A LC-MS profiling method reveals a route for apocarotenoids glycosylation and shows its induction by high light stress in Arabidopsis†

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Apocarotenoids glycosylation serves as a valve regulating carotenoid homeostasis in plants and may contribute to plants response to photo-oxidative stress. However, an analytical method that allows comprehensive and sensitive profiling of glycosylated apocarotenoids (GAPOs) is still missing. We developed an efficient ultra-high performance liquid chromatography-high resolution-mass spectrometry (UHPLC-HR-MS) method to analyze 25 GAPOs present in carotenoid-accumulating *E. coli* cells and plant tissues. Optimized HR-heated-electrospray ionization (HESI)-MS parameters enabled, based on HR MS and tandem mass spectrometry (MS/MS) data, the identification of yet undescribed GAPOs from Arabidopsis, which include Glc-apo-11-carotenal (GAPO11), Glc-apo-13-carotenone (GAPO13), and their isomers. The identity of these compounds was confirmed by the transformation of deuterium-labelled non-hydroxylated carotene cleavage products into corresponding GAPOs *in planta*. Quantitative analysis of GAPOs in Arabidopsis showed that the levels of Glc-cyclocitral (GAPO7), Glc-cyclocitral isomer I (GAPO7I), Glc-ionone (GAPO9), Glc-ionone isomer I (GAPO9I), Glc-apo-11-carotenal isomer I (GAPO11I), Glc-apo-13-carotenone (GAPO13), and Glc-apo-13-carotenone isomers (GAPO13I, GAPO13II, and GAPO13III) significantly increase after high light (HL) treatment. This treatment led also to an obvious increase in the levels of most carotene- and all xanthophyll-derived apocarotenoids detected in our system. Our work demonstrates for the first time that HL stress induces apocarotenoid glycosylation in Arabidopsis and unravels a novel plant metabolic pathway that leads from carotene cleavage products to GAPOs that are identical with xanthophyll derived GAPOs. Thus, our new approach allows sensitive and reliable profiling of GAPOs, which is crucial for understanding the function of apocarotenoid glycosylation in plants and its role in the acclimation to HL stress.

1. Introduction

Carotenoids are mostly C₄₀ terpenoids equipped with an extended conjugated double bond system. These pigments are synthesized in all photosynthetic organisms and many heterotrophic microorganisms.^{1–3} Carotenoids are divided into carotenes that consist of hydrocarbons and their oxygen-containing derivatives, the xanthophylls. In plants, carotenoids are essential constituents of photosystems, indispensable for light-harvesting and photoprotection. In addition, they determine the fluidity of plastid membranes and are accumulated in many flowers and fruits to attract animals for pollination and seed dispersal.^{3–5} Carotenoids exert also a more general function as precursor for hormones, regulatory molecules and further important metabolites in all taxa. Known examples for such carotenoid-derivatives are the plant hormones abscisic acid (ABA)⁶ and strigolactone (SL)⁷, retinoids, such as retinal and retinol (vitamin A)⁸, the fungal pheromone

trisporic acid⁹, the plant stress signal β -cyclocitral,^{10,11} and the citrus pigment citraurin¹². Very recently, the importance of carotenoid-derived metabolites in regulating plant development has been further corroborated by the discovery of anchorene and zaxinone that act as growth regulators in Arabidopsis and rice, respectively (Jia et al., Wang et al.).

All carotenoid-derived compounds mentioned above arise by oxidative cleavage of double bonds in carotenoid-backbone, which generates a large set of carbonyl products called apocarotenoids. This oxidation/cleavage reaction is catalyzed by carotenoid cleavage dioxygenases (CCDs)^{13,14} or by less-specific enzymes (e.g. lipoxygenases and peroxidases)¹⁵. However, carotenoid cleavage can also take place without enzymatic catalysis, as non-enzymatic oxidation processes triggered, for instance, by reactive oxygen species (ROSs)¹⁶. Apocarotenoids are frequently further metabolized by enzymes, such as CCDs that catalyze secondary cleavage reactions, aldehyde and alcohol dehydrogenases, or cytochrome P450 enzymes. These metabolic steps are required to generate the bioactive compounds, e.g. the plant hormones ABA and SLs, retinol/retinoic acid, and the fungal pigment neurosporaxanthin^{6–8,17}, or to convert the high-reactive apocarotenoid-aldehydes into less reactive acids, as supposed for the cyanobacterial *Synechocystis* aldehyde

