorescence Fm (obtained with an 800 ms pulse of intense white light) and the initial fluorescence level, Fo (obtained with a 2 s pulse of far-red light). Fm' is the maximum fluorescence level measured in the light, and ΔF is the difference between Fm' and the actual steady-state fluorescence level in the light.

Chlorophyll fluorescence spectra from leaf discs were measured at 77 K in liquid nitrogen using a Perkin Elmer LS50 spectrofluorimeter using an excitation light at 440 nm; 77 K fluorescence > 700 nm emanates from PSI antenna pigments, while fluorescence <700 nm originates from PSII antenna pigments (Rijgersberg et al. [1979](#)).

RNA isolation and qRT-PCR

Total RNA was isolated from 100 mg leaves using the Nucleospin® RNA Plant kit (Macherey-Nagel). The concentration was measured on a NanoDrop2000 (Thermo Scientific, USA). First strand cDNA was synthesized from 1 μg total RNA using the PrimeScript™ Reverse Transcriptase kit (Takara, Japan). qRT-PCR was performed on a Lightcycler 480 Real-Time PCR system (Roche, Switzerland). Three microlitres of a reaction mixture comprising SYBR Green I Master (Roche, Switzerland), 10 μM each of forward and reverse primers and water, was added to 2 μL of a 10-fold diluted cDNA sample in a 384 well plate. The PCR programme used was: 95 °C for 10 min, then 45 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 15 s. At least three biological replicates were performed for each gene tested. Primers for all genes examined (Supporting information Table S1) were designed using Primer3Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). ACTIN-2 (ACT2) was used as reference gene for the normalization of gene expression levels.

Confocal microscopy

Plants of the $P_{MBS1}:MBS1-GFP$ line, in the *mbs1* mutant background (Shao et al. 2013), were treated with β -cc or with high PFD ($1500 \mu\text{mol m}^{-2} \text{s}^{-1}$). Leaves from 5-week-old plants were mounted in water, on a microscope slide, and the images were acquired using a ZEISS LSM780 laser scanning confocal imaging system. The excitation and emission wavelengths were 488 nm and 500–520 nm, respectively, for GFP fluorescence; 488 nm and 650–750 nm, respectively, for chlorophyll autofluorescence; 405 nm and 440–550 nm for DAPI fluorescence (4',6-diamidino-2-phenylindole). DAPI was used at a concentration of $1 \mu\text{g mL}^{-1}$. High-definition images were acquired (1024×1024 , 20 \times objective) and analysed using the Fiji—ImageJ bundle software (<http://fiji.sc/Fiji>). All experiments were performed at least in triplicate, and the images represent typical examples.

Protein extraction and antibody revelation

Total proteins were extracted from leaves of $P_{MBS1}:MBS1-GFP$ plants, treated with β -cc or water, by grinding in liquid nitrogen and subsequent resuspension in 50 mM Tris pH 8, 0.1% Triton X100, 1 mM PMSF, 50 mM β -mercaptoethanol. Proteins were subjected to SDS-PAGE (13% polyacrylamide) and then electrotransferred onto nitrocellulose membranes (0.45 mm; Biotrace NT). Bound proteins were subsequently probed with the anti-GFP monoclonal antibody (Roche) and visualized with 1:10 000 diluted anti-mouse IgG-IRDye700 antibody (Invitrogen). Detection was done by the Odyssey infrared imaging system (Li-Cor Biosciences).

Results

MBS1 and plant resistance to high light stress

MBS1 deficiency in Arabidopsis is associated with a decrease in plant phototolerance resulting in pale leaf phenotype, early senescence and lack of anthocyanin accumulation in the *mbs1* mutants under high light stress conditions (Shao et al. 2013). In Fig. 1, 5-week-old Arabidopsis plants were transferred to high photon flux density (PFD) and low temperature conditions ($1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, 7 °C) for 2 d to assess their resistance to photooxidative stress. *mbs1* knockout mutant plants were noticeably more susceptible to this treatment than WT, as depicted by increased lipid peroxidation measured by autoluminescence imaging (Fig. 1a) and analyses of ROS-induced hydroxy fatty acid (HOTE) (Fig. 1b). Furthermore, a decrease in the PSII photochemical efficiency measured by the Fv/Fm chlorophyll fluorescence ratio (Fig. 1c) was appreciable in the mutant compared to the WT. In standard conditions, WT and *mbs1* did not differ in terms of Fv/Fm (0.79 in both genotypes, as also shown by Shao et al. 2013) and lipid peroxidation (HOTE < 2 nmol g⁻¹ fresh weight, see also Fig. 2c). These results corroborate the involvement of MBS1 in the regulation of plant resistance to high light stress and, in particular, in the protection of thylakoid membranes against photooxidative damage.

