Light-induced electron transfer and ATP synthesis in a carotene synthesizing insect

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A singular adaptive phenotype of a parthenogenetic insect species (Acyrthosiphon pisum) was selected in cold conditions and is characterized by a remarkable apparition of a greenish colour. The aphid pigments involve carotenoid genes well defined in chloroplasts and cyanobacteria and amazingly present in the aphid genome, likely by lateral transfer during evolution. The abundant carotenoid synthesis in aphids suggests strongly that a major and unknown physiological role is related to these compounds beyond their canonical anti-oxidant properties. We report here that the capture of light energy in living aphids results in the photo-induced electron transfer from excited chromophores to acceptor molecules. The redox potentials of molecules involved in this process would be compatible with the reduction of the NADH coenzyme. This appears as an archaic photosynthetic system consisting of photo-emmitted electrons that are in fine funnelled into the mitochondrial reducing power in order to synthesize ATP molecules.

Carotenoids are initially formed by the condensation of two geranylgeranyl diphosphate molecules (GGPP, C20) joined at their ends. The resulting lycopene, a key intermediate linear molecule, is then modified by the addition of a carbon ring at one or both ends (see figure 1). Carotenoids constitute a large group of compounds (the chemical structure of 750 derived molecules has been determined from plants, algae, bacteria and invertebrates and diverges by minor modifications). The addition of epoxy groups or alkyl groups to the basic structure generates the predominant derivatives. These modifications add to the multiple cis/trans isomers of the polyene double bonds in the aliphatic chain explain the vast repertoire of the carotenoid family. Moreover, a large number of derivative products, like the conjugated complex epoxy carotenoid/α-Tocopherol and multiple esterified forms with hydrophilic glycosilic groups, have been reported. The carotene basic structure is highly hydrophobic and included in the lipid bilayer. On the other hand, non-covalent binding to haemolymph circulating proteins, which makes them water soluble and/or transportable, represents a substantial fraction of the total carotenoid load in aphids. These molecules display yellow to orange colours but chemical modification and/or protein binding can trigger changes towards green or brownish. Their functions are well documented in plant photosynthesis where they harvest light energy for the chlorophyll and are scavenger for reactive singlet oxygen. One other striking function of carotene derivatives is that their cleavage leads to retinal which is part of eye photoreceptors in all the taxa.

*A. pisum* aphids reproduce parthenogenetically in spring and summer whereas sexual morphs emerge only in autumn. We have shown that epigenetic mechanisms in clonality context can be recruited in order to achieve fitness in unfavourable climate conditions, concomitant in some cases with colour changes. More recently, authors have reported that the aphid genome harbours the genes required for carotenoid synthesis like in plants, algae and fungi, which makes this insect species unique in the insect class (see Supplementary Data, figures S1 and S2). Aphids seem equipped for processing the full carotene synthesis instead of taking it from their diet (aphids suck the plant phloem, which *a priori* excludes the uptake of the hydrophobic carotenoid molecules). *A. pisum* therefore can exhibit an heavy load of carotene conferring strong orange colour depending on the environmental context. However, many other pigments are synthesized in this species as well. The best known molecules are the polycyclic/polyphenolic aphins derivatives (xantho-, erythro- and proto-aphins), which display red (alkaline pH) or yellow (neutral pH) colours and strong yellow fluorescence when they are excited by...
never occurred within the actual mother. Importantly, the switch to the cold adapted lineage from (8°C) conditions successfully selected a viable and robust colony of green variants (figure 1). By chance, progenies of the unique parthenogenetic founder mother were placed in different unfavourable environments in order to select viable and robust variants in clonality context.

Surprisingly, a cold adapted strain was obtained after a process of selection and presents a singular greenish pigmentation. The panel of carotene molecules found in the green and orange phenotypes has been extensively analyzed by mass spectrometry after extraction/chromatographic isolation and also by Raman imaging technology directly on living aphids to reveal the carotene signature. Putative physiological functions that might use carotenoid molecules have been investigated. The free electrons generated by photo-activated carotenoids and/or other pigments appeared to be transferrable to the reducing power machinery [like the reduction of NAD(P)⁺ to NAD(P)Ｈ] followed by a subsequent mitochondrial ATP synthesis. This report describes experimental data that argue for a role of chromophores as part of an archaic photosynthetic mechanism in insects.

