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ORIGINAL PAPER

Characterization of safranal-induced morphological changes and salt stress alleviation in lettuce seedlings

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Abstract

Volatile organic compounds of plant origin can be used to develop biostimulants and plant-growth regulators. While screening volatile compounds from several plant species, safranal (2,6,6-trimethylcyclohexa-1,3-diene-1-carbaldehyde), an apocarotenoid produced by saffron (*Crocus sativus* L.), effectively enhanced primary root (PR) elongation in lettuce (*Lactuca sativa* L. cv. Legacy) seedlings. Therefore, we aimed to examine the effects of exogenous safranal treatment on the morphological and physiological characteristics of lettuce seedlings. The data showed that, three days after treatment with 19 µmol/L safranal in the dark, lettuce seedlings showed a marked increase in PR length, whereas hypocotyl growth was suppressed. Based on transcriptomic and qRT-PCR analyses, a total of 114 gene expression alterations due to safranal application were detected, including upregulations of ethylene and abscisic acid (ABA) biosynthesis genes. Additionally, safranal promoted ethylene production in both roots and hypocotyls; however, $AgNO_3$, an ethylene-signalling inhibitor, did not affect PR or hypocotyl length after safranal treatment. Safranal slightly increased root ABA content. Exogenous treatment with 1 µmol/L fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl] pyridin-4(1 H)-one), a carotenoid-biosynthesis inhibitor, did not significantly affect safranal-induced changes in PR or hypocotyl length. Safranal alleviated NaCl (50 mmol/L)-induced inhibition of PR elongation and cell death in lettuce seedlings. Moreover, it mitigated the NaCl-induced increase in root O_2^- and H_2O_2 content. Altogether, our findings suggest that exogenous safranal regulated PR and hypocotyl lengths. These effects seemingly occur through mechanisms independent of ethylene, ABA, or reactive oxygen species. Safranal apparently alleviated salinity stress by reducing oxidative damage.

Keywords Apocarotenoids · Safranal · Volatile organic compounds (VOCs) · Salinity stress

Abbreviations

ABA	Abscisic acid
ACC	1-aminocyclo- propane-1-carboxylic acid
ACS	1-aminocyclo- propane-1-carboxylic (ACC) synthase
DXS	Deoxy-D-xylulose 5-phosphate synthase
DMSO	Deoxy-D-xylulose 5-phosphate synthase
DXS	Dimethyl sulfoxide
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
FW	Fresh weight
LR	Lateral root
MEP	2-C-methyl-D-erythritol-4-phosphate
NCED	9-*cis*-epoxycarotenoid dioxygenase
PGR	Plant growth regulator
PR	Primary root length
ROS	Reactive oxygen species
SLs	Strigolactones
VOCs	Volatile organic compounds

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Introduction

Abiotic stresses, such as extreme temperature, drought, and salinity, are significant factors that decrease crop yield (Savvides et al. 2016). Especially, salinity stress limits crop yield in 20% of cultivated areas (Shrivastava and Kumar 2015). NaCl, a soil saline, is highly soluble in water and induces multiple stresses in plants (Munns and Tester 2008; Fu et al. 2019). NaCl-induced stresses (e.g., osmotic, ionic, and oxidative stress) inhibit cell division, elongation, and cell wall hardening in roots (Neumann et al. 1994; Jiang et al. 2016), following the delayed formation of root architecture (Liu et al. 2015; Jiang et al. 2016; Fu et al. 2019).

An increase in food production by approximately 50% is required because of global population growth, according to a Food and Agriculture Organization report (FAO 2017). However, crop yields have already reached a plateau in conventional methodology; the rates of yield increase have declined rapidly in rice of eastern Asia and wheat of northwest Europe, which accounts for 30% of total global grain production (Grassini et al. 2013). Modern agriculture requires further technologies to alleviate abiotic stress and increase crop yield.

Low-molecular-weight secondary metabolites produced by plants often exhibit allelopathic activity and affect multiple stressors, including pathogens, herbivores, and other plants (Quiroga et al. 2001; Baldwin et al. 2006; Ramakrishna and Ravishankar 2011). Plant-derived volatile organic compounds (VOCs) are used for communication between plants and other living organisms because their physical properties allow them to reach remote locations quickly (Heil and Silva Bueno 2007). Several recent reports have suggested that plant-derived VOCs play a role in the stress response and plant development (Loreto and Schnitzler 2010; Velikova et al. 2011; Brilli et al. 2019). One plant-derived VOC, *trans*-2-hexenal, alleviates heat stress (Yamauchi et al. 2015) and has been commercialized as Suzumidori®. Exogenous treatment with (*E*)-2-hexenal induces the gene expression of heat shock proteins, which stabilize protein conformation and enhance heat stress tolerance. Another plant-derived VOC, sesquiterpene β-caryophyllene (*1R*,*4E*,*9 S*)-4,11,11-trimethyl-8-methylidenebicyclo [7.2.0] undec-4-ene), directly interacts with the transcriptional repressor TOPLESS and induces the expression of defense response genes (Nagashima et al. 2019). Additionally, β-caryophyllene promotes root development (Minerdi et al. 2011). Thus, plant-derived VOCs play an important role in plant survival and could be utilized as novel plant growth regulators (PGRs) and biostimulants in the future. However, the biological functions and modes of action are not fully understood (Sako et al. 2021).

In this study, we screened several plant species that produce VOCs, with root growth-promoting activity and examined the effects of individual VOCs on root growth. In particular, we found that safranal (2,6,6-trimethylcyclohexa-1,3-diene-1-carbaldehyde) produced by saffron (*Crocus sativus* L.) showed high activity in promoting primary root (PR) elongation in lettuce (*Lactuca sativa* L. cv. Legacy) seedlings. However, safranal effects on plant growth and abiotic stress remain poorly understood. Therefore, we conducted experiments to characterise the regulatory effects of safranal on lettuce root and hypocotyl elongation, and salt stress-induced damage.

Materials and methods

Plant material and treatment condition

Lettuce (*L. sativa* L. cv. Legacy; Takii Co., Kyoto, Japan) seeds were stored at 4 °C in the dark until use. They were placed on filter paper (ADVANTEC, Tokyo, Japan) in 90 mm Petri dishes, and 3 mL of distilled water was added. The seeds were incubated at 22–25 °C for 18–36 h in the dark. Five pregerminated seeds were transferred into a 30 mL glass vial (AZ ONE, Tokyo, Japan) with filter paper followed by the addition of 450 µL of distilled water. Safranal (Toronto Research Chemicals, North York, Ontario, Canada), isophorone (3,5,5-Trimethylcyclohex-2-en-1-one), or 4-ketoisophorone (2,6,6-Trimethyl-2-cyclohexene-1,4-dione) was diluted in hexane (FUJIFILM Wako Pure Chemical, Osaka, Japan). Ten microliters of each authentic compound diluted with hexane was spotted onto a piece of filter paper (1 × 4 cm) using a syringe (SGE Analytical Science, Ringwood, Victoria, Australia), and then the paper was immediately shaken 10 times to evaporate hexane. For the authentic compound-untreated control, 10 µL of hexane was spotted on a filter paper and the filter paper was immediately shaken 10 times to evaporate the hexane, as described above. The filter paper was sandwiched between a silicone rubber plug and glass vial, which was tightly sealed with an aluminum cap.

