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RESEARCH PAPER

Mutation of *Arabidopsis HY1* causes UV-C hypersensitivity by impairing carotenoid and flavonoid biosynthesis and the down-regulation of antioxidant defence

Yanjie Xie, Daokun Xu, Weiti Cui and Wenbiao Shen*

College of Life Sciences, Co. Laboratory of Nanjing Agricultural University and Carl Zeiss Far East, Nanjing Agricultural University, Nanjing 210095, China

* To whom correspondence should be addressed. E-mail: wbshenh@njau.edu.cn

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Abstract

Previous pharmacological results confirmed that haem oxygenase-1 (HO-1) is involved in protection of cells against ultraviolet (UV)-induced oxidative damage in soybean [*Glycine max* (L.) Merr.] seedlings, but there remains a lack of genetic evidence. In this study, the link between *Arabidopsis thaliana* HO-1 (HY1) and UV-C tolerance was investigated at the genetic and molecular levels. The maximum inducible expression of *HY1* in wild-type *Arabidopsis* was observed following UV-C irradiation. UV-C sensitivity was not observed in *ho2*, *ho3*, and *ho4* single and double mutants. However, the *HY1* mutant exhibited UV-C hypersensitivity, consistent with the observed decreases in chlorophyll content, and carotenoid and flavonoid metabolism, as well as the down-regulation of antioxidant defences, thereby resulting in severe oxidative damage. The addition of the carbon monoxide donor carbon monoxide-releasing molecule-2 (CORM-2), in particular, and bilirubin (BR), two catalytic by-products of HY1, partially rescued the UV-C hypersensitivity, and other responses appeared in the *hy1* mutant. Transcription factors involved in the synthesis of flavonoid or UV responses were induced by UV-C, but reduced in the *hy1* mutant. Overall, the findings showed that mutation of HY1 triggered UV-C hypersensitivity, by impairing carotenoid and flavonoid synthesis and antioxidant defences.

Key words: Antioxidant defences, *Arabidopsis*, carotenoid, flavonoid, *hy1*, UV-C hypersensitivity.

Introduction

Although most harmful solar ultraviolet (UV)-C is effectively eliminated by the stratospheric ozone layer (Kazan and Manners, 2011), artificial UV-C irradiation is widely adopted for disinfection processes, including preventing cyanobacterial blooms, in many drinking water treatment plants (Bin Alam *et al.*, 2001; USEPA, 2006; Sakai *et al.*, 2009). UV-C stress, sharing the ELONGATED HYPOCOTYL5 (HY5)-mediated signalling pathway involved in UV-B signalling (Castells *et al.*, 2010), is also an effective induction model for studying mechanisms of oxidative stress and programmed cell death (Renzing *et al.*, 1996; Gao *et al.*, 2008; Balestrazzi *et al.*, 2009; Saxena *et al.*, 2011). As it is highly energetic, UV-C radiation has deleterious effects on plant cells, including DNA damage, oxidation of cellular components, and impaired chloroplast function and thus reduced photosynthesis (Booij-James *et al.*, 2000). Numerous studies have examined the different strategies adopted by plants to cope with the detrimental photooxidative processes caused by excessive UV radiation, including the regulation of flavonoid metabolism and the size of the xanthophyll cycle pool, as well as the manipulation of a variety of reactive oxygen species (ROS) scavengers (Landry *et al.*, 1995; Kirchgeßner *et al.*, 2003; Close *et al.*, 2007). Nonetheless, the detailed molecular and biochemical mechanisms of the signalling pathways by which plants sense and relay UV-C signals remain to be elucidated.

Flavonoids are considered antioxidant molecules, which may serve as free radical scavengers by locating and

neutralizing radicals before they can damage plant cells. It has been suggested that non-pigmented flavonoids play a central role in protecting against harmful UV light and excess visible light (Buer *et al.*, 2010). The regulation of flavonoid biosynthesis in *Arabidopsis thaliana* has been well described by characterizing T-DNA insertion mutants (Qin *et al.*, 2007; Buer *et al.*, 2010; Castells *et al.*, 2010; Supplementary Fig. S1 available at *JXB* online). The flavonoid biosynthetic pathway is a branch of the general phenylpropanoid pathway, the first committed step of which is catalysed by chalcone synthase (CHS). Subsequent reactions catalysed by chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), and flavonol synthase (FLS) lead to production of the basic flavonols, kaempferol and quercetin (Supplementary Fig. S1 available at *JXB* online). Genetic evidence has further confirmed that the *Arabidopsis* mutants *transparent testa-4* (*tt4*; deficient in *CHS*) and *transparent testa-5* (*tt5*; deficient in *CHI*) are unable to accumulate flavonoids, and are therefore more sensitive to UV-B and UV-C (Li *et al.*, 1993; Filkowski *et al.*, 2004). Both when growing normally and when exposed to high UV levels, fine regulation of flavonoid biosynthesis is accomplished by integrated modulation of multiple transcriptional factors, including WRKY, basic leucine zipper (bZIP), MADS-box, R2R3-MYB, and basic helix–loop–helix (bHLH) factors (Ramsay and Glover, 2005; Stracke *et al.*, 2007, 2010). Under both visible light and UV-B, the bZIP transcriptional regulator HY5 is linked to *CHS* gene activation and accumulation of flavonoids (Oravecz *et al.*, 2006). Recent studies have confirmed that the HY5-dependent regulation of the gene expression of the PRODUCTION OF FLAVONOL GLYCOSIDES (PFG) family of R2R3-MYB transcriptional factors, including PFG1/MYB12, PFG2/MYB11, and PEF3/MYB111, contributes to the establishment of UV-B tolerance (Stracke *et al.*, 2010).

Other complementary and redundant mechanisms have also been shown to provide protection against photooxidative damage caused by UV irradiation, including carotenoid metabolism and a series of antioxidant defence enzymes responsible for ROS scavenging (Bartley and Scolnik, 1995). Using forward and reverse genetic approaches, the main pathway for carotenoid biosynthesis in *Arabidopsis* has been identified, and includes geranylgeranyl pyrophosphate synthase (GPS), phytoene synthase (PSY), phytoene desaturase (PDS), and zeta-carotene desaturase (ZDS). Enzymatic antioxidant systems also play a role in the efficient scavenging of ROS, including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). Further studies have shown that a *Neurospora crassa* mutant which is null for *sod-1*, encoding copper-zinc SOD, showed hypersensitivity to near-UV irradiation (Chary *et al.*, 1994).

In animals, haem oxygenase (HO; EC 1.14.99.3) is a microsomal enzyme committed to haem degradation. Haem moieties from haemoproteins are cleaved by HO at the α-meso-carbon bridge, generating equimolar amounts of ferrous iron, carbon monoxide (CO), and biliverdin (BV). BV is subsequently reduced to bilirubin (BR) by cytosolic biliverdin reductase (Otterbein *et al.*, 2003). To date, three isoforms (HO-1, HO-2, and HO-3) have been identified in animals. HO-1 has been confirmed as an inducible isoform by numerous stimuli, including haem as well as non-haem stimuli such as heavy metals, hormones, endotoxin, cytokines, ROS, heat shock, and UV-A/B/C irradiation (Applegate *et al.*, 1991; Prawan *et al.*, 2005; Loboda *et al.*, 2008). Various HO-1 inducers have provided further support for the conclusion that HO-1, besides its role in haem degradation, may play an important role as a dynamic sensor of cellular oxidative stress and in maintaining cellular homeostasis (Bilban *et al.*, 2008; Calabrese *et al.*, 2010).

Several lines of evidence confirm the presence of HO in plants (Shekhawat and Verma, 2010). For example, in *Arabidopsis*, a small family of HOs with four members is classified into two subfamilies. The HO1 subfamily includes HY1, HO3, and HO4, while HO2 is the only member of the HO2 subfamily. It has been shown that *HY1* is clearly the most highly expressed gene, followed by *HO2*, with both *HO3* and *HO4* expressed at low levels (Matsumoto *et al.*, 2004). However, recent research has confirmed that HO2 is not a true HO (Gisk *et al.*, 2010). Besides its participation in light signalling (Muramoto *et al.*, 1999; Gisk *et al.*, 2010; Shekhawat and Verma, 2010), plant HO and its catalytic product CO have been implicated in stomatal aperture regulation (Cao *et al.*, 2007), root development (Xuan *et al.*, 2008; Cao *et al.*, 2011; Xu *et al.*, 2011), and plant tolerance or acclimation to salinity (Xie *et al.*, 2008, 2011*a*, *b*), heavy metal exposure (Han *et al.*, 2008), and UV-B irradiation (Yannarelli *et al.*, 2006). For example, HO-1/CO mediates abscisic acid (ABA)-induced stomatal closure (Cao *et al.*, 2007) and auxin-induced adventitious root development (Xuan *et al.*, 2008). Importantly, the inducible response of HO-1 to the oxidative stress generated by UV-B exposure was first reported in soybean plants (Yannarelli *et al.*, 2006). However, besides its antioxidant behaviour, the detailed roles of UV-induced HO-1 in plants and the underlying signalling mechanisms remain unclear.

