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Cytokinin-mediated leaf manipulation by a leafminer caterpillar

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A large number of hypotheses have been proposed to explain the adaptive significance and evolution of the endophagous-feeding mode, nutritional benefits being considered to be one of the main advantages. Leaf-mining insects should feed on most nutritional tissues and avoid tissues with high structural and/or biochemical plant defences. This selective feeding behaviour could furthermore be reinforced by manipulating the plant physiology, as suggested by the autumnal formation of 'green islands' around mining caterpillars in yellow leaves. The question we address here is how such metabolic manipulation occurs and what the nutritional consequences for the insect are. We report a large accumulation of cytokinins in the mined tissues which is responsible for the preservation of functional nutrient-rich green tissues at a time when leaves are otherwise turning yellow. The analogy with other plant manipulating organisms, in particular galling insects, is striking.

Keywords: leafminer; cytokinins; green island; nutrient acquisition; endophytic organisms

1. INTRODUCTION

Leaf mining is a means by which some plant-eating insects consume live foliage while simultaneously dwelling inside it. A large number of hypotheses have been proposed to explain the adaptive significance and the evolution of this endophagous-feeding mode. The mine presumably functions as a shelter from the detrimental effects of the physical environment, as a protection from attack by natural enemies, enables an increased development rate due to higher temperatures and is potentially a means of making food choices at a micro-scale (Connor & Taverner 1997; Pincebourde & Casas 2006; Pincebourde *et al.* 2006). Following this 'selective feeding hypothesis', many authors have shown that leafminers select those plant tissues with a high nutrient content and avoid those tissues with a high structural and/or chemical defence (Hering 1951; Feeny 1970; Connor & Taverner 1997; Scheirs *et al.* 2001).

Leafminers develop in a small confined space with limited availability of nutritive tissue. They might also strongly suffer from alteration of leaf physiology caused, for example, by premature leaf abscission or leaf senescence. Indeed, unlike gall-forming insects—an other main type of endophytophagous insects—leaf mining does not lead to the proliferation of undifferentiated nutrient-rich plant tissues.

The observation that larval attacks may be responsible for the formation of autumnal 'green islands', green leaf tissues around larvae, is very old and well known (e.g. Tubeuf 1916). Furthermore, empirical evidence also suggests a primary role of cytokinins in 'green island' formation by leafminers (Englebrecht *et al.* 1969). These phytohormones play a crucial role in controlling leaf senescence and have been clearly shown to be involved in plant–bacteria, plant–fungi and plant–galling insects interactions (Barry *et al.* 1984; Smart *et al.* 1991; Leicht 1994; Gan & Amasino 1995).

A markedly visible sign of leaf senescence is the gradual loss of chlorophyll in autumn and concomitant yellowing, but senescence is also a metabolic turning point biochemically characterized by the transition from nutrient assimilation to nutrient remobilization (Masclaux *et al.* 2000; Soudry *et al.* 2005). Leafminers are therefore potentially deprived from the required nutrients for completing their development before winter.

Here, we report that leafminers manipulate their environment using three types of cytokinins. This system allows leafminers to generate a microenvironment with all the nutrient supply needed for their survival in an otherwise senescent environment.

2. MATERIAL AND METHODS

Phyllonorycter blancardella F. (Lepidoptera: Gracillariidae) is a polyvoltine leaf-mining microlepidopteran. The first three larval instars are sap feeders and set the outline of the mine and, therefore, the total surface available to later stages. The two following instars are selective tissue feeders.

Samples were collected early in autumn 2005 on 12-year-old apple (*Malus domestica* Borkh.) cv. 'Allstars' trees grown in a biologically managed orchard. Collected leaves were directly dissected on site, frozen on dry ice and stored at -80°C until analysis. Samples were controlled for leaf age and natural exposition in the orchard (Sun versus shade—position in the rows). Mined age (only one mine per leaf) was also controlled and corresponds to L4 tissue-feeding larvae.