Safranal concentrations in the glass vials were calculated assuming that these chemicals completely volatilized from the filter papers because the residual safranal concentration in the filter paper was very low (< 1.2% or 4%, respectively) under these experimental conditions when it was measured using high performance liquid chromatography.

Root and shoot growth assay

Lettuce seedlings were treated with 0, 2, 9, 19, 47, and 94 µmol/L safranal for 3 d at 25 °C in the dark as described in

Sect. 2.1. Thereafter, the lengths of the PR and hypocotyls of the seedlings were measured. Bioassays were performed to evaluate root growth under salinity stress conditions. Lettuce seedlings were treated with safranal (0 or 19 µmol/L) and subsequently grown in 50 mmol/L NaCl aqueous solution for three days at 25 °C under continuous dark or light conditions (50–75 $\mu mol \cdot m^{-2}s^{-1}$). PR length and shoot fresh weight (FW) were determined under light conditions, whereas PR and hypocotyl length were measured in the dark.

Evans blue staining

Cell viability was evaluated using Evans blue staining (Tamás et al. 2004; Sunohara and Matsumoto 2008). Pregerminated lettuce seeds were co-treated with 19 µmol/L safranal and 50 mmol/L NaCl for 3–5 d at 25 °C under light or dark conditions as described in Sect. 2.1. All parts of the roots were taken from the treated lettuce seedlings and stained with a 0.25% (w/v) aqueous solution of Evans blue (Fujifilm Wako Pure Chemicals, Osaka, Japan) for 1 h at room temperature. Stained roots were washed twice with distilled water for 15 min. Morphological observations were performed using BZ-X710 (KEYENCE, Osaka, Japan). The Evans blue taken into the lettuce roots was extracted using 500 µL of dimethyl sulfoxide (DMSO, FUJIFILM Wako Pure Chemical) for 2 d in the dark at 25 °C. The absorbance of Evans blue was measured at 600 nm.

Transcriptome analysis

Pregerminated lettuce seeds were treated with 0 or 19 µmol/L safranal for 2 d, as described in Sect. 2.1. After sampling 75 individuals from safranal-treated or untreated seedlings, the lettuce roots were cut using a scalpel (Kai Industries Co., Gifu, Japan) and ground using a pestle in a mortar containing liquid nitrogen. Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA-seq with no biological replicates was performed at the Biotechnology Research Institute (Tokyo, Japan) for next-generation sequencing (NextSeq 500; Illumina, San Diego, CA, USA). RNA-seq data were deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive, under accession number DRA012238. The RNA-seq reads were mapped to the lettuce reference genome (*L. sativa* cv. Salinas (v8.0) using STAR (Dobin et al. 2013). Genome and annotation data were downloaded from CoGe (https://genomevolution.org/coge/). The mapped data were quantified using featureCounts (Liao et al. 2014) to generate gene expression counts. Differential gene expression analyses were performed using the EdgeR, Bioconductor package (Robinson et al. 2010). Differences between the safranal-treated and non-treated samples were considered significant when the fold change was ≥2. MapMan bin codes were used to create functional classifications (Usadel et al. 2005).

Quantitative RT-PCR (RT-qPCR) analysis

Germinated lettuce seeds were treated with 0 or 19 µmol/L safranal for 2 d, as described in Sect. 2.1. Root segments were excised from the seedlings, as described in Sect. 2.4. The roots were ground vigorously using a mortar and pestle containing liquid nitrogen, and total RNA was extracted using the Plant Total RNA Extraction Miniprep System (Viogene, Taipei, Taiwan) according to the manufacturer's protocol. The concentration and quality of the RNA were determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was diluted in distilled water to 1 µg/µL, and Oligo(dT)20 primer (TOYOBO, Tokyo, Japan) was added. Heat denaturation was performed at 65 °C for 5 min using a thermal cycler (2720 Thermal Cycler, Applied Biosystems, Foster City, CA, USA), and the samples were cooled on ice for 5 min. A master mix was prepared using dNTPs mixture, 5×RT Buffer, RNase Inhibitor, and ReverTra Ace (TOYOBO) and added to the samples. The cDNA was synthesized under the following conditions: 42 °C for 60 min and 99 °C for 5 min. The primers were purchased from FASMAC (Kanagawa, Japan). The genome sequence was based on the Lettuce Genome Resource (https://lgr.genomecenter.ucdavis.edu/), and primers were designed using Primer3Plus (https://primer3plus.com/). Online Resource, Table S1 shows the sequences of the primers used in this experiment. Each primer and THUNDERBIRD SYBR qPCR Mix (TOYOBO) were added to the cDNA, and qRT-PCR was performed using a real-time PCR machine (CFX Connect Real-time PCR Detection System, Bio-Rad, Hercules, CA, USA) as follows: 95 °C for 3 min, 95 °C for 10 s, and 55 °C for 30 s, 39 cycles. The gene encoding actin (GenBank ID: LOC 111889264) was used as the internal standard. Fold-change values were calculated using the ΔΔCt method (Pfaffl 2001).

Quantification of ethylene

Ethylene was quantified as previously reported (Sunohara et al. 2010). Lettuce seedlings were grown as described in Sect. 2.1. Thereafter, the roots and hypocotyls were separated from the seedlings and transferred into 5 mL glass vials containing filter paper. A 150 µL volume of distilled water (DW) was added to the filter paper. The amount of DW added to the vial was the minimum amount of water needed to keep the tissue moist and not completely submerged. The

glass vials were then tightly sealed using a silicone rubber plug and aluminum cap. After the glass vials were incubated at 25 °C for 24 h in the dark, 1 mL of gas in the headspace of the vials was analyzed by gas chromatography (GC, GC-17 A, Shimadzu, Kyoto, Japan). This experiment was conducted under the following conditions: detector; FID, carrier gas; N_2, at a flow rate of 50 mL/min, column; glass column (2.6 mm × 2 m) packed with Unipak S (GL Science, Tokyo, Japan), column temperature; 60 °C, injection temperature; 120 °C, and detector temperature; 120 °C. An ethylene standard was used to construct calibration curves.

Treatment with the ethylene signaling inhibitor

To evaluate the involvement of ethylene in safranal-induced PR elongation, the ethylene signaling inhibitor, $AgNO_3$ (FUJIFILM Wako Pure Chemical), was used (Bradford et al. 1982; Kumar et al. 2009). Germinated lettuce seeds were co-treated with $AgNO_3$ (0.5 µmol/L) and safranal (0 or 19 µmol/L). After the seedlings were grown for 3 d at 25 °C in the dark, the PR and hypocotyl lengths were measured.