**Results**

Ten orange adult aphids were placed each day at 8°C for five months before we obtained a viable and robust colony of green variants (figure 1). By chance, progenies of the orange adult aphids did not survive at 8°C. Most larvae died from larval stage 1 to stage 4. Cold (8°C) conditions successfully selected a viable and robust green aphid lineage from orange descendants of a 22°C-adapted unique founder mother. Importantly, the switch to the cold adapted green variant never occurred within the actual orange adults, which excludes a direct enzymatic induction. The green phenotype is heritable in the conditions in which it emerged, but its singular pigmentation fades away when it is placed back in optimal conditions at 22°C. This demonstrates that a clonal aphid population under pressure of selection is able to generate complex traits guiding environmental fitness and underlying the recruitment of gene networks. The scenario precludes allele selection as an explanation (success of the phenotypic adaptation too fast for implying a Darwinian process) and strongly supports the hypothesis of an epigenetic regulation. The mechanism might reside in the extensive DNA methylation as the molecular cue to transmit complex traits in the framework of an unchanged genome.

This selection process is summarized in figure 1. On the other hand, if the pink/orange pigmentation is dominant at 22°C in optimal conditions (low population density and abundant resources), the declining conditions (rarefaction of resources, high population density) trigger the progressive disappearance of the pink/orange phenotype and its replacement by pale/white/yellow colours (figure 1). In such case, the colour plasticity (colour shift orange to pale/white/yellow) is proportionally induced by the increase of population density and the rarefaction of resources. The pale/white/yellow phenotype reflects an unfavourable environment and might be referred as survival forms that have turned down some less essential biochemical processes to minimize energy cost.

Following the intriguing discovery of the carotene synthesis genes in the aphid genome, we undertook an extensive analysis of carotenoid molecules by Raman spectrometry imaging and mass spectrometry technology in the framework of this genetic/epigenetic context. We took advantage of the rapid crystallisation of carotene molecules to isolate and to solubilise them in ethanol/acetone. A long centrifugation (9,300 x g for 1 hour) of PBS buffered extract of aphids triggers the formation of a pure orange crystallized precipitate at the top of the aqueous phase. Spectral absorbance properties of this precipitate were analyzed and found to be in accordance with carotenoid molecules. A comparative absorbance profile between the extracts from the green and pale orange phenotype is presented in figure 2. As expected, the decrease of spectral absorbance of the pale aphid acetone/ethanol extract in the wave lengths of carotenoid absorption is shown to be identical to the absorbance profile of the crystalline precipitate.
molecules absorption was spectacular compared to the green aphid extract (Figure 2).

Imaging of resonance Raman spectrometry allowing non-destructive molecular motif identification and quantification was performed to detect structural elements of carotene molecules directly on living aphids. A 488 nm laser wavelength corresponding to the maximum wavelength of carotene absorption was used to excite the polyene motifs (Raman conditions: 1.2 to 12 mW/1–3 seconds). The shift values (the shifts of the Stokes (lower energy) and Anti-Stokes (higher energy) Raman light scattering correspond to a vibrational mode of a structural motif in a molecule) are expressed as cm$^{-1}$. The carotene signature corresponding to the three peaks obtained at 1,520 cm$^{-1}$ (assigned to the C=C stretching vibration), 1,157 cm$^{-1}$ (CH-CH) and 1,005 cm$^{-1}$ (CH-CH$_3$) were always found in living aphids even though these molecules are part of a complex biological matrix (figure 3). The laser beam of the Raman imaging apparatus was also directed on the crystals spontaneously formed after crushing adult orange aphids (figure 3). Moreover a Raman imaging control was carried out using the reddish/brown aphid eyes, known to contain, as any eye in all the taxa, a strong concentration of retinal. Retinal (vitamin A) conserves the structural motifs of carotene by the fact that it is the enzymatic conversion product of carotenoid molecules. The three peaks corresponding to the Raman signature of carotene were unambiguously obtained with living aphids and crystals, which suggests high concentration of these compounds (figures 3 and 4). Interestingly, a stronger intensity of the peaks was consistently found with the green compared to the orange phenotype (40% increase) (figure 4). Moreover, the method was able to follow the carotene synthesis in the developing embryos where the signals were correlated with the apparition of the orange pigmentation (see Supplementary Data, figure S3).