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dehydrogenase.¹⁸ Apocarotenoid glycosylation is a further, but less investigated modification of apocarotenoids, which can be catalyzed by family 1 glycosyltransferases^{19,20}. The generation of glycosylated apocarotenoids (GAPO) might be related to detoxification and enables the storage and transport of apocarotenoids in plants^{19,21}. In addition, apocarotenoid glycosylation is thought to act as a valve regulating plants carotenoid homeostasis²². For instance, the enhanced pathway flux upon constitutive overexpression of the key carotenoid biosynthesis enzyme phytoene synthase in *Arabidopsis* caused an increase of C₁₃ GAPOs content in leaves²². Saffron flowers provide a further example for the accumulation of glycosylated apocarotenoids, which results from an induction of the zeaxanthin cleavage dioxygenase that initiates the formation of crocetin glycosides and picrocrocin²³, and UDP-glucosyl transferases (UGT) that mediate the required apocarotenoid glycosylation²⁴. In addition, a diversity of xanthophyll-derived GAPOs are accumulated in fruits (e.g., grapevine and peach) and flowers (e.g., *Boronia megastigma*), suggesting high abundance of apocarotenoid glycosylation^{25–27}.

Particularly under HL conditions, plant photosynthesis inevitably generates highly reactive intermediates that can cause oxidative damage of the photosynthetic apparatus, resulting in a decrease of photosynthesis efficiency²⁸. Thus, HL stress triggers the generation of ROSs, such as H₂O₂ in photosystem I^{29,30} and singlet oxygen (¹O₂) in photosystem II^{31,32}, which damage cells and can ultimately lead to plant death^{33,34}. To survive under unfavourable light conditions, plants have evolved several efficient protective mechanisms, including enzymatic (e.g., superoxide dismutase, dehydroascorbate reductase, glutathione peroxidase, glutathione reductase, and glutathione S-transferase) and non-enzymatic anti-oxidation systems^{35–38}. Carotenoids can quench excitation energy from singlet- or triplet-state chlorophylls and decrease the risk of ¹O₂ generation^{39,40}. Moreover, carotenoids also convey the ¹O₂ stress signal to the nucleus, to trigger the expression of ¹O₂ responsive genes required for protecting the cell from oxidation and for acclimation. This transduction occurs via water soluble apocarotenoids, such as cyclocitral, that arise by oxidative cleavage of carotenoids^{10,11}. Therefore, synthesis and catabolism of carotenoids are crucial element in the mechanisms utilized by plants to cope with ROSs³⁶. Recent studies showed that ¹O₂ induces the expression of UGT genes in different plant species, including *Arabidopsis*, rice, and green algae⁴¹, indicating a role of apocarotenoid glycosylation in plant's response to HL stress. However, little is known about the effect of HL on apocarotenoid glycosylation.

Understanding the biological function of GAPOs and determining their role in plant's response to photo-oxidative stress require an analytical method that enables sensitive and reliable determination of these compounds. In this work, we developed a UHPLC-HESI-MS method for the analysis of carotenoid-derived GAPOs with hydrocarbon chain lengths ranging from C₁₀ to C₃₀. For this purpose, we firstly optimized MS conditions to increase the sensitivity of MS detection of GAPOs from engineered, zeaxanthin diglucoside accumulating *E. coli* cells and *Arabidopsis*. Next, we improved the UHPLC

resolution of individual compounds to significantly enhance the accuracy of GAPOs identification. Finally, we conducted feeding experiments in planta to confirm the identity of *Arabidopsis* GAPOs. Using this optimized method, we quantitatively determined the profile of GAPOs in *Arabidopsis* under normal conditions and upon HL treatment.

2. Experimental

2.1. Chemicals

Methanol (LC-MS grade), acetonitrile (LC-MS grade), 2-propanol (LC-MS grade), and formic acid (LC-MS grade) were obtained from Sigma-Aldrich (Germany). Water (LC-MS grade), acetone (HPLC grade), ethyl acetate (HPLC grade), and dichloromethane (HPLC grade) were provided by VWR International, LLC. (USA). Butylated hydroxytoluene (BHT, 99%) was purchased from Sigma-Aldrich (Germany). Deuterium-labeled apocarotenoid standards including D₁- β -cyclocitral, D₃- β -ionone, D₃- β -apo-11-carotenal, D₃- β -apo-13-carotenone, D₃- β -apo-15-carotenal, D₃- β -apo-14'-carotenal, D₃- β -apo-12'-carotenal, D₃- β -apo-10'-carotenal, D₃- β -apo-8'-carotenal, and D₃-3-OH- β -apo-13-carotenone were synthesized by Buchem B.V. (Netherlands).

