β-cyclocitral upregulates salicylic acid signalling to enhance excess light acclimation in Arabidopsis

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Abstract

β-cyclocitral (β-CC), a volatile oxidized derivative of β-carotene, can upregulate the expression of defence genes to enhance excess light (EL) acclimation. However, the signalling cascades underlying this process remain unclear. In this study, salicylic acid (SA) is involved in alleviating damage to promote β-CC-enhanced EL acclimation. In early stages of EL illumination, β-CC pretreatment induced SA accumulation and impeded reactive oxygen species (ROS) production in the chloroplast. A comparative analysis of two SA synthesis pathways in Arabidopsis revealed that SA concentration mainly increased via the isochorismate synthase 1 (ICS1)-mediated isochorismate pathway, which depended on essential regulative function of enhanced disease susceptibility 1 (EDS1). Further results showed that, in the process of β-CC-enhanced EL acclimation, nuclear localization of nonexpressor of pathogenesis-related genes 1 (NPR1) was regulated by SA accumulation and NPR1 induced subsequent transcriptional reprogramming of gluthathione-S-transferase 5 (GST5) and GST13 implicated in detoxification. In summary, β-CC-induced SA synthesis contributes to EL acclimation response by decreasing ROS production in the chloroplast, promoting nuclear localization of NPR1, and upregulating GST transcriptional expression. This process is a possible molecular regulative mechanism of β-CC-enhanced EL acclimation.

Key words: Arabidopsis, EDS1, EL acclimation, GST, ICS1, NPR1, ROS, SA, β-CC.

Introduction

As the primary energy source of photosynthesis, light provides signals to regulate diverse mechanisms of plant growth and development, which is essential for plant survival (Chen et al., 2004; Foyer et al., 2012). However, excess light (EL), which is higher than the amount of light needed by plants to undergo photosynthesis, can trigger imbalanced reactive oxygen species (ROS) metabolism in the photosynthetic system (PSI and PSII) and subsequently damage DNA, proteins and lipids. This damage is often manifested as chlorosis, bleaching or bronzing of leaves. (Karpinski et al., 1999; Apel and Hirt, 2004). To avoid damage caused by EL, plants have evolved sophisticated photoprotective mechanisms, including light capture reduction through chloroplast avoidance movements or nastic leaf movements, dissipation of excess absorbed energy through water cycle and energy-dependent non-photochemical quenching (NPQ), and adaptation to light quality through a state transition. (Li et al., 2009; Takahashi and Badger, 2011; Gordon et al., 2012; Kruk and Szymanska,
Among these mechanisms, acclimation response is crucial for plant survival. Acclimation can be controlled by several retrograde signal transduction events to increase the efficiency of defence mechanisms or to develop physiological, biochemical and structural changes to prevent damage to plants; these mechanisms are initiated by EL perception. Extensive research has shown that some models operate these signalling pathways in acclimation to EL, such as SAL1-PAP and oxidative by-products of β-carotene, including β-cyclocitrin (β-CC) and dihydroactinidiolide (Estavillo et al., 2011; Ramel et al., 2012b; Shumbe et al., 2014). β-CC is a volatile oxidized derivative of β-carotene that quenches singlet oxygen (1O2) during EL stress (Ramel et al., 2012a). β-CC containing an α, β-unsaturated carbonyl is identified as a reactive electrophilic species (RES), which may transmit signals and modulate the expression of cell survival genes at low levels (Farmer and Davoine, 2007; Mueller and Berger, 2009). Low β-CC levels also regulate gene transcription; the most pronounced effects have been observed in transcript levels of cell survival- and stress-related genes, which can change protein abundance and activity to enhance EL acclimation (Ramel et al., 2012b, 2013a; Havaux, 2014). Among β-CC-upregulated genes, glutathione-S-transferase (GST) is overrepresented (Ramel et al., 2012b). Constitutive GST overexpression sufficiently enhances 1O2 resistance to light stress in Chlamydomonas reinhardtii (Ledford et al., 2007). The GST enzyme performs different functional roles with the tripeptide glutathione as a co-substrate or co-enzyme. GST can also promote glutathione dependent catalytic functions; as a result, toxic products are eliminated and organic hydroperoxides are reduced to maintain cell survival during biotic and abiotic stress (Dixon et al., 2002). However, the molecular mechanism by which β-CC regulates GST transcription remains unclear.

As an important phytohormone, salicylic acid (SA) is involved in various regulatory signalling pathways, particularly in plant immunity response (An and Mou, 2011). Chemicals and compounds create a signalling network that induces SA accumulation and activates downstream protein to regulate defence gene expression in plant defence systems (Park et al., 2007; Truman et al., 2007; Chaturvedi et al., 2012; Shah and Zeier, 2013). SA is also implicated in systemic acquired acculation and signalling crosstalk between light stress and immune reactions in plants (Szczylnska-Hebda et al., 2010; Karpinski et al., 2013; Trotta et al., 2014).