The extensive comparative analysis of these molecules between the green and the orange aphids has been performed by mass spectrometry (after chromatographic isolation), in order to quantify few intermediate components in the cascade of carotenoid synthesis. The major components of the carotene family found in the green and orange aphids is reported in table 1. A substantial increase of concentration of trans- and trans- carotene is observed in the green variant compared to the orange (8.3 ± 1.2 versus 4.0 ± 1.9 and 12.6 ± 1.5 versus 6.5 ± 1.8 mg.100 g$^{-1}$ respectively). At the opposite, the cis-torulene is drastically increased in the orange phenotype (10.7 ± 3.4 versus 3.0 ± 0.6 mg.100 g$^{-1}$) whereas the trans-torulene (a precursor metabolite) was roughly unchanged (Table 2). Therefore, the mass spectrum analysis confirmed the trend observed by Raman analysis (see Supplementary Data, figure S4).

One well documented role regarding these compounds are the annihilation of singlet oxygen and radical scavengers in plant photosynthesis along with the light harvesting function of chlorophyll$^b$.

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**Figure 2 | Absorbance properties of green, orange and pale aphid ethanol extracts.** The orange pigments were extracted as indicated in Methods. Briefly, 250 mg of adult aphids were centrifuged in Ringer’s buffer and the orange layer was collected. This precipitate was then solubilized in ethanol/acetone (75/25). A comparative spectrum of absorbance was carried out from 320 to 700 nm (A), at 600 nm, 650 nm and 700 nm (B) and at 425 nm, 450 nm and 480 nm (specific peaks of carotene absorbance) (C). A comparative measure was carried out at 295 nm (peak of absorbance of phytoene, a precursor of carotene) and at 450 nm (peak of carotene absorbance) with the orange crystallized precipitate obtained with the green and orange aphid extracts (D). The means of three independent experiments are shown in (A) and bars in (B), (C), (D) represent the average of three experiments $\pm$ S.E (comparison orange versus green, $P<0.002$). More pigment is present in green than in orange aphids.
Because strong carotenoid concentration was observed especially in the green and orange larval forms (despite a high level of variation between individuals in the same colonies when food resources are declining), we tried to unravel some putative physiological functions beside their canonical anti-oxidant properties. We investigated the hypothesis that the photon energy might excite the π delocalized electrons of the carotene polynene structure and trigger an electron transfer to acceptor molecules. Adult aphids (green, orange and white phenotypes) and larvae from orange mothers were placed in dark or left in light photoperiodicity (18/6 hours). Furthermore, these conditioned aphids were returned under light photoperiodicity (18/6 hours) after a dark episode. The striking data show that ATP synthesis is sensitive to light, but differs among the orange (marked effects), green (little effects likely because a strong lipid load in this variant acts as a metabolic reserve) and white phenotypes (no change). Results are summarized in figure 5 and support the concept of photo-conditioning of ATP synthesis in some environmentally shaped variants.

To get more insight about the light-dependent reduction-oxidation (redox) process, orange aphid extracts were used to reduce tetrazolium salts (MTT) in presence or absence of light. Although the effect was moderate, an increase of MTT reduction in presence of light was obtained with the orange, but not with the white extract (figure 6). This trend was also obtained with orange embryos incubated with MTT and exposed to light whereas the white embryos in the same conditions display a weak fluctuation of the basal level (figure 6). The same results were observed when the experiments were conducted with pure molecules. Briefly, 100 µl of MTT solubilized in water were placed on a layer of dry β-carotene and illuminated by a regular electric light. The reduction of MTT in blue precipitated formazan was observed as the result of a capture of free electrons generated by the photoactivated carotene, which suggests that the energy of these free electrons is high enough to pass the barrier of the tetrazolium redox potential (see Supplementary Data, figure S5).

Finally the balance NAD+/NADH was measured in the light versus dark context. A series of experiments shows unambiguously a
significant increase of the reduced co-enzyme level in particulate fraction enriched in mitochondria when the orange aphids are exposed to light (figure 7). Intriguingly, a drastic decrease in NAD⁺ (oxidized) concentration was found in the soluble fraction of the extract when the orange aphids were maintained in dark, suggesting that its synthesis is partly controlled by light. By contrast and as expected, the white aphid variants display weak levels of NAD⁺/NADH, which seem little affected by light (figure 7). Together these data reinforce the hypothesis that light, through biological membranes enriched in pigments, triggers a reducing power that in fine is captured by co-enzymes like NAD⁺. This reduced co-enzyme is known to transit inside mitochondria through a shuttle mechanism and deliver electrons for the respiratory chain machinery ending with the H⁺ inflow-driven ATP synthesis\(^{15-17}\). Amazingly, we observe that carotene molecules are disposed as a bilayer under the cuticle from 0 to 40 μM in depth, suggesting that this structure might present an optimal efficiency to harvest light energy (figure 7).