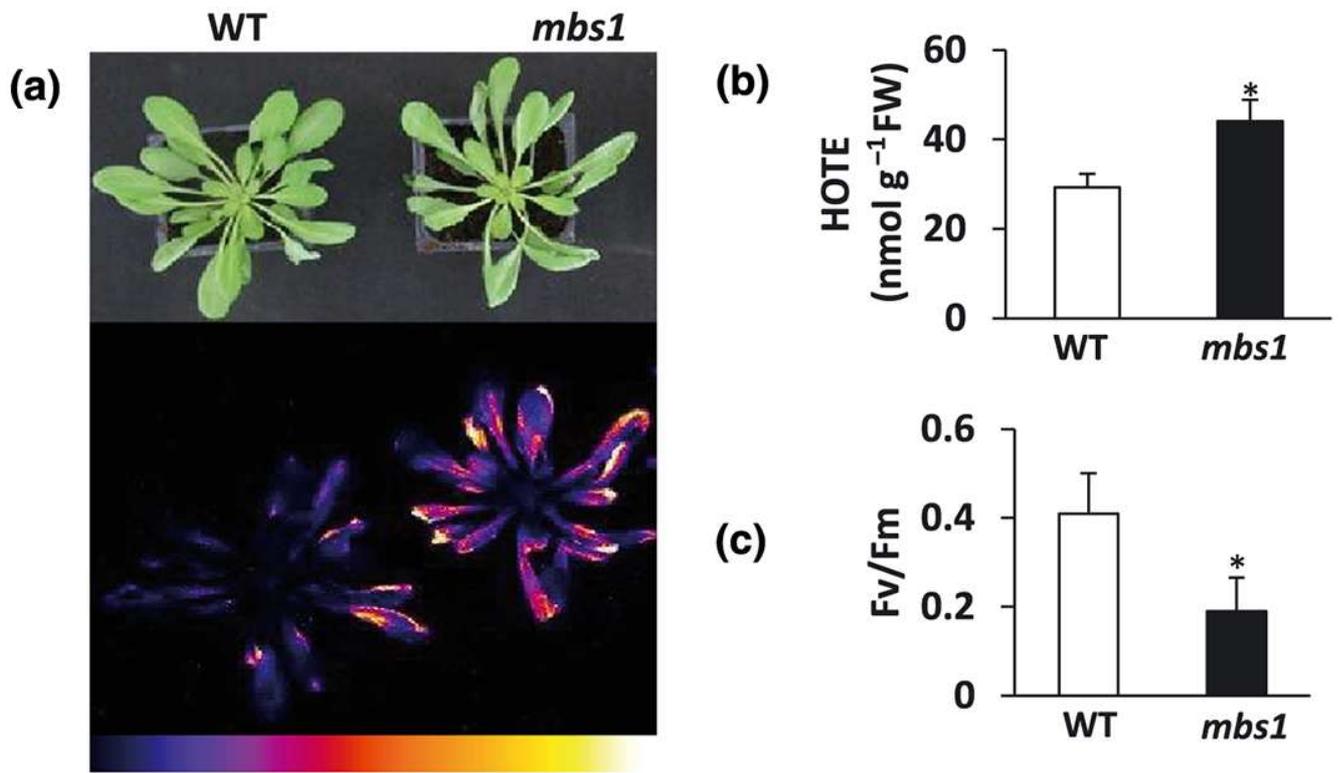


Figure 1.

*Involvement of MBS1 in protection against high light stress. Lipid peroxidation in WT and *mbs1* mutant plants after 28 h exposure to high light ($1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low temperature ($7^\circ\text{C}/18^\circ\text{C}$ day/night) monitored by autoluminescence imaging (a) and HPLC quantification of ROS-induced HOTEs (b). The colour scale in panel A indicates signal intensity ranging from zero (black) to saturation (white). (c) Maximum quantum yield of PSII photochemistry determined by the Fv/Fm chlorophyll fluorescence ratio after high light stress. * indicates significant difference between *mbs1* and WT at $P < 0.05$ (Student's t-test). Data are mean values of 4 (b) or 7 (c) measurements + SD.*

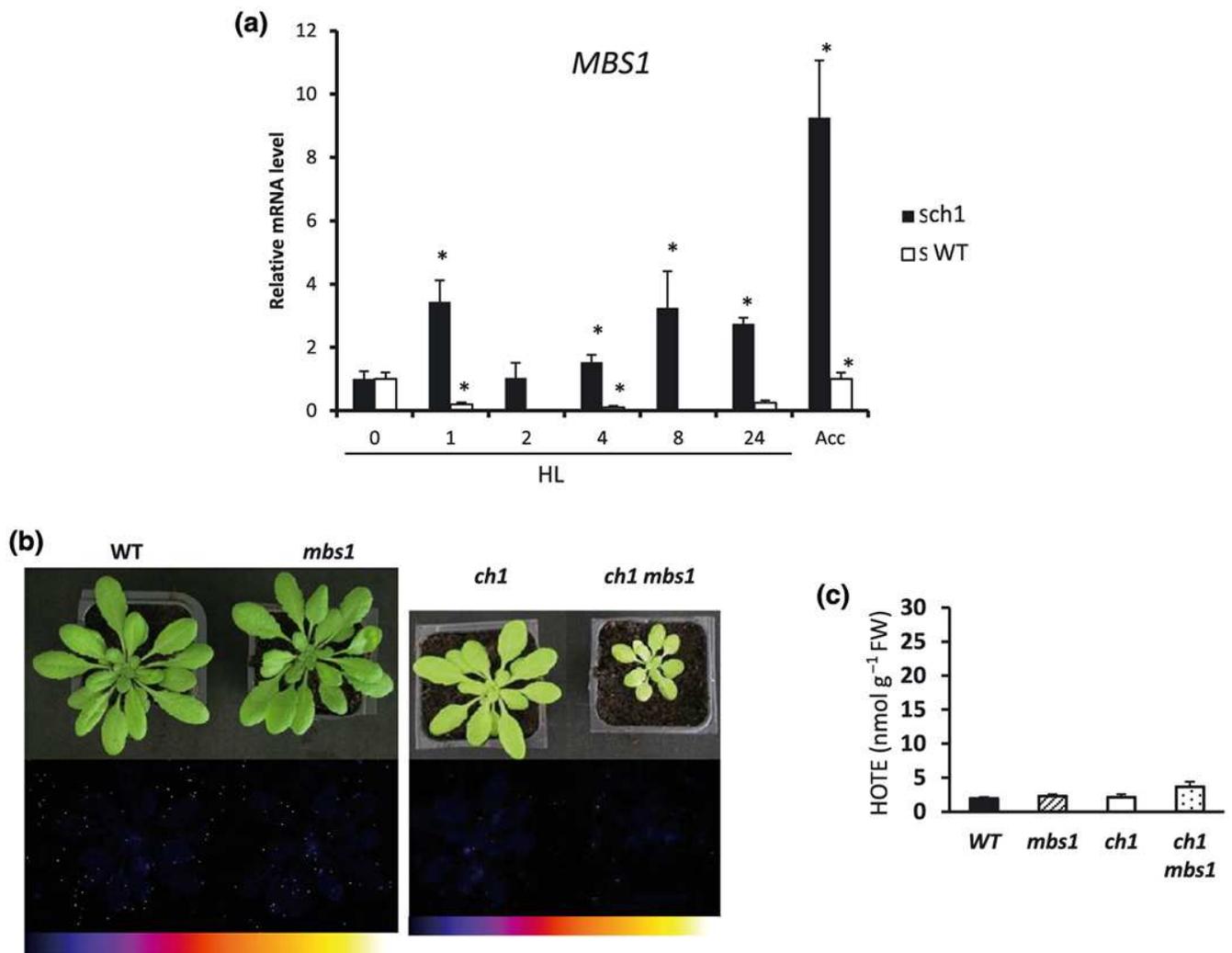


Figure 2.

Effects of the *mbs1* mutation in the *ch1* genetic background. (a) Transcript profile of *MBS1* in WT and in the *ch1* mutant exposed to high light and low temperature (HL = 1200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 8 °C, 1, 2, 4, 8 and 24 h), and acclimation (Acc) conditions (450 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 20 °C, 2 d). * represents significant difference between time 0 and the light treatments at $P < 0.05$ (Student's *t*-test). (b) Characterization of the *mbs1* single mutant and the *ch1 mbs1* double mutant grown under short-day conditions (120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PFD; 8 h/16 h, day/night photoperiod; 22 °C/18 °C, day/night temperature; 65% relative humidity) for 5 or 7 weeks relative to WT and *ch1*, respectively. At the bottom: lipid peroxidation detected by autoluminescence imaging. The colour scale represents the signal intensity ranging from 0 (black) to saturation (white). (c) HPLC quantification of ROS-induced HOTE levels in non-stressed plants. Data (a, c) are mean values of 3 biological replicates + SD.