In order to investigate the possible interconnection between HO signalling and UV-C response in plants, UV-C-induced phenotypic changes in the seedlings of *hy1-100*/*ho2*/*ho3*/*ho4* single and/or double mutants and seedlings of a *HY1* overexpression line were compared. It was found that only *HY1* mutation (*hy1-100*) leads to UV-C hypersensitivity, consistent with observed decreases in flavonoid and carotenoid contents, and severe oxidative damage. Some contrasting responses, such as the enhancement of the contents of chlorophyll *a*, chlorophyll *b*, and carotenoid, were observed in the *HY1* overexpression line, compared with the wild-type plants. Moreover, the data showed that the application of the CO donor carbon monoxide-releasing molecule-2 (CORM-2; Desmard *et al.*, 2011; García-Arnandis *et al.*, 2011) and BR, two catalytic by-products of HO, could reverse the above responses, including the UV-C hypersensitivity phenotype and the down-regulation of corresponding genes in the carotenoid and flavonoid biosynthesis pathways as well as antioxidant defences, in the *hy1-100* mutant, with

CORM-2 being especially effective . Transcriptional factors involved in the synthesis of flavonoid and UV responses, including *HY5*, *HY5-homologue* (*HYH*), *MYB11*, and *MYB12*, were induced by UV-C but reduced in the *hy1-100* mutant.

Materials and methods

Plant materials, growth conditions, and chemicals

Arabidopsis thaliana hy1-100 (CS236, Col-0), *hy1* (CS67, L*er*), *ho2* (SALK_025840, Col-0), *ho3* (SALK_034321, Col-0), and *ho4* (SALK_044934, Col-0) mutants were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/abrc), and L*er* ecotype seeds were provided by C.Y. Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, China). The homogenous *HY1* overexpression line *35S:HY1-4* was used according to Xie *et al.* (2011*b*). Seeds were surface-sterilized and washed three times with sterile water for 20 min, then cultured in Petri dishes on solid Murashige and Skoog (MS) medium (pH 5.8). Plates containing seeds were kept at 4 °C for 2 d, and then transferred into a growth chamber with a 16/8 h (23/18 °C) day/night regime and 120 μmol m^{-2} s^{-1} irradiation.

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless stated otherwise. The chemicals used for treatments were CORM-2 (0.1 μM), BR (100 μM), and $FeSO_4$ (100 μM) (Cao *et al.*, 2011; Desmard *et al.*, 2011; García-Arnandis *et al.*, 2011; Wu *et al.*, 2011). The concentrations of CORM-2 and BR used in this study were determined in pilot experiments from which maximal induced responses were obtained.

Generation of the ho2/3, ho2/4, *and* ho3/4 *mutants*

Homozygous T-DNA insertion lines for *ho2* (SALK_025840), *ho3* (SALK_034321), and *ho4* (SALK_044934) that prevented accumulation of the full-length corresponding mRNAs had been previously identified (Xie *et al.*, 2011*b*). To generate the *ho2/3*, *ho2/4*, and *ho3/4* double mutants, pollen of either *ho2* or *ho3* was transferred to the stigmas of emasculated flowers of *ho3* and *ho4* mutants. F_1 plants were self-pollinated, and the resulting F_2 individuals were genotyped using PCR primers specific for the presence of the double mutants (Supplementary Table S1 at *JXB* online).

UV-C tolerance assay and phenotype analysis

For UV-C tolerance analysis, 5-day-old seedlings were irradiated with 1.8 (0.5 W m^{-2} for 1 h), 3.6 (0.5 W m^{-2} for 2 h), or 5.4 (0.5 W m^{-2} for 3 h) kJ m^{-2} of UV-C (λ=254 nm) using a HL-2000 HybriLinker UV Crosslinker (UVP, USA), according to Castells *et al.* (2010), with some modifications. Alternatively, *Arabidopsis* seeds were cultured in MS medium with or without 0.1 μM CORM-2, 100 μM BR, and 100 μM Fe^{2+} pre-treatments for 5 d. Afterwards, seedlings were exposed (+) or not (–) to UV-C irradiation. After irradiation, plants were immediately returned to the growth chamber under 120 μmol m^{-2} s^{-1} light irradiation. The phenotypes, including the fresh weight and primary root growth rate (Castells *et al.*, 2010; Xie *et al.*, 2011*b*), were then observed 5 days later.

Pigment analysis

To determine UV-absorbing pigments, seedling shoot or root tissues of each ecotype were ground in liquid nitrogen and extracted in methanol (1 ml per 0.1 g fresh weight). After centrifugation for 10 min at 14 000 *g*, the supernatant was diluted in methanol (1:10), and the absorbance was scanned between 220 nm and 450 nm using a DU 800 UV/VIS spectrophotometer (Beckman Coulter, USA). Flavonoids were extracted (Bieza and Lois, 2001) and quantified following Li *et al.* (1993) and Tossi *et al.* (2011). Chlorophyll *a*/*b* and carotenoids were extracted from leaves using 95% ethanol and quantified as described by Lichtenthaler (1987).

Histochemical staining and determination of the content of thiobarbituric acid-reactive substances (TBARS)

Histochemical detection of lipid peroxidation was performed with Schiff's reagent as described by Han *et al.* (2008). *Arabidopsis* roots stained with Schiff's reagent were washed thoroughly, then observed and photographed under a light microscope (model Stemi 2000-C; Carl Zeiss, Germany). Lipid peroxidation of seedlings was also estimated by measuring the amount of TBARS as previously described (Xie *et al.*, 2008).

Real-time RT-PCR analysis

Total RNA was isolated using Trizol reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Real-time quantitative reverse transcription-PCRs (RT-PCRs) were performed using a Mastercycler® ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) with SYBR® *Premix Ex Taq*™ (TaKaRa Bio Inc., China) according to the manufacturer's instructions. Using specific primers (Supplementary Table. S2 at *JXB* online), the expression levels of the genes are presented as values relative to the corresponding control samples at the indicated times or under the indicated conditions, after normalization to *actin2/7* (accession number NM_121018) transcript levels.

Statistical analysis

Data are shown as the means ±SE (standard error) from at least three independent experimental replications. Both the analysis of variance (ANOVA) and multiple comparison were used in data analysis. In particular, the data of different genotype samples upon UV treatments were analysed using a general linear model for a completely randomized design with UV irradiation levels as one fixed factor. Differences in the phenotypic indicators among the wild-type, mutant, or overexpression lines were analysed by a nested ANOVA, with genotypes nested within UV irradiation treatments [model: phenotypic indicators = replication + treatment + sample (treatment)] or within recuperation times [model: phenotypic indicators = replication + time + sample (time)], followed by a multiple comparison. A threshold of $P < 0.05$ was set as the level of significance in tests. All the above procedures were programmed to run in a SAS version 9.1 environment (SAS Institute, Cary, NC, USA.

Results

UV-C hypersensitivity and HO *transcripts*

A previous study showed the involvement of *Arabidopsis HY1* in salt acclimation (Xie *et al.*, 2011*b*). In this study, to assess the putative connection between UV-C hypersensitivity and *HO*, UV-C-induced phenotypic changes in *hy1-100*, *ho2*, *ho3*, and *ho4* mutants were first analysed. The results shown in Fig. 1A confirmed that the growth of seedlings of the wild type (Col-0) is progressively inhibited with increasing levels of UV-C exposure (Castells *et al.*, 2010). To identify the possible role of each HO in this process, the *HY1*, *HO2*, *HO3*, and *HO4* mRNAs present in the wild type were investigated using quantitative RT-PCR (Fig. 1B). Compared with the UV-C-free condition, the maximal

inducible expression of *HY1* was observed in response to increasing doses of UV-C irradiation, while no inducible response was observed in *HO2*, *HO3*, and *HO4* transcripts when exposed to 1.8 kJ m^{-2} UV-C. Although *HO2*, *HO3*, and *HO4* were moderately induced by 3.6 kJ m^{-2} UV-C, levels of these transcripts then decreased at exposures of 5.4 kJ m^{-2} UV-C.