Discussion

Aphid lycopene cyclase and phytoene synthase are enzymes fused in one unique protein with two distinct catalytic activities. This rare hybrid has been previously described in Phycomyces\(^ {18}\). Surprisingly, to our knowledge genes like phytoene dehydrogenase and lycopene cyclase/phytoene synthase, key enzymes in the cascade of carotenoid synthesis, seem to be present in different locations of the aphid genome (see Supplementary Data, figure S1).

The existence of this fused gene in the aphid genome highlights the regulation of the two enzymatic activities by a unique promoter. This suggests a probable in situ carotene synthesis and at this stage makes the aphids unique in the insect class\(^ {12}\). On the other side, the availability of free carotenoid pigments in phloem sap on which the aphids feed is very unlikely, because of their hydrophobicity. Our data suggest strongly that the environmentally-guided synthesis of these compounds in aphids plays a role in absorption of sun light and in electron transfer to mitochondrial protein complexes. This is corroborated by the fact that the emergence of sexuals, life history traits and metabolism are highly dependent on photoperiodicity in this species\(^ {9,10}\). To argue in favour of this scenario, the photoconditioning of the Pieris brassicae caterpillar mediated by pterobilin, an abundant pigment found in their integument, has been described to drive a light-dependent production of ATP\(^ {17}\). Moreover, the accumulation of carotenoid compounds in different caterpillar species has been well documented and currently associated with canonical antioxidant properties, although other putative functions have not been investigated up to date\(^ {14,19}\). Insects, except aphids, do not synthesize these molecules, but absorb them by food uptake from the chloroplasts of plants/algae\(^ {20}\). The polyenic-conjugated structure of carotenoids (C=C alternated with C=C leading to π delocalised electrons)
appears an efficient mode to transport electrons across the lipid bilayer, putatively funnelling them to the mitochondrial redox machinery. This might end up with another level of regulation of ATP synthesis. The electron transport chain might partly use the electrons from photo-activatable pigments. The linear polyene motif of carotene might act as a "molecular wire" in electron transfer to acceptors across the bilayer membranes. Furthermore, aphids synthesize other pigments like the aphins for which the physiological roles are little known. Their redox properties based on a polyphenolic structure and the inherent π delocalized electrons make them solid candidates to fulfill the same functions as those attributed to carotenoids (see Supplementary Data, figure S6). A large panel of pigments might act as a light and energy harvesting system leading to a photon-triggered electron separation and subsequent transfer to an acceptor. To corroborate this hypothesis and to confirm our data, the photoreduction of NAD\(^+\) by light-excited chromatophores, extracted from a purple colored proteobacterium that synthesizes carotenoids, has been reported. Furthermore and independently, the photoreduction of NAD\(^+\) in presence of metal complexes and organic compounds that donate electrons has been also described. An artificial photosynthetic system like self-assembling of a mix of phenyl dipeptides, porphyrin and metal as platinum was successfully tested to transfer electrons to NADP\(^+\) via light excitation. Finally, the photopotential and photocurrent generated by carotene molecules and chlorophyll have been compared and authors report that the efficiency of electron transfer to an electrode was higher with the illumination of carotene than of chlorophyll. In parallel experiments, by building triad molecules (carotene, porphyrin, quinone and/or fullerene), authors have generated a strong electron separation from carotene (after light absorption) for the benefit of the quinone or fullerene component. This chemical system was designed as a model to study the redox properties of carotene because the photo-excited electrons in this compound are extremely short lived to be tracked. To this regard, a system composed of β-carotene placed inside carbon nanotubes was used to study the light harvesting and chemical capture of energy. Moreover and more importantly, the photocconversion of GFP protein (concomitant to a shift from green to red color) and the photo-induced electron transfer from GFP to tetrazolium, quinone, FMN\(^-\) and NAD\(^+\) has been observed and reported. The redox potential of NAD\(^+\) is quite high [E\(^f\) = −0.32 v], which suggests that many proteins such as GFP under light absorption are able to donate electrons with a level of energy compatible to pass the redox barrier of NAD\(^+\). Therefore, the auto fluorescence of insects coming from numerous endogenous molecules might be concomitant with electron separation, captured subsequently by oxidized co-enzymes. The aphins (erythoaphin, xanthoaphin and proto aphins), which are polymeric and polyphenolic compounds known to complex metal as Fe\(^{2+}\), might participate