Growth, development and photoacclimation are regulated by *MBS1* in a high ¹O₂ environment

The *chlorina1* (*ch1*) *Arabidopsis* mutant is a ¹O₂-overproducing mutant that has recently emerged as a useful tool to study the signalling role of ¹O₂ *in vivo* (Laloi & Havaux 2015). The increased release of ¹O₂ from the PSII reaction centres in *ch1* is associated with changes in the expression of nuclear genes compared to WT. Enhanced production of ¹O₂ was observed in the *ch1* mutant relative to WT even in low light (Ramel et al. 2013a), as confirmed under the PFD used in this study to grow *ch1* plants (120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) (Supporting Information Fig. S1). Depending on the levels of ¹O₂ production induced by light, the ¹O₂-triggered signalling pathway in *ch1* was found to lead either to cell death (at high PFDs and high ¹O₂ levels) or to an acclimation process (at low PFDs). We assessed transcript levels of *MBS1* in the *ch1* mutant exposed to different light conditions: high light stress (1200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 10 °C) for several hours or photoacclimation (450 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 20 °C). *MBS1* was induced during high light stress and even more during photoacclimation (Fig. 2a). By contrast, in WT plants, *MBS1* was induced neither by high light treatments nor by treatments that led to acclimation in the *ch1* mutant. Rather, we observed a repression of *MBS1* in WT leaves exposed to high light stress. The ¹O₂ induction of *MBS1* in *ch1* leaves was qualitatively similar to the

expression profile of the $^1\text{O}_2$ marker gene AAA (Supporting information, Fig. S2), except for the acclimation treatment that induced lower expression levels of AAA compared to stress conditions (24 h). Both genes were transiently induced after 1 h exposure to high light stress and were also induced after stress treatments longer than 4 h. The results presented in Fig. 2a delineate for the first time *MBS1* as a $^1\text{O}_2$ responsive gene which may play a vital role in acclimation to high levels of $^1\text{O}_2$.

To better relate the susceptible phenotype observed in the *mbs1* mutant during high light stress (Fig. 1) to $^1\text{O}_2$, and also to confirm the phenotype observed in the *mbs1* mutant after high light stress in a typical high $^1\text{O}_2$ background, the *mbs1* mutant was crossed with the *ch1* mutant. Homozygous lines of the *ch1 mbs1* double mutant showed a drastically impaired growth phenotype compared to the *ch1* mutant, at $\sim 120 \mu\text{mol m}^{-2} \text{s}^{-1}$ PFD (Fig. 2b, top panel). This phenotype was not rescued by lowering the PFD to a very low level ($\sim 60 \mu\text{mol m}^{-2} \text{s}^{-1}$ in short day) (data not shown). This growth inhibition took place without any apparent oxidative damage to lipids (Fig. 2b, bottom panel, and Fig. 2c). Interestingly, this phenotype corresponded to a lower maximum PSII photochemical efficiency measured by the F_v/F_m ratio in non-stressed *ch1 mbs1* plants compared to *ch1* plants (< 0.4 in *ch1 mbs1* versus 0.8 in *ch1*, Fig. 3a). Moreover, the quantum yield of photosynthetic electron transport ($\Delta F/F_m'$) was noticeably reduced in *ch1 mbs1* leaves compared to *ch1* leaves (Fig. 3b) in a large range of PFDs up to $6000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Chlorophyll fluorescence spectra (Fig. 3c) measured at 77 K showed that PSII (monitored by F_{690}) was decreased relative to PSI (F_{720}) in the double mutant *ch1 mbs1* compared to the single mutant *ch1*. Data of Fig. 3 show that the photosynthetic apparatus was altered in *ch1 mbs1* leaves compared to *ch1* leaves. In contrast with the *ch1 mbs1* double mutant, the *mbs1* single mutant did not differ in terms of growth and development from WT (Fig. 2b).

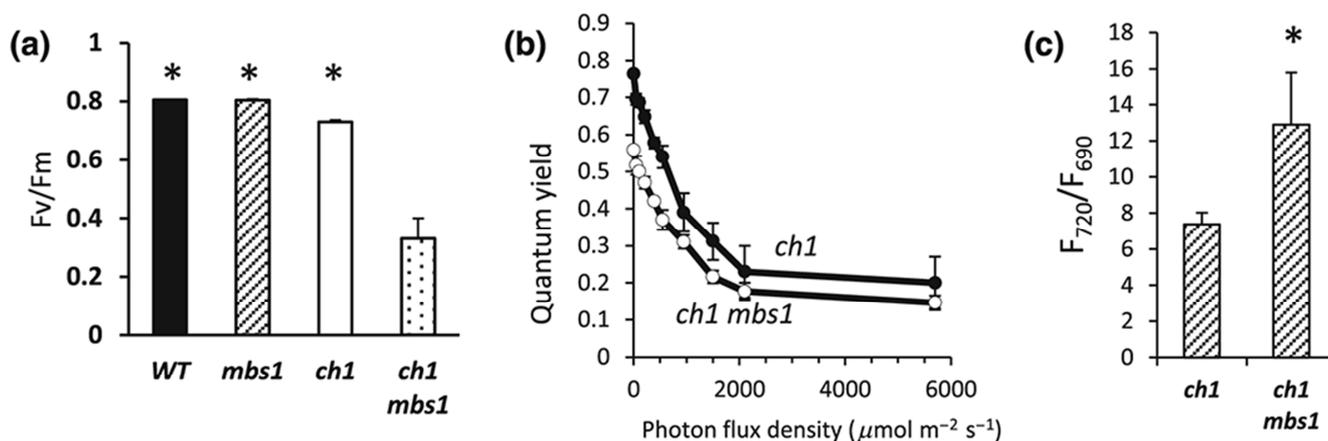


Figure 3.

*Photosynthetic characteristics of *ch1* and *ch1 mbs1* leaves. (a) PSII photochemical efficiency measured by the F_v/F_m ratio from non-stressed plants (WT, *mbs1* and *ch1* single mutants and *ch1 mbs1* double mutant). Data are mean values of nine measurements + SD. * represents significant difference with *ch1 mbs1* plants at $P < 0.05$ (Student's *t*-test). (b) Light dependence of the quantum yield of photosynthetic electron transport in non-stressed *ch1* and *ch1 mbs1* leaves. Data are mean values of three biological replicates + SD. (c) Ratio between 77 K chlorophyll fluorescence emission at 720 nm (F_{720}) and 77 K fluorescence emission at 690 nm (F_{690}) in *ch1* and *ch1 mbs1* leaves. * indicates significant difference with *ch1* plants at $P < 0.05$ (Student's *t*-test).*

We also germinated seeds on solid MS-1/2 medium and analysed root growth. As shown in Fig. 4, root growth of the *ch1 mbs1* double mutant was drastically inhibited compared to WT, *ch1* and *mbs1*. Interestingly, this difference was cancelled when sucrose was added to the growth medium. This supports the idea that the growth inhibition of *ch1 mbs1* relative to *ch1* is related to photosynthesis inhibition and reduced carbon assimilation. The results of Figs 2-4 thus suggest also a role of the MBS1 protein in the regulation of growth and development in conditions of chronic production of low levels of $^1\text{O}_2$ as in the *ch1* mutant in low light.

