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Repellent and Attractive Effects of α-, β-, and Dihydro-β- Ionone to Generalist and Specialist Herbivores

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Abstract In plants, the oxidative cleavage of carotenoid substrates produces volatile apocarotenoids, including α-ionone, β-ionone, and dihydro-β-ionone, compounds that are important in herbivore-plant communication. For example, β-ionone is part of an induced defense in canola, *Brassica napus*, and is released following wounding by herbivores. The objectives of the research were to evaluate whether these volatile compounds would: 1) be released in higher quantities from plants through the over-expression of the *carotenoid cleavage dioxygenase1* (*CCD1*) gene and 2) cause herbivores to be repelled or attracted to over-expressing plants relative to the wild-type. *In vivo* dynamic headspace collection of volatiles coupled with gas chromatography–mass spectrometry was used to determine volatile organic compounds (VOC) in the headspace of the *Arabidopsis thaliana* ecotype Columbia-0 (L.) over-expressing the *AtCCD1* gene. The analytical method allowed the detection of β-ionone in the *Arabidopsis* headspace where emission rates ranged between 2 and 5-fold higher compared to the wild type, thus corroborating the *in vivo* enhancement of gene expression. A two chamber choice test between wild type and *AtCCD1* plants revealed that crucifer flea beetle *Phyllotreta cruciferae* (Goeze) adults were repelled by the *AtCCD1* plants with the highest transcription and β-ionone levels. α-Ionone and dihydro-β-ionone were not found in the headspace analysis, but solutions of the three compounds were tested in the concentration range of β-ionone found in the *Arabidopsis* headspace (0.05 to 0.5 ng/μl) in order to assess their biological activity with crucifer flea beetle, two spotted spider mite *Tetranychus urticae* (Koch), and silverleaf whiteflies *Bemisia tabaci* (Gennadius). Choice bioassays demonstrated that β-ionone has a strong repellent effect toward both the flea beetle and the spider mite, and significant oviposition deterrence to whiteflies. In contrast, dihydro-β-ionone had attractant properties, especially to the crucifer flea beetle, while α-ionone did not show any significant activity. These findings demonstrate how regulating genes of the carotenoid pathway can increase herbivore deterrent volatiles, a novel tool for insect pest management.

Keywords Ionone · Apocarotenoid · Bioassay · Dynamic headspace · Repellent · Attractive

Introduction

Carotenoids are a class of terpenoid compounds, mainly of plant origin, that play roles not only in plant defense, but also in plant growth and development (Fernández-García et al. 2012). Carotenoids are in constant turnover involving continuous biosynthesis and catabolism, and are substrates for the carotene cleavage dioxygenases (CCD) that catalyze cleavage of carotenoids to apocarotenoids (Peter and Russell 2001). These secondary metabolites formed due to the action of several different CCDs are of special interest because of their possible biological functions including herbivore feeding deterrence. In the model plant *Arabidopsis thaliana* Columbia-0,

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there are nine members in the gene family that regulate CCD activity. The genes *CCD* 1, 4, 7, and 8 and *NCED* 2, 3, 5, 6, and 9 (Harrison and Bugg 2014) encode CCD and 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzymes that selectively cleave double bonds of the conjugated system of carotene molecules, and they are present in many plants. Depending upon the position of the cleavage among the substrate molecules, ketones and aldehyde derivatives can be produced. Therefore, the over-expression of the *CCD* genes and testing the volatile organic compounds (VOC) produced may improve our understanding of their importance to the plant and the surrounding environment.

The CCD1 enzyme cleaves a broad range of carotenoids, such as lycopene, β-carotene, δ-carotene, zeaxanthin, violaxanthin, and neoxanthin, to generate aldehydes and ketones that are volatile aroma compounds (Auldridge et al. 2006; Schwartz et al. 2001; Simkin et al. 2004a). In *Petunia hybrida* Juss., CCD1 controls the emission of β-ionone (Simkin et al. 2004b), and in tomato, *Lycopersicon esculentum* Mill., CCD1 generates flavor volatiles such as geranylacetone, pseudoionone, and β-ionone (Simkin et al. 2004a). Some of the VOC that are commonly known to be produced by CCD1 include β-ionone, α-ionone, 3-hydroxy-β-ionone, pseudoionone, geranylacetone, and 6-methyl-5-hepten-2-one (Vogel et al. 2008).

Of particular interest are the volatiles present in the headspace of *Arabidopsis* modified to overexpress the genes *CCD* and *NCED*, and the effect these VOC have on herbivore behavior. Previous findings by our group explained the action of the CCD1 enzyme and demonstrated that *CCD1* overexpression reduces the feeding damage from the crucifer flea beetle *Phyllotreta cruciferae* Goeze (Wei et al. 2011). *Arabidopsis* plants overexpressing the *CCD1* gene released higher levels of β-ionone compared to the wild type, which was thought to account for the reduced feeding activity. The focus of the present study was to identify other VOC present in the headspace of *CCD1* over-expressing *Arabidopsis* plants and to determine the role of those VOC in the overall attractive and repellent effect on herbivore insects. To accomplish this, a dynamic headspace method was developed for the *in vivo* extraction of VOC from the headspace of *Arabidopsis* followed by the analyses and identification of these VOC using gas chromatography coupled with mass spectrometry (GC/MS). The VOC analyses identified aromatic and hydrocarbon derivatives as well as β-ionone. These VOC are derived mainly from four biosynthetic classes: terpenoids, fatty acid catabolites, aromatics, and amino acids (Das et al. 2013) and are recognized to have multiple functions including growth regulation and plant protection.

In the case of the apocarotenoids, α-ionone, β-ionone, and dihydro-β-ionone, these versatile aroma chemicals compose the unique fragrance of the violet *Viola arvensis* Murray, *V. odorata* L., and *V. reichenbachiana* Rchb. flowers, and play roles in the making of perfumes and fragrances (Victoria 2005). The floral bouquet of *Philodendron adamantinum* Schott, which is dominated by dihydro-β-ionone, is responsible for attracting the beetles *Erioscelis emarginata* Mannerheim that facilitate the pollination process (Pereira et al. 2014). Production of β-ionone was part of the induced defense by canola flowers *Brassica napus* when plants were wounded by *P. cruciferae* (Gruber et al. 2009). β-Ionone together with a range of terpenoids such as linalool, myricene, and pinene appeared to synergistically attract the western corn rootworm, *Diabrotica virgifera virgifera* LeConte females in maize fields (Hammack 2001), while α-ionone and α-ionol have been described as effective male attractants for solanum fruit fly *Bractocera latifrons* Hendel (Ishida et al. 2008).