Figure 6 | Tetrazolium (MTT) reduction by orange aphid extract. 100 µl of tetrazolium solution (1 mM in water) were placed on a glass slide in which 10 µl of orange aphid extracts were added. The system was irradiated by visible light (A) for 30 min or kept in dark (B). Then, the medium was delicately washed out. Top: The photos show the border of the spots where the formazan precipitation is more intense. Unambiguously, an increase of MTT reduction, measured as formazan precipitation on the glass, is observed under light (A). Middle: higher magnification of the photograph above. Bottom: The light exposure of strongly pigmented ovarioles in presence of MTT (1 mM in water) is compared with white/pale ovarioles in the same conditions as above. Produced formazan by orange or white aphid extract (100 µg protein) under light or kept in dark was measured after solubilization in acid/ethanol (C and D). The representations are the mean of three separate experiments.
to this phenomenon. We suggest that these complex pigments constitute a system of photo-induced electron transfer that (due to their high energy) are able to reduce efficiently NAD$^+$ bound to membrane proteins ending in fine with ATP synthesis.

Methods

Maintenance and propagation of aphids. The pea aphid *Acyrthosiphon pisum* belongs to the order Homoptera (Aphididae family) and feeds on the *Vicia faba* plant. Aphids were maintained on *V. faba* in cages in an incubation room at about 22°C/18°C and a light/dark photoperiodicity of 16/8 hours. Aphids were raised at 8°C to select a predominant phenotype (green body color). The white phenotype was induced immediately by increased density of population and by declining plant resources at room temperature.

Reagents and commercial kits for ATP dosage and NAD$^+$/NADH assay. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma Aldrich. The balance and determinations of NAD$^+$/NADH were carried out with the ultrasensitive colorimetric kit purchased from Bioassay Systems, Hayward CA USA (catalog n. ECND-100). Finally the ATP dosage was performed with the kit FLASC purchased from Sigma Aldrich.

ATP determination: Detailed information regarding the protocol used for figure 5. A. Dark exposure of adult aphids: Aphids were raised in light/dark periods (16/8 hours) then submitted to the following conditioning: adult aphids (10–12 days after birth for the green and 7–10 days for the orange aphids, according to their temperature-dependent life cycle) were placed in dark, then the full population is tested after two days (1 and 2; 5 and 6) for ATP content or alternatively were kept in light two days more as control (3 and 4; 7 and 8) before the measure of ATP content. ATP determination was also obtained with the content of one white aphid from a cohort placed in the same conditions as the corresponding lane described above.

B. Dark exposure of larvae: Orange aphid mothers were raised in light/dark periods (16/8 hours), then after birth the larvae were conditioned as indicated in legend to figure. The standards roughly represent 50 pmoles (blue) to 250 pmoles (red).

C. Comparative time course of the decline of ATP content in embryos and larvae placed in dark: The full ovarioles were dissected from orange aphid mothers kept in dark according to the indicated timing, then analyzed for their ATP content. A comparative determination was performed with the larvae placed in dark after birth from orange aphid mothers raised in light. The determinations were conducted with the ovarioles from three mothers and with five larvae.

D. Light-induced ATP synthesis after a dark exposure episode of orange adult aphids: 1 to 8 are parallel experiments carried out with the descendants of a unique orange founder mother. The determinations were normalized with protein content.

E. Comparative determination of ATP between green, orange and white adult aphids: 1 to 8 parallel experiments carried out with the descendants of a unique orange founder mother. The determinations were normalized with protein content.

F. Light-induced ATP synthesis after a dark exposure episode in larvae: Top: orange mothers were placed in dark and first instar larvae were tested at day 0 (1), at day 2 (2) and day 3 (3). Down: adult orange mothers were placed three days in dark, then placed back in light (day 0). Emerged progenies (first instar larva) were tested at day 0 (1), at day 2 (2) and finally at day 3 (3). Determinations were normalized with protein content. Standards roughly represent 20 pmoles (green) to 100 pmoles (red).