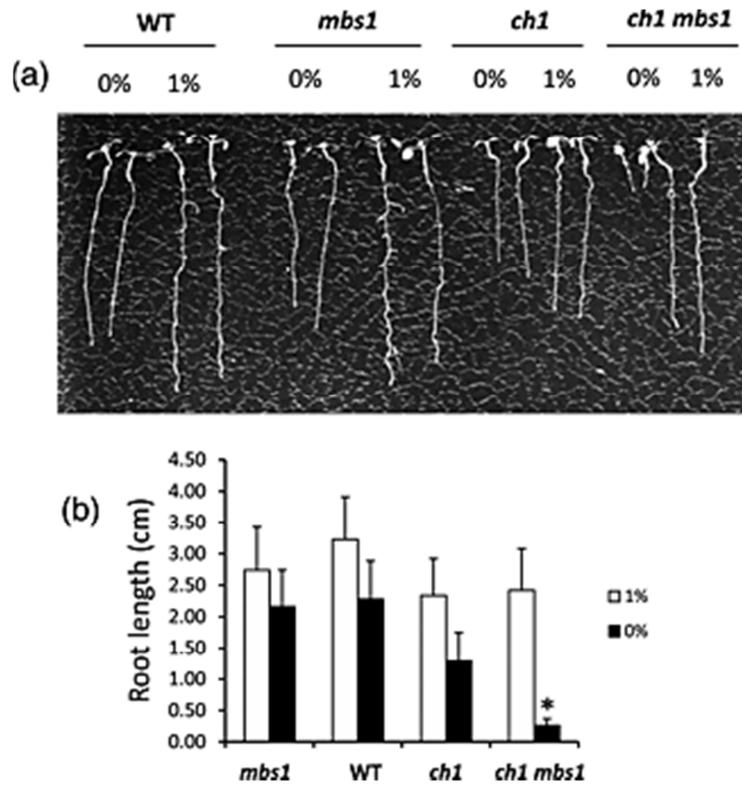


Figure 4.

Root growth phenotype of WT, *ch1* and *mbs1* single mutants and *ch1 mbs1* double mutant. Seedlings were grown for 11 d on solid growth medium with or without sucrose (0% and 1%, respectively). (a) Picture of representative seedlings. Scale bar = 1 cm. (b) Root length (in cm) + SD. * indicates significant difference between the two conditions (with and without sucrose) at $P < 0.05$ (Student's *t*-test).

As mentioned above, the *ch1* mutant can acclimate to high light and $^1\text{O}_2$ stress by pre-exposure to a moderately elevated PFD (e.g. $450 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 20°C) that induces a moderate production of $^1\text{O}_2$ (Ramel et al. 2013a). As confirmed in Fig. 5, acclimated *ch1* plants (*ch1-A/S*) were highly resistant to a subsequent light stress treatment that caused extensive damage and lipid peroxidation in non-acclimated plants (*ch1-S*). In contrast to *ch1*, the double mutant *ch1 mbs1* was unable to fully acclimate to high light (Fig. 5a,b): leaf bleaching and lipid peroxidation after high light stress were noticeably more pronounced in double mutant plants pre-exposed to the acclimation treatment (*ch1 mbs1-A/S*) compared to *ch1* plants after acclimation (*ch1-A/S*). It should be noted that, because of the marked growth impairment in the *ch1 mbs1* mutant (Fig. 2), plants at a comparable developmental stage were chosen for this experiment. The results of Fig. 5 confirm the importance of MBS1 in photoacclimation.

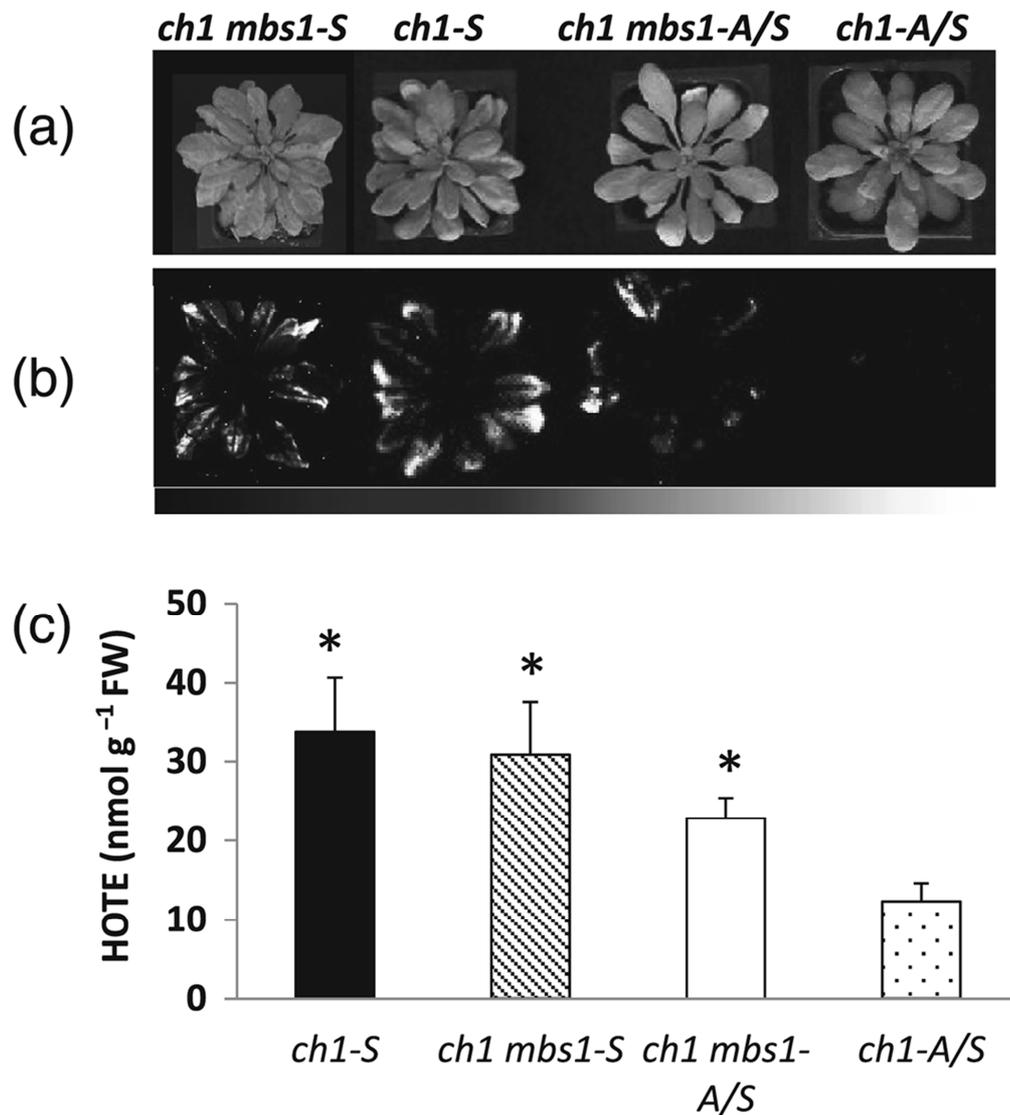


Figure 5.