2.2. Sample solution preparation

Internal standard 1 (IS1) solution (D₃-3-OH- β -apo-13-carotenone, 6.6 ng/mL) was prepared for quantitation of GAPOs with 0.1% (w/v) BHT in methanol. Internal standard 2 (IS2) solution (D₁- β -cyclocitral, D₃- β -ionone, D₃- β -apo-11-carotenal, D₃- β -apo-13-carotenone, D₃- β -apo-15-carotenal, D₃- β -apo-14'-carotenal, D₃- β -apo-12'-carotenal, D₃- β -apo-10'-carotenal, D₃- β -apo-8'-carotenal, and D₃-3-OH- β -apo-13-carotenone, 100 ng/mL each compound) was prepared for quantification of apocarotenoids with ethyl acetate. Deuterium-labelled apocarotenoid standard solutions including D₁- β -cyclocitral, D₃- β -ionone, D₃- β -apo-11-carotenal, and D₃- β -apo-13-carotenone at concentration of 20 mM were prepared with acetone.

In vivo assays, Top10 competent *E. coli* strains were transformed with the plasmid pPL376 carrying the entire *Pantoea agglomerans* (formerly *Erwinia herbicola*) carotenoid gene cluster that leads to the accumulation of zeaxanthin diglucoside (Fig. 1, i)⁴². 15 mL of overnight cultures were inoculated into 1 L of 2x YT medium and incubated at 28 °C for 16 h in the dark. *E. coli* cells were harvested by centrifugation at 10875 g for 15 min. Pellets were re-suspended in 50 mL acetone, following repeated sonication. After centrifugation at 3220 g for 10 min, supernatants containing GAPOs were dried under vacuum. Residue was re-dissolved in 100 μ L of acetonitrile/water (60/40, v/v) and filtered through a 0.22 μ m filter before LC-MS analysis.

Sterilized *Arabidopsis* (ecotype Columbia) seeds were kept in the dark for 3 days at 4 °C. Seeds were then sown on half strength MS (with 0.5 % sucrose + 1% agar, 0.5 g·L⁻¹ MES, pH 5.7) plates. *Arabidopsis* seedlings were grown in growth chambers under long day conditions (16 h light/8 h dark, 22 °C, 60% relative humidity, light density: 200 μ mol photons·m⁻²·s⁻¹) for 10 days. Seedlings were then randomly separated into two

groups and exposed for 6 h to HL (light density of 1200 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) or normal light (light density of 200 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). For feeding experiments, 10 days old Arabidopsis seedlings were collected and immersed in half strength MS containing different deuterium-labelled apocarotenoids (D_1 - β -cyclocitral, D_3 - β -ionone, D_3 - β -apo-11-carotenal, and D_3 - β -apo-13-carotenone) at 20 μM concentration in darkness for 6 h. After harvest, Arabidopsis seedlings were lyophilized, powdered and stored at -20°C . Approximate 15 mg tissue powder was extracted with 500 μL of IS1 solution in an ultrasound bath (Branson 3510 ultrasonic bath) for 15 min. Then the extraction solution was centrifuged for 5 min at 18407 g at 4°C . The resulting supernatant was collected, and the pellet was re-extracted with 500 μL of methanol containing 0.1% (w/v) BHT. The two supernatants were combined and dried under vacuum. The residue was re-dissolved in 100 μL of acetonitrile/water (60/40, v/v) and filtered through a 0.22 μm filter before LC-MS analysis.

Analysis of apocarotenoids from Arabidopsis was performed according to the protocol published in the work⁴³. Briefly, approximate 15 mg tissue powder spiked with 10 μL of IS2 was extracted twice with methanol containing 0.1% BHT in an ultrasound bath. After centrifugation, the two supernatants were combined and dried under vacuum. The residue was re-dissolved in 100 μL of acetonitrile/water (90/10, v/v) and filtered through a 0.22 μm filter before LC-MS analysis.

