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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 NAD^+ coenzyme. This appears as an archaic photosynthetic system consisting of photo-emitted 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[1,2]. 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)[3]. The addition of epoxy groups or alkyl groups to the basic structure generates the predominant derivatives. These modifications added to the multiple *cis*/*trans* isomers of the polyene double bonds in the aliphatic chain explain the vast repertoire of the carotenoid family[4,5]. 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[4,6]. 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[7,8]. These molecules display yellow to orange colours but chemical modification and/or protein binding can trigger changes towards green or brownish[7]. Their functions are well documented in plant photosynthesis where they harvest light energy for the chlorophyll and are scavenger for reactive singlet oxygen[5]. One other striking function of carotene derivatives is that their cleavage leads to retinal which is part of eye photoreceptors in all the taxa[6].

Acyrthosiphon pisum aphids reproduce parthenogenetically in spring and summer whereas sexual morphs emerge only in autumn[9,10]. 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[11]. 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[12] (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

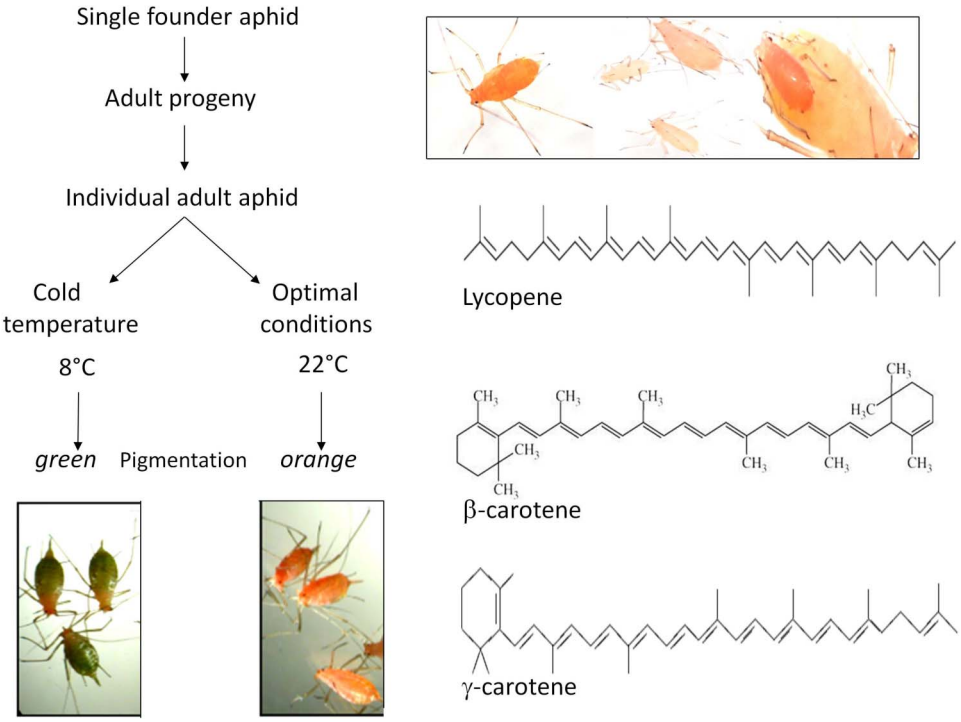

Figure 1 | Protocol scheme used to generate the *green* variant. The graph on the left summarizes the protocol used to select the *green* variant from a unique parthenogenetic founder mother. The photographs show the differences between the *green*, *orange* and *pale* adult aphid phenotypes. The *white* phenotype is directly induced by high population density and rarefaction of resources. The *green* phenotype was the result of a process of selection in cold conditions. All these variants were the descendants of a unique *orange* founder mother adapted to optimal conditions (22°C). Lycopene, β-carotene and γ-carotene are represented.