To the best of our knowledge, a comparison of insect behavior in response to structurally similar chemical analogs is a novel approach for determining which gene to preferentially manipulate for a desired VOC production. The objectives of this study were to: 1) determine the behavioral response of herbivores to the volatiles produced by the over-expression of selected *CCD* genes at different steps in the carotenoid biosynthetic pathway and 2) determine which VOC, specifically the apocarotenoids, α-ionone (**1**), β-ionone (**2**), and dihydro-β-ionone (**3**) (Fig. 1), are produced preferentially by the enzymes controlled by the genes. These compounds have similar chemical structures derived from a 2, 6, 6-trimethylcyclohexyl carbon skeleton with a butyl side-chain located at the C-1 position and bearing a carbonyl group. The difference between the isomers α-ionone and β-ionone is the location of the endocyclic double bond, which for the case of α-ionone is at the C-2 position of the ring and C-1 for the β-ionone. Dihydro-β-ionone shares the same structure with β-ionone but without the exocyclic C-3 double bond on the alkyl side-chain. The presence of each apocarotenoid in the *Arabidopsis* headspace and the relative response of the herbivores will assist in the determination of which chemicals, genes, and pathways are most relevant to plant defense or attractiveness.

Methods and Materials

Reagents The analytical standards, β-ionone, α-ionone, dihydro-β-ionone ≥90 %, β-pinene, α-pinene, and (1*R*)-(+)-α-pinene, along with dichloromethane (DCM) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Acetone was purchased from Caledon (Georgetown, ON, Canada).

Plant Transformation and Growth Conditions Preparation of constructs and *AtCCD1* plant transformation from 3-wk-old wild type *Arabidopsis* were performed according to published methods (Wei et al. 2011). PCR was performed to

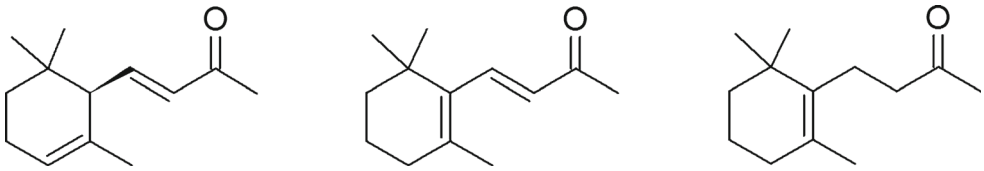

Fig. 1 Chemical structures of apocarotenoids α-ionone (**1**), β-ionone (**2**), and dihydro-β-ionone (**3**)

1 **2** **3**

confirm the presence of the corresponding transgene in eight lines of the first generation of plants (T1). In order to confirm the transcript levels of the *CCD1* transgenes, qRT-PCR was performed on the T3 generation of the transgenic plants. The transcript levels of independent lines for each transgene were tested, and the transcript levels were compared to those of wild type plants. Lines having higher levels of expression were used for the volatile studies. Recombination of the gene from pENTRD to pMDC-32 was performed by using Gateway® LR Clonase® II enzyme mix according to the manufacturer's instruction (Life Technologies, USA). Transformation of the TOP10 *E. coli* cells was carried out by electroporation. The constructs then were transformed into *Agrobacterium tumefaciens* cells (GV3101 strain; containing rifampicin and gentamycin resistance) with electroporation. Electroporation was performed by using the Gene Pulser® Cuvette (BioRad Laboratories, Canada) with 0.1 cm electrode gap using MicroPulser™ (BioRad Laboratories, Canada). The electroporation settings used for TOP10 and GV3101 were 1.79 and 2.18 kV, respectively, for six milliseconds.

Arabidopsis plants over-expressing the *AtCCD* genes and wild type were grown in 4-inch plastic pots containing Promix-BX soil at the London Research and Development Centre (LoRDC), Agriculture and Agri-Food Canada (AAFC), London ON, Canada. A total of 10 to 15 seeds were sowed onto pre-moistened soil, and the surface of the soil was given adequate moisture. Once the trays containing the pots were covered with a plastic dome to maintain humidity, they were placed at 4 °C in the dark for 3–4 d to stratify the seeds. Subsequently, the trays were transferred to a growth room maintained at a controlled environment of 20 °C ± 3 °C with a photoperiod of 16:8 h L:D light intensity of 95–130 μmol/m^2/s, and 70 % RH. The plastic domes were removed 1 wk after the seeds germinated. Seedlings were watered three times a week, on every alternate day. Once the plants were 2 weeks old, they were supplemented with All-purpose Fertilizer 20-20-20 (1 mg/l), which was added to the soil on a bi-weekly basis. Six-wk-old plants with flowers were used for the *in vivo* volatile collection.

Plant Volatile Collection System Cylindrical 46 × 26 cm inert quartz glass chambers with a circular glass base (PEGASUS, Cambridge, ON, Canada) (Supplemental Fig. 1) were used to enclose and collect volatiles from 6-wk-old wild type and transgenic *Arabidopsis* plants. Experiments were done using six chambers simultaneously. Chambers were placed in a growth incubator (Conviron 20, Winnipeg, MB, Canada) that was maintained at 22 ± 3 °C with a photoperiod of 16:8 h L:D and 55 % RH. Before plant headspace analysis, a petri dish filled with activated carbon (Fisher Scientific, Toronto, ON, Canada) was placed in the chambers for 24 hr to absorb any traces of ambient volatiles. Compressed air from a pressurised air tank (Air Liquide UN 1002, Montreal, QC, Canada) was passed through inert Tygon vacuum tubing, 3/8 inch ID × 7/8 inch OD (Cole-Parmer, Montreal, QC) at a flow rate of 100 ml/min and a manifold to push air into the six chambers. Poropak Q sorbent tubes (SKC Inc., PA, U.S.A.) containing ethylvinylbenzene-divinylbenzene beds were connected at the outlet of the chamber to adsorb VOC.

Every headspace collection of four transgenic plants from the same line was performed along with one wild type and one empty chamber as a control. After a 24 hr period, samples were eluted from the columns with 3 ml DCM HPLC grade. The eluent then was concentrated to exactly 0.25 ml by passing the samples under a stream of nitrogen gas. An internal standard, 2-octanone, was added to the samples at a final concentration of 20 μg/ml.

