Dihydroactinidiolide, a High Light-Induced β-Carotene Derivative that Can Regulate Gene Expression and Photoacclimation in *Arabidopsis*

Dear Editor,

The physiological functions of carotenoids in plants go beyond their traditional roles as accessory light-harvesting pigments, natural colorants, and quenchers of triplet chlorophyll and singlet oxygen ($^1O_2$). Recent studies have indeed emphasized the functional role of molecules derived from carotenoids as phytohormones (Ruyter-Spira et al., 2013) or messengers in stress signaling pathways (Havaux, 2014). In particular, chemical quenching of $^1O_2$ by carotenoids within the photosystems involves oxidation of the carotenoid molecules, generating a variety of oxidized products (Ramel et al., 2012). β-Cyclocitral, a volatile C7 derivative of β-carotene, is one such molecule produced during high light stress, which was found to induce changes in the expression of $^1O_2$-responsive genes (Ramel et al., 2012). Moreover, the β-cyclocitral-dependent gene reprogramming was associated with an increased tolerance of the plants to photooxidative stress. These effects appeared to be specific to β-cyclocitral since they were not observed with β-ionone, a C9-oxidized derivative of β-carotene, which was not able to induce or repress the expression of $^1O_2$ gene markers. Based on those results, it was proposed that β-cyclocitral is a plastid messenger involved in the chloroplast-to-nucleus $^1O_2$ signaling pathway leading to acclimation to high light stress (Ramel et al., 2012). However, *in vitro* $^1O_2$ oxidation of β-carotene is known to produce other volatile compounds besides β-cyclocitral and β-ionone, such as dihydroactinidiolide (dhA, Figure 1A) and α-ionene (Ramel et al., 2012). The dhA molecule is a lactone (cyclic ester) resulting from the secondary oxidation of β-ionone through the intermediate 5,6-epoxy-β-ionone (Havaux, 2014). Both dhA and α-ionene were detected in plant leaves and fruits (e.g. Del Mar Caja et al., 2009; Ramel et al., 2012). Interestingly, dhA, but not α-ionene, was reported to accumulate in *Arabidopsis* leaves under high light stress (Ramel et al., 2012).

The dhA molecule contains a carbonyl group that can react with nucleophilic structures in macromolecules, providing this compound with a high potential reactivity. Actually, dhA is known to be a bioactive molecule in animals. It is a component of pheromones in insects, such as red fire ants (Rocca et al., 1983) and in mammals such as the Cat and the Red Fox (Albone, 1975). dhA was also found to exhibit cytotoxic effects against cancer cell lines (Malek et al., 2009). In contrast, much less is known on the actions of dhA in vascular plants. Nevertheless, dhA was identified as a major component of ethyl acetate extracts of cyanobacteria or aquatic macrophytes which inhibit seed germination and seedling growth (Stevens and Merrill, 1980). This compound has also been identified in wheat glumes where it was suggested to act as a germination inhibitor (Kato et al., 2003).

Figure 1B shows that dhA, quantified by GC–MS (Supplementary Data) is present in control *Arabidopsis* leaves at a concentration of around 3 ng g^{-1} fresh weight. When *Arabidopsis* plants were exposed to photooxidative stress conditions (high light and low temperature), dhA was found to rapidly accumulate in leaves, reaching a concentration of ~45 ng g^{-1} after 8 h (Figure 1B). To check the possible involvement of dhA in gene regulation, we exposed *Arabidopsis* plants to volatile dhA in an airtight Plexiglass chamber, using a protocole that has been described previously (Ramel et al., 2012) (Supplementary Data). Two different volumes (100 and 200 µl) of pure dhA were applied on cotton wicks placed in the closed chambers, increasing the dhA concentration in the atmosphere from 0 to 0.18 and 0.23 p.p.m., respectively, after 4 h. These treatments induced a rise in the internal dhA content of *Arabidopsis* leaves to levels (~40 ng g^{-1}) in the concentration range measured in high light-treated plants (Figure 1C). In Figure 1D and 1E, we examined the effect of volatile dhA on genes whose expression is known to be affected by $^1O_2$. At3g25250 (encoding the kinase OXIDATIVE SIGNAL INDUCIBLE 1), At2g33380 (encoding RESPONSIVE TO DESSICATION 20), At2g29450 (coding for a glutathione transferase GSTU5), At2g15490 (encoding the UDP-glycosyltransferase 73B4), and At3g50970 (encoding LOW TEMPERATURE INDUCED 30) (Figure 1D) were shown to be induced by $^1O_2$ in the *Arabidopsis flu* and *ch1* mutants (op den Camp et al., 2003; Ramel et al., 2013). Conversely, the genes At1g44446 (encoding CHLORINA 1) and At3g14210 (encoding EPITHIOSPECIFIER MODIFIER 1) (Figure 1E) have been reported to be noticeably repressed by $^1O_2$. In this study, gene expressions