UV light[7]. Their physiological roles in aphids and other insects are yet poorly documented. In this report, we analyzed the carotenoid components in few variants presenting singular pigmentation (greenish, orange and pale yellow/white). The aphid descendants of a unique founder mother were placed in different unfavourable environments in order to select viable and robust variants in clonality context[11]. 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[11]. 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[11]. 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 spectrum 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 were 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

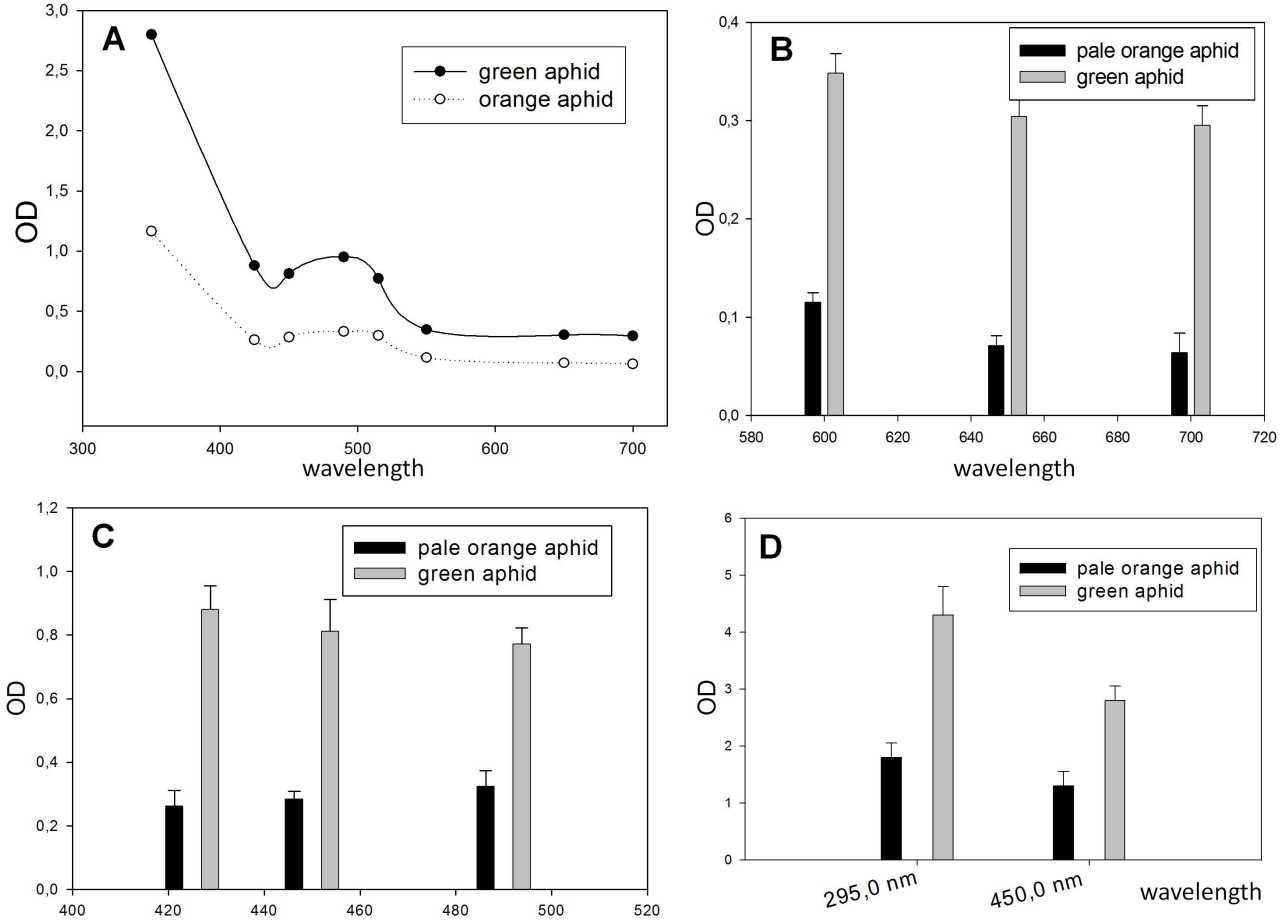

Figure 2 | Absorbance properties of *green*, *orange* and *pale* aphid ethanol extracts. The orange pigments were extracted as indicated in Methods. Briefly, 250 mg of adult aphids were centrifuged in Ringer's buffer and the orange layer was collected. This precipitate was then solubilized in ethanol/acetone (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 +/− S.E (comparison *orange versus green*, $P < 0.002$). More pigment is present in *green* than in *orange* aphids.

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-CH3) 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*-β and *trans*-γ 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 μg.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 μg.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[5].