In order to study the spatial variation of cytokinins and nutrient concentrations, mined tissues, ipsilateral tissues (leaf tissues situated on the same side of the main vein as the mine) and contralateral tissues (leaf tissues on the opposite side of the main vein as the mine) were dissected according to figure 1. Leaf-miner insects and frass were also removed from the mine. Non-infected green and yellow apple leaves were dissected as previously and used as a control.

(a) *Cytokinin quantification in mined and non-mined tissues according to leaf senescence*

Leaf samples were lyophilized and pulverized. Similar amounts of control, mined, ipsilateral and contralateral tissues were extracted overnight in aqueous methanol containing butylated hydroxytoluene as an antioxidant.

(i) *Purification*

Purification was performed using a nitrocellulose prefilter (4.5 μm, Sartorius, Germany) connected to an Oasis cartridge (Waters, USA) with a Teflon filter (0.2 μm, Sartorius, Germany) at the outlet.

(ii) *Separation*

Eluates were reduced by rotary evaporation, taken up with acidified water and injected into a reverse-phase HPLC column (Waters, USA). After sample injection, the column was eluted at 1 ml min^{-1} with a convex gradient of acidified water and pure acetonitrile (HPLC grade) until the eluant was 100% acetonitrile. Retention time of cytokinins was determined by separate and pooled injections of pure zeatin (Z), ribosyl zeatin (ZR), isopentenyladenine (iP) and isopentenyl adenosine (iPA) (Sigma, USA) as standards. Fractions centred on the retention time of each cytokinin were collected and evaporated to dryness in a speed-vac concentrator (Savant, USA).

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Figure 1. An apple tree leaf infected with the tentiform leafminer *Phyllonorycter blancardella* larva in autumn. The 'Green island' (feeding area) exhibits intact green chlorophyll-containing tissues, while the remaining leaf tissues undergo leaf senescence. The white spots on the mine are feeding windows, where all but the epidermis has been consumed by the caterpillar. Letters show areas used for cytokinin and nutrient content analysis: 'a' mined, 'b' ipsilateral and 'c' contralateral plant tissues.

(iii) *Quantification*
Aliquots of each fraction corresponding to the retention time of each hormone standard were subjected to enzyme-linked immunosorbent assay (ELISA) as previously published (Jourdain *et al.* 1997). ELISA quantification could be done owing to a strict separation of each compound by HPLC fractionation. Optical densities were measured at 405 nm. See electronic supplementary materials for details.

(**b**) ***Nutrient quantification in mined and non-mined tissues according to leaf senescence***
Quantification of the amount of nutrients in leaf samples was carried out using colorimetric techniques successfully used for plant material (Yemm & Willis 1954; Jones *et al.* 1989). Briefly, samples were lyophilized, weighed and ground. Nutrients were measured using a spectrophotometer after reaction with Anthrone reagent for sugars and Bradford reagent for proteins. See electronic supplementary materials for details.

(**c**) ***Statistical analysis***
Statistical analyses were performed using R v. 2.3.0 software. Data were analysed by non-parametric Kruskal–Wallis tests followed by Behrens–Fisher *post hoc* tests.

3. RESULTS

(a) *Cytokinin content of mined area exceeds that of non-infected or control areas*

To evaluate the role of phytohormones on 'green island' formation, we compared cytokinin contents in mined and non-mined tissues (figure 2). As expected, cytokinin content decreases with leaf senescence, some compounds such as isopentenyladenine, falling below the detection level (green versus yellow controls). However, the global cytokinin content of mined areas far exceeded that of uninfected areas (ipsi- and contralateral) and controls (green and yellow uninfected leaves). This phenomenon is particularly true for isopentenyl adenosine and isopentenyladenine, both compounds being at the beginning of the synthesis pathway of cytokinins. High levels of cytokinins are also observed in the near vicinity of the mine, ipsilateral tissues showing an amount of iP higher than contralateral tissues.