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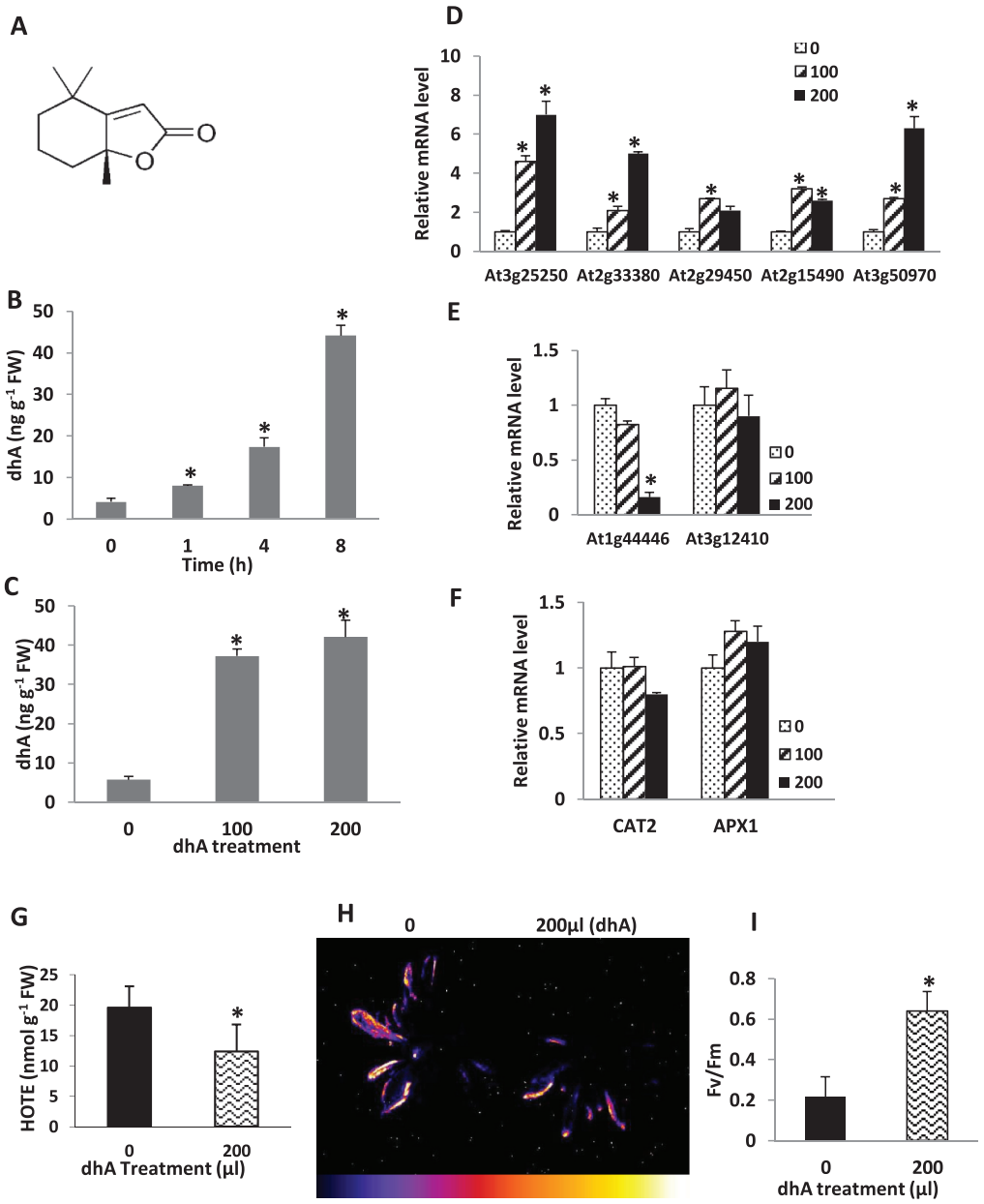