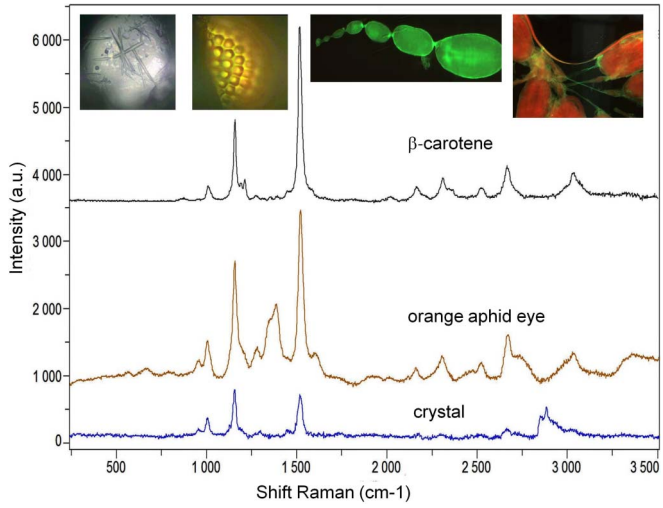

Figure 3 | Carotene signature in aphid eyes and spontaneous crystals by Raman imaging. A 488 nm laser excitation of the Raman spectrometry was used. A control with pure β-carotene is shown. Two other spectra are shown: the microscope laser was focused on an eye and on spontaneous crystals obtained after crushing adult orange aphids in PBS solution. The 1,550, 1,150 and 1,005 cm^{-1} shifts correspond respectively to the C=C, CH-CH and CH-CH3 motifs. Conditions of Raman: 488 nm; 1.2 mW; 3 s. The panels at the top represent spontaneous crystals, an eye, and embryos (mature orange embryos in a germaria *plus* an ovariole stained in green with anti HRP[10]). β-carotene is present in whole aphids and in aphid eyes.

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 polyene 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.

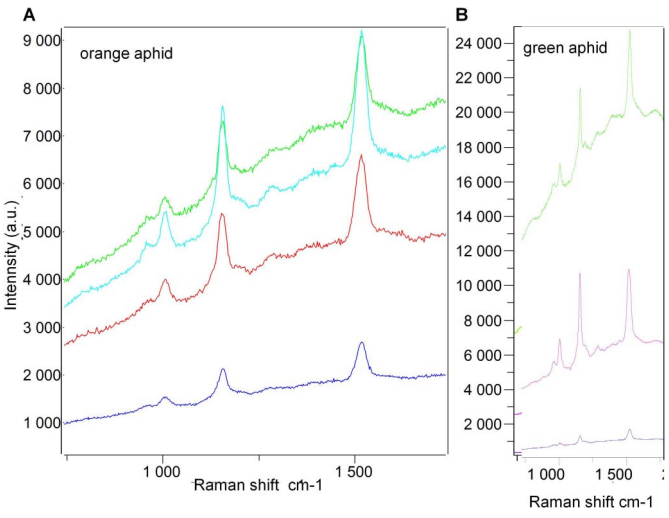

Figure 4 | Carotene signature in aphid adults corresponding to the *green* and *orange* phenotype by Raman imaging. Individual *orange* (A) and *green* (B) living aphid phenotypes (see figure 1 for details) were analysed according to the same protocol as in figure 3. Each assay was conducted with individual adult aphids ten days after the birth of the first instar larva. A significant increase of the intensity of the three identified signals (1,550, 1,150 and 1,005 cm^{-1} shifts) was consistently observed for the *green* phenotype indicating a stronger carotenoid pigmentation. Each colour represents the measures with different individual adult aphids. Raman conditions: 488 nm; 1.2 mW; 1 s.

Table 1 | Comparative quantification of carotenoid molecules in the *green* and *orange* aphid phenotype by LC-MS/MS. Characteristics of identified carotenoids of *orange* and *green* aphids: the molecules were extracted as indicated in Methods and then submitted to HPLC separation and mass spectrum analysis. Strong differences are observed between the two environmentally selected variants originated from a unique parthenogenetic founder mother. No significative differences were observed for *all-trans*-torulene and 3,4-dehydrolycopene as they were for the other compounds analysed

Peak	Tr (min)	λ_{max}	% III/II	M	Compound*
1	35.0	(425), 453, 477	15	536	*all-trans*-β-carotene
2	46.3	436, 463, 492	40	536	*all-trans*-γ-carotene
3	52.7	380, 458, 483, 515	43	534	*cis*-torulene
4	59.3	378, 463, 488, 520	50	534	*all-trans*-torulene
5	63.5	380, 465, 492, 525	60	534	3,4-dehydrolycopene

*Tentative identification.