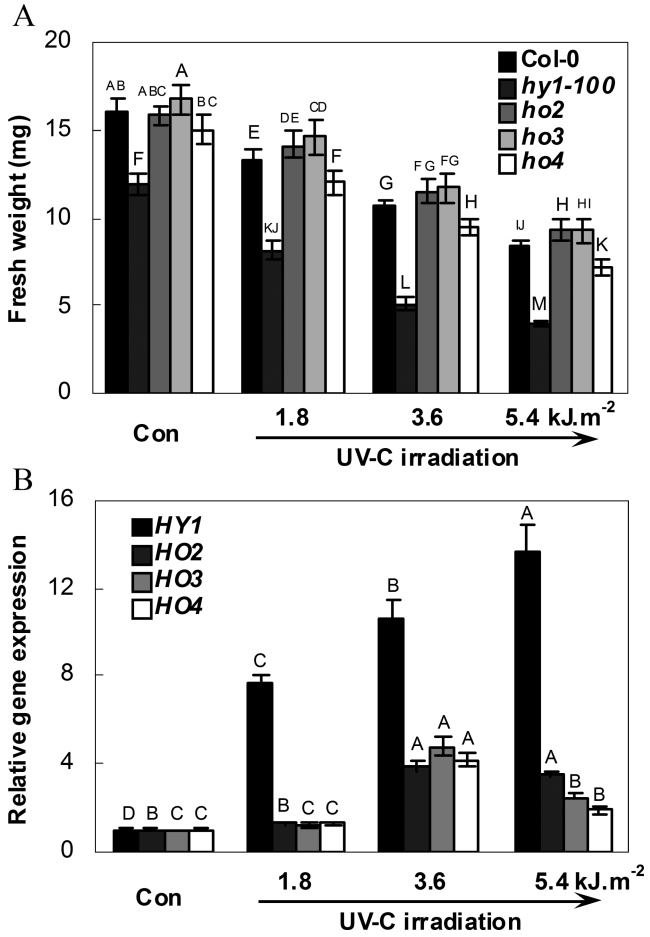

Fig. 1. Seedling growth of the wild-type (Col-0), and *hy1-100*, *ho2*, *ho3*, and *ho4* mutants (A) and expression analysis of four *HO* genes of wild-type roots (B) in response to increasing doses of UV-C irradiation. After exposure to corresponding doses of UV-C, seedlings of each ecotype were transferred to normal growth conditions for 5 d, after which the fresh weight per seedling was evaluated (A). After irradiation with different doses of UV-C, transcript levels of *HY1* (At2g26670), *HO2* (At2g26550), *HO3* (At1g69720), and *HO4* (At1g58300) of wild-type roots were then analysed using real-time PCR (B). The levels of *HO* transcripts are presented relative to the wild type in control conditions (Con, without UV-C irradiation). Data are means ±SE from three independent experiments. (A) Mean values were compared by a multiple comparison on the basis of the nested ANOVA, with genotypes nested within UV irradiation treatments [model: phenotypic indicator=replication+treatment+sample (treatment)]. Mean values with different letters denote a significant difference according to multiple comparison ($P < 0.05$). (B) Bars with different letters were significantly different within the different UV irradiation treatments at $P < 0.05$ according to multiple comparison.

Further observation showed that the fresh weight of the *hy1-100* mutant was 25.78±1.8% less than that of the wild type (Col-0) under normal growth conditions (Con, Fig. 1A). The relative decreases in fresh weight in wild-type and *hy1-100* mutant plants upon UV-C exposure were then compared. The results further showed that fresh weight inhibition (mean ±SE) of *hy1-100* mutant plants was 30.7±4.8, 57.0±3.0 and 66.5±1.3% at exposures of 1.8, 3.6, and 5.4 kJ m^{-2}, respectively, in comparison with 16.7±3.5, 33.3±2.3, and 47.1±1.6% in wild-type plants (original data are shown in Fig. 1A), suggesting that the *hy1-100* mutant was hypersensitive to UV-C. In comparison, no such severe inhibition appeared in the *ho4* mutant. However, similar and/or slightly increased fresh weight was observed in *ho2* and *ho3* mutants, in comparison with the wild-type plants. Further redundancy tests showed that in contrast to the responses of *hy1-100*, the double mutants *ho2/3*, *ho2/4*, and *ho3/4* did not show UV-C hypersensitivity, as revealed by changes in relative primary root growth, compared with the wild type (Fig. 2). These data clearly indicate that loss of HY1 increases sensitivity to UV-C irradiation.

This conclusion was further confirmed by measuring the chlorophyll *a*, chlorophyll *b*, and carotenoid contents after UV-C exposure. The transgenic overexpressing plant line *35S:HY1-4* (Xie *et al.*, 2011*b*) was used as a positive control. In the experiments, in the wild-type UV-C irradiation resulted in dose-dependent losses of chlorophyll *a* and *b*, and reduced carotenoid contents were observed (Fig. 3A). The *hy1-100* mutant exhibited a similar UV-C-hypersensitive tendency, consistent with the observed inhibition of seedling growth (Fig. 1) and changes in relative primary root growth (Fig. 2). Similarly, the *Arabidopsis hy1* mutant (L*er* background) also displayed a UV-C-hypersensitive

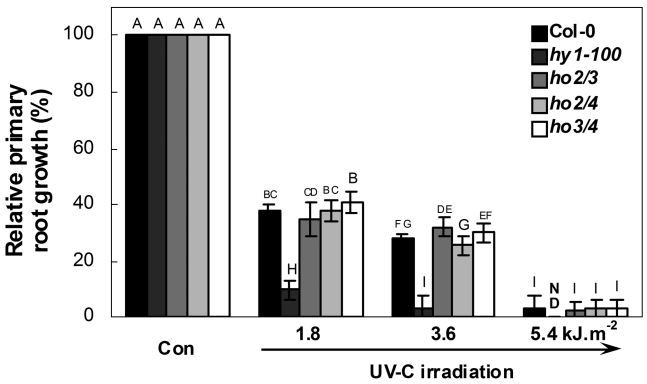

Fig. 2. Redundancy analysis of *HO2*, *HO3*, and *HO4*. After exposure to increasing doses of UV-C and transfer to normal growth conditions for 5 d, the primary root growth rate of each ecotype was measured, taking the growth rate of each ecotype grown in MS medium (Con) as 100%. Data are means ±SE from three independent experiments. Mean values were compared by a multiple comparison on the basis of the nested ANOVA, with genotypes nested within UV irradiation treatments [model: phenotypic indicator=replication+treatment+sample (treatment)]. Mean values with different letters denote a significant difference according to multiple comparison ($P < 0.05$). ND, not detected.