In plants, two distinct pathways of SA biosynthesis have been identified by classic biochemical studies and mutant-based genetic analysis (Chen et al., 2009). In one pathway, SA is synthesized from cinnamate through the rate-limiting enzymatic reaction catalysed by phenylalanine ammonia-lyase (PAL) (Lee et al., 1995), in the other pathway, SA is produced via an isochorismate synthase (ICS1)-mediated isochorismate pathway related to the process involved in bacterial metabolism (Wildermuth et al., 2001). In SA signalling pathways during the plant immune response, enhanced disease susceptibility 1 (EDS1), a lipase-like protein, plays a critical role in modulation of SA accumulation (Falk et al., 1999; Chandra-Shekara et al., 2004; Xiao et al., 2005). EDS1 is localized in both the cytoplasm and the nucleus, where it forms complexes with other defence co-regulators such as PAD4 and SAG101. A part of the cytoplasmic EDS1 pool is redistributed to the nucleus during pathogen attack, as a result, transcriptional reprogramming of relevant genes (e.g. ICS1, PAD4 and PBS3) of SA biosynthesis and signalling is stimulated (Garcia et al., 2010). Gene transcription reprogramming associated with SA is mainly attributed to the transcription co-activator nonexpressor of pathogenesis-related genes 1 (NPR1) (Mou et al., 2003). NPR1 remains as a stable oligomer in the cytoplasm because of Cys residues that facilitate disulphide bonding between two residues. SA accumulation can also alter cellular redox status; as a consequence, NPR1 disulphide bonds are reduced and the NPR1 monomer is released (Kinkema, et al., 2000). This monomer is then transferred to the nucleus and interacts with TGA transcription factors to activate defence-related gene transcripts (Johnson et al., 2003; Mou et al., 2003). Therefore, this nucleocytoplasmic localization of NPR1 is necessary to establish a successful defence response.

This study aimed to explore a possible molecular mechanism of SA signalling during β-CC-enhanced EL acclimation, including SA biosynthesis, subsequent inhibition of ROS accumulation in the chloroplast, activation of NPR1 translocation and upregulation of GST expression. Our results provide a reference for future studies on β-CC as a signalling intermediate linking the cytoplasm and nucleus during increasing tolerance of Arabidopsis to photooxidative stress under EL; this increase in tolerance is essential for plant growth.

Materials and methods

Plant material and treatments
Seeds of wild-type (WT) (ecotype Columbia) Arabidopsis thaliana, Arabidopsis thaliana NahG pal1-2, npr1, eds1-2, sid2-2 (Wang et al., 2011), ICS1:GUS transgenic Arabidopsis (Hunter et al., 2013) and 35S:NPR1-GFP transgenic Arabidopsis (Kinkema et al., 2000) were sterilized and grown in soil culture with 16/8 h light/dark cycle (120 μmol photons m−2 s−1) and 54% relative humidity at 22°C. Three four-week-old plants were used for experiments.

Injection of the leaf with 100 μl of SA at a concentration of 0.5 mM (Mateo et al., 2006) was achieved by pressing firmly into the lower epidermis with a 1 ml disposable syringe. The treatment of β-CC and EL was performed according to Ramel et al. (2012b). Briefly, the mature Arabidopsis plants were placed for 4 h in a transparent airtight box (~22 l) installed in a growth chamber under controlled conditions of light and temperature (120 μmol photons m−2 s−1, 22°C). In the airtight box, 50 μl of a pure β-CC compound were deposited on a wick of cotton, using distilled water instead for the control condition, with subsequent transfer to an EL growth chamber (1200 μmol photons m−2 s−1, 18°C).

Measurement of chlorophyll fluorescence and content
The maximum operating efficiency of PSII photochemistry (the ratio of variable fluorescence/maximal fluorescence, Fv/Fm) was measured in dark by chlorophyll fluorometry using a PAM fluorometer (Walz GmbH, Effeltrich, Germany), as previously described (Havaux et al., 2005). Chlorophyll was extracted from the leaves by boiling them in 95% ethanol at 80°C. Absorbance was recorded at 664 nm and
648 nm, and total chlorophyll concentration was calculated according to the methods described previously in Chai et al. (2014).

Detection of lipid peroxidation
Malondialdehyde (MDA) is the secondary metabolite of lipid peroxidation that is used as an indicator of lipid peroxidation levels. MDA is measured by the thiobarbituric acid test (Sunkar et al., 2003). Briefly, the leaves with different treatments were ground in 1 mL of chilled reagent [0.25% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid]. After incubation at 90°C for 20 min, the extracts were cooled at room temperature and centrifuged at 12,000 × g for 15 min. The absorbance of the supernatant was measured at 532 nm, subtracting the value for nonspecific absorption at 600 nm using an LS 55 Ultraviolet Spectrophotometer (PerkinElmer, LS55, UK).

Detection of ROS production
The detached Arabidopsis leaves were incubated with H$_2$DCFDA at a final concentration of 5 μM for 1 h in dark. The DCF fluorescence and chloroplast autofluorescence were visualized under the Zeiss LSM 510 (LSM510/ConfoCor2, Carl-Zeiss, Jena, Germany). The 488 nm line of an Ar-ion laser was used, the DCF signal was visualized with excitation at 488 nm and emission at 600–550 nm using a bandpass filter and 408 μm pinhole, and chloroplast autofluorescence (488 nm excitation) was visualized at 650 nm with a long pass filter and 408 μm pinhole. Laser intensity was adjusted to the lowest level that retained a significant signal-to-noise ratio. All images were taken with the 100× oil-immersion objectives on the Zeiss LSM 510. The quantitative analysis of ROS production was performed with fluorescence images by Zeiss Rel 4.2 image processing software and intensity of chloroplast autofluorescence in selected areas of leaves was equal. The average value of DCF fluorescence intensity for each experiment was obtained from five independent samples.