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

Table 2 | Quantification ($\mu g.100\ g^{-1}$) of carotenoids of *orange* and *green* aphids

Compound	*Green* aphids	*Orange* aphids
all-trans-β-carotene	8.3 ± 1.2^a	4.0 ± 1.9^b
all-trans-γ-carotene	12.6 ± 1.5^a	6.5 ± 1.8^b
cis-torulene	3.0 ± 0.6^a	10.7 ± 3.4^b
all-trans-torulene	9.1 ± 1.3^a	9.9 ± 1.5^a
3,4-dehydrolycopene	5.7 ± 1.7^a	9.1 ± 3.9^a

Values are means ± SD of three independent determinations. Values within a row labeled with different letters are significantly different (Newman–Keuls, $P < 0.05$).

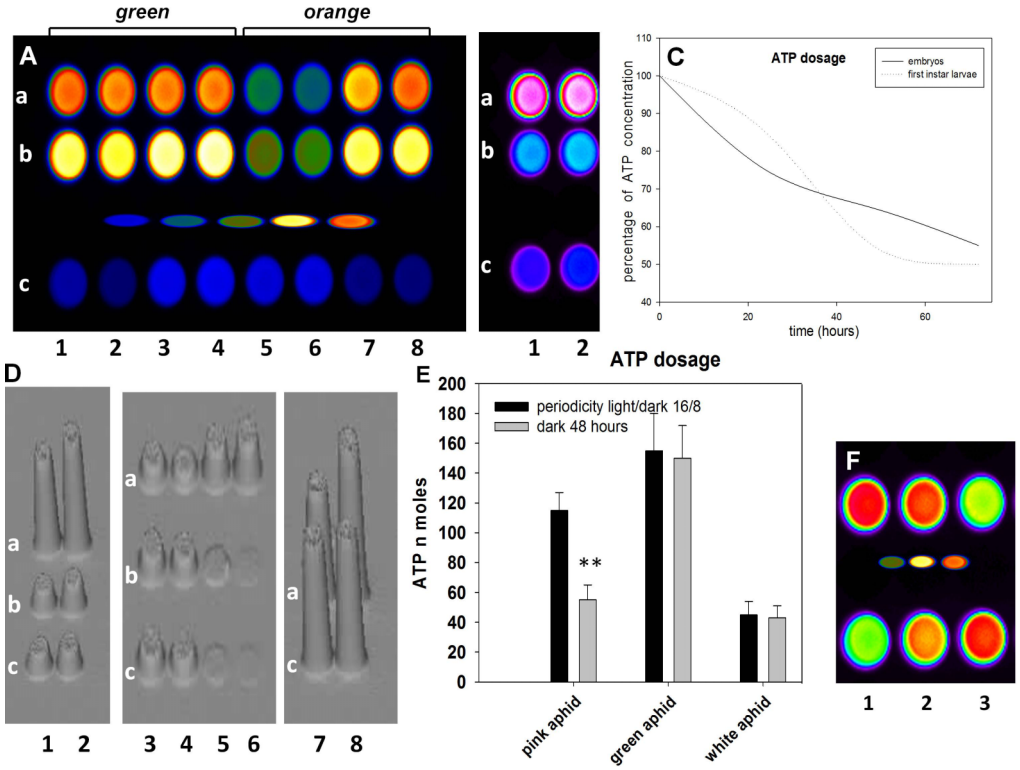

Figure 5 | ATP dosage in dark conditioned aphids. (*A*). *Dark exposure of adult aphids.* Aphids were placed in dark, then tested after two days (1 and 2; 5 and 6) or alternatively were kept in light two days more as control (3 and 4; 7 and 8) before the measure of ATP content. 1–4 and 5–8 are the separate ATP determinations obtained with *green* aphids and *orange* aphids. a and b: ATP dosage determined with the content of 5 and 1 aphids, respectively. c: ATP determination obtained with the content of one *white* aphid. The standards roughly represent 50 pmoles (blue) to 250 pmoles (red). (*B*). *Dark exposure of larvae.* Larvae from orange aphid were kept two days in light (a) or alternatively two days in dark (b). A control *white* larva aphid kept two days in light after birth is shown (c). The determinations were done with 5 larvae. (*C*). *Comparative time course of the decline of ATP content in embryos and larvae placed in dark.* (see methods for experimental design). (*D*) *Light-induced ATP synthesis after a dark exposure episode of orange adult aphids.* 1, 2 are ATP dosages from separate experiments. (a) light-exposure control. (b) and (c) are the dark exposure for two and three days, respectively. 3 and 4 are *orange* adult aphids placed two days in dark (a, b, c). 5 and 6 are the same aphids than in 3 and 4 placed back in light for one day (a) or kept one day (b) or two days more (c) in dark. 7 and 8 are the light rescue of ATP content three days after a dark exposure of two days (c) *versus* the light control (a). (*E*) *Comparative determination of ATP between green, orange and white adult aphids in dark or light.* Bars represent the mean of three different experiments +/−S.E. (** $P<0.005$). (*F*) *Light-induced ATP synthesis after a dark exposure episode in larvae. Top*: First instar larvae were tested at day 0 (1), at day 2 (2) and day 3 (3). *Down*: Emerged progenies were tested at day 0 (1), at day 2 (2) and finally at day 3 (3).

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[13–15]. Amazingly, we observe that carotene molecules are disposed as a bilayer under the cuticule 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*[16]. 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[18,19]. Insects, except aphids, do not synthesize these molecules, but absorb them by food uptake from the chloroplasts of plants/algae[1,2]. The polyenic-conjugated structure of carotenoids (C=C alternated with C-C leading to π delocalised electrons)