Treatment with the carotenoid-biosynthesis inhibitor

To evaluate the involvement of endogenous abscisic acid (ABA) in safranal-induced PR elongation, the carotenoid-biosysnthesis inhibitor, fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]pyridin-4-one, FUJIFILM Wako Pure Chemical) was used to decrease endogenous ABA content (Gamble and Mullet 1986; Rasmussen et al. 2012). Fluridone was diluted with DMSO, and germinated lettuce seeds were co-treated with fluridone (1 µmol/L) and safranal (0 or 19 µmol/L). The final concentration of DMSO was 0.1% (v/v). After the seedlings were grown for 3 d at 25 °C in the dark, the PR and hypocotyl lengths were measured.

Quantification of ABA

The amount of endogenous ABA was quantified using previously reported methods (Chiwocha et al. 2003; Pan et al. 2010) with some modifications. Approximately 100 mg of 3-day-old lettuce roots treated with 0 or 19 µmol/L safranal and grown in the dark, as described in Sect. 2.1, was frozen in liquid nitrogen and ground vigorously. Methanol (350 µL) was added to the sample powder, which was then permeated using a mixer (MT-360, Tomy Seiko, Tokyo, Japan). After centrifugation (15000 rpm, 10 min), 200 µL of the supernatant was diluted in 2 mL of 1% (v/v) formic acid (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and 5 µL of 250 ppb (+)-[$^2H_6$] ABA (d6-ABA, OlChemIm sro, Olomouc, Czech Republic) was added as an internal standard. The sample solution was loaded onto an InertSep® C18 column (filler volume; 100 mg, particle size; 45 µm, GL Sciences) equilibrated with 1% (v/v) formic acid and eluted with 1 mL of acetonitrile (Nacalai Tesque). The extracted solution was dried with nitrogen gas, and the sample was dissolved in 30 µL of methanol. Endogenous ABA was quantified using a high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Nexera HPLC system coupled with an LC-MS-8045, Shimadzu) equipped with COSMOSIL 2.5C_{18}-MS-II column (75 × 2.0 mm i.d., particle size; 2.5 µm, Nacalai Tesuque) under the following conditions: Mobile phase A; 0.1% (v/v) formic acid in water, Mobile phase B; acetonitrile, 10–60% linear gradient of B for 8 min at a flow rate of 0.4 mL/min, and column oven; 40 °C. Mass spectrometry was simultaneously performed under negative-ion mode via electrospray ionization (interface voltage; 3 kV, interface temperature; 300 °C, DL temperature; 250 °C, heat block temperature; 400 °C, nebulizing gas; 3 L/min, drying gas; 10 L/min, and heating gas; 10 L/min). ABA and d6-ABA ions were detected by multiple reaction monitoring as follows: *m/z* 263 $[M-H]^- \rightarrow$ 153 (Q1 pre bias; 13 V, collision cell energy [CE]; 10 V, Q3 pre bias; 29 V) and *m/z* 269 $[M-H]^- \rightarrow$ 159 (Q1 pre bias; 13 V, CE; 10 V, Q3 pre bias; 29 V). The amount of ABA was estimated from the peak area and concentration ratios using a standard curve obtained from authentic ABA (Combi-Blocks, San Diego, CA, USA) and d6-ABA.

Oxidation of epinephrine by O_2^-

The oxidation of epinephrine by O_2^- was measured as previously reported (Krasuska et al. 2016), with some modifications. Lettuce roots treated with 19 µmol/L safranal and/or 50 mmol/L NaCl were frozen in liquid nitrogen and ground to a fine powder. The powder was homogenised with 500 µL of 2% polyvinylpolypyrrolidone (w/v) (Nacalai Tesque) in Tris-HCl (pH 7.5). After centrifugation (12000 g, 20 min), 350 µL of the supernatant, 350 µL of reaction solution (50 mM Tris-HCl, pH 7.5), and 350 µL of 15 mM epinephrine (Sigma-Aldrich) were mixed. The subsequent decrease in epinephrine as it became oxidised to adrenochrome by O_2^- was measured at 480 nm. (E = 4.02 mM^{-1} cm^{-1}).

Quantification of H_2O_2

The amount of H_2O_2 was measured as described previously (Jana and Choudhuri 1982), with some modifications. Lettuce roots treated with 19 µmol/L safranal and/or 50 mmol/L NaCl were frozen in liquid nitrogen and ground to a fine powder. The powder was homogenized with 500 µL of potassium phosphate buffer (pH 6.5). After centrifugation (6000 g, 25 min), 390 µL of supernatant was mixed with

130 µL of 0.1% (v/v) titanium chloride (FUJIFILM Wako Pure Chemical) in 20% (v/v) H_2SO_4. The mixture was centrifuged again at 6000 g for 25 min, and the absorbance of H_2O_2 was measured at 410 nm ($E = 0.28\ \mu M^{-1}\ cm^{-1}$).

Statistical analysis

Except for transcriptome analysis, all experiments were performed at least in triplicate. Transcriptome analysis was conducted once with no replicates. Statistical analysis was performed using the multcomp package (Hothorn et al. 2008) in R (version 4.04), and differences were analyzed using Student's *t*-test, Welch's *t*-test, Dunnett's test, Dunnett's T3 test, Tukey-Kramer test, or the linear mixed model. Differences were considered statistically significant at $P < 0.05$.

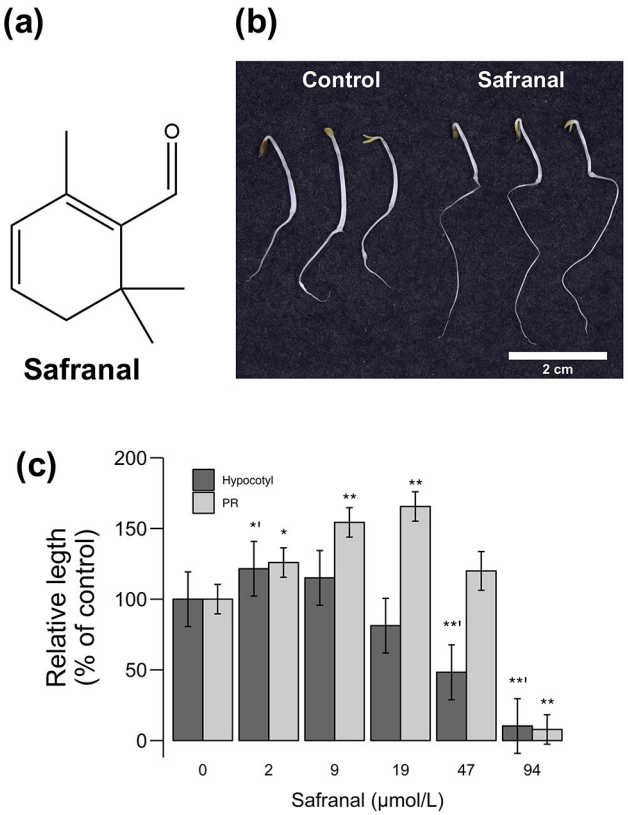

Fig. 1 Effects of safranal on lettuce seedling growth. (**a**) Chemical structure of safranal; (**b**) Untreated (control) and safranal-treated (19 µmol/L, 3 d in the dark) lettuce seedlings; (**c**) Effects of safranal on PR and hypocotyl length in lettuce seedlings, at 3 d after treatment in the dark. Error bars indicate 95% confidence interval for difference, $*P < 0.05$, $**P < 0.01$ (the linear mixed model)