GC/MS and Volatile Analysis Two microliters of plant volatile extracts were injected using an auto-sampler into the 7890 A gas chromatograph system (Agilent Technologies, Mississauga, ON, Canada), and components were separated in a 30 m × 0.25 mm i.d. 0.25 μm HP-5MS, 5 % (*w*/*v*) phenyl siloxane fused silica capillary column in the pulsed splitless mode. Pressure was set at 25 psi until 0.5 min, and the purge flow to the split vent was adjusted at 40 ml/min for 1 min. Compounds were detected in a 5975C inert XL EI/CI MSD with triple-axis (Agilent Technologies). The carrier gas used was Helium (12.445 psi). The voltage used in the EMV mode was relative, and the resulting EMV was 1376. Oven temperature was maintained at 30 °C for 1 min, then increased at 5 °C/min to 200 °C, and held for 1 min at this temperature. The total run time for each sample was 36 min.

Volatile compounds were identified using Automated Mass Spectral Deconvolution and Identification System (AMDIS) GC/MS analysis (Version 2.71, June 12, 2012), with the Mass Spectral Search Program National Institute of Standards and

Technology (NIST) (Version 2.0 g, December 4, 2012). The GC/MS spectra of 16 samples were deconvoluted using the freely available AMDIS software, with the following parameters: adjacent peak subtraction = 2; Resolution – High; Sensitivity – Very High; Shape Requirements – Medium. A library of known compounds was compiled within AMDIS using the NIST database as a reference. This method has also been applied to identify tomato headspace volatiles by using the AMDIS library as a reference in our previous work (Caceres et al. 2015).

Volatile data of 4 replicates is presented as a percentage area, relative to the area of the internal standard 2-octanone. To quantify β-ionone emissions, a calibration curve with the following concentrations was made; 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 ng/μl ($R^2 = 0.985$).

Crucifer Flea Beetle Two Chamber Choice Test Adult flea beetles were collected in sweep nets from Canola fields at the LoRDC, AAFC, London, ON, during the summer of 2012. After field collection, crucifer flea beetles were transferred into 50 cm^3 cages, and the day before the experiment 15 insects at a time were gently aspirated into a 50 ml Erlenmeyer flask.

Choice experiments were performed by connecting two volatile collection chambers (Supplemental Fig. 2). Each chamber enclosed a pot containing 8 *Arabidopsis* plants, one with wild type plants and the other *AtCCD1* plants. A trial began when 15 adult crucifer flea beetles were introduced at the center of the bridge between the two chambers, and after 24 hr, the number of insects that had moved into each chamber was counted. Six trials, with new plants each time, were performed at 24 ± 3 °C, 65 ± 5 % RH.

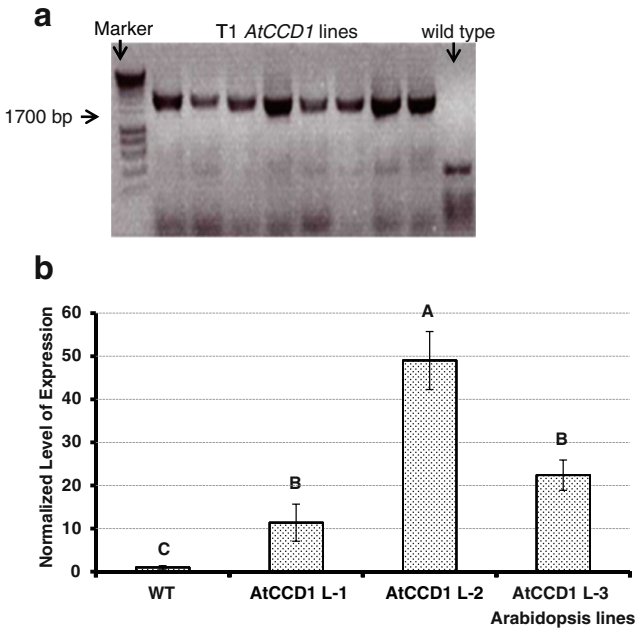

Fig. 2 PCR gel for eight lines of T1 *AtCCD1* (**a**) and the expression level of T3 *AtCCD1* by RTq-PCR (**b**). Error bars represent the standard error of four technical replicates. Bars with different letters are significantly different (One-way ANOVA, Tukey's Test, $P < 0.05$)

Crucifer Flea Beetle Y-tube Olfactometer Test The attraction to solutions of pure standards α-, β-, and dihydro- ionone was conducted with individual adult flea beetles in a Y-tube olfactometer (Supplemental Fig. 3). One hundred microliters of 0.3 ng/μl of apocarotenoid were added into a 10 ml beaker and used as a reference, as this was the average concentration of β-ionone measured in the headspace of two lines of *Arabidopsis* plants overexpressing the *AtCCD1* gene. The inside diameter of the glass Y-tube was 2.8 cm, and the length of the tube from the base to the Y-juncture, was 15.5 cm. A pump (BOC Edwards E2M 1.5, Mississauga, ON, Canada) was used to push air into a charcoal and water trap and then into two flow meters to control the air flow (50 ml/min) into two 500 ml erlenmeyer flasks. One flask contained 20 μl of the apocarotenoid solution in a 10 ml beaker and the other one was empty. Air passed through the flask containing the ionone solution and then through one arm of the Y-tube. Simultaneously, air passed through the empty control flask into the other arm of the Y-tube. Each trial began when an adult flea beetle was placed 8 cm past the junction of the two arms, followed by observations of the direction of movement into either arm over a 5-min period (lab conditions of 22 ± 3 °C, 45 ± 5 % RH). The experiment was repeated three times using ten flea beetles/experiment, and potential left or right turning bias was controlled by switching the positioning of the treatment and control arms after each group of five flea beetles had been tested.

Spider Mite Paper Clamp Bio-assay Solutions of α-, β-, and dihydro-β- ionone in acetone were used to test the response of the adult two-spotted spider mite *Tetranychus urticae* Koch. Spider mites were reared on California red kidney beans *Phaseolus vulgaris* L. plants in a growth room at 24 ± 3 °C, 65 ± 5 % RH and with a photoperiod of 16:8 h L:D at AAFC London. The bioassay was conducted at room conditions of 24 ± 2 °C and 35 ± 5 % RH using a modified procedure (Snyder et al. 1993, 2011) in a sterile fume hood inside a styrofoam box with one open side through which observations could be made. The apparatus consisted of two strips of Whatman qualitative #1 filter paper (1.0 × 0.75 cm), (Sigma-Aldrich, Oakville, ON, Canada) held in a small size paper clamp. At each end of the filter paper, 20 μl of apocarotenoid solution (0.05, 0.1, 0.3, and 0.5 ng/μl) and 20 μl of acetone control were pipetted. An adult spider mite was placed at the middle of the filter paper bridge (1.5 × 0.5 cm) connecting the two papers. For each set of drops, three spider mites were tested on a bridge, and every treatment was repeated ten times with observations of the spider mite movement recorded up to 1 min.