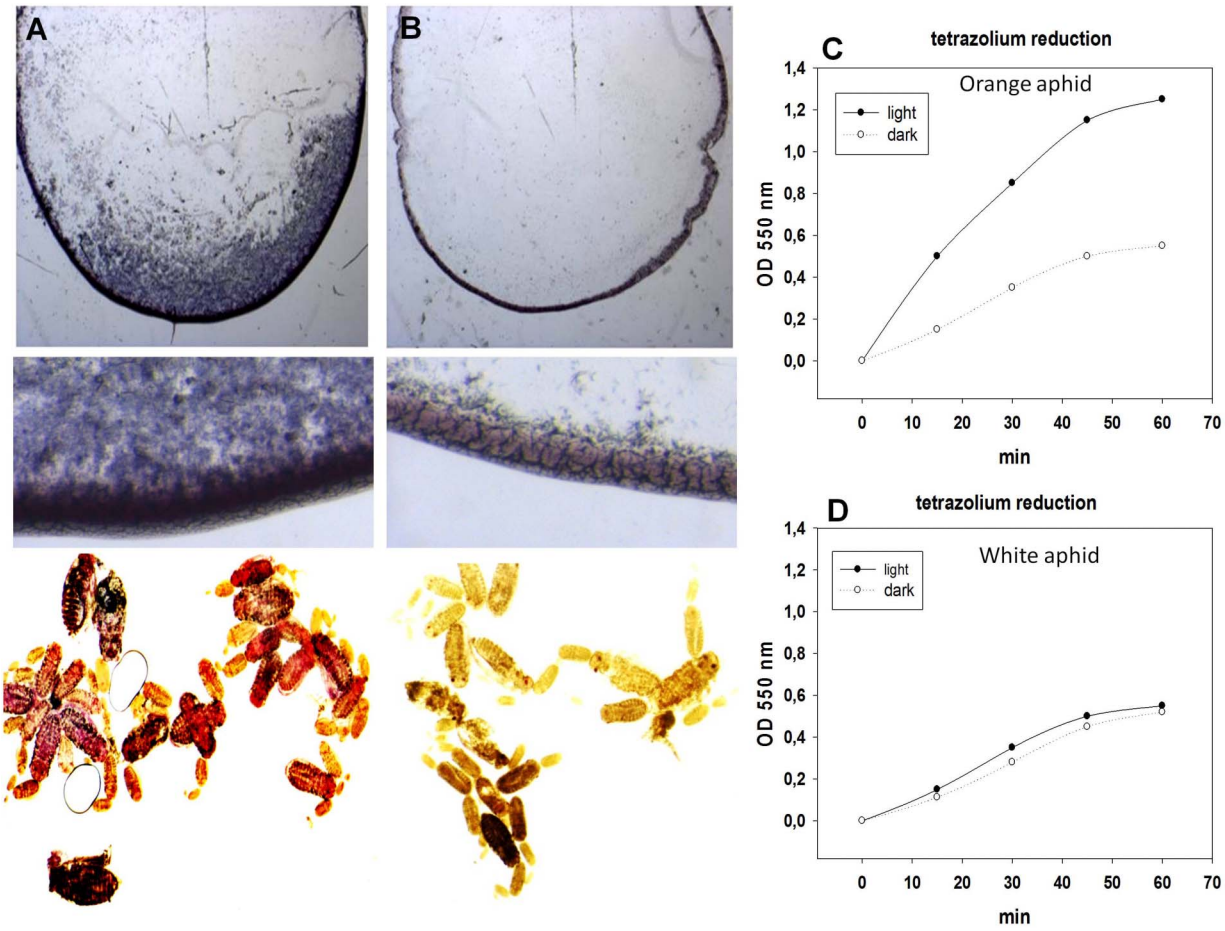

Figure 6 | Tetrazolium (MTT) reduction by *orange* aphid extract. 100 µl of tetrazolium solution (1 mM in water) were placed on a glass slide in which 10 µl of *orange* aphid extracts were added. The system was irradiated by visible light (A) for 30 min or kept in dark (B). Then, the medium was delicately 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.

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[9]. 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[20]. Furthermore and independently, the photoreduction of NAD^+ in presence of metal complexes and organic compounds that donate electrons has been also described[21]. An artificial photosynthetic system like self-assembling of a mix of phenyl dipeptides, porphyrin and metal as platinium was successfully tested to transfer electrons to $NADP^+$ *via* light excitation[22,23]. 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[24]. 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[25]. 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[25]. 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[25]. Moreover and more importantly, the photoconversion 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[26]. The redox potential of NAD^+ is quite high [$E^\circ = -0{,}32$ 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 polycyclic and polyphenolic compounds known to complex metal as Fe^{2+}, might participate