Quantitation of SA
The total SA including conjugate and free SA was extracted according to the method described in Xing et al. (2013). The extracted total SA was analysed by high performance liquid chromatography (HPLC) using fluorescence detectors, with the excitation wavelength at 294 nm and the emission wavelength at 426 nm.

Detection of GUS activity
The ICS1:GUS transgenic Arabidopsis seedling leaves with different treatments were harvested. Leaves were fixed in acetone-H$_2$O (9:1) individually for 20 min at room temperature, then fixed samples were washed in staining buffer (50 mM sodium phosphate buffer pH 7.0, 0.2% Triton X-100, 2 mM potassium ferrocyanide, and 2 mM potassium ferricyanide) three times on ice. Fixed leaves were gently vacuum-infiltrated for 15 min with a staining solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid) and staining buffer. Samples were incubated at 37°C overnight. The indigo stain develops and indicates regions where the GUS reporter gene has been expressed. Stained leaf samples were boiled in 70% ethanol for 5 min to remove chlorophyll and then photographed.

Total RNA isolation and quantitative reverse transcript-PCR (qRT-PCR)
Total RNA was extracted from detached Arabidopsis leaves using the TRIZOL reagent (Invitrogen) according to the specifications of the manufacturer. The concentration and purity of RNA was determined by measuring the optical density (OD) at 260 nm. The RNA samples were reverse transcribed into first-strand cDNA using a PrimeScript RT reagent kit (Takara) and cDNA were used as a template in PCR reactions with gene-specific primers (designed and synthesized by Sangon Biotech, Shanghai). qRT-PCR was performed using the Roche light Cycler™ 2.0 Real-time PCR Detection System. Reactions were performed in triplicate for each sample, and expression levels were normalized against UBQ1, which is identified as the most stable control gene among GAPDH, UBQ1 and ACTIN2 using GeNorm software (Vandesompele et al., 2002). The primers used are listed in Supplementary Table S1.

Enzyme activity assays
Enzyme PAL was extracted from leaf material after treatments according to Xing et al. (2013), and PAL activity was assayed by measuring absorbance at 290 nm because of the release of trans-cinnamate. PAL activity was recorded using an LS 55 Ultraviolet Spectrophotometer (PerkinElmer, LS55, UK) to calculate the production of trans-cinnamate.

Crude protein of enzyme GST was extracted from leaf material after treatments. A sample was ground into a fine powder with liquid nitrogen (IN$_2$) and homogenized in extracts buffer containing 20 mM Tris-Hcl, 1 mM EDTA and 5 mM DTT, (NH)$_4$SO$_4$ was added to the resulting supernatant to 80% saturation and the protein precipitate was recovered by centrifugation (12,000 × g for 20 min) and stored at −80°C. Spectrophotometric assays were used to determine GST-specific activity toward the reaction between 1-chloro-2-dinitrobenzene (CDNB) and glutathione. Activity was determined by measuring changes in absorbance at 340 nm within 3 min using an LS 55 Ultraviolet Spectrophotometer (PerkinElmer, LS55, UK) and calculating the production of CDNB-glutathione compound in each minute and 1 mg sample protein.

Protein concentration was assayed with bovine serum albumin as the standard using the method of Bradford (Chai et al., 2014).

Observation of NPR1-GFP subcellular localization
Three-week-old NPR1-GFP Arabidopsis leaves after different treatments were mounted in distilled water and imaged with Zeiss LSM 510 for guard cell assays (Chai et al., 2014; Pavé et al., 2005). GFP fluorescence was captured following excitation at 488 nm and detection at 505 to 550 nm using a band-pass filter, and chloroplast autofluorescence (488 nm excitation) was visualized at 650 nm with a long-pass filter.

Protein extraction and western blot assay
Nuclear fractionation was performed based on the protocol described by Kinkema et al., (2000). NPR1 gel blots of nucleus and cytoplasm were probed with antibody raised against NPR1 (a gift from Dr Xinnian Dong, Duke University, NC, USA) at a 1:1000 dilution and using anti-histone and anti-β-actin antibodies, respectively, as nuclear and cytosolic markers. The above protein extracts can be stored at −20°C set aside.

Total proteins were extracted from Arabidopsis leaves at the indicated time points after different treatments by grinding in IN$_2$ and resuspending the powder in extraction buffer [50 mM Tris-HCl, pH 6.8, 10% glycerol, 4% (w/v) SDS, 50 mM DTT, 1% (w/v) PVPP and a protease inhibitor cocktail]. The extract was boiled for 10 min and then centrifuged at 10,000 rpm for 20 min to remove cell debris and other impurities. Western blot was analysed with primary antibodies: anti-EDSI (a gift from Dr Jane E. Parker, Max-Planck Institute, Germany) and anti-β-actin. Then antibody-bound proteins were detected using a horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody by an Odyssey two-colour infrared imaging system (Li-Cor, Inc., Lincoln, NE, USA).