2.3. UHPLC-MS conditions

A Dionex Ultimate 3000 UHPLC system coupled with a HR Q-Orbitrap-MS (Q-Exactive plus MS, Thermo Scientific) with a HESI source in positive mode was used for identification and quantification of GAPOs. GAPOs were separated on a Waters ACQUITY UPLC BEH C_{18} column (100 $\times$ 2.1 mm, 1.7 μm) with a Waters UPLC BEH C_{18} guard column (5 $\times$ 2.1 mm, 1.7 μm). Chromatographic column temperature was maintained at 35°C . The flow rate was 200 $\mu\text{L}\cdot\text{min}^{-1}$. The injection volume was 10 μL . The optimized mobile phases A (water/acetonitrile/formic acid, 95/5/0.2, v/v/v) and B (acetonitrile/formic acid, 100/0.2, v/v) were employed for eluting GAPOs with the gradient program: 0–17 min, 5% B to 100% B; followed by washing with 100% B and equilibration with 95% A. The HESI source parameters were as follows: sheath gas flow rate, 30 arbitrary units; auxiliary gas flow rate, 10 arbitrary units; spray voltage, 4.0 kV; capillary temperature, 250°C ; S-lens RF level, 50; and auxiliary gas heater temperature, 350°C . The full scan (FS) MS parameters included scan range of 150 to 900 m/z , resolution of 280,000, AGC target of $3\text{e}6$, and maximum inject time of 150 ms. The parallel reaction monitoring (PRM) MS conditions included scan range of 80 to 900 m/z , resolution of 35,000, AGC target of $1\text{e}6$, maximum inject time of 150 ms, and NCE of 10 eV. UHPLC-MS conditions published in the work⁴³ were used for quantitative analysis of Arabidopsis apocarotenoids.

2.4. Method application and data analysis.

This method described in the above sections was applied to investigate the effect of HL stress on apocarotenoid glycosylation in Arabidopsis. In this work, glycosylated

Table 1. PRM transitions for the quantification of 10 endogenous GAPOs from Arabidopsis.

Name	RT (min)	Precursor ion $[\text{M}+\text{H}]^+ (m/z)$	Product ion $[\text{M}+\text{H}]^+ (m/z)$
GAPO7	5.09	331.17513	169.12231
GAPO7I	4.95	331.17513	169.12231
GAPO9	6.31	371.20643	209.15361
GAPO9I	6.37	371.20643	209.15361
GAPO11	7.61	397.22208	235.16926
GAPO11I	8.03	397.22208	235.16926
GAPO13	8.88	437.25338	275.20056
GAPO13I	9.41	437.25338	275.20056
GAPO13II	9.51	437.25338	275.20056
GAPO13III	9.80	437.25338	275.20056

apocarotenoids in Arabidopsis samples were identified and assigned by using high-resolution MS and MS/MS data, supported by the identification of glycosylated apocarotenoids present in *E. coli* strain that accumulates zeaxanthin-diglycoside. The UHPLC-HR-MS/MS method in PRM mode was employed for quantification of endogenous GAPOs. PRM transitions are shown in Table 1. GAPOs amounts were calculated as follows: Relative level $[\text{target GAPO}] = \text{Area} [\text{target GAPO}] / \text{Area} [\text{spiked IS}] \times \text{Amount} [\text{spiked IS}] / \text{Mass} [\text{dry weight}]$. UHPLC-HR-MS method with FS mode was employed in the quantification of endogenous apocarotenoids, T-test was used to analyze data and compare groups.