MBS1 is required for acclimation of the *ch1* mutant to high light stress. Leaf bleaching (a) and lipid peroxidation (b and c) induced by high light stress ($1200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 8°C) in *ch1* and *ch1 mbs1* plants pre-exposed to an acclimation treatment (2 d at $\text{PFD } 450 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 20°C) (*ch1-A/S* and *ch1 mbs1-A/S*, respectively). Non-acclimated *ch1* and *ch1 mbs1* plants after high light stress are also shown as controls (*ch1-S* and *ch1 mbs1-S*, respectively). Because of the differences in growth rate between *ch1* and *ch1 mbs1* (see Fig. 5B), *ch1* plants aged 8 weeks are compared with *ch1 mbs1* plants aged 12 weeks. The grey scale in panel A indicates signal intensity ranging from zero (black) to saturation (white). * indicates significant difference with *ch1-A/S* at $P < 0.05$ (Student's *t*-test). Data in (c) are mean values of ROS-induced HOTE of at least 3 measurements + SD.

Role of MBS1 in β -cc-mediated photoprotection

The role of the β -carotene oxidation product, β -cc, in mediation of plant resistance to high light stress has been established: WT plants treated with volatile β -cc were found to exhibit changes in the expression of $^1\text{O}_2$ -responsive genes which led to increased tolerance to high light stress (Ramel et al. 2012). Whole Arabidopsis plants were exposed for 4 h to volatile β -cc, as previously described (Ramel et al. 2012): a given amount of β -cc ($100 \mu\text{L}$) was let to vaporize in a hermetically closed chamber (volume, 22.3 L). GC-MS analyses of the gas phase in the airtight box indicated a β -cc concentration in the range $10\text{--}20 \mu\text{g L}^{-1}$. Control plants were treated similarly, except that β -cc was replaced by distilled water. In the WT and the *MBS1* OE line, the β -cc-treatment led to a substantial decrease in lipid peroxidation, measured by autoluminescence imaging (Fig. 6a,d) and HPLC quantification of HOTE (Fig. 6b), and a noticeable increase in PSII photochemical efficiency (Fig. 6c), compared to the corresponding untreated plants under high light stress. In striking contrast, *mbs1* plants treated with β -cc showed similar levels of lipid peroxidation as untreated plants after high light stress (Fig. 6a,b). This was supported by similar extents of inhibition of PSII photochemical efficiency in β -cc-treated and

untreated *mbs1* plants after high light stress (Fig. 6c). Interestingly, the effects of β -cc on lipid peroxidation were observed for ROS-induced production of HOTEs (Fig. 6c), while LOX-induced lipid peroxidation was modulated neither by β -cc nor by the *mbs1* mutation (Supporting information, Fig. S3). Taken together, these results highlight the pivotal role of MBS1 in mediating signal transfer from β -cc to the nucleus to elicit tolerance to high light stress. In agreement with Shao et al. (2013), the effects of high light stress appeared to be less marked in the MBS1-overexpressing plants compared to WT plant: less leaves exhibited a luminescence signal related to lipid peroxidation and the HOTE levels were lower in MBS1 OE leaves (Fig. 6). This result supports and strengthens the correlation between MBS1 levels and tolerance to high light. However, after the β -cc treatment, both WT and MBS1 OE plants exhibited a high phototolerance. The WT levels of MBS1 appear thus to be sufficient for the β -cc-dependent increase in the tolerance to the high light stress conditions used in this study.

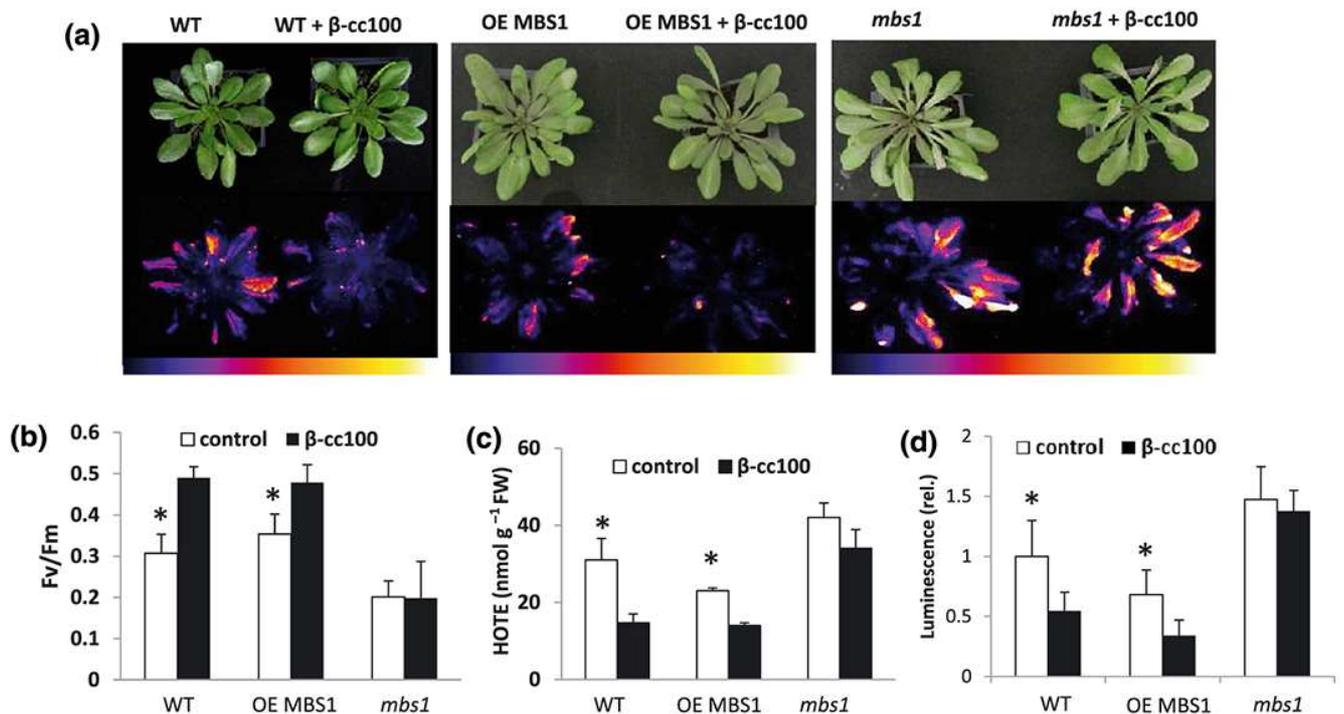


Figure 6.