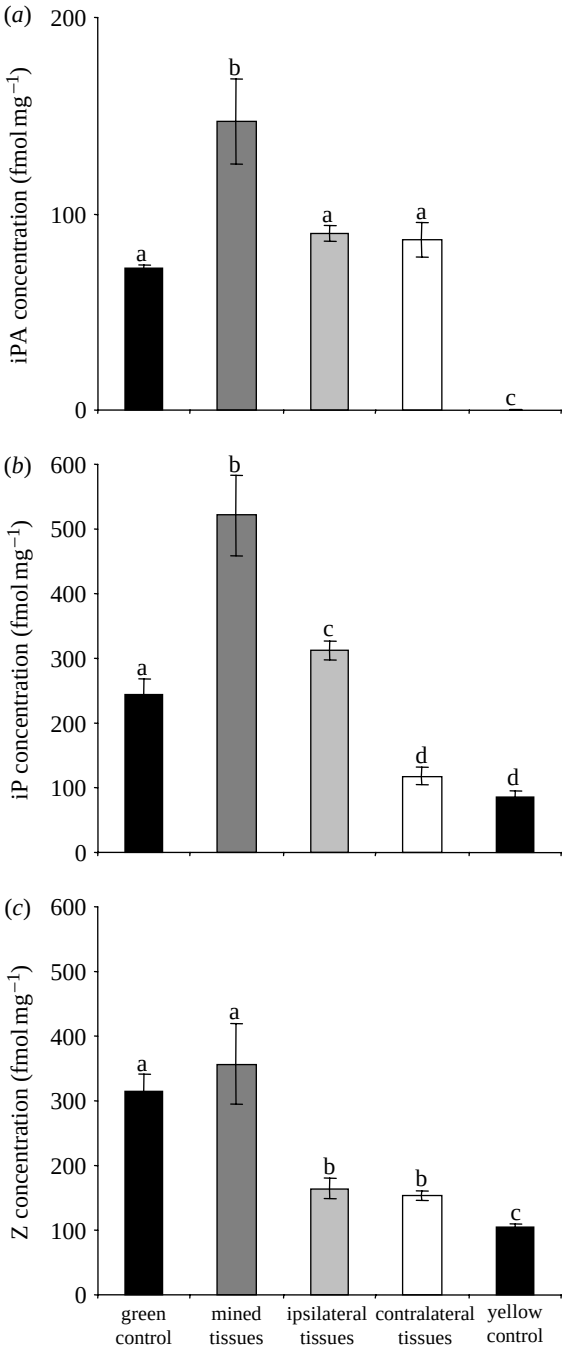

Figure 2. Spatial changes in the levels of cytokinins of senescing apple leaves. (*a*) Isopentenyladenosine, (*b*) isopentenyladenine and (*c*) zeatin concentrations in mined, ipsilateral and contralateral plant tissues are expressed as means ± s.e. Non-infected green or yellow apple leaves were used as a control. Homologous groups are indicated by identical characters (Kruskal–Wallis test: iP, $p<0.01$; iPA, $p<0.05$; Z, $p<0.01$).

(b) *Nutrient content of mined tissues does not decrease in senescing leaves*

We next conducted experiments investigating the nutritional benefits of the hormonal manipulation. Both food quality parameters decreased in senescing leaves falling from 135.56 ± 25.35 to 53.25 ± 4.27 μg mg^{-1} for sugars ($n=15$) and from 29.10 ± 0.91 to 16.22 ± 1.67 μg mg^{-1} for proteins ($n=15$; figure 3). Mined areas showed similar content of carbohydrates and proteins as uninfected green tissues. Carbohydrate and protein amounts in distant