3. Results and discussion

3.1. Optimization of LC-MS conditions for GAPOs analysis

To achieve the authentic identification of GAPOs from zeaxanthin diglycoside accumulating *E. coli* cells and Arabidopsis, GAPOs were analyzed using the a UPLC C_{18} column under optimized UHPLC conditions enabling clear separation of all detected GAPOs within 17 min (Fig. 1 and Fig. S1 in ESI[†]). The MS parameters were subsequently optimized to achieve a high sensitivity of GAPOs MS detection, especially for the quantification of GAPOs with short hydrocarbon chain present in Arabidopsis. Five of HESI source parameters, including sheath gas flow rate, auxiliary gas flow rate, spray voltage, capillary temperature, and auxiliary gas heater temperature, were optimized by comparing peak areas of GAPOs detected under different conditions. The results unravelled that capillary temperature, among all HESI source parameters, as the most sensitive factor for the detection of GAPOs with short hydrocarbon chain (e.g., GAPO9, GAPO11, and GAPO13). The intensity of MS response signals of GAPOs with short hydrocarbon chain at capillary temperature of 250°C were around two fold of those at high capillary temperature (320°C) (Fig. 2). Although there was a little loss of sensitivity in the detection of long hydrocarbon chain GAPOs (GAPO12', GAPO10', and GAPO8'), capillary temperature of 250°C was the most suitable for GAPOs determination. The other results obtained (Fig. S2 in ESI[†]) show that the following conditions are optimal for MS detection of GAPOs at capillary temperature of 250°C , sheath gas flow rate (30 arbitrary units), auxiliary gas

flow rate (10 arbitrary units), spray voltage (4.0 kV), and auxiliary gas heater temperature (350 °C).

3.2. Identification of GAPOs from engineered *E. coli* and *Arabidopsis*

To identify GAPOs from *E. coli* cells engineered to accumulate zeaxanthin diglucoside and *Arabidopsis*, we established a personal GAPOs database including GAPOs name, chemical formula, theoretical accurate mass, and feature fragment ion, to search HR MS and MS/MS data. Selected GAPOs candidates were further filtered to remove false ones by matching of isotope distribution and feature product ion. We employed HR-HESI-Obitrap-MS/MS with a resolving power of 280,000, which can achieve a low ppm mass accuracy and resolve the fine structure of isotopes for targeted analytes to determine their correct elemental formula⁴⁴. We also applied MS/MS to induce the fragmentation of candidate compounds, which was used to elucidate the structure of GAPOs. For example, there were two chromatographic peaks obtained from the FS extracted ion chromatogram (EIC) of protonated Glc- β -apo-13-carotenone (GAPO13) ion at m/z 437.25338 (theoretical accurate mass) with an error window of 1 ppm (Fig. 3A upper). The accurate

masses of protonated ions from peak 1 at retention time (RT) 8.86 min and peak 2 at RT 9.51 min were m/z 437.25391 and m/z 437.25348, respectively, which indicate that the two compounds have the same elemental formula of $C_{24}H_{36}O_7$ containing 7 of unsaturated degrees. Moreover, their PRM EIC of ions pair at m/z 437>275 had the same pattern as that in FS EIC (Fig. 3A down). In the MS/MS spectra, the feature ion at m/z 275.20053 reflected OH- β -apo-13-carotenone moiety via a loss of a glucose group (162 u) from precursor ion, confirming the assignment of endogenous Glc- β -apo-13-carotenone (GAPO13) and GAPO13 isomer (GAPO13II) extracted from engineered *E. coli* cells.

An adequate good chromatographic separation of GAPOs significantly increases the capability of identifying GAPOs from plants. For example, we identified four endogenous GAPO13 isomers from *Arabidopsis*, which were confirmed by HR MS/MS data (Fig. 3B upper and Fig. 3C left). Feeding experiments performed with D3- β -apo-13-carotenone in *Arabidopsis* unravelled four chromatographic peaks of deuterium-labelled GAPO13 and three corresponding isomers (D3-GAPO13 at RT 8.85 min, D3-GAPO13I at RT 9.38 min, D3-GAPO13II at RT 9.50

Fig. 1. UHPLC-HR-MS analysis of GAPOs from engineered *E. coli* cells accumulating zeaxanthin diglucoside using the optimized mobile phases and eluting gradient. Mobile phases consisted of water/acetonitrile (95/5, v/v, A) and acetonitrile (B) both containing 0.2% formic acid. The gradient was as follows: 0–17 min, 5%–100% B; 17–21 min, 100% B; 21–22 min, 100%–5% B; 22–25 min, 5% B. a, Glc- β -ionone (GAPO9); b, Glc- β -apo-11-carotenal (GAPO11); c, Glc- β -apo-13-carotenone (GAPO13); d, Glc- β -apo-15-carotenal (GAPO15); e, Glc- β -apo-14'-carotenal (GAPO14'); f, Glc- β -apo-12'-carotenal (GAPO12'); g, Glc- β -apo-10'-carotenal (GAPO10'); h, Glc- β -apo-8'-carotenal (GAPO8'); and i, Zeaxanthin diglucoside.