Effect of β -cc treatment on the photosensitivity of WT, *mbs1* mutant and MBS1 overexpressing (OE) plants. (a) Lipid peroxidation monitored by autoluminescence in WT, MBS1 OE lines and *mbs1* knockout lines after treatment with 100 μ L β -cc for 4 h, followed by exposure to high light and low temperature (1500 μ mol m⁻² s⁻¹ PFD, 7 °C/18 °C day/night, respectively). Control plants were treated with 100 μ L H₂O. The colour scale indicates the signal intensity ranging from 0 (black) to saturation (white). (b) PSII photochemical efficiency measured by the Fv/Fm chlorophyll fluorescence ratio, (c) lipid peroxidation measured by HPLC quantification of ROS-induced HOTEs and (d) quantification of the autoluminescence signals from WT, MBS1 OE and *mbs1* mutant leaves after β -cc treatment followed by high light stress relative to control plants. * indicates significant difference between β -cc-treated and untreated plants at $P < 0.05$ (Student's *t*-test). Data in (b) and (c,d) are mean values of seven and minimum 4 measurements + SD, respectively.

We then examined whether MBS1 deficiency affects the regulation of gene expression by β -cc. Figure 7 shows that β -cc induced a modification in the expression profile of ¹O₂ specific marker genes (*AAA* and *LT130*) and ROS responsive transcription factors (*ZAT12* and *WRKY40*) in WT. The *mbs1* mutation drastically perturbed the expression profile of those genes, with a marked reduction of the expression levels induced by 50 μ L β -cc and a significant increase or decrease at the highest β -cc concentration (100 μ L). We also examined the responses of a number of H₂O₂-responsive genes to β -cc in WT and *mbs1*: *CAT2* and *APX1* (Fig. 7), and *MAPK6* and *PTI1-4* (Supporting Information Fig. S4). The two latter genes have been shown to be specific markers of H₂O₂, exhibiting a marked up-regulation (> $\times 7$) in Arabidopsis leaves treated with H₂O₂ and no response to ¹O₂ (Shumbe et al. 2016). However, an increase in gene expression of a factor of 7 for those genes is rather low compared to the gene expression levels that can be reached by the ¹O₂ marker genes used here (*AAA* and *LT130*) which can reach much higher expression levels ($\times 20$ and $\times 15$, Fig. 7). This sensitivity issue

must be taken into account in the interpretation of the transcriptomic data. As expected (Ramel et al. 2012), the effect of β -cc on the expression levels of the H_2O_2 marker genes was modest ($< \times 2$), except for *PTI1-4* which was repressed by the highest concentration of β -cc. In all cases, *MBS1* deficiency had relatively little effect on the expression profile of H_2O_2 -responsive genes, thus supporting the idea that *MBS1* as well as β -cc are specific $^1\text{O}_2$ signal mediators.

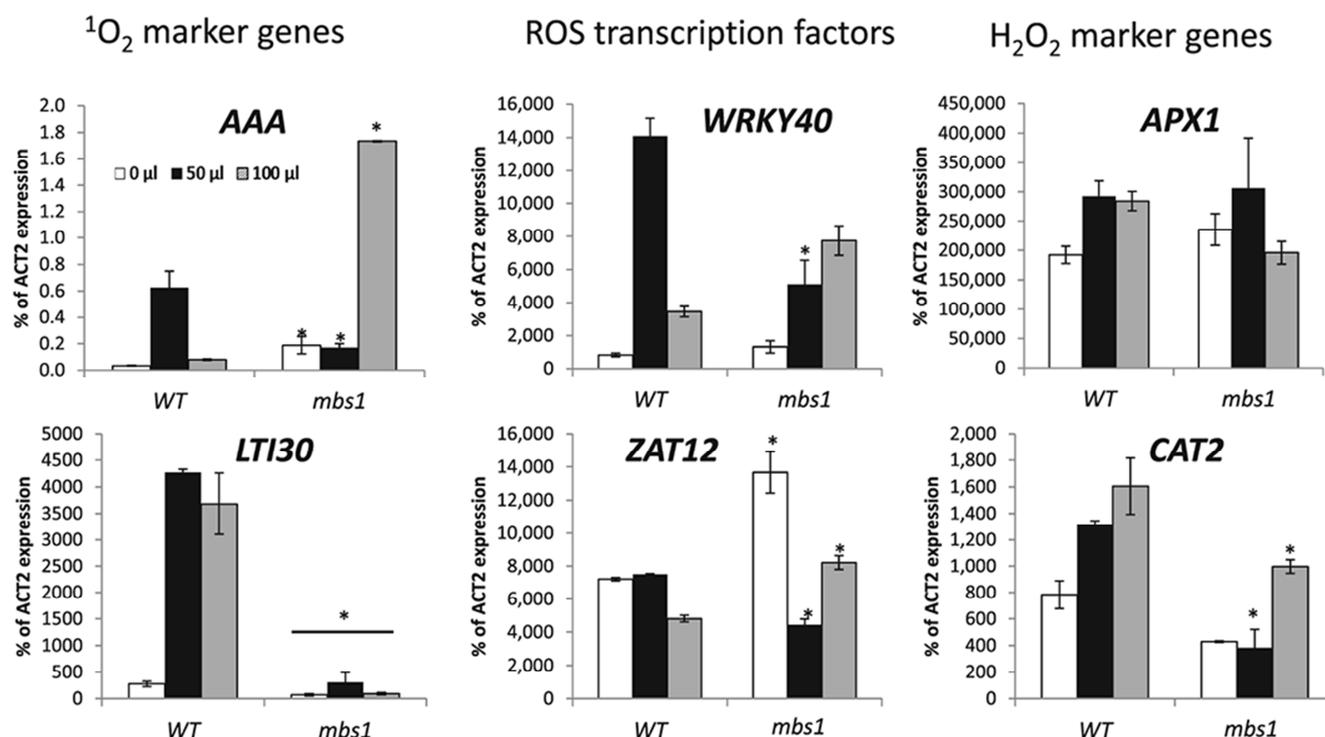


Figure 7.

Effects of β -cyclocitral and the *mbs1* mutation on gene expression. qRT-PCR measurements of transcript accumulation of $^1\text{O}_2$ -specific marker genes (*AAA* and *LTI30*), ROS-responsive transcription factors (*ZAT12* and *WRKY40*) and H_2O_2 -specific marker genes (*CAT2* and *APX1*) in WT and *mbs1* mutant plants after treatment with 50 μL and 100 μL of β -cc. Data were normalized on the expression level of the housekeeper gene *ACT2*. * indicates significant difference between WT and *mbs1* at $P < 0.05$ (Student's *t*-test). Data are mean values of at least 3 biological replicates + SD.