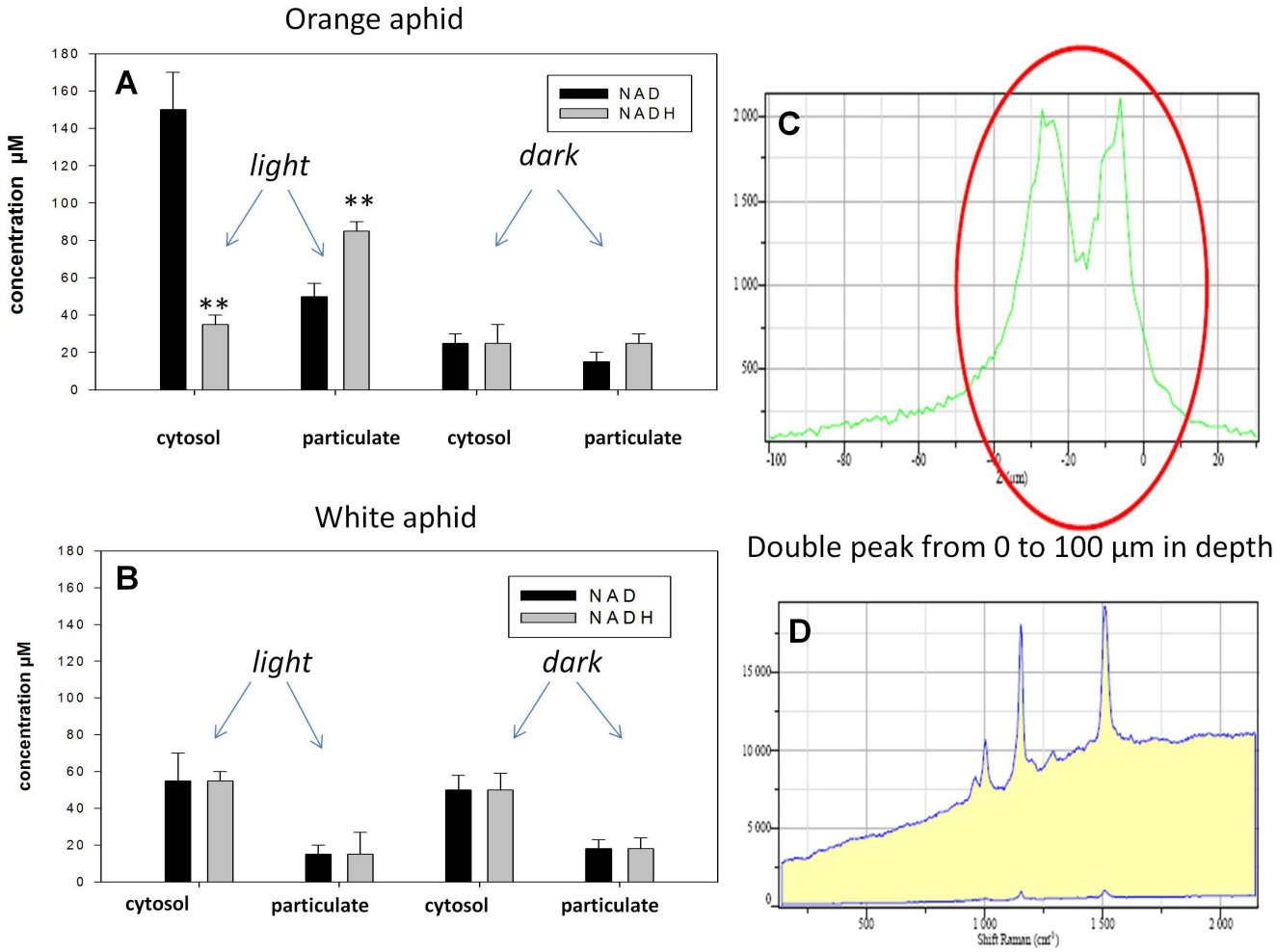

Figure 7 | NAD$^+$/NADH balance in cytosol and particulate (mitochondria) fractions in dark- and light-conditioned aphids. The dosage was performed with *orange* (A) and *white* (B) aphids conditioned in light or dark using methyphenylsulfonate and tetrazolium salts (see Methods). Briefly, 50 adult aphids (*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 +/− 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).

to this phenomenon[7]. 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 +/−3°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 in dark or light: The determinations were carried out with the ATP extraction from ten adult aphids for each experiment. 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).

Extraction of pigments for spectral analysis. Aphids were extracted with Ringer's buffer[27] (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 added. 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, 450 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 compound[28]).

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[29,30]. 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 methods[31,32]. Carotenoids were separated along a C30 column (250 × 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 MTBE as eluent C. Flow rate was fixed at 1 mL/min and the column temperature was set at 25°C. A gradient program was performed: 0–2 min, 40% A/60% B, isocratic elution; 2–5 min, 20% A/80% B; 5–10 min, 4% A/81% B/15% C; 10–60 min, 4% A/11% B/ 