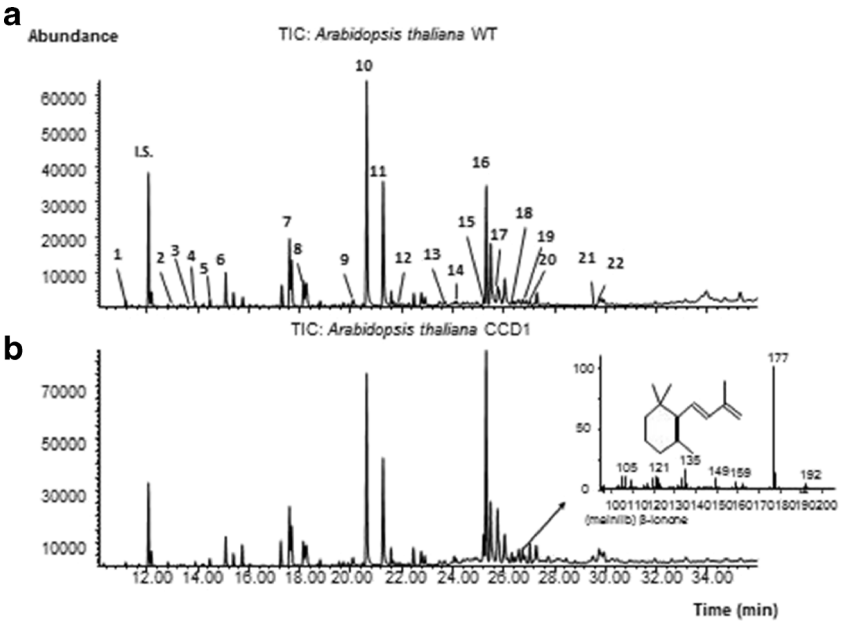

Fig. 3 Volatile profiles of *Arabidopsis thaliana* (ecotype Col-0) at the flowering stage. Volatiles from wild type (**a**) and *AtCCD*1 line 2 (**b**) were detected by dynamic headspace-GC/MS. The figures show the representative TIC obtained with a HP-5MS capillary column. The peak numbering refers to volatile compounds listed in Table 1. Insert shows the mass spectrum from the data base for β-ionone (compound 19)

Silverleaf Whitefly Oviposition Bioassay A colony of silverleaf whiteflies *Bemisia tabaci* Gennadius was reared on greenhouse Poinsettia *Euphorbia pulcherrima* Wild. ex Klotzsch plants at the Vineland Research and Innovation Centre, Vineland, ON. Samples received at AAFC London included approximately 200 adult silverleaf whiteflies that were held in a growth chamber at 24±3 °C, 60±5 % RH, 16:8 L:D until experiments were performed. A Potter Precision Laboratory Spray Tower 120 cm height, 36 cm width and 36 cm depth (Burkard Scientific, Uxbridge, UK) was used to spray detached tomato leaves with the ionone solutions. The tower was operated at 5.0 psi of pressure and calibrated by spraying 5 ml of concentrated (0.05 to 0.5 ng/μl) solutions of α-, β-, and dihydro- β- ionone onto 9.5 cm diam petri dishes. Samples were recovered by washing the petri dishes with five ml of acetone to yield samples that could be injected (2 μl) into a GC/MS port for detection and quantification as previously described. Two detached wild type Micro-Tom tomato *Solanum lycopersicum* cv. leaves (approximately 11 cm^2) were used for the control and treatment as follows: control leaves were sprayed with 5 ml acetone, and the treated leaves were sprayed with 5 ml of an ionone solution. Leaf stems were put into 3 ml rosette vials with water to preserve leaves during the bioassay period. The control leaf, treated leaf, and 20 mixed sex adult whiteflies were held in a 2 L beaker covered with Parafilm "M" laboratory film (Neenah, WI, USA), with several small holes punctured by needle. Beakers were kept in an incubator chamber at 24 ±3 °C, 60±5 % RH and 16:8 L:D for a 24 hr period, and then chilled at 4 °C. The leaves were examined for eggs, and the number on each counted using a stereo microscope (Olympus SZX). The oviposition deterrence index (ODI) was calculated using a formula ODI=[(T-C)/(T+C)]×100, where T is the number of eggs counted on the tomato leaf containing the ionone treatment, and C the number of eggs counted on the leaf treated with acetone (Baldin et al. 2013). The ODI is considered to reflect attraction if the values are positive, or deterrence when values are negative.

Statistical Analysis Statistical differences in the *CCD1* transcript levels, the concentration of β-ionone released by the wild type and transgenic plants and between treatment groups in the herbivore bioassays were determined by one-way analysis of variance (ANOVA) using Proc GLM with Tukey's Studentized Range (TSR) test. Statistical differences for the transformed Asin $(\times/100)^{1/2}$ oviposition deterrence index (ODI) with silverleaf whiteflies were determined by two-way ANOVA. All analyses were performed using the Statistical Analysis System (SAS) (Server Interface Ver. 2.03, SAS Institute, Cary, NC, USA).

Results

Generating Transgenic *Arabidopsis* Lines Overexpressing AtCCD1 Primary transgenic *Arabidopsis* plants were analyzed first by PCR to confirm the presence of the 1700 bp *AtCCD1* transgene (Fig. 2a). These plants then were used to generate homozygous third generation (T3) plants for further experimentation to reduce the influence of segregation. Expression levels of the transgene were determined with qRT-PCR analysis. CCD1 expression was determined in 4-week-old rosette leaves. Three T3 homozygous lines were found to have significantly higher ($d.f.=3,8$; $F=34.59$; $P=<0.001$) *CCD1* transcript levels compared to wild type

(11, 49, and 22-fold higher, respectively) and were chosen for further investigation by volatile analysis.

Volatile Analysis Six-week old flowering *Arabidopsis* plants representing both transgenic and non-transgenic plants released a similar spectrum of volatiles into the chamber's headspace. The Wiley and the NIST database were used to identify 22 VOC in the three lines of the AtCCD1 and the wild type plants, mainly aromatic derivatives, alcohols, monoterpenes, and sesquiterpenes (Fig. 3, Table 1).