Together with β -cc, the lactone dihydroactinidiolide (produced by $^1\text{O}_2$ oxidation of the β -carotene derivative β -ionone) has been shown to induce changes in the expression of $^1\text{O}_2$ responsive genes (Shumbe et al. 2015). In Fig. S5 (Supporting Information), we show that volatile dihydroactinidiolide induced the expression of the $^1\text{O}_2$ marker genes *AAA*, *OX11* and *RD20* in WT, while the *mbs1* mutant was unresponsive to this treatment. In contrast, the *mbs1* mutation had no effect on the expression of H_2O_2 responsive genes (*CAT2* and *APX1*) in dihydroactinidiolide-treated plants. The data suggest that *MBS1* is a common player in both the β -cc and the dihydroactinidiolide signalling pathways downstream of $^1\text{O}_2$.

β -cyclocitral increases *MBS1* protein levels

The *MBS1*-GFP protein was analysed under different conditions in the *mbs1* mutant line complemented with the *MBS1*-GFP fusion gene under the control of the native *MBS1* promoter ($P_{MBS1}:MBS1\text{-GFP}/mbs1$ line). GFP fluorescence associated with the expression of this gene is visible mainly in the epidermis (Shao et al. 2013). In normal light, *MBS1*-GFP fluorescence was found in the cytoplasm and in the nucleus of epidermal cells (Fig. 8a, Supporting Information Fig. S6). The nuclear localization was confirmed using DAPI (4',6-diamidino-2-phenylindole), a DNA-specific fluorescent marker. As previously reported by Shao et al. (2013), high PFDs induced a noticeable accumulation of the *MBS1* protein as shown by the increased GFP fluorescence in Supporting Information Fig. S5. Interestingly, the treatment of the plants with β -cc was sufficient to enhance *MBS1*-GFP fluorescence, even at low PFDs (Fig. 8a). Quantification of GFP fluorescence in the nuclei showed that fluorescence intensity was almost doubled after the β -cc treatment (Fig. 8b). *MBS1*-GFP abundance was

also estimated by western blotting using an anti-GFP antibody (Fig. 8c). The immunoblots confirmed the analysis of the fluorescence levels in the images, with β -cc increasing the intensity of the MBS1 band at 38 kDa. Thus, our results show a regulation of MBS1 protein abundance by β -cc, similar to the regulation by high light, placing the carotenoid metabolite upstream of MBS1.

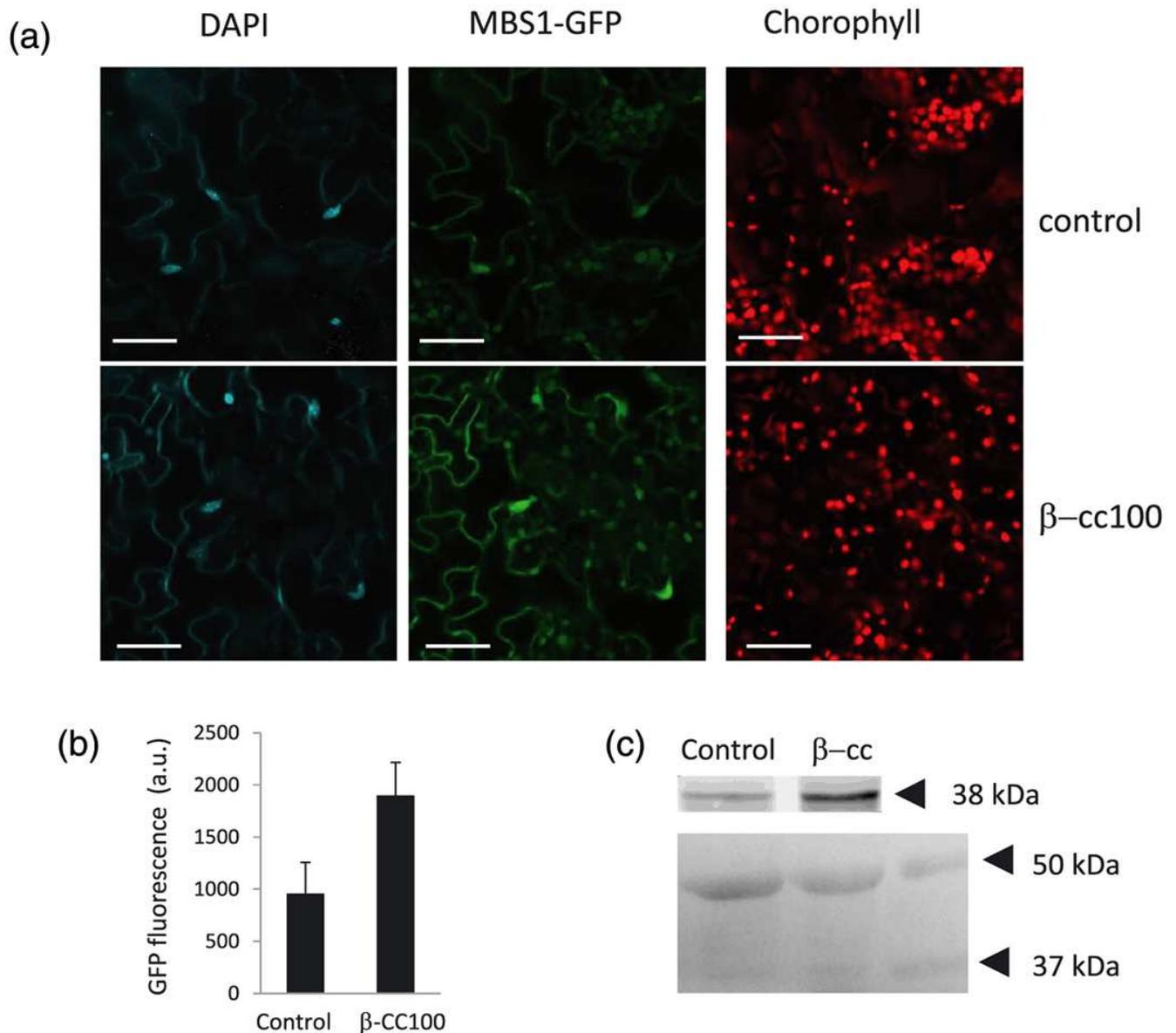


Figure 8.