Results and discussion

Effect of safranal on seedling growth

First, we screened plant species producing VOCs with plant growth-promoting activities, using the dish-pack method described by Fujii et al. (2005). The results showed that volatiles generated from powdered samples (1 g FW) of saffron (*Crocus sativus* L.), *Curcuma longa* L., and *Coriandrum sarivum* L. contain compounds that promote PR elongation in lettuce seedlings 3 days after treatment in the dark (Online Resource, Table S2). Volatile emissions from saffron pistils increased PR elongation by up to 33%, 46%, and 33% in lettuce seedlings incubated in wells at 5.8, 8.2, and 9.2 cm away from the saffron-supplement wells, respectively. Gas chromatography-mass spectrometry (GC-MS) detected isophorone, 4-ketoisophorone, 2,6,6-trimethyl-1,4-cyclohexanedione, and safranal as candidate volatiles in saffron pistils (Online Resource, Fig. S1).

Safranal is one of apocarotenoids known to act as PGRs, similar to ABA or strigolactones (SLs) (Online Resource, Fig. S5), which are biosynthesized via the cleavage of carotenoids. Safranal has an α-β unsaturated carbonyl structure. The α-β unsaturated carbonyl structure is known to be highly reactive with protein thiol groups via a Michael addition type of reaction (Amslinger 2010), and one of compounds with such structure reportedly to have strong biological activity (Izumi et al. 2021). Further, safranal allegedly inhibits plant growth by inducing the overproduction of reactive oxygen species (ROS) in lettuce seedlings and protoplasts (Mardani et al. 2019; Mardani-Korrani et al. 2020). However, detailed effects of safranal on plant growth have not yet been reported.

Therefore, in the next experiment, the effects of the authentic compounds on lettuce seedling growth were also evaluated. In lettuce growth in the dark, 2, 9, and 19 µmol/L safranal treatments promoted PR length, while 94 µmol/L safranal treatment shortened PRs (Fig. 1c). The most effective concentration of safranal to promote PR elongation was 19 µmol/L, with the PR about 60% longer than control seedlings. In lettuce hypocotyls, safranal treatment with 2 µmol/L promoted hypocotyl length by about 20%, but there was a concentration-dependent trend toward shorter hypocotyls at concentrations above 9 µmol/L, with 47 or 94 µmol/L safranal treatment significantly shorter (Fig. 1c). These results indicate that under these experimental conditions, the effective concentration ranges of safranal that promote PR and hypocotyl length differ, and that its effect on PR length is higher than that on hypocotyl length. In contrast to safranal, isophorone or 4-ketoisophorone did not increase PR and hypocotyl length in lettuce seedlings 3 days after treatment in the dark (Online Materials, Fig. S2).

ABA, one of the apocarotenoids as well as safranal, is crucial for plant adaptive response to biotic and abiotic stress conditions, regulation of water uptake, dormancy, and stomatal closure (Dodd 2013; Merilo et al. 2015; Tuan et al. 2018). Furthermore, another apocarotenoid, SLs also play important roles in the root architecture and shoot branching (Xie et al. 2010; Saeed et al. 2017). Recently, three additional apocarotenoids have been identified as positive regulators of root growth, namely zaxinone (Wang et al. 2019, 2020; Ablazov et al. 2020), anchorene (Jia et al. 2019), and β-cyclocitral (2,6,6-trimethylcyclohexene-1-carbaldehyde) (Dickinson et al. 2019). Therefore, we hypotheseized that safranal may be a new PGR among apocarotenoids; hence, here, we aimed to examine safranal-induced morphological and physiological changes in plants.

The effects of safranal on the root growth of maize (*Zea maize*) and Arabidopsis (*Arabidopsis thaliana*) seedlings were also examined. In maize, safranal tended to increase the number of lateral roots (LRs), although the effect was not significant (Online Resource, Fig. S3b). Meanwhile, in Arabidopsis, safranal (6 µmol/L) significantly enhanced LR development (Online Resource, Fig. S4b), although PR elongation was not affected (data not shown). These results suggest that safranal acts as a positive regulator of root growth in various plant species. In lettuce seedlings, the promotion of PR elongation was most effective at a concentration of 19 µmol/L (Fig. 1b and c). Therefore, we focused mainly on the roots after 19 µmol/L safranal treatment to investigate physiological changes in the following experiments.

Characterization of safranal-induced altered gene expression in lettuce roots

To gain a broad understanding of how safranal regulates plant growth, transcriptome analysis was performed using safranal-treated lettuce roots. In all, 114 gene expression alterations were detected in lettuce roots at 48 h after safranal (19 µmol/L) treatment (Online Resource, Table S3); the expression levels of 75 genes were upregulated, whereas those of 39 genes were downregulated. When these genes were classified by function, the number of genes related to hormone metabolism was the highest (eight genes) among the 114 genes whose expression was altered as a result of safranal treatment (Fig. 2). Among plant hormone biosynthesis-related genes, those involved in ethylene and ABA biosynthesis were found among the upregulated genes. The expression levels of *1-aminocyclo-propane-1-carboxylic (ACC) synthase* (*ACS*), which encodes a key enzyme in ethylene biosynthesis (Chae and Kieber 2005), and *9-cis-epoxycarotenoid dioxygenase 3* (*NCED3*), which encodes the rate-limiting enzyme in ABA biosynthesis (Seo and Koshiba 2002), were upregulated (Online Resource, Table S3). In addition, we focused on the transcriptional changes of *ACS* and *NCED3* in the RNA-seq analysis and performed qRT-PCR to confirm the results and to quantify the transcript levels. When 19 µmol/L safranal was applied to lettuce seedlings for 48 h, the transcript levels of *ACS* and *NCED3* were increased 18.5- and 1.5-fold, respectively (Online Resource, Fig S6). These results suggest that safranal regulates biosynthesis of ethylene and ABA. Regarding other plant hormone-related genes, *auxin efflux carrier component5-like (PIN5)*, which regulates auxin flux, was