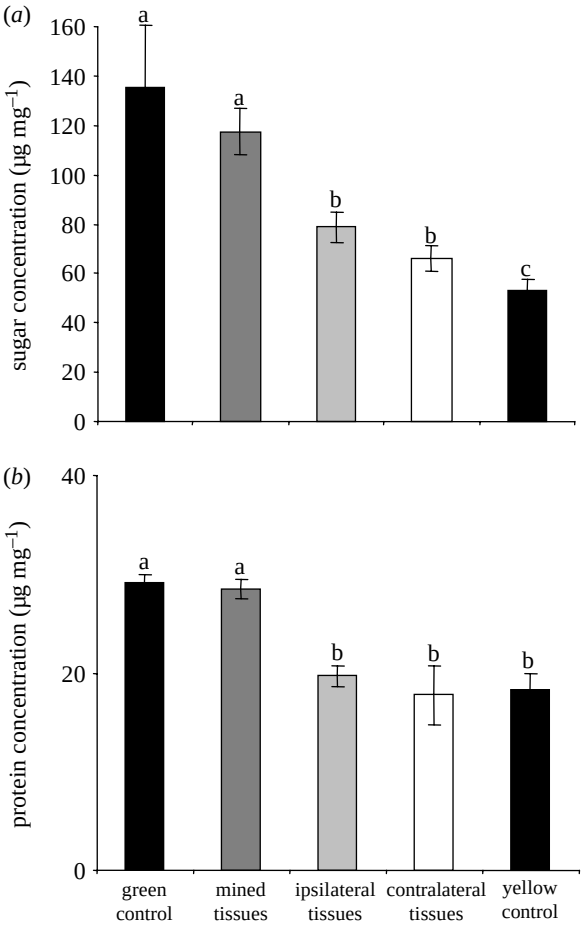

Figure 3. Spatial changes in the levels of (*a*) sugars and (*b*) proteins of senescing apple leaves. Results for mined, ipsilateral and contralateral plant tissues are expressed as means ± s.e. Non-infected green or yellow apple leaves were used as a control. Homologous groups are indicated by identical characters (Kruskal–Wallis test: *sugars*, $p < 0.01$; *proteins*, $p < 0.05$).

regions of infected leaves were similar or slightly higher than uninfected yellow controls.

4. DISCUSSION

'Green islands' are characterized by similar or even greater levels of zeatin, isopenteyladenine and isopentenyl adenosine than non-senescing green leaves. This explains the stay-green phenotype of mined areas, while other regions of the same leaf are turning yellow. The origin of these cytokinins is however currently unknown and several hypotheses can be proposed. First, high levels of cytokinins in mined areas could result from the overexpression of the plant genes which produce cytokinins in the infected zone. Oral secretions of feeding insects contain many specific compounds able to interact with plant gene expression as shown in the upregulation of plant defences (e.g. Arimura *et al.* 2005). Second, cytokinins in mined tissues could result from an accumulation process rather than a synthesis *per se*. Feeding activity of leafminers is known to alter biophysical properties of leaves and consequently respiration and transpiration rates (Pincebourde *et al.* 2006). Such processes strongly impact the flow of nutrients within a leaf and could potentially result in the accumulation of cytokinins in mined areas. Third, insects themselves could potentially synthesize cytokinins. Large quantities of cytokinins have already been measured in labial glands of leafminers (Engelbrecht *et al.* 1969) and the ability to produce cytokinins has been shown in several plant-associated organisms like fungi and bacteria (Barry *et al.* 1984; Stevens & Berry 1988; Morris 1995).

During the senescence of apple tree leaves, the levels of proteins and carbohydrates decrease, but mined tissues displayed a similar level of both proteins and sugars as non-senescing uninfected green leaves. Owing to the maintenance of the photosynthetic apparatus within the mine—as a direct consequence of high levels of phytohormones—natural production of sugars and proteins 'on site' cannot be excluded. However, modifications of the water flow and accumulation of nutrient-attracting cytokinins in the mined area could favour the translocation of nutrients from the leaf to the mine as well. In any case, both processes result in a net accumulation of nutrients in the mined tissues with potential competition between the plant and the mine for nutrients originating from senescing cells (change in the source–sink relationships—Balibrea *et al.* 2004; Wingler *et al.* 2006).