85% C; 60–71 min, 100% B; 71–72 min, back to the initial conditions for re-equilibration. The injection volume was 10 µL and the detection was monitored from 250 to 600 nm. After passing through the flow cell of the diode array detector the column eluate was split and 0.5 ml were directed to the ion trap of a LCQ mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, California, USA). Experiments were performed in positive ion mode. Scan range was 100–2000 and scan rate 1 scan/s. The desolvation temperature was set at 25°C.

High performance liquid chromatography analysis of carotenoids. Carotenoids were analysed by HPLC using an Agilent 1100 System (Massy, France). The column and gradient conditions were the same as used in mass spectrometry analysis. The injection volume was 20 µL. Absorbance was followed at 290, 350, 400, 450 and 470 nm using an Agilent 1100 photodiode array detector. An Agilent Chemstation Plus software was used for data analysis. Each analysis was made in triplicates. All carotenoid concentrations were expressed in β-carotene equivalent.

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Author contributions statements

J. C. V. performed the Raman experiments, A. D. did the selection of aphid variants, P. B. and C. M. did the HPLC and mass spectrum analysis of carotene, J. C. V., P. B. and A. R. designed the experiments, M. C. contributed to the experimental work, J. C. V., M. C. and A. R. wrote the manuscript.

Additional information

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