Results
SA is involved in β-CC-enhanced EL acclimation in Arabidopsis
When exposed to EL, β-CC enhances the tolerance of Arabidopsis to photooxidative stress by preserving PSIi
photochemical efficiency and by reducing chlorophyll bleaching (Ramel et al., 2012b). To investigate the involvement of the β-CC signalling pathway in EL acclimation, plants were exposed to β-CC for 4 h in an airtight box and subsequently to EL (1200 μmol photons m⁻² s⁻¹, 18°C) stress conditions. After the plants were illuminated for 36 h, the WT leaves turned bronze and wilted, by contrast, β-CC-pretreated leaves showed minute bronze lesions (Fig. 1A). The same bronze lesions were observed in SA-deficient NahG transgenic Arabidopsis that constitutively express the bacterial salicylate hydroxylase and convert SA to catechol, thus, low SA levels were detected in leaves. And damage in β-CC-treated NahG leaves was not significantly alleviated (Fig. 1A). SA-pretreated WT leaves displayed less developed bronze lesions than those in WT leaves. This process became more significant because of β-CC (Fig. 1A). In addition, maximum operating efficiency of PSII photochemistry (Fv/Fm) and chlorophyll content were higher in β-CC-pretreated WT leaves but not in NahG transgenic leaves than those in untreated WT plants; this high level was more evident in β-CC- and SA-treated WT when these plants were exposed to EL illumination (Fig. 1B, C). MDA content, secondary metabolites and the index of lipid peroxidation in β-CC- or SA-pretreated WT leaves were lower than those in untreated WT and NahG transgenic leaves in response to EL (Fig. 1D). These results showed that NahG transgenic Arabidopsis and WT plants were more susceptible to EL than β-CC-treated WT plants. Therefore, β-CC can enhance EL tolerance, and SA is implicated in this process.

**SA inhibits ROS accumulation in the chloroplast in β-CC-enhanced EL acclimation**

ROS burst in chloroplasts in the early phase of EL illumination is the original mechanism that reduces photochemical efficiency and bleaches chlorophyll during leaf tissue damage (Apel and Hirt 2004). We performed LSM to detect ROS localization based on high DCF fluorescence produced from the non-fluorescent compound H₂DCFDA in the presence of ROS in leaf tissue. This procedure was also conducted to confirm whether β-CC depends on SA to inhibit ROS accumulation in the chloroplast in response to EL. In EL illumination, bright green fluorescence was observed in chloroplasts in WT leaves (Fig. 2A); a similar DCF fluorescence signal was detected in NahG leaves (Fig. 2B). However, the fluorescence signal was reduced in WT leaves that were treated with β-CC alone or SA; this reduction was more significant in β-CC- and SA-pretreated leaves (Fig. 2A, C). Fluorescence signals were negligible in untreated plants (control) and β-CC-treated leaves (Fig 2A–C). These results suggested that SA reduced...
ROS production in the chloroplasts in β-CC-enhanced EL illumination. ROS production was also investigated at the population levels in the early phase of EL illumination. The DCF fluorescence intensity of different areas selected in the leaves was statistically analysed to assess the difference in ROS levels between populations (Fig. 2D). EL challenge caused an evident increase in ROS generation, which peaked at 12 h and then declined slightly without returning to the baseline level in the entire assessment period (Fig. 2D). At each time point of EL illumination, ROS production in NahG transgenic leaves was similar to that in WT leaves. However, the ROS levels in β-CC- or SA-treated WT leaves were lower than that in WT leaves; more significant low ROS levels were observed in SA- and β-CC-treated WT leaves (Fig. 2B). These results indicated that β-CC depended on SA to reduce ROS production in the early phase of EL illumination. Therefore, β-CC inhibited ROS accumulation in chloroplasts when plants were challenged by EL; indeed, SA is essential for this process.

β-CC upregulates SA concentration in EL acclimation

SA participated in β-CC-induced EL acclimation. To confirm whether β-CC can regulate SA, SA concentration was examined in WT plants with or without β-CC treatment in the early phase of EL illumination (Fig. 3). Using HPLC, it was revealed that the total SA concentration increased after the plants were subjected to EL illumination. A 2-fold increase in SA content in β-CC-treated leaves was observed at 6 h of EL exposure, and the highest content exhibited a 5-fold increase at 12 h EL exposure (Fig. 3). Compared with β-CC-treated leaves, WT leaves demonstrated a slight increase at 6 h of EL exposure, and the SA content of WT leaves was lower than that of β-CC-treated leaves at each time point (Fig. 3). These data confirmed that β-CC can increase SA concentration under EL illumination.

SA biosynthesis mainly depends on ICS1-mediated isochorismate pathway

Two SA synthesis pathways have been reported in plants: one pathway is regulated by PAL and the other is regulated by ICS1 (Chen et al., 2009). We previously observed that the SA concentration in β-CC-treated Arabidopsis plants increased within 6 h and peaked at 12 h exposure to EL (Fig. 3). Therefore, to investigate the mechanism by which β-CC-induced SA is synthesized in Arabidopsis under EL, SA levels were measured using SA induction deficient 2 (sid2-2) mutants and pal1-2 mutants, which have a defect in ICS1 and PAL, respectively. The total SA levels under EL illumination were significantly reduced in sid2-2 mutants treated with β-CC compared with WT plants. By contrast, no evident differences were observed between pal1-2 and WT plants with various treatments (Fig. 4A); these results indicated that β-CC-induced SA synthesis occurred through a pathway involving ICS1. To obtain insights into the regulation of β-CC in SA synthesis, ICS1 and PAL expression were examined. ICS1 transcription increased in the leaves treated with β-CC only or subjected to direct EL illumination and increased more significantly in the leaves both treated with β-CC and subjected to EL. By comparison, PAL transcript levels slightly increased (Fig. 4B), suggesting that β-CC mainly induced ICS1 accumulation to upregulate SA biosynthesis. Transgenic plants harbouring ICS1:β-glucuronidase (GUS) gene fusion constructs were treated with β-CC and EL. GUS activity increased more in ICS1:GUS-transgenic leaves treated with β-CC and EL compared with that in the leaves with either β-CC treatment alone or subjected to direct EL illumination (Fig. 4C). Thus, β-CC could promote ICS1 expression to accumulate SA; however, PAL may not participate in this process. To determine whether PAL regulates β-CC-induced SA biosynthesis, we analysed PAL enzyme activity. The results showed no significant changes after WT leaves treated or untreated with β-CC were subjected to EL illumination (Fig. 4D). Therefore, the ICS1-mediated isochorismate pathway is mainly responsible for SA biosynthesis in response to β-CC-enhanced EL acclimation.