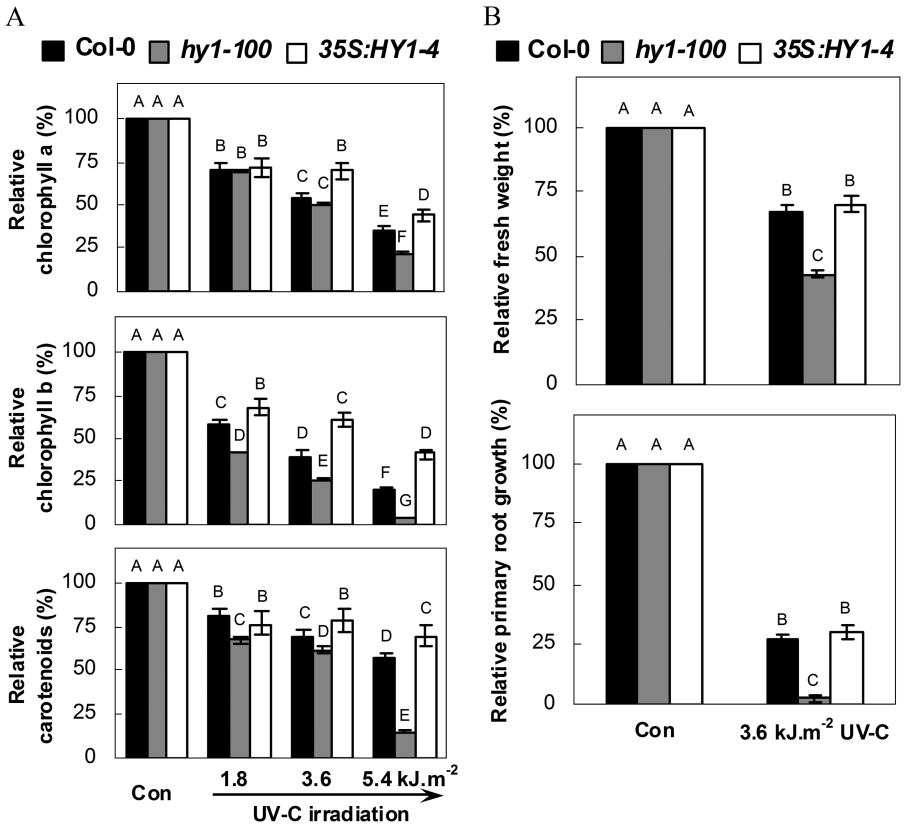

Fig. 3. Effects of UV-C irradiation on relative chlorophyll *a*, chlorophyll *b*, and carotenoid contents (A) and seedling growth (B) of wild-type, *hy1-100* mutant, and *HY1*-overexpressing line *35S:HY1-4* seedlings. After exposure to increasing doses of UV-C, seedlings of each ecotype were transferred to normal growth conditions for 5 d, and measured (n=15). Corresponding seedlings without UV-C irradiation were regarded as a control (Con, 100%). Data are means ±SE from three independent experiments. Mean values were compared by a multiple comparison on the basis of the nested ANOVA, with genotypes nested within UV irradiation treatments [model: phenotypic indicator=replication+treatment+sample (treatment)]. Mean values with different letters denote a significant difference according to multiple comparison ($P < 0.05$).

phenotype (Supplementary Fig. S2 at *JXB* online). Contrasting responses, such as the alleviation of chlorophyll *a*, chlorophyll *b*, and carotenoid loss, were observed in the *HY1* overexpression line. However, no significant differences were observed between the wild-type plants and the overexpressing plant line *35S:HY1-4* regarding changes in the inhibition of seedling growth or relative primary root growth (Fig. 3B). Taken together, these results further confirm that *hy1* mediates *Arabidopsis* UV-C hypersensitivity. Therefore, a moderate dose of UV-C irradiation (3.6 kJ m^{-2}) was used to investigate the role of *hy1* in UV-C hypersensitivity throughout this study.

Changes in carotenoid biosynthetic genes

Results from microarray data showed four representative carotenoid biosynthetic genes, namely *ZDS*, *GPS*, *PSY* (in particular), and *PDS1*, which were induced in wild-type seedling roots during the 24 h period of UV-B irradiation (Supplementary Fig. S3 at *JXB* online). These inducible genes were then compared in order to study further the mechanisms of *hy1*-mediated UV-C hypersensitivity. A similar inducible phenomenon was observed in the wild type after 3 d recuperation following treatment with 3.6 kJ m^{-2} UV-C (Fig. 4). However, the expression of the above four genes was down-regulated in the *hy1-100* mutant during the recovery test compared with the wild type, consistent with the changes in carotenoid contents (Fig. 3A).

Changes in flavonoid metabolism

It is well known that flavonoids can act as sunscreen pigments to reduce the penetration of UV irradiation (Castells *et al.*, 2010). Production of flavonoids from phenylalanine and acetyl-CoA is stimulated by increased UV-C (Filkowski *et al.*, 2004). In the next experiment, it was shown that under normal growth conditions, *hy1-100* extracts from shoot and root tissues (the latter in particular) had significantly lower levels of UV-absorbing compounds in the 220–450 nm range, the UV-A, UV-B, and part of the UV-C wavelength range (Supplementary Fig. S4A at *JXB* online). In particular, the absorbance measurement at 330 nm showed a 53.9% decrease in flavonoid contents in the root tissue of *hy1-100* compared with the wild type (Con; Fig. 5). This finding also perfectly matches the changes in the four representative flavonoid biosynthetic genes, namely *CHS* (in particular), *CHI*, *F3H*, and *FLS* (Supplementary Fig. S4B at *JXB* online). When irradiated,

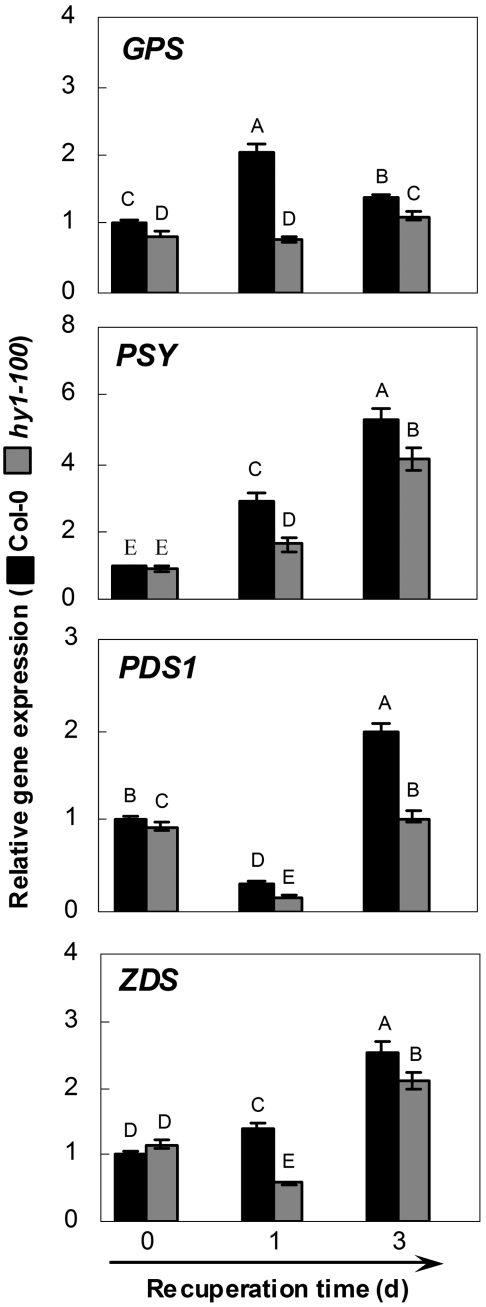

Fig. 4. Effects of UV-C irradiation on the gene expression in wild-type and *hy1-100* mutant seedling roots. The transcript levels of representative carotenoid biosynthetic genes *Geranylgeranyl Pyrophosphate Synthase* (*GPS*; At2g34630), *Phytoene synthase* (*PSY*; At5g17230), *Phytoene Desaturase1* (*PDS1*; At4g14210), and *Zeta-carotene desaturase* (*ZDS*; At3g04870) analysed by real-time PCR. After exposure to 3.6 kJ m^{-2} of UV-C, seedlings of each ecotype were allowed to recuperate under normal growth conditions, and samples were collected at the indicated times. Expression levels of each gene are presented relative to wild-type samples at 0 d. Mean values were compared by a multiple comparison on the basis of the nested ANOVA, with genotypes nested within different treatment times [model: phenotypic indicator=replication+time+sample (time)]. Mean values with different letters denote a significant difference according to multiple comparison ($P < 0.05$).

the decrease of flavonoid in the *hy1-100* mutant, with a reduction to 21.4% compared with the wild type, was greater compared with the *HY1* overexpression line, with 67.9% compared with the wild type.

To determine whether the modulation of *CHS*, and other representative genes of the flavonoid biosynthetic pathway, in *hy1-100* mutant seedling roots could explain these changes in flavonoid contents, quantitative RT-PCR analysis of *CHS*, *CHI*, *F3H*, and *FLS* mRNAs was performed on the same root extracts. This analysis showed that, compared with the wild type, expression of *CHS* is significantly down-regulated in *hy1-100* roots exposed to UV-C irradiation, while *F3H* is unaffected, and *CHI* and *FLS* are moderately decreased (Fig. 6A). Similarly, further analysis revealed that expression of *HY5*, *HYH*, and *MYB11/12*, the genes encoding four transcription factors responsible for the regulation of flavonoid biosynthesis and UV responses, was reduced to 57.3±6.7, 32.5±4.8, 76.3±8.8. and 28.5±2.5%, respectively, compared with the wild type (Fig. 6B). Therefore, the down-regulated expression of early functional and related transcription factor genes in the flavonoid synthetic pathway may result in decreased levels of both kaempferol and quercetin flavonoids, which serve as precursors for the downstream flavonol compounds (Supplementary Fig. S1 at *JXB* online).