One peak that eluted at 26.87 min and that increased the most in the transgenic plants was identified as β-ionone by the database and by an analytical standard. At the flowering stage, transgenic plants released up to 4.5-fold higher amounts of β-ionone relative to wild type plants ($d.f=3$; $F=18.96$; $P<0.001$) (Fig. 4). Of the 22 volatiles detected, 13 were produced at the same level in the WT and AtCCD1 lines. Peak area relative to the internal standard 2-octanone showed that the most abundant components in headspace from both wild type and transgenic plants were the aromatic derivatives 2,4-dimethylacetophenone (**10**) and 4-ethyl acetophenone (**11**). Each compound at similar levels: 2,4-dimethylacetophenone was released from L-1 and L-2 transgenic plants at 1.1 and 1.3 fold-higher than wild type, while L-3 plants released at a 0.94 proportion compared to wild type; 4-ethyl acetophenone, with a relative peak area of 11.3 in wild type headspace, was released by L-1, L-2 and L-3 plants in a 1:1 ratio.

The compounds that were approximately two-fold lower in emission from the transgenic compared to wild type plants included: the aromatic compound ethanone,1-(2,3-dihydro-1H-inden-5-yl) (**13**), β-chamingrene (**20**), isocaryophyllene (**21**), and caryophyllene epoxide (**22**) . In contrast, α-cubebene (**14**) levels were approximately two-fold higher in *AtCCD1* L-3 compared to the wild type, and thujopsene (**17**) was approximately two-fold higher from L-2 and L-3 plants.

Table 1 Relative peak area percentage (± S.E.) of volatile compounds identified by the NIST library with AMDIS of wild type and *CCD1 Arabidopsis thaliana* lines ($N=4$)

No.	RT (min)	Compound	Chemical group	Peak area % relative to internal standard 2-octanone (±SE) $N=4$			
				WT[a]	*AtCCD1* L-1	*AtCCD1* L-2	*AtCCD1* L-3
1	11.13	Benzaldehide	Aromatic	0.65 (0.02)	0.64 (0.09)	0.58 (0.04)	0.76 (0.06)
2	12.99	2,4-Nonadiyene	Un. Hydrocarbon	0.12 (0.01)	tr	0.17 (0.00)	tr
3	13.65	3-undecyne	Un. Hydrocarbon	tr	tr	tr	0.06 (0.04)
4	13.86	Benzene-1,4-diethyl	Aromatic	0.41 (0.03)	0.39 (0.01)	0.41 (0.01)	0.36 (0.03)
5	14.44	Acetophenone	Aromatic	0.79 (0.01)	0.64 (0.04)	0.66 (0.04)	0.61 (0.06)
6	15.05	Benzene-1-methyl-3-ethyl	Aromatic	2.59 (0.01)	1.89 (0.01)	1.92 (0.01)	1.86 (0.03)
7	17.57	Benzaldehide-4-ethyl	Aromatic	5.40 (0.04)	5.39 (1.06)	3.41 (2.06)	4.36 (2.08)
8	18.12	Isoxylaldehyde	Aromatic	2.07 (0.05)	2.53 (0.57)	2.56 (0.57)	2.50 (0.59)
9	20.06	3-cyclohexene-1-ol-5-methylene-6-(methylethenyl)	Alcohol	0.87 (0.07)	0.72 (0.18)	0.75 (0.26)	0.69 (0.02)
10	20.59	Acetophenone-2,4-dimethyl	Aromatic	19.5 (0.02)	12.3 (3.02)	11.3 (4.02)	10.4 (3.05)
11	21.25	Acetophenone-4-ethyl	Aromatic	9.02 (1.05)	11.4 (1.24)	6.4 (0.99)	8.66 (2.37)
12	21.91	3-buten-2-one-4-phenyl	Aromatic	0.33 (0.08)	0.27 (0.01)	0.29 (0.01)	0.24 (0.03)
13	23.67	Ethanone-1-(2,3-dihydro-1H-inden-5-yl)	Aromatic	0.95 (0.16)	0.59 (0.03)	0.61 (0.03)	0.56 (0.05)
14	24.04	α-cubebene	Sesquiterpene	0.70 (0.24)	1.04 (0.02)	1.01 (0.02)	1.05 (0.05)
15	25.20	α-thujene	Monoterpene	0.76 (0.08)	0.51 (0.19)	0.54 (0.19)	0.48 (0.21)
16	25.31	Caryophyllene	Sesquiterpene	9.33 (0.12)	10.0 (1.02)	13.28 (1.02)	9.97 (1.05)
17	25.74	Thujopsene	Sesquiterpene	2.18 (0.30)	2.26 (0.24)	3.28 (0.24)	4.24 (0.26)
18	26.31	Humulene	Sesquiterpene	0.07 (0.02)	0.05 (0.01)	0.08 (0.01)	0.02 (0.03)
19	**26.87**	**β-ionone**	**Apocarotenoid**	**0.43 (0.36)**	**0.56 (0.28)**	**1.02 (0.26)**	**1.26 (0.14)**
20	27.02	β-chamingrene	Sesquiterpene	0.60 (0.04)	0.37 (0.03)	0.39 (0.03)	0.34 (0.05)
21	29.50	Isocaryophyllene	Sesquiterpene	0.19 (0.04)	0.10 (0.03)	0.12 (0.03)	0.17 (0.05)
22	29.73	Caryophyllene epoxide	Sesquiterpene	0.63 (0.04)	0.27 (0.03)	0.29 (0.07)	0.24 (0.09)