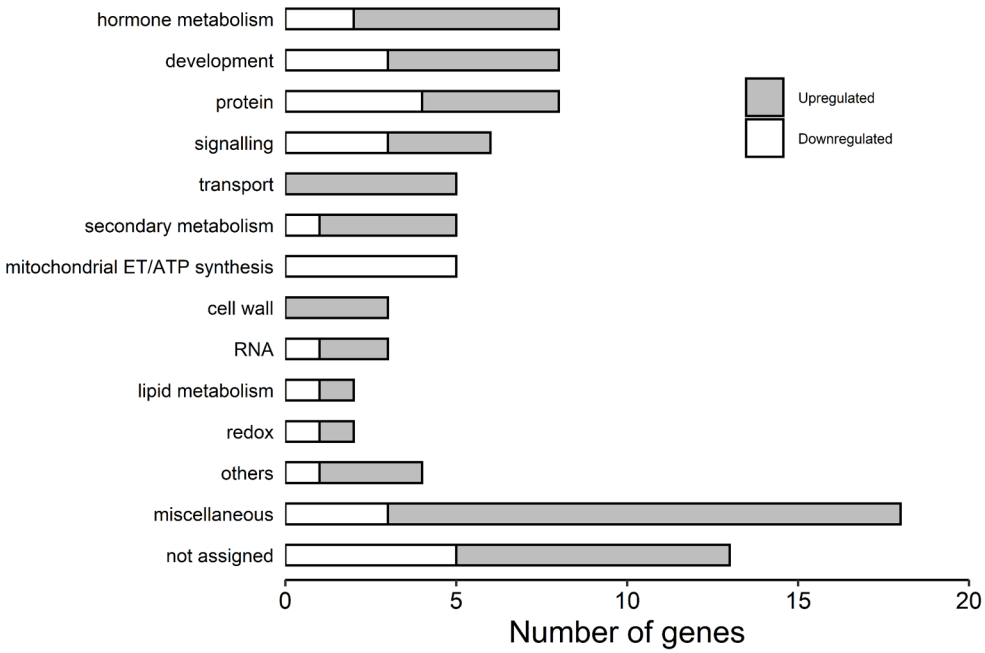

Fig. 2 Functional classification of altered gene expression in lettuce roots treated with safranal. Total RNA was extracted from lettuce roots 48 h after safranal treatment (19 µmol/L). Functional classification of the identified genes was performed using the MapMan bin code (http://mapman.gabipd.org/)

suppressed after safranal treatment. It has been reported that the PIN5 mutation did not alter root growth (Di et al. 2023). Furthermore, β-cyclocitral, a structural analogue of safranal, reportedly promotes auxin-independent PR elongation in Arabidopsis (Dickinson et al. 2019). Therefore, in subsequent experiments, we focused on the relationship between ethylene or ABA and safranal-induced PR elongation.

Effect of safranal on ethylene production

To examine the relationship between safranal-induced morphological changes and ethylene biosynthesis, ethylene production was quantified in safranal-treated lettuce seedling. Although this ethylene measurement may underestimate the amount of ethylene produced from the tissue because small amount of water was added to the vial to prevent tissue drying, under these experimental conditions, ethylene production increased by approximately 2- and 1.3-fold in the roots and hypocotyls of lettuce seedlings, respectively, after 3 d of safranal treatment in the dark (Fig. 3a). These results indicate that safranal enhanced ethylene biosynthesis in lettuce seedlings.

Ethylene regulates PR development via crosstalk with other hormones, such as auxin and brassinosteroids, which are significant factors in determining PR length (Qin et al. 2019). A tight coordination between ethylene and these hormones also regulates hypocotyl growth (De Grauwe et al. 2005). In this study, safranal inhibited hypocotyl elongation in lettuce grown in the dark (Fig. 1b, and c). Therefore, we investigated the role of ethylene in the safranal-induced morphological changes in lettuce seedlings using ethylene inhibitors. Figure 3b shows the effects of $AgNO_3$, an ethylene signalling inhibitor, on PR and hypocotyl length in lettuce seedlings treated with safranal (19 µmol/L) for 3 d. $AgNO_3$ (0.5 µmol/L) had no significant effect on safranal-induced alterations in root and hypocotyl growth under the experimental conditions used here (Fig. 3b). In addition, $AgNO_3$ (0.5 µmol/L) did not change ethylene production in safranal-treated lettuce seedlings (data not shown). $AgNO_3$ is known to inhibit the action of ethylene (Beyer 1976). This occurs via a process whereby silver ions replace copper ions on ethylene receptors, occupying the receptors and acting to inhibit ethylene signaling (Jakubowicz et al. 2010). These findings suggest that safranal may not primarily regulate PR or hypocotyl elongation via the mediation of ethylene.

Effect of safranal on endogenous ABA content

The results of RNA-seq and qRT-PCR analyses indicated that safranal enhanced ABA biosynthesis. Therefore, we examined the effect of safranal on the endogenous ABA content in lettuce roots. Endogenous ABA content increased by approximately 1.5-fold in lettuce roots treated with 19 µmol/L safranal for 3 d in the dark (Fig. 4a).

ABA regulates biphasic root growth, and at low concentrations (<0.1 µmol/L), it promotes PR elongation and LR development (Li et al. 2017; Miao et al. 2021). Therefore, we examined the effect of fluridone and safranal co-treatment on PR elongation to investigate whether an increase in ABA is involved in the safranal-induced elongation of PR. Fluridone blocks the early stages of the carotenoid pathway, thereby inhibiting the production of carotene and its apocarotenoid derivatives, including SLs and ABA (Gamble and Mullet 1986; Rasmussen et al. 2012). The endogenous ABA content decreased in lettuce roots grown under 1 µmol/L fluridone and 19 µmol/L safranal co-treatment

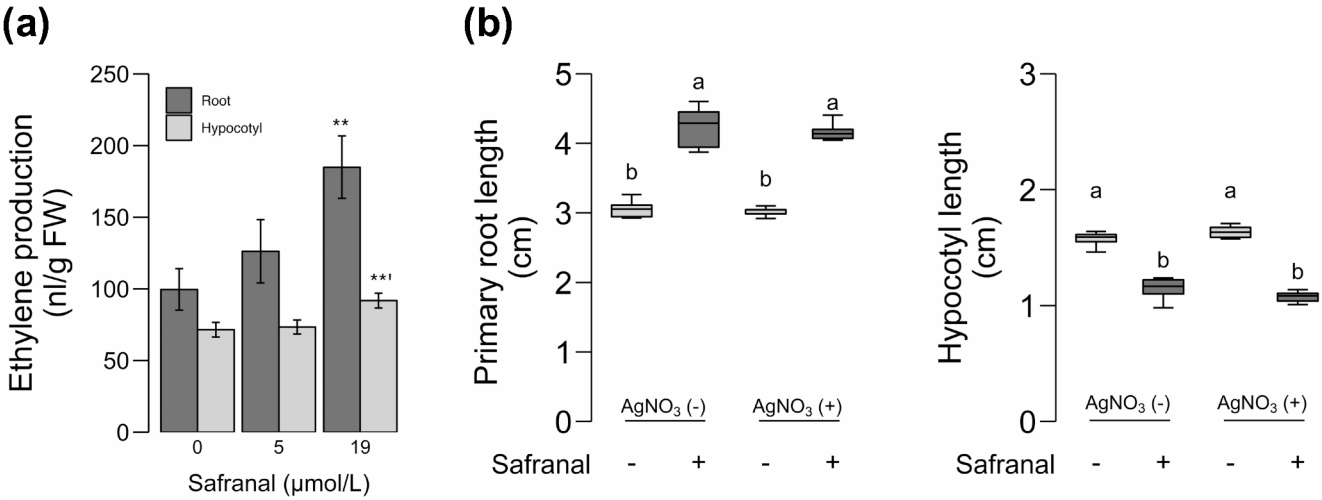

Fig. 3 Effects of safranal on ethylene biosynthesis and involvement of ethylene in the compound-induced morphological changes in lettuce seedlings. (**a**) Ethylene production from roots and hypocotyls of safranal (19 µmol/L)-treated lettuce seedlings 3 d after treatment. Error bar means ± SE, $^{**}P<0.01$ (Dunnett's test); (**b**) Effect of $AgNO_3$, an ethylene signaling inhibitor, on PR and hypocotyl length in lettuce seedlings treated with 19 µmol/L safranal for 3 d. Different letters indicate significant difference at $P<0.05$ (Tukey-Kramer test)