EDS1 plays a significant role in mediating β-CC-induced SA synthesis under EL

As a regulator of SA, EDS1 is involved in plant resistance responses (Falk et al., 1999). The phenotype and other related parameters of WT and EDS1-less Arabidopsis mutant (eds1-2) plants were investigated to determine whether EDS1 is involved in β-CC-enhanced EL acclimation. Poor survival was observed in eds1-2 leaves with or without β-CC treatment compared with that in β-CC-pretreated WT when plants were subjected to EL illumination (Fig. 5A). Fv/Fm and chlorophyll content were lower in eds1-2 mutants than those in β-CC-pretreated WT plants; and Fv/Fm and chlorophyll content in eds1-2 mutants were similar to those in WT leaves under EL illumination (Fig. 5B, C). The MDA content in eds1-2 plants did not differ from that of WT leaves (Fig. 5D). This finding is consistent with the phenotype shown in Fig. 5A. Additionally, western blot analysis revealed that EDS1 was greatly increased after the plants were exposed to EL illumination and then peaked at 12 h. EDS1 concentration in WT plants was lower than that in β-CC-treated plants at each time point of EL illumination (Supplementary Fig. S1A, B). These results suggested that EDS1 participated in β-CC-enhanced EL acclimation.

To verify functions of EDS1 on SA synthesis, previously described eds1-2 mutants and WT plants were used to measure total SA concentration. Lower total SA levels were observed in eds1-2 leaves with or without β-CC treatment than that in WT leaves when plants were subjected to EL illumination (Fig. 5E). The transcript levels of ICS1, which is the main enzyme regulating β-CC-induced SA synthesis, were determined through qRT-PCR. Consistent with the total SA concentration, ICS1 expression levels were evidently reduced in eds1-2 mutants with various treatments (Fig. 5F). Therefore, EDS1 is involved in SA accumulation by regulating the transcript expression of ICS1 in response to β-CC-induced EL acclimation.
Fig. 2. Effects of β-CC on ROS production during the early phase of EL illumination. The localization of ROS production was observed in (A) WT leaves, (B) NahG transgenic leaves and (C) exogenous SA-pretreated WT leaves. The detached leaves were stained with 5 μm H2DCFDA for 1 h in dark. Samples were observed by LSM. DCF and chlorophyll fluorescence images were recorded, and fluorescence intensity of DCF and chlorophyll were measured. Bars, 10 μm. (D) DCF fluorescence intensity in WT, NahG transgenic and exogenous SA-pretreated WT leaves. Quantitative analysis of ROS production was measured using Zeiss Rel3.2 image processing software (Zeiss, Germany). a.u., arbitrary units. The detached leaves were treated as follows: control, 50 μl distilled water in airtight box for 4 h; β-CC, 50 ul poured into an airtight box for 4 h; EL, 1200 μmol photons m⁻² s⁻¹ for 12 h; β-CC+EL, EL illumination for 12 h after β-CC treatment. Asterisks (Asterisks (*) indicate a significant difference from distilled water-treated WT leaves at *P<0.05 or **P<0.01 (Student’s t test). Data represent means ±SD for five independent experiments and each experiment was performed on five leaves. (This figure is available in colour at JXB online.)
SA plays an important role in β-cyclocitrinal-enhanced EL acclimation

β-CC regulates the nuclear localization of NPR1 through SA under EL

SA accumulation induces the translocation of NPR1 to regulate defence gene expression in plant systemic acquired resistance of plants (Kinkema et al., 2000; Tada et al., 2008). In this study, NPR1-GFP fluorescence appeared in the nucleus of stomatal guard cells in the leaves of 35S::NPR1-GFP plants treated with β-CC or EL. Stronger fluorescence was detected in the nucleus of leaves pretreated with β-CC and subjected to EL illumination; fluorescence was primarily detected in the cytoplasm without any treatments by Zeiss LSM 510 (Fig. 6A). This result suggested that NPR1 could transfer from cytoplasm to nucleus during β-CC-enhanced EL acclimation. Western blot analysis was also performed to evaluate the subcellular localization of NPR1. A high increase in nuclear NPR1 and an evident decrease in cytoplasmic NPR1 were observed in β-CC-treated WT after these plants were subjected to EL illumination; by contrast, a slight increase in nuclear NPR1 and a week decrease in cytoplasmic NPR1 were found in leaves individually treated with β-CC or EL (Fig. 6B). These results illustrated that β-CC induced NPR1

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**Fig. 3.** β-CC upregulates total SA concentration following EL illumination in WT plants. WT plants were pretreated by β-CC or distilled water and then transferred to EL exposure. The samples were collected at indicated time point under EL. The SA concentration was examined by HPLC with a fluorescence detector: 296 nm excitation and 426 nm emission. Asterisks (*) indicate a significant difference from distilled water-treated WT leaves at *P*<0.05 or **P*<0.01 (Student’s t test). Data represent means ±SD for three independent experiments.