Figure 1 Dihydroactinidiolide and Gene Expression in *Arabidopsis*.

(A) Molecular structure of dihydroactinidiolide (dhA).

(B) dhA accumulation in *Arabidopsis* plants subjected to high light stress (1500 µmol photons m^{-2} s^{-1}, 7°C). Data are mean values of three or four measurements + SD.

(C) dhA accumulation in leaves of *Arabidopsis* plants treated with two concentrations of volatile dhA (100 µl and 200 µl) in a closed chamber for 4 h. Data are mean values of three or four measurements + SD.

(D, E) Effect of the dhA treatments on the expression of $^1O_2$-responsive genes. All values are normalized to the value of control treatment, which was assigned the value of 1. Data are mean values of three independent experiments + SD.

(F) Effects of the dhA treatments on the expression of two H_2O_2-responsive genes. Data are mean values of three independent experiments + SD.

(G) Lipid peroxidation, as measured by the levels of hydroxy linolenic acid (HOTE, hydroxy-octadecatrienoic acid), in *Arabidopsis* plants exposed to high light stress (1500 µmol photons m^{-2} s^{-1} and 7°C) for 2 d. Data are mean values of four independent experiments + SD. HOTE concentration before stress was about 5 nmol g^{-1} fresh weight (FW).

(H) Autoluminescence imaging of lipid peroxidation in dhA-treated and -untreated plants exposed to high light stress.

were analyzed by quantitative RT–PCR (Supplementary Data). The data presented in Figure 1D indicate that the expression of all selected $^1O_2$-inducible genes was noticeably induced by dhA, with a clear dose response for At3g25250, At2g33380, and At3g50970. The inducing effects of dhA on the genes At2g29450 and At2g15490 did not significantly differ between the two dhA concentrations, suggesting a saturation phenomenon. The five genes up-regulated by dhA are also inducible by β-cyclocitral (Ramel et al., 2012). The $^1O_2$-repressible gene At1g44446 was drastically repressed by dhA, while the expression of At3g14210 did not significantly change with dhA (Figure 1E). Both genes are known to be down-regulated by β-cyclocitral (Ramel et al., 2012). The decrease in At1g44446 gene transcripts by dhA was correlated with the dhA concentration. Similarly to β-cyclocitral, dhA was unable to trigger the expression of the H_2O_2-inducible genes CAT2 (catalase 2, At4g35090) and APX1 (ascorbate peroxidase 1, At1g07890) (Figure 1F).

The results presented in Figure 1 demonstrate that the carotenoid derivative dhA rapidly accumulates in *Arabidopsis* leaves exposed to high light stress, reaching concentrations that are able to trigger changes in the expression of $^1O_2$-responsive genes. One can thus conclude that dhA is a new gene regulator derived from carotenoids that plays a role in the chloroplast retrograde signaling of $^1O_2$ stress. As a corollary, the previously identified signal molecule, β-cyclocitral, is not the sole carotenoid-derived messenger involved in the $^1O_2$ signaling pathway. Actually, it is likely that β-cyclocitral and dhA are part of a larger group of reactive electrophile species (RES) that collectively stimulate $^1O_2$-specific responses and activate acclimation to photooxidative stress. The transcriptomic response *in vivo* may thus be a complex integration of multiple signals from various carotenoid RES.