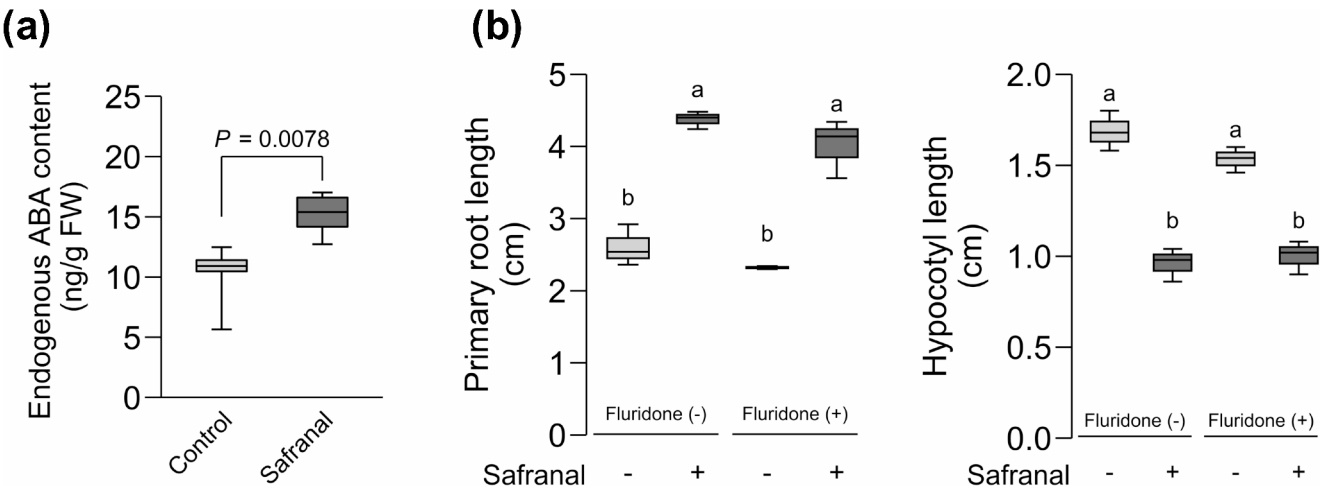

Fig. 4 Effect of safranal on ABA biosynthesis and involvement of ABA in the compound-induced morphological and physiological changes in lettuce seedlings. (**a**) Effect of safranal on endogenous ABA content in lettuce roots. Endogenous ABA content was measured in lettuce seedlings treated with safranal (19 µmol/L) for 3 d in the dark. $P=0.0078$ (Student's *t*-test); (**b**) Effect of fluridone (1 µmol/L), a carotenoid- biosynthesis inhibitor, on safranal (19 µmol/L)-induced alterations of PR or hypocotyl growth in lettuce seedlings. Different letters indicate significant difference at $P<0.05$ (Tukey-Kramer test)

(Online Resource, Fig. S7). However, exogenous treatment with fluridone (1 µmol/L) did not significantly affect the PR elongation-promoting effect of safranal (19 µmol/L) in lettuce seedlings, although there was a tendency to suppress it (Fig. 4b). In addition, exogenous treatment with fluridone (1 µmol/L) did not affect the safranal-induced suppression of hypocotyl elongation in the dark (Fig. 4b). These results suggest that an increase in endogenous ABA is not the main factor driving safranal-induced morphological changes in PR or hypocotyls in lettuce seedlings grown in the dark.

Effect of safranal on seedling growth under salinity stress

Volatile apocarotenoids are released when plants are exposed to abiotic stress (Lamikanra et al. 2002). Therefore, these compounds may play an important role under abiotic stress conditions. To determine whether exogenous safranal alleviates salt stress, and to further investigate the effect of light, we examined the effect of safranal treatment on the growth of NaCl-treated lettuce seedlings under dark (Fig. 5a) and light conditions (Fig. 5b). Safranal (19 µmol/L) remarkably mitigated NaCl (50 mmol/L) -induced inhibition of PR elongation in the dark (Fig. 5a and c). The positive effect of safranal was more prominent in NaCl-treated lettuce seedlings than in un-NaCl-treated seedlings ($P<0.0001$). However, 50 mmol/L NaCl enhanced hypocotyl elongation by approximately 1.5-fold, while safranal reduced NaCl-enhanced hypocotyl elongation in the dark (Fig. 5c). On the other hand, under light conditions, 19 µmol/L safranal also enhanced PR elongation in both NaCl (50 mmol/L)-treated and un-treated lettuce seedlings at 3 d after safranal treatment (Fig. 5b and d); however, safranal reduced the shoot FW of NaCl (50 mmol/L)-treated seedlings. As for effects on shoot growth of NaCl-untreated seedlings, 19 µmol/L safranal did not significantly reduced shoot FW under light conditions, but it did hypocotyl length in the dark (Figs. 1b and c and 5c and d). These results suggest that exogenous safranal promoted PR elongation in lettuce seedlings at the cost of suppressing hypocotyl elongation; furthermore, safranal mitigated the salt stress-induced inhibition of PR elongation, particularly under dark conditions. These differential effects of safranal under contrasting light conditions suggest that safranal affected the light-mediated control over seedling morphogenesis.

Salinity stress induces servere damage, especially to root tips, due to hardening, which inhibits root growth (Neumann et al. 1994). In general, under salt stress conditions, NaCl concentrations at the soil surface are higher than those at the subsurface. Thus, safranal-induced promotion of PR elongation may be an important trait for mitigating damage under salt stress, especially in outdoor saline environments.