**Fig. 4.** Biosynthesis pathways of β-CC-induced SA in response to EL. (A) Total SA concentration was examined in WT, sid2-2 and pal1-2 leaves. (B) Transcript levels of ICS1 and PAL were examined in WT plants. (C) ICS1:GUS transgenic Arabidopsis plants were stained with X-Glu for GUS activity. Non-treated WT and 35S:GUS plants are included as controls. (D) The PAL activity was detected in WT plants with or without β-CC treatment during EL illumination. SA concentration and total RNA were extracted and ICS1:GUS was stained from the leaves of full-grown Arabidopsis after the following treatments: control, 50 μl distilled water in an airtight box for 4 h; β-CC, 50 μl poured into an airtight box for 4 h: EL, 1200 μmol photons m⁻² s⁻¹ for 12 h; β-CC+EL, EL illumination for 12 h after β-CC treatment. Asterisks (*) indicate a significant difference from distilled water-treated WT leaves at *P*<0.05 or **P*<0.01 (Student’s t test). Data represent means ±SD for three independent experiments. (This figure is available in colour at JXB online.)
to transfer from the cytoplasm to the nucleus in response to EL. It has already been demonstrated that β-CC promoted SA accumulation in response to EL acclimation (Fig. 3). To further determine whether cellular distribution of NPR1 is affected by SA accumulation, NPR1 concentration was measured in the nucleus and the cytoplasm of NahG transgenic plants and exogenous SA-pretreated WT plants. Western blot results showed that NPR1 concentration did not evidently change between the nucleus and the cytoplasm of NahG transgenic leaves treated with or without β-CC, when these plants were subjected to EL (Fig. 6C). By contrast, NPR1 concentration evidently increased in the nucleus and decreased in the cytoplasm of exogenous SA-pretreated leaves with β-CC treatment or EL illumination alone; and this result was more significant in SA pretreated levels treated with β-CC and subjected to EL (Fig. 6D). Together, these data implied that SA can promote the nuclear localization of NPR1 in β-CC enhanced EL acclimation.

NPR1 is responsible for GST expression during β-CC-induced EL acclimation

Among β-CC-unregulated defence genes, GST encodes an important detoxification enzyme to promote plant survival (Dixon et al., 2002). GST5 and GST13 are $^{18}$O$_2$-responsive genes under EL (Ramel et al., 2012b). Analysis of expression levels revealed that those of GST5 and GST13 increased in

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**Fig. 5.** EDS1 is indispensable for β-CC-induced SA under EL exposure. (A) The phenotypes of detached leaves observed in WT plants and eds1-2 mutant plants exposed to EL at 0 h and 36 h after β-CC treatment or not. (B) Maximum operating efficiency of PSII photochemistry, (C) chlorophyll content and (D) MDA content were examined in leaves at the times indicated. (E) Total SA content and (F) ICS1 expression in eds1-2 and WT plants after the following treatments: control, 50 μl distilled water in airtight box for 4 h; β-CC, 50 μl pour in an airtight box for 4 h; EL, 1200 μmol photons m$^{-2}$ s$^{-1}$ for 12 h; β-CC+EL, EL illumination for 12 h after β-CC treatment. Asterisks (*) indicate a significant difference from distilled water-treated WT leaves at *$P$<0.05 or **$P$<0.01 (Student's t test). Data represent means ±SD of three independent experiments. (This figure is available in colour at JXB online.)
SA plays an important role in β-cyclocitrinal-enhanced EL acclimation

WT plants exposed to EL illumination; the increased expression levels of *GST5* and *GST13* were more evident in WT plants treated with β-CC (Fig. 7A, B). Conversely, such a reinforcing effect was ruled out in NahG transgenic plants and amplified in exogenous SA-treated WTs (Fig. 7A, B); thus, β-CC could promote *GST5* and *GST13* transcripts in response

![Image](image-url)
subjected to EL (Fig. 7C). The upregulation of GST activity exhibited the same pattern as the induction of GST5 and GST13 transcripts in NahG transgenic plants and exogenous SA-pretreated plants (Fig. 7C). These data demonstrated that gene transcription and activity of GST were upregulated during β-CC-enhanced EL acclimation.

The previous studies have demonstrated that SA can induce NPR1 accumulation in nucleus (Fig. 6). As a transcription coactivator, NPR1 can modulate defence gene transcription (Mou et al., 2003; Wang et al., 2006). So it was speculated whether elevated GST expression and GST activity depended on NPR1 function when plants were exposed to EL after these plants were treated with β-CC. GST5 and GST13 transcript levels and GST activity were measured in npr1 mutants with β-CC treatment and EL illumination. The upregulated GST5 and GST13 transcript levels and GST activity were both clearly suppressed in npr1 under various treatments (Fig. 7A–C). Taken together, these results confirmed that β-CC regulated NPR1 to promote GST transcription and subsequently increase GST activity in response to EL acclimation.