Tr Trace; NIST with AMDIS identification

Table shows the apocarotenoid β-ionone in bold

[a] $n=8$

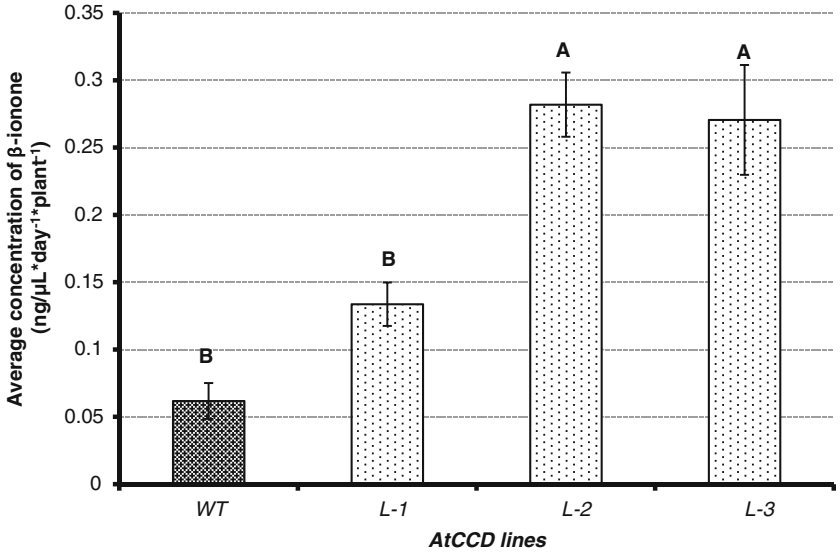

Fig. 4 Emission rates of the apocarotenoid β-ionone (mean ± S.E.) detected in the wild type and *AtCCD* lines from the *Arabidopsis* plants after 24 hr headspace collection. Peak areas obtained from the TIC were divided by the number of plants contained in a pot. Bars with different letters are significantly different (One-way ANOVA, Tukey's Test, $P < 0.05$). $N = 4$

The most abundant sesquiterpene was caryophyllene (**16**) with a relative percentage peak area of 9.33 in wild type and 10.0, 13.3, and 9.97 in the corresponding transgenic lines, or less than a 1.5-fold difference in emission levels. Thujopsene (**17**) was the second most abundant sesquiterpene with a peak are percentage of 2.18 in the wild type, while the *AtCCD1* L-1, L-2, and L-3 were 1, 1.5 and 2-fold higher, respectively. Humulene (**18**) was 3.5 fold-higher in the wild type compared to the L-3, but was found at the same level in the other two lines.

Insect Bioassays

Crucifer Flea Beetle Two Chamber Choice Test The trials completed with the connected two chambers (Supplemental Fig. 2) containing a wild type plant and one of the corresponding transgenic lines showed that a greater number of flea beetles moved towards the wild type rather than to the *AtCCD1* L-2 plants (*d.f.* = *1,10* $F = 16.38$; $P = 0.002$) (Fig. 5). Similar behaviour was observed in the comparison of wild type with *AtCCD1* L-3 plants but was not significantly different (*d.f.* = *1, 10*; $F = 2.26$; $P = 0.16$) for that line or for the *AtCCD1* L-1 (*d.f.* = *1,10*; $F = 1.16$; $P = 0.30$).

Crucifer Flea Beetle Y-tube Olfactometer Test The flea beetles consistently moved towards the control Y-tube arm (air only) rather than toward the arm containing β-ionone (*d.f.* = *1,4*; $F = 52.76$; $P = 0.002$) (Fig. 6). Flea beetles did not show a significant difference in response when given the choice between α-ionone and air (*d.f.* = *1,4*; $F = 1.66$; $P = 0.260$), but more beetles were attracted to the arm with dihydro-β-ionone (*d.f* = *1,4*; $F = 12.84$; $P = 0.023$).

Two Spotted Spider Mite Paper Clamp Bioassay A dose-dependent negative effect was observed with the adult spider mite exposed to β-ionone in the 0.05 to 0.5 ng/μl range (*d.f.* = *3,36*; $F = 3.57$: $P = 0.02$) (Fig. 7). In contrast, there was no significant difference observed with α-ionone (*d.f.* = *3,36*; $F = 0.76$: $P = 0.52$) or dihydro-β-ionone (*d.f.* = *3,36*; $F = 0.50$: $P = 0.68$).

Silverleaf Whitefly Oviposition Deterrence A measure of oviposition deterrence determined that β-ionone acted as a deterrent at 0.05, 0.1, and 0.5 ng/μl treatments, with an average oviposition deterrence index value of −55, −65, and −34, respectively (Fig. 8). The oviposition deterrence index when

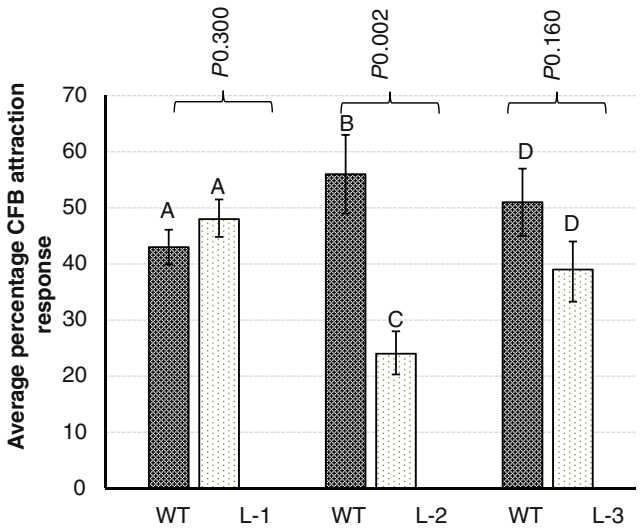

Fig. 5 Average percent crucifer flea beetle attraction response (± S.E.) to wild type plants vs *AtCCD1* L-1, L-2, and L-3 in a two chambers choice test. Bars with different letters are significantly different from the corresponding wild type. (One-way ANOVA, Tukey's Test $P < 0.05$). $N = 6$

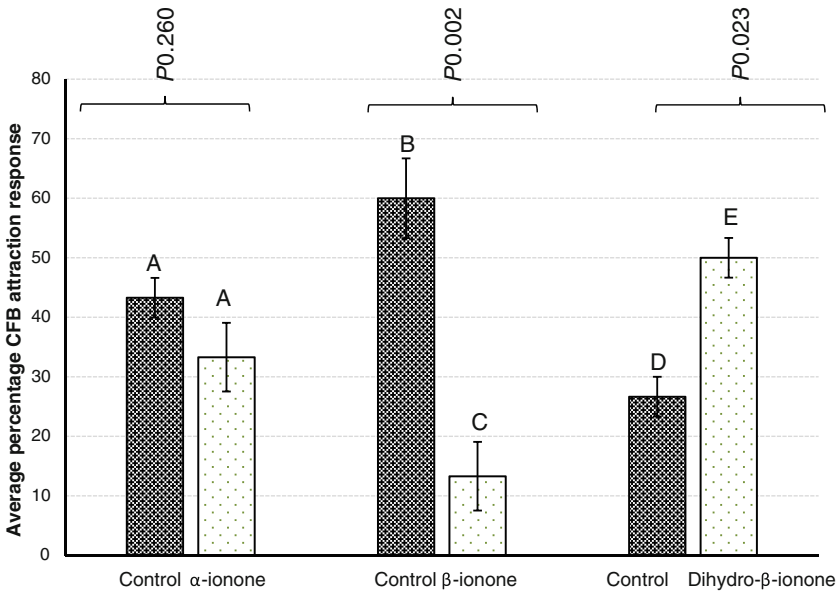

Fig. 6 The average percent (± S.E.) attraction response of crucifer flea beetle to 0.3 ng/µl of three ionone compounds using the Y-tube olfactometer ($N = 3$). Bars with different letters are significantly different from the corresponding control (One-way ANOVA, Tukey's Test, $P < 0.05$). $N = 3$