The changes in gene expression induced by $^1O_2$ are known to lead either to programmed cell death or to acclimation. The cell death process is dependent on the proteins EXECUTER 1 and 2 (Lee et al., 2007) while the acclimation process does not rely on EXECUTER and involves β-cyclocitral (Ramel et al., 2013). *Arabidopsis* plants treated with volatile β-cyclocitral were observed to be substantially more tolerant to high light stress than untreated plants, with lipid peroxidation and PSII photoinhibition strongly reduced (Ramel et al., 2012). The same phenomenon was found with dhA: lipid peroxidation after high light stress, monitored by the levels of hydroperoxy linolenic acid (measured after conversion of the peroxides into alcohols by reduction with triphenyl phosphine; see Supplementary Data), was significantly lowered in dhA-treated *Arabidopsis* plants compared to control plants (Figure 1G). Lipid peroxides were also analyzed by autoluminescence imaging (Ramel et al., 2013). The images shown in Figure 1H are consistent with the HOTE data: fewer leaves were luminescent after high light stress in dhA-treated plants compared to untreated plants, indicating less accumulation of lipid peroxides. We also observed that PSII photoinhibition, measured by the decrease in the chlorophyll fluorescence ratio Fv/Fm, was mitigated in plants treated with volatile dhA (Figure 1I).

As previously reported in *Arabidopsis* plants treated with volatile β-cyclocitral (Ramel et al., 2012), the EXECUTER proteins are not involved in the dhA-dependent signaling pathway. Indeed, dhA-induced changes in gene expression were not inhibited in the *ex1 ex2* double mutant deficient in both EXECUTER proteins (Supplemental Figure 1) compared to wild-type (Figure 1D).

Singlet oxygen is produced from triplet excited chlorophylls in the PSII reaction center where β-carotene is located. Oxidation of β-carotene by $^1O_2$ is thus an early event in the responses of plants to excess light energy. The signal molecules generated by this oxidation are upstream of the $^1O_2$ signaling pathway and can be considered as primary sensors of light stress. β-Cyclocitral and dhA are volatile compounds and have therefore the potential to convey information out of the chloroplast. However, the exact mode of action of those compounds is unknown. As RES, they can react with nucleophilic groups in macromolecules (Havaux, 2014). However, although the electrophilicity of β-ionone is higher than that of β-cyclocitral, it is unable to induce or repress $^1O_2$-specific genes (Ramel et al., 2012). This suggests that the role of carotenoid-derived molecules in the signaling of $^1O_2$ is probably more specific than a general RES response. As mentioned above, dhA is a lactone, and this chemical species is known to have signaling functions, especially in bacteria. For instance, acyl-L-homoserine lactone is involved in the concerted expression of genes associated with the so-called quorum sensing by interacting with regulatory proteins (Camilli and Bassler, 2006). In *Streptomyces*, γ-butyrolactones serve as signaling molecules in the regulation of antibiotic biosynthesis pathway (Takano, 2006); they bind to receptor proteins, they inhibit their binding to specific DNA targets and, since most of the γ-butyrolactone receptors act as repressors, they induce target genes. Whether receptors of carotenoid RES are present in chloroplasts is completely unknown. Determination of the primary targets of the dhA lactone and of other carotenoid RES in plant cells is clearly a major research challenge for the future.

(I) Maximal PSII photochemistry (Fv/Fm) in leaves of dhA-treated and -untreated plants exposed to high light stress. Data are mean values of eight measurements + SD. Panels (B, C, D, E, G, I): * indicates significant difference between control and dhA-treated plants at $P < 0.05$ (Student's *t*-test).

SUPPLEMENTARY DATA

Supplementary data are available at *Molecular Plant Online*.

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