*MBS1-GFP protein levels after treatment with β -cc. (a) Effect of β -cc (100 μ L) on the levels of MBS1-GFP under the control of the MBS1 native promoter in Arabidopsis epidermal cells (P_{MBS1} :MBS1-GFP/*mbs1* line). Scale bars indicate 45 μ m. Chlorophyll fluorescence from chloroplasts is shown in red on the right panels and DAPI fluorescence from DNA is shown in blue on the left panels. The control and β -cc images were obtained with the same laser light intensity. (b) Effect of β -cc on GFP fluorescence intensity in the nuclei. (c) Effect of β -cc on GFP-MBS1 abundance measured by western blotting using an anti-GFP antibody. At the bottom: Ponceau S-stained blot as a loading control, with two protein molecular weight markers at 37 and 50 kDa.*

Discussion

MBS1 is a small zinc finger protein previously identified to play a significant role in the regulation of tolerance to high light stress (Shao et al. 2013). In this work, we confirm and extend this role of the MBS1 protein and reveal a likely molecular cause of the susceptible phenotype of the *mbs1* mutant under high light stress, by quantifying the extent of lipid peroxidation and the effect on PSII photochemistry (Fig. 1). Phototolerance controlled by MBS1 was specifically attributed to the regulation of $^1\text{O}_2$ signalling. Several arguments have been advanced to support this conclusion. First, MBS1 participates in the regulation of $^1\text{O}_2$ -responsive gene

expression, with the absence of the protein resulting in a drastically perturbed expression profile of $^1\text{O}_2$ marker genes. Second, based on the fact that MBS1 was identified from a screen based on exposure of algal cultures to methylene blue (Shao et al. 2013), a photosensitizer which triggers the production of $^1\text{O}_2$ (Shao et al. 2007), the phenotype of the *mbs1* mutant observed under high light stress could easily be associated to $^1\text{O}_2$ effects. This fact was further confirmed in this work by providing evidence of an up-regulation of *MBS1* expression in a $^1\text{O}_2$ -rich environment (the *ch1* mutant exposed to high light; Fig. 2a), as opposed to the lack of a similar induction in WT plants exposed to high light (Shao et al. 2013), where the effects of other ROS are appreciable and $^1\text{O}_2$ production is lower than in the *ch1* mutant. Therefore, we can confidently infer that MBS1 acts as a regulator of $^1\text{O}_2$ signalling, thus also explaining the decrease in tolerance to high light in the absence of this protein.

The *ch1 mbs1* double mutant displayed a drastic perturbation in shoot and root growth (Figs 2 and 4). Interestingly, this growth retardation corresponded to a decrease in PSII photochemical efficiency (Figs 2 and 3) without any lipid peroxidation and apparent photooxidative damage (Fig. 2) and was rescued by supplementing seedlings with sugar (Fig. 4). This leads us to suggest that MBS1 is also involved in the mediation of the $^1\text{O}_2$ signal for growth and development. The *ch1* mutant under normal growth conditions is known to produce higher amounts of $^1\text{O}_2$ than WT (Dall'Osto et al. 2010; Ramel et al. 2013a; see also Supporting Information Fig. S1). Moreover, as previously shown (Shao et al. 2013), the *mbs1* mutant produces substantially higher amounts of $^1\text{O}_2$ in high light compared to WT, and this was attributed to the inability to efficiently induce the antioxidant defence system which detoxifies $^1\text{O}_2$. Thus, in the *ch1 mbs1* double mutant exposed to low light, the slight increase in the concentration of $^1\text{O}_2$ might not be sufficient to cause damage to lipids, but may be sufficient to cause perturbations in signalling processes for growth and development, because of the fact that the *ch1 mbs1* double mutant can neither sense $^1\text{O}_2$ nor transduce the $^1\text{O}_2$ signal, nor efficiently detoxify the $^1\text{O}_2$ produced. Moreover, the inability of the *ch1 mbs1* double mutant to increase its resistance to $^1\text{O}_2$ stress after exposure to moderately elevated PFD (Fig. 5) confirmed the essential role of MBS1 in photoacclimation. This idea is also supported by the marked up-regulation of the *MBS1* transcript in the *ch1* mutant exposed to photoacclimation conditions (Fig. 2a), in line with a vital role of MBS1 in this process.

MBS1 is essential for β -cc-mediated $^1\text{O}_2$ signal transduction during high light stress

We previously identified the β -carotene oxidation product β -cc as one of the early $^1\text{O}_2$ -signalling mediators in plants under high light stress (Ramel et al. 2012). β -cc was able to cause changes in the expression of $^1\text{O}_2$ -specific genes in a manner similar to high light stress, ultimately leading to acclimation of WT plants to high light. This β -cc-dependent acclimation mechanism was previously shown to be independent of the EX1 protein (Ramel et al. 2012). In contrast, the present study has revealed the dependence of β -cc-signalling on MBS1, with *mbs1* mutant plants treated with β -cc being equally susceptible to high light stress as untreated plants, whereas WT and MBS1-overexpressing plants acclimated to high light after the treatment. Interestingly, these results also provide evidence to confirm the involvement of MBS1 in the regulation of tolerance to high light stress in that untreated MBS1 OE plants (Fig. 6a) were more tolerant to high light stress compared to WT plants (Fig. 6b). The involvement of MBS1 in β -cc-mediated $^1\text{O}_2$ -signalling was further supported by the fact that the absence of MBS1 in the *mbs1* mutant led to de-regulation of specific $^1\text{O}_2$ -responsive markers and ROS-responsive transcription factors ($^1\text{O}_2$ inclusive), but not H_2O_2 -responsive markers (Fig. 7, Supporting Information Fig. S5) in response to β -cc. The same phenomenon was observed with another $^1\text{O}_2$ signalling molecule, dihydroactinidiolide (Supporting Information Fig. S4). The location of β -carotene in the PSII reaction centre close to the site of production of $^1\text{O}_2$ led to the discovery that, upon quenching of $^1\text{O}_2$ by β -carotene, β -cc and dihydroactinidiolide are formed as primary $^1\text{O}_2$ signal mediators (Ramel et al. 2012; Shumbe et al. 2014). With the implication of MBS1 in signal transduction mediated by β -cc and dihydroactinidiolide, it is evident that this protein is situated downstream of β -cc in the $^1\text{O}_2$ signalling pathway, a conclusion that is also in agreement with the cytosolic and nuclear localization of MBS1 (Shao et al. 2003). In addition, we observed an increase in the MBS1-GFP protein levels after β -cc treatment, similar to the previously reported induction by high light (Shao et al. 2013) that we also confirmed. Altogether, these findings confirm that β -cc acts through a high-light-like response in WT plants and, more importantly, allow us to shed light on a part of the likely molecular mechanisms downstream of β -cc, in that an increased MBS1 protein level is sufficient to achieve an enhanced tolerance to high light.

Conclusions

A clear picture on the mechanisms of $^1\text{O}_2$ signalling in plants is yet to be drawn. However, based on the chemical properties of this ROS, it is clear that chloroplast-to-nucleus retrograde signalling of $^1\text{O}_2$ requires mediators (Farmer & Davoine, 2007; Triantaphylides & Havaux, 2009). A couple of these mediators have been identified lately, but how one relates with another to propagate the signal to the nucleus is a mystery yet to be unraveled. This study brings to light the role of MBS1 as a $^1\text{O}_2$ signal mediator which regulates tolerance to high light stress and photoacclimation, and places MBS1 as the first identified downstream partner in the β -cc-mediated signalling pathway. It also assigns another function to the MBS1 protein in the regulation of $^1\text{O}_2$ signalling for growth and development. Further studies are required to identify and characterize other molecular targets downstream of β -cc in the pathway and their interaction with MBS1 to better appreciate both the architecture and the physiological role of this retrograde signalling pathway.

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