Changes in hy1*-mediated oxidative damage and ROS-scavenging enzyme genes*

To determine whether wild-type and *hy1-100* mutant plants differ in terms of oxidative stress induced by UV-C

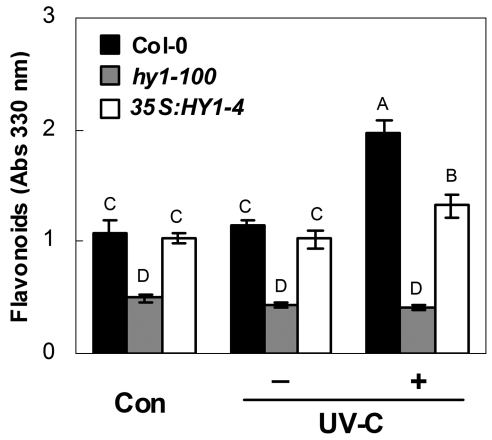

Fig. 5. Effects of 3.6 kJ m^{-2} UV-C irradiation on the flavonoid contents of wild-type, *hy1-100* mutant, and *HY1*-overexpressing line *35S:HY1-4* seedling roots. Five-day-old seedlings were exposed (+) or not (–) to UV-C irradiation. Afterwards, the flavonoid contents were determined. Samples before UV-C irradiation constituted the control (Con). Data are means ±SE from three independent experiments. Mean values were compared by a multiple comparison on the basis of the nested ANOVA, with genotypes nested within UV irradiation treatments [model: phenotypic indicator=replication+treatment+sample (treatment)]. Mean values with different letters denote a significant difference according to multiple comparison ($P < 0.05$).

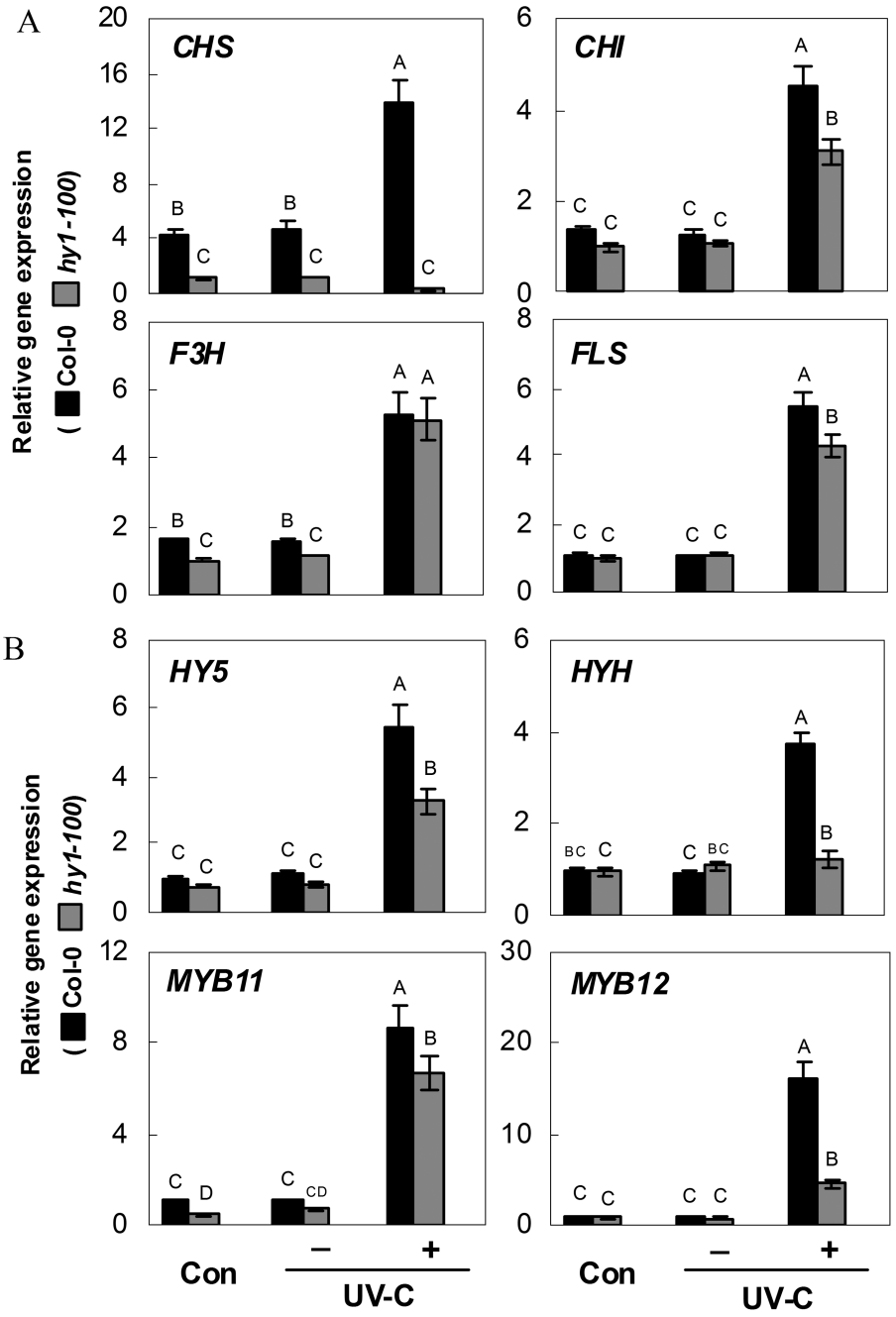

Fig. 6. Effects of 3.6 kJ m^{-2} UV-C irradiation on the expression of genes encoding representative enzymes in the flavonoid biosynthetic pathway (A; *Chalcone Synthase*, *CHS*, At5g13930; *Chalcone Isomerase*, *CHI*, At3g55120; *Flavanone 3-Hydroxylase*, *F3H*, At3g51240; and *Flavonol Synthase*, *FLS*, At5g08640) and related transcription factors (B; *Elongated Hypocotyl5*, *HY5*, At5g11260; *HY5 homologue*, *HYH*, At3g17609; *MYB11*, At3g62610; and *MYB12*, At2g47460) in wild-type and *hy1-100* mutant seedling roots. Five-day-old seedlings were exposed (+) or not (–) to UV-C irradiation. Samples before UV-C irradiation constituted the control (Con). Expression levels of each gene are presented relative to the *hy1-100* mutant under normal growth conditions. Mean values were compared by a multiple comparison on the basis of the nested ANOVA, with genotypes nested within UV irradiation treatments [model: phenotypic indicator=replication+treatment+sample (treatment)]. Mean values with different letters denote a significant difference according to multiple comparison ($P < 0.05$).

irradiation, assessment of lipid peroxidation in root tissues was performed using histochemical staining with Schiff's reagent. As shown in Fig. 7A, lipid peroxidation staining in the wild-type or *hy1-100* plants upon UV-C irradiation was more extensive than that under control conditions. The changes in the amount of TBARS showed that lipid peroxidation in the *hy1-100* mutant increases to a greater extent than that in the wild type in response to UV-C