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Figure 7 | NAD$^+$/NADH balance in cytosol and particulate (mitochondria) fractions in dark- and light-conditioned aphids. The dosage was performed with orange (A) and white (B) aphids conditioned in light or dark using methyphenylsulfonate and tetrazolium salts (see Methods). Briefly, 50 adult aphids (orange and white phenotypes), kept in dark for three days or alternatively kept in light photoperiodicity (18/6 hours), were grinded and assayed for the NAD$^+$/NADH balance. The experiments were repeated three times. Bars are the mean $\pm$ S.E. **P < 0.005. The Raman imaging between 0 to 100 μm in depth was performed and shows a double layer structure of carotenoid compounds (C). A control carotene signature in Raman imaging corresponding to the analysis in (C) is shown in (D).
Extraction of pigments for spectral analysis. Aphids were extracted with Ringer's buffer\(^{29}\) (20 adult orange and green variants) in a glass potter and centrifuged 20 min at 13,400 x g. The supernatants were normalized with the weight of the pellet and submitted to spectral absorbance from 290 to 700 nm. We observed at the top of the water phase an orange intense layer. Another orange layer, found at the top of the pellet, was collected with a glass pipette in 100 µl of water and re submitted to centrifugation. The orange layers at the top of the water phase were collected. The crude isolation of these orange pigments was facilitated by their known rapid crystallisation. This orange precipitate after crystallisation was solubilized in acetone/ethanol (25/75) and analyzed by spectrometry of absorbance. The obtained spectral absorbance properties confirmed to be in accordance with carotenoid molecules (peak of absorbance at 425 and 480 nm). On the other hand, the green pigment was found for two thirds in the membrane (hydrophobic compounds extracted by ethanol or ether) and for one third in the soluble fraction (bound to proteins as hydrophobic compounds or esterified with carbohydrates). The molecular structure of this green pigment is still unknown due to its complexity (probable polyphenolic compounds)\(^{29}\).

Raman imaging spectrometry. The Raman analysis was conducted with living animals. The equipment was a spectrophotometer Labram HR800 Horiba Jobin-Yvon. An argon ion laser beam was focused on the sample by using a 100x objective (NA 0.9) for crystal analyses and a 50x LWD objective (NA=0.45) for aphid analyses and Raman back scattered light was collected by the same objectives. Then, we estimated the analysed area to about 1 square µm with 100x and about 10 square µm with 50x.

Extraction of carotenoids for spectrometry analysis. Carotenoid extraction was adapted from previous work\(^ {29}\). One gram of aphids previously milled in liquid N\(_2\) was added to 80 mg of MgCO\(_3\) in 15 ml of extraction solvent (ethanol/hexane, 4:3 v/v, containing 0.1% of BHT as antioxidant) and stirred for 5 min. The residue was separated from the liquid phase by filtration with a filter funnel (porosity N\(_{2})\) and washed successively with 15 ml of the above solvent, 15 ml of ethanol and 15 ml of hexane. Organic phases were transferred to a separating funnel and successively washed with 40 mL of 10% sodium chloride and 2 x 40 mL of distilled water. The aqueous layer was removed. The hexanic phase was dried under anhydrous sodium sulphate, filtered and evaporated to dryness at 40°C in a rotary evaporator. The residue was dissolved in 250 µl dichloromethane and 250 µl MTBE/methanol (80:20, v/v). Samples were placed in amber vials before chromatographic analysis.

Liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) analysis of carotenoids. The HPLC apparatus was a Surveyor plus model equipped of an autosampler, a PDA detector and LC pumps (Thermo Electron Corporation, San Jose, CA, USA). Carotenoids were analysed according to previously published method\(^{29,30}\). Carotenoids were separated along a C30 column (250 x 4.6 mm, 5 µm particle size), YMC (EUROP, GmbH). The mobile phases were water/20 mM ammonium acetate as eluent A, methanol/20 mM ammonium acetate as eluent B and particle size), YMC (EUROP, GmbH). The mobile phases were water/20 mM ammonium acetate as eluent A, methanol/20 mM ammonium acetate as eluent B and 2. Hirschberg, J. Carotenoid biosynthesis in flowering plants. Current opinion in plant biology 4, 210–218 (2001).

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