**Discussion**

Although studies have focused on the mechanisms of EL acclimation in *Arabidopsis*, the specific mechanism by which β-CC increases EL tolerance is unknown. β-CC is a RES with strong biological effects, such as induction of defence genes, activation of detoxification responses and growth inhibition (Mueller and Berger, 2009). In general, β-CC may modify highly sensitive proteins and alters activity or function of these proteins because RES exhibits lipophilicity and thiol-reactivity; as a result, cellular toxic and mutagenicity may occur (Farmer and Davoine, 2007). However, low β-CC levels may modulate expression of cell survival-related genes and may contribute to survival during EL acclimation (Ramel et al., 2012b). Phytohormones are also implicated in the complex retrograde signalling of light acclimation. SA is an important phytohormone that improves plant resistance in various biotic and abiotic stresses. Our study showed that β-CC regulated SA signalling to enhance EL acclimation.

β-CC is dependent on SA to enhance EL acclimation

SA functions as a signalling molecule that regulates multiple physiological processes, including plant growth, development, senescence and death, particularly in the induction of defence mechanisms (Lee et al., 1995; Chen et al., 2009; An and Mou 2011). SA is also involved in signalling of abiotic stresses, such as heat stress, low temperature, or ozone and EL exposure. In these signalling pathways, SA regulates the redox balance and protects plants from oxidative damage, thereby enhancing stress acclimation (Sharma et al., 1996; Larkindale and Knight, 2002; Scott et al., 2004). Our research found that β-CC-enhanced EL acclimation did not occur in SA-deficient NahG transgenic plants but became more evident in SA-pretreated WT plants; the result indicated that SA participated in this process (Fig. 1). It has been demonstrated

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**Fig. 7.** GST5 and GST13 expression and GST activity in response to β-CC enhanced EL acclimation. Expression of (A) GST5 and (B) GST13 and (C) GST activity were measured in WT, NahG, exogenous SA-pretreated WT and npr1 plants. Total RNA and enzymes were extracted from leaves after the following treatments: control, 50 μl distilled water in airtight box for 4h; β-CC, 50 μl poured into an airtight box for 4h; EL, 1200 μmol photons m m⁻² s⁻¹ for 12h; β-CC+EL, EL illumination for 12 after β-CC treatment. Asterisks (*) indicate a significant difference from distilled water-treated WT leaves at P<0.05 or **P<0.01 (Student’s t test). Data represent means ± SD for three independent experiments.
that the effect of SA depends on numerous factors, such as the degree of stress and SA levels of plants (Ogawa et al., 2007). We found that the total SA levels in β-CC-pretreated leaves significantly increased until 12 h in the early phase of EL illumination when lethal damage did not occur in plants (Fig. 3). These results confirmed that β-CC induced SA accumulation to enhance EL acclimation.

SA signalling inhibits ROS production in β-CC-enhanced EL acclimation

The excess amount of absorbed light energy cannot be used for photosynthesis when plants are exposed to EL; as a result, ROS, such as hydrogen peroxide and $^{1}$O$_{2}$, are generated in chloroplasts (Karpinski et al., 1999; Apel and Hirt, 2004). Plants have evolved sophisticated photoprotective mechanisms to scavenge these ROS or to repair the damaged cell components, such as energy-dependent NPQ, to repair antioxidant systems and to regulate the fate of retrograde signalling (Havaux et al., 2005; Gordon et al., 2012; Kruk et al., 2012). In retrograde signalling, constitutive SA accumulation in plant cells can enhance catalase, Cu/Zn superoxide dismutase and glutathione reductase activities to affect glutathione and H$_{2}$O$_{2}$ pool size and maintain cellular redox status (Mateo et al., 2006; Ogawa et al., 2007). This provides evidence for the indirect role of SA in scavenging ROS of EL acclimation-associated processes. Our results also confirmed that SA is essential for the process in which β-CC decreased ROS production in chloroplasts under EL (Fig. 2). Increased SA signalling may also activate antioxidant enzymes to decrease ROS production in response to β-CC-enhanced EL acclimation.

ICS1 contributes to SA accumulation dependent on EDS1 in β-CC-enhanced EL acclimation

The role of the SA signalling pathway in the induction of defence mechanisms in plants has been extensively investigated. SA is derived from two distinct enzymatic reaction pathways: the PAL-mediated phenylalanine pathway and the ICS1-mediated isochorismate pathway (Lee et al., 1995; Wildermuth et al., 2001; Chen et al., 2009). Our results showed that ICS1 contributed to SA biosynthesis (Fig. 4A). ICS1 is implicated in the regulation of optimal photosynthesis and chloroplast-to-nucleus retrograde signalling responding to light acclimation and defence immune responses (Mateo et al., 2006; Gawroński et al., 2013). SA accumulation is accompanied by an increase in ICSI expression (Wildermuth et al., 2001; Ogawa et al., 2007). ICSI expression was significantly increased during the peak of SA accumulation in our study (Fig. 3B, C). SA is also synthesized via the phenylalanine pathway in response to biotic and abiotic stresses (Ogawa, et al., 2005; Xing et al., 2013). Although a slight increase in the amount of PAL transcripts was observed in our study (Fig. 3B), PAL activity remained unchanged in leaves treated with β-CC during EL illumination (Fig. 3D); indeed, PAL did not affect SA synthesis in β-CC-enhanced EL acclimation. Nevertheless, the function of PAL may affect SA accumulation during SA-promoted cell death under severe stress (Lee et al., 1995; Xing et al., 2013).