Fig. 2. Effect of the capillary temperature on MS response signals of GAPOs. $n = 3$, mean $\pm$ SD.

Fig. 3. Identification of GAPOs and their isomers from engineered *E. coli* and Arabidopsis using UHPLC-HESI-MS/MS. (A), HR FS EIC of GAPO13 with $[M+H]^+$ ion at m/z 437.25338 (blue), and HR PRM EIC of GAPO13 with ion pair at m/z 437>275 (red) from the engineered *E. coli*. (B), HR PRM EIC of endogenous GAPO13 and its isomers with ion pair at m/z 437>275 (upper) from Arabidopsis, and D₃-GAPO13 and its isomers with ion pair at m/z 440>278 (down) from Arabidopsis fed with D₃-apo-13-carotenone. (C), HR MS/MS spectra of endogenous GAPO13 and its isomers identified from Arabidopsis (left), and D₃-GAPO13 and its isomers from Arabidopsis fed with D₃-apo-13-carotenone (right). The error window is 1 ppm.

min, and D₃-GAPO13III at RT 9.76 min) in the PRM EIC of ions pair at m/z 440>278, which co-eluted with endogenous GAPO13 and its isomers (Fig. 3B). In their MS/MS spectra, the feature ion at m/z 278 in the MS/MS spectra, which reflects the D₃-OH-apo-13-carotenone moiety of D₃-GAPO13, corroborated the identification of D₃-GAPO13 and its isomers (Fig. 3C right). The formation of deuterium-labelled GAPOs, including D₁-GAPO7, D₃-GAPO9, D₃-GAPO11, D₃-GAPO13, and their isomers from the corresponding deuterium-labelled apocarotenoids (D₁- β -cyclocitral, D₃- β -ionone, D₃- β -apo-11-carotenal, and D₃- β -apo-13-carotenone), in Arabidopsis (Fig. 3, Fig. S3 and S4 in ESI[†]), not only confirmed the identification of endogenous GAPOs, but also demonstrated that non-hydroxylated apocarotenals/apocarotenones can be converted into GAPOs that are identical with xanthophyll-derived GAPOs. This result unravelled a novel metabolic path that leads from apocarotenals/apocarotenones to GAPOs via hydroxylated intermediates. It can be speculated that this metabolic process is a general mechanism utilized by plants to maintain the concentration of apocarotenals/apocarotenones at certain levels.

LC retention time of GAPOs on UPLC C18 column allowed the construction of a linear regression model with goodness of fit ($r^2 > 0.997$) by plotting carbon number versus RT (Fig. S5 in ESI[†]), supporting the identification of GAPOs^{43,45}. Taken together, application of the analytical strategy mentioned above led to the identification of 25 endogenous GAPOs and their isomers from engineered *E. coli* and Arabidopsis (Table S1 in ESI[†]), including GAPO11 and GAPO13 that were identified for the first time from green tissues.

3.3. Effect of HL stress on apocarotenoid glycosylation in Arabidopsis

Major part of our knowledge on apocarotenoid glycosylation has been generated from studies on the formation and quantification of very few GAPOs, such as picrocrocin and crocin which are responsible for the taste and colour of saffron, respectively⁴⁶, C₁₃ apocarotenoid glycosides, and C₃₀ glycosyl-apocypenals. These studies relied on different analytical methods, i.e. LC-electrospray ionization-MS, LC-atmospheric pressure chemical ionization-MS, and hydrolysis of glycol-conjugates coupled with gas chromatography-MS, which were used to analyze saffron GAPOs,^{47,48} bacterial long chain glycosyl-apocypenals,⁴⁹ and C₁₃ apocarotenoids glycosides from Arabidopsis and grape berries,^{22,50,51} respectively. However, a comprehensive and sensitive method needed for profiling of GAPOs and for investigating the general biological function of apocarotenoid glycosylation is still missing.