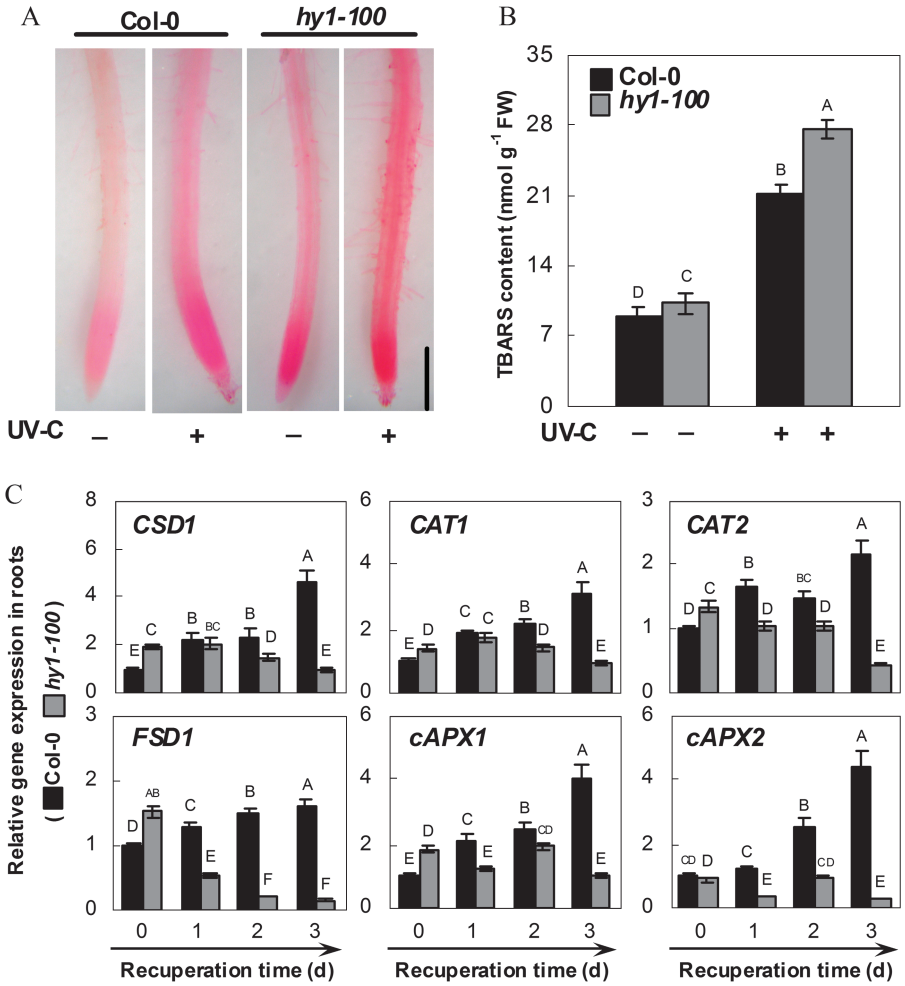

Fig. 7. Effect of 3.6 kJ m^{-2} UV-C irradiation on lipid peroxidation (A), TBARS content (B), and the expression of antioxidant defence genes (C) in the wild-type and the *hy1-100* mutant. Five-day-old seedlings were exposed (+) or not (–) to UV-C irradiation. Seedlings of each ecotype were allowed to recuperate under normal growth conditions for 2 d. Afterwards, the TBARS content was determined (B). After another 1 d, corresponding roots were stained with Schiff's reagent, and immediately photographed under a light microscope. Bar=0.5 mm (A). Transcript levels of *Copper/zinc Superoxide Dismutase* (*CSD1*; At1g08830), *Catalase1* (*CAT1*; At1g20630), *Catalase2* (*CAT2*; At4g35090); *Fe Superoxide Dismutase1* (*FSD1*; At4g25100), *cytosolic Ascorbate Peroxidase1* (*cAPX1*; At1g07890), and *cytosolic Ascorbate Peroxidase2* (*cAPX2*; At3g09640) of root tissues of each ecotype were analysed by real-time PCR at the indicated times (C). Expression levels of each gene are presented relative to wild-type samples at 0 d. Mean values were compared by a multiple comparison on the basis of the nested ANOVA, with genotypes nested within UV irradiation treatments [model: phenotypic indicator=replication+treatment+sample (treatment)] (B) or within UV irradiation treatment times [model: phenotypic indicator=replication+time+sample (time)] (C). Mean values with different letters denote a significant difference according to multiple comparison ($P < 0.05$).

irradiation (Fig. 7B). These results indicate that *hy1-100* plants are less effective in counteracting UV-C-induced lipid peroxidation than wild-type plants.

Because the data indicated a possible link between *hy1-100* and UV-C-triggered lipid peroxidation, the responses of representative antioxidant enzyme genes to UV-C were tested. Time-course analysis showed that compared with the control samples (T0), significant increases in the transcripts of *CSD1*, *CAT1*, *CAT2*, *FSD1*, *cAPX1*, and *cAPX2* were observed in wild-type seedling root (Fig. 7C) and shoot (Supplementary Fig. S5 at *JXB* online) tissues, after 3 d of recuperation. In contrast, compared with the wild type, significant down-regulation was seen in *hy1-100* mutants, all of which was consistent with the histochemical staining and TBARS content results obtained previously (Fig. 7A, B).

Mutation of HY1-induced responses was rescued by CO and BR

If the loss of HY1 function increases sensitivity to UV-C irradiation, feeding *hy1-100* mutant plants with HO by-products might, at least partially, rescue UV-C-induced degradation of chlorophyll as well as the inhibition of primary root growth. As expected, exposure to the CO donor, CORM-2, significantly alleviated the decrease in chlorophyll (Fig. 8A), carotenoid, and flavonoid contents

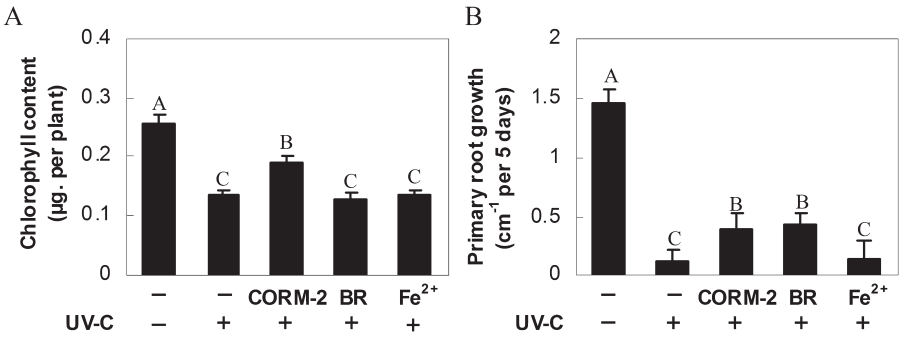

Fig. 8. Effects of carbon monoxide-releasing molecule-2 (CORM-2), bilirubin (BR), and $FeSO_4$ (Fe^{2+}) pre-treatments on *hy1-100* seedling growth under 3.6 kJ m^{-2} UV-C irradiation. *Arabidopsis* seeds were cultured in MS medium with or without 0.1 μM CORM-2, 100 μM BR, and 100 μM Fe^{2+} for 5 d. Afterwards, seedlings were exposed (+) or not (–) to UV-C irradiation. Chlorophyll content (A) and primary root growth rate (B) were then determined after another 5 d growth under normal conditions. Data are means ±SE from three independent experiments. Bars with different letters are significantly different compared with the control sample without UV-C irradiation at the $P < 0.05$ level according to multiple comparison.

(Fig. 9A, B), and the inhibition of primary root growth (Fig. 8B), although no obvious response was found to the addition of Fe^{2+}. Meanwhile, treatment with BR only alleviated the inhibition of primary root growth and the decrease in flavonoid content. After the addition of CORM-2, there were comparative increases in the levels of *GPS* and *PDS1*, and *CHS* and *CHI* mRNA in *hy1-100* seedlings. Meanwhile, only *CHI* transcript was induced in BR-treated samples (Fig. 9B). In contrast, no significant changes were observed in the carotenoid and flavonoid synthetic genes in Fe^{2+}-treated mutant plants subjected to UV-C stress, compared with those stressed but not treated with Fe^{2+}. These changes in synthetic gene expression were consistent with the partial restoration of carotenoid content and synthesis of flavonoids following UV-C irradiation.

Changes in the antioxidant enzyme genes exhibited similar patterns (Fig. 9C). For example, in comparison with the stress alone samples, *CAT1*, *CAT2*, *cAPX1*, and *cAPX2* transcripts were induced significantly in CORM-2-treated mutant roots. BR also brought about increases in *CAT1*, *CAT2*, and *cAPX2* mRNA levels. However, no obvious difference was observed when plants were treated with Fe^{2+} together with UV-C stress.

Discussion

HY1, an indispensable endogenous modulator of plant UV-C tolerance

UV irradiation causes non-specific damage responses in living organisms, including decreased biomass accumulation, acceleration of chlorophyll degradation, and overproduction of ROS (Gao and Zhang, 2008; Gao *et al.*, 2008; He *et al.*, 2008; Castells *et al.*, 2010; Tao *et al.*, 2010). Previous pharmacological tests have shown that HO-1 is involved in soybean defence against the oxidative stress generated by UV-B radiation, and that ROS are involved in the mechanisms inducing HO-1 (Yannarelli *et al.*, 2006; Santa-Cruz *et al.*, 2010). In this study, several consequences of UV-C hypersensitivity were determined (Bashandy *et al.*, 2009; Castells *et al.*, 2010), including inhibition of the increase in fresh weight (Fig. 1) and relative primary root growth (Fig. 2), and the degradation of chlorophyll *a*/*b* (Fig. 3). It was discovered that HY1 plays a central role, acting as an indispensable endogenous modulator of plant UV-C tolerance. This conclusion was supported by genetic and pharmacological evidence. For example, compared with *ho2*/*ho3*/*ho4* mutants, the maximal UV-C-hypersensitive phenotype was observed in *hy1-100* plants (Col-0 background; Fig. 1). The *Arabidopsis hy1* mutant from the L*er* background also displayed a similar hypersensitive phenotype (Supplementary Fig. S2 at *JXB* online). The maximal inducible expression of *HY1* in the wild type increased in a dose-dependent manner with increasing doses of UV-C, compared with *HO2*/*HO3*/*HO4* transcripts. A functional redundancy analysis of *ho2*/*3*, *ho2*/*4*, and *ho3*/*4* double mutants further confirmed that UV-C-induced maximal hypersensitivity was *hy1* specific (Fig. 2). As expected (Cao *et al.*, 2011; Wu *et al.*, 2011), the addition of the CO donors CORM-2 (in particular) and BR, two catalytic by-products of HY1, could partially rescue the hypersensitive phenotype of the *hy1* mutant (Fig. 8). Together, these results indicate that the *Arabidopsis HY1* mutation is required for UV-C hypersensitivity.