The maintenance of functional green tissues is, of course, of considerable ecological value to the development of the larvae as it allows the insect to maintain a favourable nutritional environment in an otherwise degenerating context. The 'green island', enriched in cytokinins, enables the larvae to retain nutrients within the mine and to undergo a long development later in the season. Interestingly, remote parts of infected leaves have higher concentration of cytokinins than uninfested yellow leaves (except for iP). Yellow leaves also totally lack iPA, while this class of cytokinin is present in all tissues of infected leaves. Both results are a clear proof of manipulation of the plant physiology by the herbivore and over a much larger scale than the mine itself. The observed metabolic changes taking place in the mine and the manipulation of plant physiology induced by the insect strengthens the hypothesis that mines behave independently from the hosting leaf, operating a metabolic machinery of their own. Similar conclusions have been drawn with galling insects also known to produce galling tissues with high levels of cytokinins (e.g. Leicht 1994; Carol & Davies 2001). Despite great morphological differences between galls and mines, our results suggest a probable generalized process associated with the colonization of plants by endophagous organisms. Such intimate associations probably facilitated biochemical and hormonal cross-talk between insects and plants, setting the ground for host-plant manipulation by insects.

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Electronic supplementary material

Cytokinins quantification. Cytokinins were measured using enzyme-linked immunosorbent assay (ELISA). Elisa quantification could be done because of a strict separation of each compound by HPLC fractionation. *iP* and *iPA* were measured using anti-*iPA* polyclonal antibodies. Zeatin riboside and Zeatin were measured using anti-*ZR* polyclonal antibodies. Briefly, ELISA was performed with microtitration plates (Nunc, Germany) coated with the antiserum appropriate to the assay being performed and incubated at room temperature in the dark overnight. Excess antiserum was removed by washing each plate four times (distilled water with 0.1% photoflo) and a limited amount of hormone standard or sample and anti-hormone antibody were then added. Blank controls and duplicates of authentic cytokinin standards in a logarithmic concentration series from 0 to 30 pM were performed on each plate. Remaining wells were used for duplicated samples of HPLC fractions. Plates were incubated 2h at 4°C in darkness. After washing, anti-hormone antibodies bound to the plate were quantified. Optical densities were measured at 405nm and values obtained from ELISAs were corrected for extraction and purification losses according to the percentage recovery of the tritiated adenine included in the sample extraction procedure.

Antisera used are specific for the two families of isoprenoid cytokinins (isopentenyladenine- and zeatin-types). The use of these immunoreagents in combined HPLC-ELISA has enabled identification and measurement of individual members of the cytokinin families naturally occurring in plants in numerous studies with the advantage over standard GC-MS methods that much smaller amounts of tissue are required (see for example, Jourdain *et al.* 1997). Cross-reactivity between the two families was remarkably low, i.e. no more than 3% but typically less than 1.5% indicating that the individual antibodies were highly selective.

Nutrient quantification. Quantification of the amount of nutrients in leaf samples was carried out using colorimetric techniques successfully used for plant material (Jones *et al.* 1989; Yemm & Willis 1954).

Sugars - Briefly, samples were lyophilized, weighed and grounded in an Eppendorf tube and 40µl of a 2% sodium sulphate and 300µl of chloroform-methanol (1:2) were added to each sample. After centrifugation at 1500rpm for 15min, sugars dissolved in the supernatant were transferred to a borosilicate tube and heated at

90°C to almost complete dryness. After adding 1ml Anthrone reagent, the tubes were placed at 90°C for 15 min, then cooled and read in a spectrophotometer at. Calibration curves were obtained using glucose.

Proteins - For proteins, lyophilized samples were weighed and grounded in an Eppendorf tube with 800µl of physiological water (0.15M NaCl) containing 0.001% Triton X-100 (Sigma, USA) and placed at 4°C for 3 days to allow time for the Triton-X to dissolve the proteins. Then 200µl of Bradford Reagent (Bio-Rad Laboratories, Germany) reactive were added and absorbance was read at 595nm after 15min incubation. Calibration curves were obtained using bovine serum albumin (Sigma, USA).