using β-ionone was significantly different compared to the index obtained when using α-ionone and dihydro-β-ionone (*d.f.* = *2,27*; *F* = *17.04*; *P* = *<0.001*) at each concentration tested. For the 0.05, 0.1, and 0.5 ng/µl ionone concentrations, the average index values observed with α-ionone were −9.5, 12.1, and −10.9, and 9.5, 8.0, and 13.7 with dihydro-β-ionone.

Discussion

Three herbivores were selected for our investigation representing different classes, orders, feeding types, and host plant preferences. The adult crucifer flea beetle, a specialist herbivore (Agrawal and Sherriffs 2001) was chosen because it feeds on Brassicacae crops including mustard, canola, cabbage, and radish, and was affected by Brassica volatiles in previous studies (Gruber et al. 2009; Wei et al. 2011). The silverleaf whitefly, a polyphagous herbivore, having a host range of 600 different plant species (Botha et al. 2007), is an important greenhouse pest that feeds on tomatoes, squash, broccoli, cauliflower, cabbage, melons, cotton, carrots, sweet potato, cucumber, pumpkin, and ornamental plants (Goolsby et al. 2005). The silverleaf whitefly female oviposits 80 to 100 eggs during its lifetime. The third herbivore, the two-spotted spider mite, is a polyphagous generalist (Dermauw et al. 2013), and is considered to be one of the most economically important spider mites, as it has been reported to feed on over 3000 host species (Attia et al. 2013).

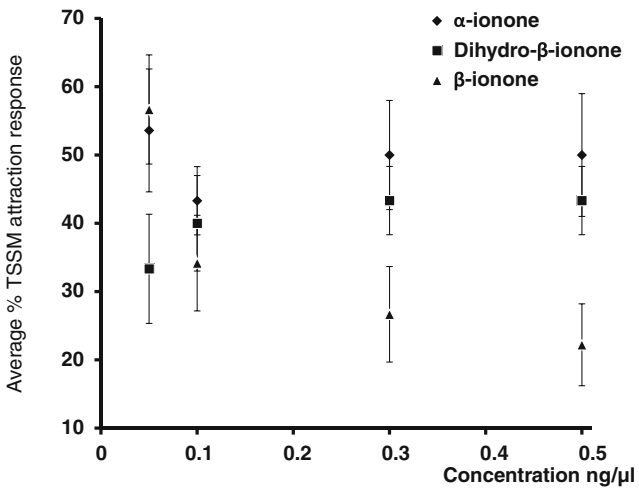

Fig. 7 The average percent (± S.E.) attraction response of two spotted spider mite adults towards α-, β-, and dihydro-β ionone concentrations using a paper clamp bio-assay ($N = 10$ per concentration). A negative dose-dependent effect was observed for β ionone (One way ANOVA, Tukey's Test, $P < 0.05$) but no significant effect ($P > 0.05$) was measured for either α- or dihydro-β ionone in the 0.05 to 0.5 concentration range

The crucifer flea beetle was used in the present study to confirm the earlier findings that *Arabidopsis* deterred feeding of a Brassica specialist (Gruber et al. 2009; Wei et al. 2011) through a repellent action of the headspace VOC, while the two spotted spider mite and the silverleaf whitefly are representative of generalists that will feed or oviposit on *Arabidopsis*, other Brassicaceae, and many other host plants.

The present study evaluated the repellent and attractive effects of VOCs from the headspace of *Arabidopsis* plants that had been genetically modified to overexpress the *CCD1* gene. The volatile compound measured in the *Arabidopsis* headspace that had the most significant increase in concentration due to *CCD1* over-expression was β-ionone. The enhancement of this apocarotenoid compound confirms the action and selectivity of the *AtCCD1* gene in the plants. This compound results from specific oxidative cleavage of the 9,10 and/or 9',10' double bond in the C_{40} β-carotenoid molecule (Wei et al. 2011).

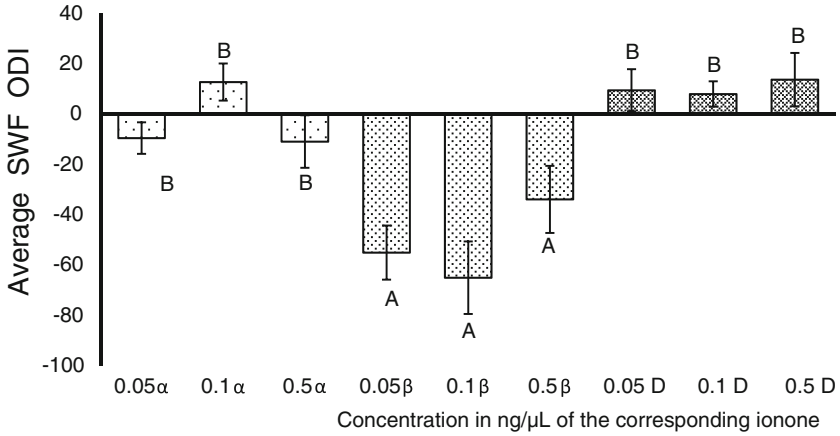

Fig. 8 Average ODI (± S.E.) with 3 concentrations of α-ionone (light shaded bars), β-ionone (*grey shaded bars*), and dihydro β- ionone (*dark shaded bars*) for silverleaf whitefly after 24 hr. Positive values mean attraction, negative values mean deterrence ($N = 3$ per concentration). Means with different letters are significant different (Two-way ANOVA, Tukey's Test, $P < 0.05$)

The VOC blends in both wild type and transgenic plants were very similar in composition (Table 1). Some volatiles (**13**, **20**, **21**, and **22**) were approximately 1.5–2.5 fold-higher in the wild type compared to the transgenic lines, while other volatiles (**14**, **17**, and **19**) were 1.5 and 2.5 times lower in the wild type. These compounds were identified in the headspace of the plants, but none was correlated with the higher repellent activity observed with the *CCD1* line (L-2) that expressed the highest β-ionone levels. To avoid bias from the blend of VOC released by the *Arabidopsis* plants, and to confirm the specific activity of β-ionone, behavioral assays were used to determine whether β-ionone alone caused a similar response to herbivores.