A very recent study has provided genetic and pharmacological evidence that *hy1-100* showed maximal sensitivity to salinity and no acclimation response, whereas plants overexpressing *HY1* (*35S:HY1-3*/*4*) showed tolerance characteristics (Xie *et al.*, 2011*b*). However, it should be noted that, except for the alleviation of chlorophyll *a*, chlorophyll *b*, and carotenoid degradation upon exposure to UV-C (Fig. 3A), no visible hyposensitivity phenotype was observed in the *HY1*-overexpressing line *35S:HY1-4* (Fig. 3B). To account for this discrepancy, it is suggested that additional factors may be required for HY1 to function in promoting plant tolerance against UV-C irradiation, and that its signalling mechanism might be different from that for salt tolerance/acclimation. Similar phenomena regarding the

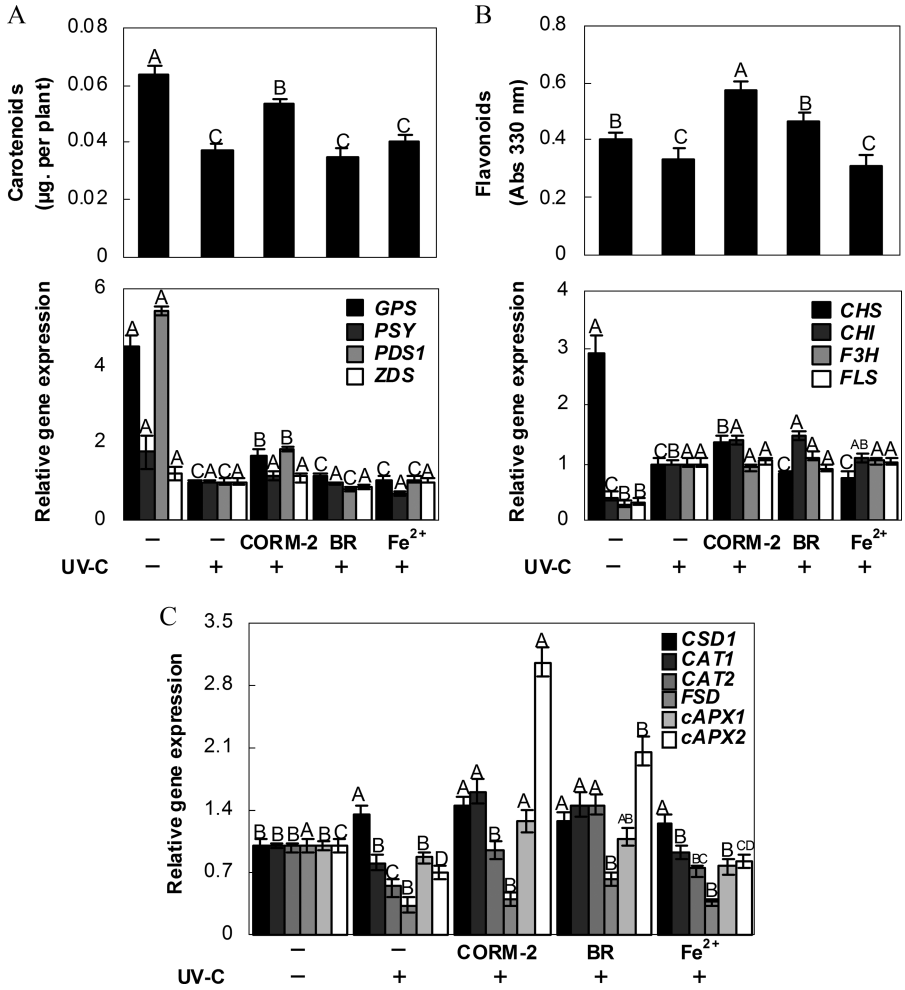

Fig. 9. Effects of CORM-2, BR, and Fe^{2+} pre-treatments on carotenoid and flavonoid metabolism as well as antioxidant enzyme genes in *hy1-100* seedlings exposed to 3.6 kJ m^{-2} UV-C irradiation. *Arabidopsis* seeds were cultured in MS medium with or without 0.1 μM CORM-2, 100 M BR, and 100 μM $FeSO_4$ for 5 d. Afterwards, seedlings were exposed (+) or not (–) to UV-C irradiation. Carotenoid (A) and flavonoid (B) contents and the expression of corresponding biosynthesis genes as well as the representative antioxidant enzyme genes (C) were determined after another 5 d growth under normal conditions. Data are means ±SE from four independent experiments. Bars with different letters are significantly different within the different treatments at the $P < 0.05$ level according to multiple comparison.

lack of a phenotypic change in a gain-of-function mutant compared with the wild type were also reported in a regulatory network involving *YAB3*, *WOX3*, and *KNOX* genes, required for leaf development in rice (Dai *et al.*, 2007). Additionally, the possibility of the fact that the protective action of HO-1 may be restricted to a narrow threshold could not be easily ruled out (Suttner and Dennery, 1999; Bauer *et al.*, 2008).

It was previously reported that *Arabidopsis* HY1 was required for phytochrome chromophore biosynthesis (Muramoto *et al.*, 1999; Davis *et al.*, 2001), and that phytochrome A and light-stable phytochromes appeared to be directly involved in the regulation of specific genes in the carotenoid biosynthetic pathway (von Lintig *et al.*, 1997). Additionally, the *yellow-green-2* mutant of tomato and the *hy1* mutant of *Arabidopsis*, both of which are defective in *HO-1*, display the reduced chlorophyll phenotype as a result of reduced 5-aminolaevulinic acid (ALA) formation, one of the two pivotal control points of tetrapyrrole biosynthesis (Terry and Kendrick, 1999; Davis *et al.*, 2001; Cornah *et al.*, 2003). These results might partially explain why the alleviation or aggravation of chlorophyll *a*, chlorophyll *b*, or carotenoid degradation on exposure to UV-C was observed in the *HY1*-overexpressing line *35S:HY1-4* or *hy1-100* mutant seedlings, respectively (Fig. 3A).

Decreases in carotenoid and flavonoid biosynthesis and antioxidant defences: three major mechanisms responsible for hy1*-induced UV-C hypersensitivity*

The degree of UV-C-induced damage to plants is strongly correlated with the efficiency of their UV-stimulated photo-oxidative protection and repair mechanisms (Bieza and Lois, 2001; Frohnmeyer and Staiger, 2003; Castells *et al.*, 2010). Plants have evolved survival mechanisms to eliminate the overproduction of ROS induced by UV irradiation, including mechanisms to modulate the metabolism of carotenoids and flavonoids, and to activate antioxidant