Taking advantage of its high sensitivity and the low matrix effect (Fig. S6 in ESI[†]), we used our UHPLC-HR-MS/MS method in PRM mode^{52,53} to determine the impact of HL stress on GAPOs pattern in Arabidopsis. It was previously reported that HL stress (4 h of illumination with 1,500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) does not lead to significant changes in levels of the four C₁₃ apocarotenoids aglycons 3-oxo- α -ionol, 3-oxo- α -ionone, 3-hydroxy-5,6-epoxy- β -ionone, and 6-hydroxy-3-oxo- α -ionone in

Fig. 4. Comparison of endogenous GAPOs abundance in Arabidopsis under normal and HL conditions. $n = 5$ in each group. Mean $\pm$ SD. T-test, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Fig. 5. Comparison of endogenous apocarotenoids abundance in Arabidopsis under normal and HL conditions. $n = 5$ in each group. Mean $\pm$ SD. T-test, * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

Arabidopsis leaves.²² However, our quantification of GAPOs upon HL treatment (Fig. 4) revealed significant and general increase in the amounts of these compounds, which also affected GAPO9 and its isomer (C_{13}) and was up to 10-fold in the case of GAPO13 and its isomers. It should be mentioned here that, under normal as well as HL conditions, levels of endogenous GAPO9 and GAPO9I were 100- to 1000-fold higher than that of other GAPOs (Fig. 4). Thus, the increased sensitivity of our LC-MS detection allowed the identification and quantification of high-abundant GAPO9 and its isomer (C_{13}) as well as of GAPOs, e.g. GAPO7, GAPO11, GAPO13, that are present at low levels in Arabidopsis. GAPOs with longer hydrocarbon chains (more than 18 carbon atoms) were not detected from Arabidopsis, which could be a result of further cleavage reactions that convert them into shorter GAPOs. Taken together, our data show for the first time that HL stress increases the levels of GAPOs and may induce apocarotenoid glycosylation in planta.

3.4. Effect of HL stress on apocarotenoids level in Arabidopsis

The enhanced GAPOs levels detected upon HL treatment raised the question on whether this stress also affects leaf apocarotenoid content. Therefore, we quantified apocarotenoids under these conditions, following our recently published method.⁴³ Fig. 5 shows that levels of five carotene-derived apocarotenoids, including β -apo-11-carotenal (APO11), β -apo-13-carotenone (APO13), β -apo-15-carotenal (APO15), β -apo-14'-carotenal (APO14'), and β -apo-12'-carotenal (APO12'), obviously increased after HL treatment. Similarly, we observed under this condition an enhancement in the levels of all hydroxylated apocarotenoids detected. In contrast, exposure to HL significantly decreased contents of β -cyclocitral (APO7) and its isomer (APO7I). This result contradicts a previous report describing an increase of β -cyclocitral content in Arabidopsis subjected to HL treatment and low temperature (7 °C).¹⁰ However, we performed our HL stress experiments under normal temperature (22 °C), which may explain the difference to the previous report and indicates that the conversion of cyclocitral is temperature-sensitive. It should be mentioned here that hydroxylated β -cyclocitral and its isomer were not detected in Arabidopsis. These findings together with the HL-induced increase in the content of GAPO7s (Fig. 4), which derive from β -cyclocitral or hydroxy- β -cyclocitral, suggest that the glycosylation of hydroxylated β -cyclocitral and the conversion of the oxidative stress signaling molecule β -cyclocitral into GAPO7s are main factors in determining the homeostasis of these C_{10} cleavage products.

Conclusions

In this work, we established a new UHPLC-HESI-MS method that enables unambiguous characterization of GAPOs from *E. coli* engineered to accumulate zeaxanthin diglucoside and Arabidopsis. For this purpose, we optimized the MS conditions to increase the sensitivity of GAPOs MS detection, which significantly enhanced the identification of low-level GAPOs isolated from plant materials. Moreover, we improved UHPLC

separation to realize the baseline separation of 25 xanthophyll-derived GAPOs in 17 min; and to enhance the capability of determining GAPOs by optimizing isomers separation. The quantitative profiling of GAPOs and apocarotenoids from *Arabidopsis* under normal and HL conditions shows that HL enhances steady state levels of apocarotenoids, with exception of cyclocitral and its isomer, and demonstrates, for the first time, that HL increases apocarotenoid glycosylation. Feeding experiments with labelled apocarotenal/apocarotenones unravelled a novel plant metabolic route that converts them into GAPOs identical with xanthophyll-derived ones. It can be assumed that apocarotenoid glycosylation is an important metabolic process that contributes to *Arabidopsis* response to HL by attaining and maintaining adequate steady-state concentrations of apocarotenoids.

Conflicts of interest

There are no conflicts to declare.

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