In this work, plant volatile collection was performed with pots containing 7 to 11 plants, but not all plants developed at the same rate, a factor that might have contributed to small variations in particular volatiles. In the case of β-ionone, the quantity was established for use in the bioassay by the total amount of compound released and contained in the headspace. α-Ionone production by the action of the carotenoid cleavage enzymes is half that of β-ionone production (Baldermann et al. 2010; Schwartz et al. 2001) but this does not explain why α-ionone was not detected as part of the headspace in the *Arabidopsis* plants. α-Ionone can be produced *in vitro* by the CCD cleavage of either α- or δ-carotene (Vogel et al. 2008), but was detected *in vivo* only in the orange-fleshed tomato fruit where δ-carotene accumulates (Lewinsohn et al. 2005). This suggests it may not be released as a volatile by *Arabidopsis*, but rather is stored in other tissues.

When crucifer flea beetles were allowed to move between wild type and *AtCCD1* plants in the two chamber choice test, the strongest response was to move away from the *AtCCD1* L-2 plants (Fig. 5). This line had the highest transcript level of *CCD1* gene (Fig. 2b), and as a consequence, the highest level of β-ionone, previously determined to act as a feeding deterrent for the flea beetles (Wei et al. 2011). Two other compounds with similar structure (α-ionone and dihydro- β-ionone) were tested at the same time in order to determine whether the observed response is structure-specific. Although this does not eliminate other plant volatiles as factors in the observed deterrence, it does examine the role of specific apocarotenoids in the plant-insect interactions. The use of single compounds tested their repellant and attractive effects in order to direct the future manipulation of the carotenoid pathway and its products for beneficial effects in insects. The Y-tube olfactometer allowed us to test β-ionone at concentrations measured in the headspace of transgenic plants, and demonstrated a significant preference by the crucifer flea beetle for the arm with air only, rather than air containing β-ionone. This not only corroborated the behavior observed in the *Arabidopsis* plant choice test, but demonstrated the clear distinction between structurally similar analogues, α-ionone and dihydro-β-ionone, at the same concentration (Fig. 6). When similar experiments were performed previously in a vertically oriented, narrow-bore glass, T-shaped olfactometer to test the crucifer flea beetle behavior with a series of volatiles found in canola *Brassica napus* variety AC Excel, the strongest response was observed with β-ionone compared to E-caryophyllene, indole, (±)-linalool, (+)-limonene, E-geraniol, and (−)-β-pinene) (Gruber et al. 2009).

Static choice tests like the paper clamp bioassay are useful in that they rely on diffusion to transport the test sample toward the test organism. The repellent effect of volatiles to the two spotted spider mite was observed when mites were in close proximity to plant leaves treated with each compound (Snyder et al. 2011). The response of the spider mite was similar to the crucifer flea beetle in that the β-ionone concentrations were repellent while α-ionone and dihydro-β-ionone were neutral or slightly attractive. This is the first evidence that β-ionone has a repellent effect against the spider mite *T. urticae*. However, other studies have demonstrated that the red-legged earth mite *Halotydeus destructor* Tucker fed less on *Trifolium glanduliferum* Boiss extracts that had high levels of β-ionone and other terpenes (Wang et al. 2005).

These results suggest that β-ionone could be an important pest management tool for the control of the two spotted spider mite, which is adapted to a wide variety of environments and has developed resistance to many pesticides (Grafton-Cardwell et al. 1987). In this research, we showed that dihydro-β-ionone and α-ionone had attraction and neutral effects, respectively, towards crucifer flea beetle and two spotted spider mite. However, uncertainty lies in whether the transgenic plants would produce volatiles like β-ionone that could also repel the third trophic level, a focus for future research.

This is the first report of an oviposition deterrent effect of β-ionone against the silverleaf whitefly and an attraction effect of the analogous α-ionone and dihydro- β-ionone. Studies of silverleaf whitefly oviposition on tomato leaves have demonstrated that the essential oils of *Artemisia camphorata* Vill., *Ageratum conyzoides* L., *Foeniculum vulgare* Mill., *Lippia alba* Mill., *Plectranthus neochilus* Schltr., and *Tagetes erecta* L. have repellent and oviposition-deterrent effects (Baldin et al. 2013). (E)-Caryophyllene (30.67 %) and the monoterpenes α-pinene (15.02 %) and α-thujene (11.70 %) were identified as the major constituents of the essential oil of *P. neochilus*, but no carotenoid derivatives were present. It has been demonstrated that oil extracts from plant sources can significantly affect oviposition and can be used as a strategy to control pests. However, this procedure requires extraction by solvents and application of the extract onto the leaves, which is impractical, especially for field trials. Since the silverleaf whitefly is an important pest in soybeans *Glycine max* L., and because current approaches for management rely heavily on development of more resistant crops (Perring et al. 1993), the future development of crops that over-express CCD1 or other carotenoid pathway genes may improve the plant resistance through oviposition deterrence. The ability to manipulate the release of chemical volatiles, including apocarotenoids, as a means of managing plant-insect interactions could provide a more sustainable natural pest control that reduces reliance on synthetic chemical pesticides. These strategies are expected to be most effective for crops grown under greenhouse conditions due to the ability to concentrate volatiles in a controlled environment.

In summary, in this work, 22 VOC were detected in the *Arabidopsis* using a non-targeted analysis with a headspace-GC/MS method. Transgenic plants over-expressing the AtCCD1 had similar profile of VOC compared to the wild type except that the AtCCD1 released higher amounts of β-ionone. This correlates with the action of the gene in the enhancement of the apocarotenoid compound. We demonstrated that enhancement of the apocarotenoid β-ionone significantly increases the repellant effect of *Arabidopsis*. The work with standards of α-ionone, β-ionone, and dihydro-β-ionone, further confirmed that the repellent effect against three herbivores was due in large part to the β-ionone and not the other volatiles present in the head space of the plants. In the future, modified plants producing natural compounds with repellent or attractive effects can be exploited to reduce reliance on synthetic pesticides.

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