To further evaluate the effects of safranal on salt-stress mitigation, Evans blue staining, which stains dead cells with a blue color, was performed (Fig. 6). In lettuce roots grown for 5 d under 50 mmol/L NaCl conditions in the dark, Evans blue uptake, representing dead cells, was approximately 1.5-fold higher than that in the non-NaCl treatments (Fig. 6a and b). After co-treatment with NaCl and safranal, the number of dead cells was lower than that after NaCl treatment alone (Fig. 6a and b). Furthermore, safranal alleviated the NaCl-induced increase in dead cells in lettuce roots grown for 5 d under light conditions after the same treatment as described above (Online Resource, Fig. S8). These results

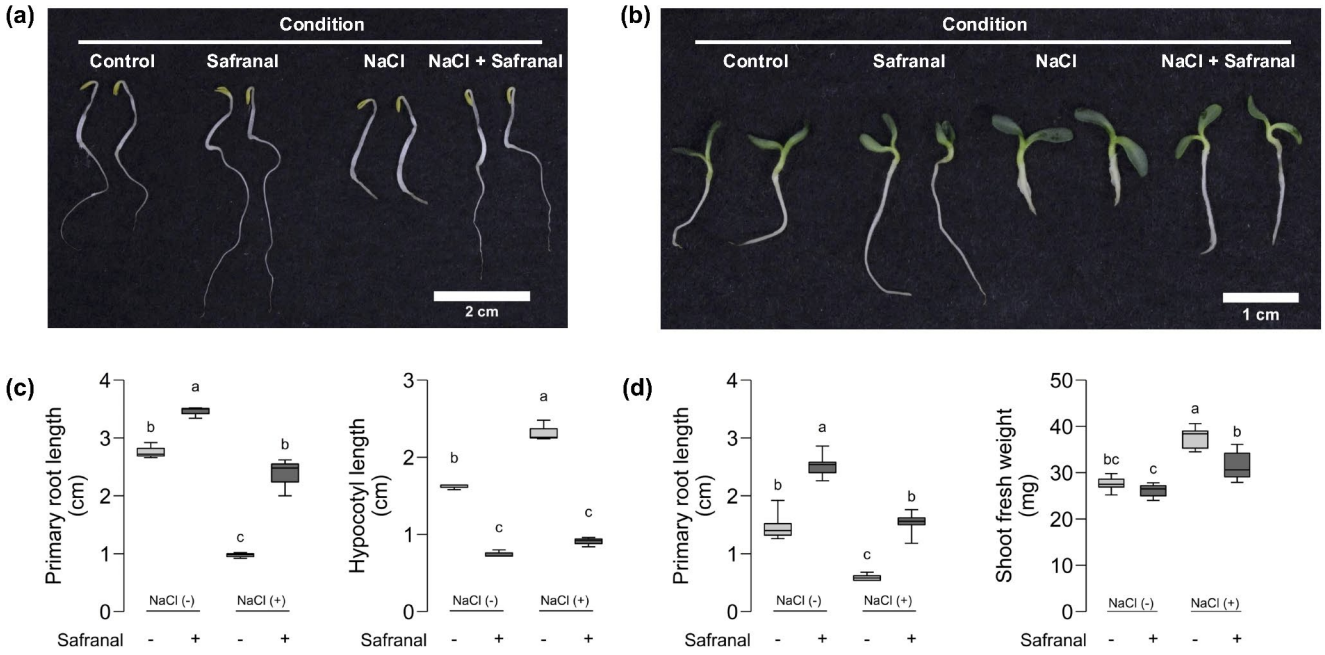

Fig. 5 Effect of safranal on lettuce seedling growth under salinity stress. PR and shoot lengths were measured in lettuce seedlings treated with 50 mmol/L NaCl for 3 d after safranal (19 µmol/L) treatment under dark (**a**) and light (**b**) conditions. Photon flux density was 50–75 $\mu mol \cdot m^{-2}s^{-1}$; (**c**) Effects of safranal on PR and hypocotyl lengths of lettuce at 3 d after safranal (0 or 19 µmol/L) and NaCl (0 or 50 mmol/L) co-treatment in the dark; (**d**) Effects of safranal on PR length and shoot FW of lettuce seedlings at 3 d after safranal (0 or 19 µmol/L) and NaCl (0 or 50 mmol/L) co-treatment in the light (50–75 $\mu mol \cdot m^{-2}s^{-1}$). Different letters indicate significant differences ($P<0.05$, Tukey-Kramer test)

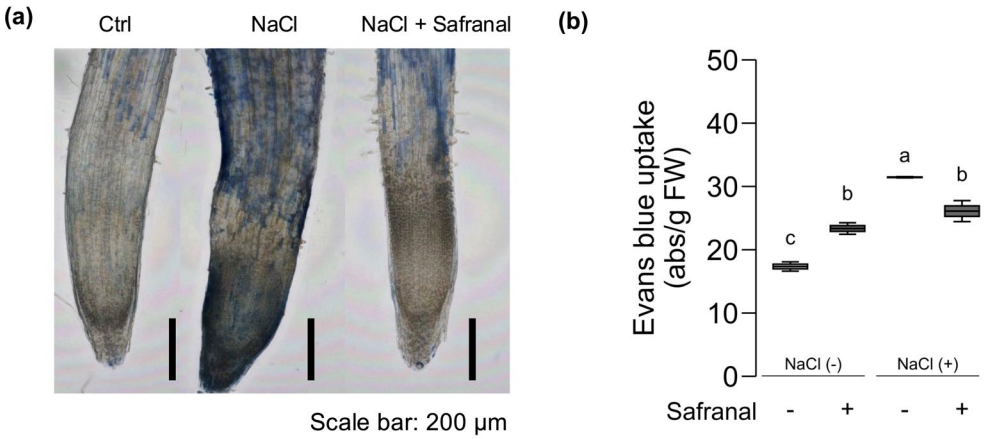

Fig. 6 Effect of safranal on the salinity stress-induced increase in the number of dead cells in lettuce roots. (**a**) Evans blue-stained lettuce roots after treatment with 50 mmol/L NaCl or co-treatment with NaCl and safranal (19 µmol/L) for 3 d in the dark. Blue; cells in which Evans Blue has been incorporated; (**b**) Quantification of Evans blue uptake by lettuce roots. Different letters indicate significant differences ($P<0.05$, the linear mixed model)

suggest that treatment with 19 µmol/L safranal mitigated the NaCl-induced increase in the number of dead cells in lettuce roots, regardless of light conditions.

Possible factors for safranal-induced cell death alleviation

Safranal alleviated salt stress-induced cell death in lettuce roots under both dark (Fig. 6) and light conditions (Online Resource, Fig. S8) conditions. However, this effect was not affected by treatment with 1 µmol/L fluridone (Online Resource, Fig. S9). Under the experimental conditions, fluridone effectively reduced endogenous ABA content after safranal treatment (Online Resource, Fig. S7). These results suggest that the increase in endogenous ABA content under safranal treatment was not the primary cause of safranal-induced alleviation of salt stress. ABA is critical for plant adaptation to osmotic and drought stress. Thus, it is needed to investigate whether the upregulation of endogenous ABA by safranal can alleviate these stresses in more detail in the future.