The immune regulator EDS1 is a critical positive regulator of plant immunity; EDS1 is localized in the cytoplasm and the nucleus where complexes with other defence coregulators (PAD4 or SAG101) are formed (Fey et al., 2001, 2005). In our study, eds1-2 mutants exhibited poor survival in response to β-CC-enhanced EL acclimation (Fig. 5A–D); this result indicated that EDS1 was involved in this process. As pathogens attack, the cytoplasmic and nuclear EDS1 pools can be redistributed and achieve balance. The coordinated nuclear and cytoplasmic activities of EDS1 are considered as an appropriately balanced immune response. In the nucleus, EDS1 may function as a transcription co-activator that interacts with transcription factors (e.g. WRKY) to regulate gene transcripts (García et al., 2010). In our study, β-CC-induced SA accumulation through ICSI was dependent on EDS1 regulation (Fig. 5E, F). Therefore, β-CC may maintain the balance between the cytoplasmic and nuclear EDS1 pools. EDS1 in the nucleus may also interact with other transcriptional regulators to reprogram ICSI expression, thereby promoting SA synthesis in response to EL acclimation.

SA-mediated nuclear accumulation of NPR1 promotes GST expression in response to β-CC-enhanced EL acclimation

NPR1 is involved in transcription reprogramming associated with SA-dependent immune responses (Wang et al., 2006). NPR1 is maintained in the cytoplasm as an oligomer formed via intermolecular disulfide bonds. Oligomeric NPR1 is depolymerized to a monomeric form; SA accumulation induces this monomeric form to translocate across the nuclear membrane to the nucleus (Mou et al., 2003). The subcellular localization of NPR1 was analysed by detecting GFP fluorescence in NPR1-GFP plants to understand the function of β-CC-induced SA response to EL. Figure 6A and Supplementary Fig. S2 show that the enhanced nuclear NPR1-GFP fluorescence was the strongest in the guard cells of β-CC-treated leaves exposed to EL illumination; thus, β-CC can promote NPR1 accumulation in the nucleus. Subcellular fractionation analyses further confirmed that β-CC-induced nuclear localization of NPR1 is dependent on SA levels during EL illumination (Fig. 6B–D).

Ramel et al. (2012b) demonstrated that β-CC-induced genes can respond to oxidative stress and participate in cellular sensing, hormone signalling and detoxification mechanisms. In these genes, GST functions in detoxification processes to enhance plant survival (Edwards et al., 2000). GST overexpression sufficiently enhances $^{1}$O$_{2}$ resistance under EL and promotes acclimation to alter the light environment in Chlamydomonas reinhardtii (Ledford et al., 2007). In our study, β-CC could increase GST5 and GST13 expression and subsequently enhanced GST activity in response to EL acclimation, which was regulated by SA (Fig. 7A–C). This result suggested that SA could increase GST expression. In plant immunity, NPR1 accumulated in the nucleus physically interacts with TGA transcription factors to form
A transcription factor-activating complex (Boyle et al., 2009). The transcription factor class-II TGA is responsible for the transcription of RES-induced defence genes, including GST (Mueller et al. 2008). We also observed that the upregulated expression of GST5 and GST13 and the increased activity of GST were abolished in npr1 mutants (Fig. 7A–C). Therefore, the interaction of NPR1 with class-II TGA transcription factors in the nucleus regulates GST expression.

SA may interact with other phytohormones to regulate β-CC-enhanced EL acclimation

Phytohormones are core messengers that participate in light acclimation and coordinate system signalling. In addition to SA, jasmonic acid (JA) is also involved in light stress response (Danon et al., 2005; Alsharafa et al., 2015). Compounds related to JA biosynthesis are upregulated during EL stress, however, most of them are suppressed during acclimation (Ramel et al., 2013b; Alsharafa et al., 2015). β-CC-activated genes, such as LOX2 and OPR1, participate in JA biosynthesis (Ramel et al., 2012b), this finding indicates JA may respond to β-CC. However, JA and SA signalling pathways function antagonistically in plant responses to severe biotic and abiotic stresses; these pathways also coordinate with ROS in plant immunity and cell death (Tsuda et al., 2009). Furthermore, JA promotes O2−-mediated cell death under EL, but the JA precursor 12-oxophytodienoic acid (OPDA/dnOPDA) can antagonize JA-promoted cell death (Danon et al., 2005; Ramel et al., 2013b). OPDA is synthesized from α-linolenic acid in chloroplasts and exert a signalling function independent of JA regulation when plants are exposed to EL stress (Stintzi et al., 2001; Danon et al., 2005). Therefore, JA affects cell death under EL stress and light acclimation response, which may be dependent on the degree of plants stress. The crosstalk between SA and JA is also an important metabolic event in EL acclimation response, but this process requires further investigation.

In conclusion, this investigation suggests that SA signalling is responsible for β-CC-enhanced EL acclimation. Figure 8 shows that β-CC induces SA accumulation under EL via the isochorismate pathway; the increased SA indirectly inhibits ROS production in the chloroplast and promotes NPR1 accumulation in the nucleus. Nuclear NPR1 may then interact with TGA transcription factors to reprogramme defence gene GST5 and GST13 transcripts and subsequently increase GST activity. As a result, this signalling response alleviates the damaging effects of EL. Our results corroborate the signalling mechanism of β-CC-enhanced EL acclimation and emphasize the important role of SA in this process.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Table S1. Primers used in this study.

Supplementary Fig. S1. The contrast in EDS1 protein levels between WT and β-CC-treated WT plants during EL illumination.

Supplementary Fig. S2. Localization of NPR1 in nucleus and cytoplasm at population level during β-CC-enhanced EL acclimation.

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References


that mediate gene responses to singlet oxygen in plants. Proceedings of the National Academy of Sciences, USA 109, 5535–5540.