defences (Li *et al.*, 1993; Gao and Zhang, 2008; Liu *et al.*, 2011; Tossi *et al.*, 2011). In plants, carotenoids are the essential group of pigments responsible for quenching the ROS (singlet oxygen, etc.) induced by exposure to UV radiation (Bartley and Scolnik, 1995). The common carotenoid biosynthetic pathway has been described, and carotenoids serve as precursors in the biosynthesis of vitamin A and ABA (Qin *et al.*, 2007). In this study, it was shown that the decreases in carotenoid content in wild-type, *hy1-100* mutant, and *HY1* overexpression line plants were approximately positively correlated with UV-C dose levels (Fig. 3A). For example, the relative carotenoid content of the *hy1-100* mutant decreased greatly (86.7%) after irradiation with 5.4 kJ m^{-2} UV-C, compared with ~42.3% in wild-type plants. In contrast, in comparison with corresponding wild-type plants, relative carotenoid levels rose notably in *HY1* overexpression line plants at the 3.6 kJ m^{-2} and 5.4 kJ m^{-2} UV-C exposures tested. In addition, four carotenoid biosynthesis genes (Fig. 4) differentially decreased in the *hy1-100* mutant seedling roots 1 d after recuperation. These results, combined with the severe chlorophyll degradation in *hy1-100* exposed to UV-C (Fig. 3A), are consistent with those reported by Moliné *et al.* (2009), who found that enhanced carotenoid biosynthesis in yeast might be an early response to combat UV exposure, and also with a previous study where nuclear-encoded photosynthetic genes were repressed in a bleached carotenoid-deficient mutant (Mayfield and Taylor, 1984). Functional complementation has also confirmed that disruption of *Arabidopsis PDS3* results in albino and dwarf phenotypes by impairing chlorophyll, carotenoid, and gibberellin biosynthesis (Qin *et al.*, 2007). In contrast, pre-treatments with CORM-2, a CO donor, significantly increased *GPS* and *PDS1* transcripts (Fig. 9A) as well as chlorophyll content (Fig. 8A), and weakened the UV-C hypersensitivity as shown by the inhibition of primary root growth in the *hy1-100* mutant (Fig. 8B). No significant responses were observed when Fe^{2+} was added. The above results clearly suggest that the UV-C hypersensitivity of *hy1-100* might be mainly due to the lack of CO production, one of HY1's enzymatic products. Additionally, the possible antioxidant role of BR in the attenuation of UV-C hypersensitivity (Figs 8, 9) cannot be easily ruled out (Noriega *et al.*, 2004; Santa-Cruz *et al.*, 2010).

Flavonoids are considered ubiquitous in plants and affect many facets of their physiology, including UV absorbance, modulation of ROS levels, defence, and hormone transport (Buer *et al.*, 2010). Genetic studies indicate that flavonoid-deficient mutants are susceptible to UV exposure. The cytoprotective functions of flavonoids are unique and are active in protecting against long-term light stress, but do not protect against rapid light intensity fluctuations (Li *et al.*, 1993; Bartley and Scolnik, 1995; Havaux and Kloppstech, 2001). These previous studies suggest that reductions in flavonoid biosynthesis might lead to UV hypersensitivity. In the second experiment in the present study, mutation of *hy1*-induced UV-C hypersensitivity was accompanied by reduced flavonoid contents (Fig. 5) as well a reduction in their biosynthesis (Fig. 6). For example, the expression level of *CHS*, which catalyses the first committed step of flavonoid biosynthesis, was obviously inhibited in the *hy1-100* mutant regardless of UV-C exposure. Similarly, four well-known *CHS*-related transcription factor genes (Stracke *et al.*, 2007), *HY5*, *HYH*, *MYB11*, and *MYB12* (in particular), displayed significantly reduced tendencies in the *hy1-100* mutant upon UV-C treatment, compared with the corresponding wild-type plants (Fig. 6B). In contrast, some of the responses noted above could be blocked by pre-treatment with CORM-2 (in particular) and BR (Fig. 9B). These results are consistent with the partial reversal of hypersensitivity in the *hy1-100* mutant (Fig. 9B), suggesting that the mutation of *Arabidopsis HY1*-induced UV-C hypersensitivity might be due to the down-regulation of flavonoid biosynthesis. A significant, but much weaker reduction in flavonoid content was observed in the *HY1* overexpression line, compared with the wild type (Fig. 5). These results also suggest that besides its fundamental role in acting as an antioxidant enzyme, as previously reported (Noriega *et al.*, 2004), HY1 itself and/or its catalytic products might be involved in flavonoid metabolism (Fig. 9B).

Previous studies reported that the up-regulation of soybean HO-1 served as a cell protection mechanism against UV-induced oxidative damage (Yannarelli *et al.*, 2006). UV irradiation is known to induce ROS generation, resulting in oxidative stress, and UV tolerance is partially correlated with an efficient antioxidant system (Gao and Zhang, 2008; Gao *et al.*, 2008). Previous work also illustrated that the *Arabidopsis* ascorbic acid-deficient mutant *vitamin c-1* (*vtc1*) was more sensitive to supplementary UV-B treatment than wild-type plants, and ascorbic acid was considered an important antioxidant for UV-B radiation (Gao and Zhang, 2008). Subsequently, it was observed that *hy1-100* mutants suffered severe oxidative stress upon UV-C irradiation, compared with the wild type, and this was confirmed by histochemical staining for the detection of lipid peroxidation in root apexes (Fig. 7A) and TBARS

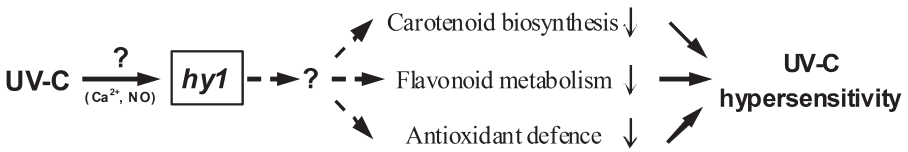

Fig. 10. Schematic representation of the proposed *hy1*-mediated UV-C hypersensitivity signalling pathway. Mutation of *HY1* triggered decreases in carotenoid and flavonoid synthesis, as well as antioxidant defence, further leading to UV-C hypersensitivity. The dashed line and the question mark denote the possible involvement of other undescribed signalling components, such as Ca^{2+} and ROS signals.

content determination (Fig. 7B). This result also suggests that mutation of *Arabidopsis HY1* makes it more vulnerable to UV-C-induced oxidative stress than wild-type plants. Meanwhile, this phenotype was ascribed to decreasing expression levels of transcripts encoding antioxidant enzymes, including *CSD1*, *CAT1/2*, *FSD1*, and *cAPX1/2* (Fig. 7C; Supplementary Fig. S5 at *JXB* online), which were further blocked differentially when CORM-2 (in particular) or BR was added, separately (Fig. 9C). This finding further confirms that HO-1 could confer beneficial cytoprotection against oxidative damage in both animals and plants (Yannarelli *et al.*, 2006; Paine *et al.*, 2010; Santa-Cruz *et al.*, 2010; Kim *et al.*, 2011).

In summary, *hy1-100*, an *Arabidopsis* mutant with a defect in *HY1*, displayed a hypersensitivity to UV-C irradiation compared with wild-type plants, as indicated by: (i) a greater inhibition of seedling growth and primary root growth; (ii) marked degradation of chlorophyll; (iii) lower biosynthesis of carotenoids and flavonoids; (iv) a greater accumulation of lipid peroxidation; and (v) the down-regulation of the antioxidant system. However, the addition of the CO donors CORM-2 (in particular) and BR, two catalytic by-products of HY1, could partially rescue the above responses in the *hy1* mutant. These genetic, molecular, and biochemical results provide the first indication, to our knowledge, that the mutation of HY1-triggered UV-C hypersensitivity is associated with decreases in carotenoid and flavonoid synthesis, as well as antioxidant defences (Fig. 10). A recently published paper has reported a causal relationship between the UV-C tolerance of tobacco plants and Ca^{2+}/H^{+} exchanger-mediated fast cytosolic Ca^{2+} extrusion (within 30 min; Shabala *et al.*, 2011). Meanwhile, ROS production is also known to be critically dependent on cytosolic Ca^{2+} levels (Lecourieux *et al.*, 2002). Although the identification of the interrelationship between the above signalling components and HY1 might be beyond the scope of this work, in view of the fact that no visible hyposensitivity phenotype was observed in the *HY1*-overexpressing line (Fig. 3B), further genetic and molecular investigations will be needed to explore the possible endogenous factors (Ca^{2+} or ROS signalling, etc.) required for HY1 function in promoting plant tolerance to UV-C irradiation.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Scheme of the flavonoid biosynthetic pathway in *Arabidopsis*.

Figure S2. Primary root growth of wild-type (L*er*) and *hy1* mutant seedlings in response to 3.6 kJ m^{-2} UV-C irradiation.

Figure S3. Representation of carotenoid biosynthetic genes in *Arabidopsis* seedling root tissues upon UV-B irradiation.

Figure S4. The *hy1-100* mutant underproduces UV-absorbing compounds.

Figure S5. Effects of 3.6 kJ m^{-2} UV-C on expression of antioxidant defence genes.

Table S1. The sequences of PCR primers for genotyping.

Table S2. The sequences of PCR primers for real-time RT-PCR.

Acknowledgements

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