To elucidate the factors that contribute to the alleviation of salinity-induced cell death by safranal treatment, we focused on ROS, which are known to be the major cause

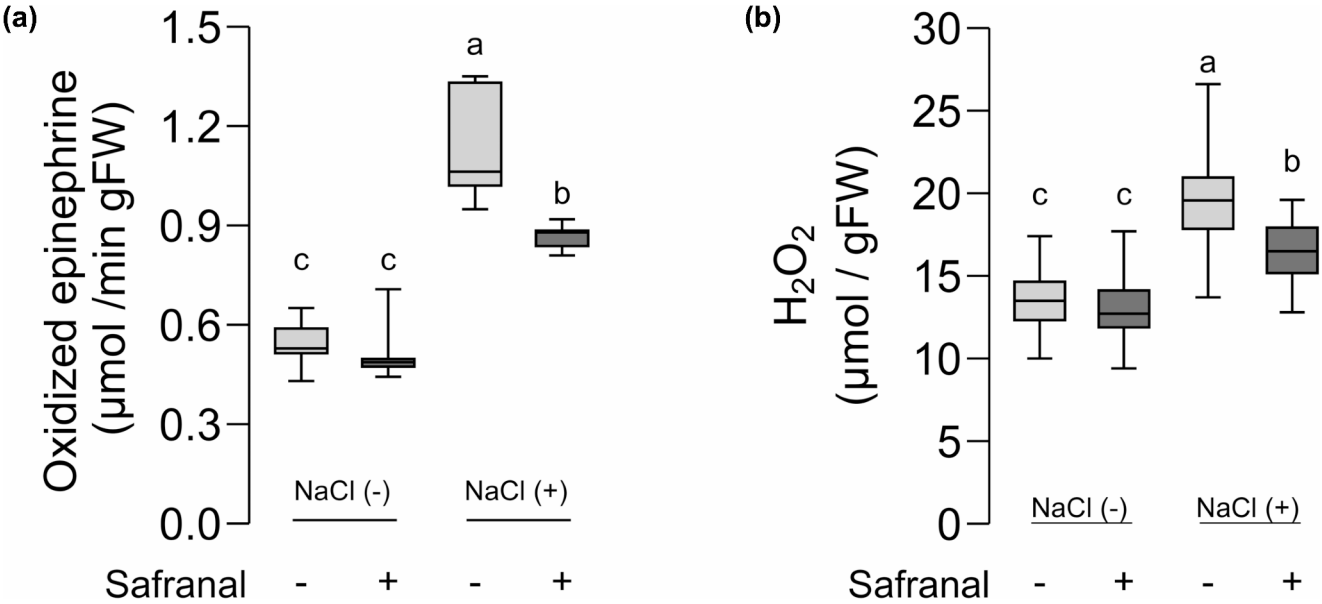

Fig. 7 Effects of safranal on salinity stress-induced generation of O_2^- (**a**) and H_2O_2 (**b**) in lettuce roots. The generation of O_2^- and H_2O_2 were measured in lettuce roots at 5 d after safranal (0 or 19 µmol/L) and NaCl (0 or 50 mmol/L) co-treatment under light conditions (50–75 µmol·m^{-2}s^{-1}). The results for O_2^- and H_2O_2 generation were statistically analyzed by Tukey-Kramer test and the linear mixed model, respectively. Different letters indicate significant differences ($P<0.05$)

of cell damage under salinity stress. Specifically, effects of safranal (19 µmol/L) treatment on the generation of O_2^- and H_2O_2 in lettuce roots treated with 50 mM NaCl for 5 d under light conditions were investigated (Fig. 7). As an indicator of O_2^- generation, oxidation of epinephrine was measured (Krasuska et al. 2016). The production of O_2^- increased under saline conditions, whereas safranal significantly reduced O_2^- generation under salinity stress (Fig. 7a). In addition, safranal reduced the amount of H_2O_2 that increased under saline conditions (Fig. 7b). These findings suggests that safranal alleviates cell death by reducing the overproduction of ROS under salt stress in lettuce roots. Conversely, safranal did not alter the level of O_2^- and H_2O_2 production under non-saline conditions (Fig. 7a and b), while it promoted PR elongation (Fig. 5c). Therefore, we inferred that O_2^- and H_2O_2 are not the primary cause of the observed enhancement of PR elongation upon safranal treatment.

Root development is regulated by ROS and several phytohormones, such as auxin, ABA, brassinosteroid, and cytokinin (Zluhan-Martínez et al. 2021). β-cyclocitral, which has a structure similar to that of safranal (Online Resource, Fig. S5), reportedly promotes PR elongation independent of auxins, ROS, or brassinosteroids (Dickinson et al. 2019). The present study also suggests that safranal-induced PR elongation is not dependent on ethylene, ABA and ROS. However, the localisation of phytohormones and ROS in roots after safranal treatment has not yet been analysed. Therefore, further analysis of their localisation may be necessary to elucidate the mechanisms by which safranal causes morphological changes.

Various plant secondary metabolites have been suggested to regulate biological activities via protein interactions (Veyel et al. 2018; Venegas-Molina et al. 2021). The safranal analogue, β-cyclocitral has been proposed to bind to 1-deoxy-D-xylulose 5-phosphate synthase (DXS), an enzyme in the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, thereby reducing the carbon flux along the MEP pathway (Mitra et al. 2021). In addition, possible safranal receptors have been reported in animals (Ali and Al-Lohedan 2018, 2019; Al-Hrout et al. 2018; Vanaei et al. 2020). Hence, the phenotypic expression of safranal-induced enhancement of PR elongation may require direct binding to proteins, such as DXS. Future research will have to identify the potential target proteins of safranal to better understand its mode of action.

Conclusions

Safranal detected by preliminary screening of VOCs emitted from saffron markedly enhanced PR elongation in lettuce seedlings under both light and dark conditions. At 19 µmol/L, safranal increased endogenous ethylene and ABA contents in lettuce seedlings. However, treatment with $AgNO_3$, an ethylene signalling inhibitor, or with fluridone, a carotenoid biosynthesis inhibitor, did not alter PR or hypocotyl length after safranal treatment, suggesting that neither

ethylene nor ABA, which were effectively increased by safranal, were the primary cause of safranal-induced morphological changes. Furthermore, safranal alleviated the salt-induced increase in ROS, cell death, and inhibition of PR elongation, but it did not affect ROS levels under non-saline conditions. These results suggest that exogenous safranal regulated PR and hypocotyl elongation, through mechanisms independent of ethylene, ABA, or ROS. Furthermore, safranal alleviated salt stress by reducing oxidative cell damage. These effects of safranal indicate its potential for use as a novel PGRs and biostimulant.

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Author contributions Yukari Sunohara conceived the study and designed the experiments. Tomomasa Oka, Yuki Fujimaru and Takehiro Minami designed and performed the experiments. Tomomasa Oka, Yuki Fujimaru, Takehiro Minami, Takuya Yamaguchi and Yukari Sunohara analyzed the data. Tomomasa Oka and Yukari Sunohara drafted the manuscript. Takuya Yamaguchi, Hiroshi Matsumoto and Yukari Sunohara supervised the experiments, and reviewed and edited the manuscript. All authors approved the manuscript.

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Data availability The datasets generated during and /or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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