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ORIGINAL ARTICLE

Dynamics and origin of cytokinins involved in plant manipulation by a leaf-mining insect

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Abstract Several herbivorous insects and plant-associated microorganisms control the phytohormonal balance, thus enabling them to successfully exploit the plant by inhibiting plant defenses and withdrawing plant resources for their own benefit. The leaf-mining moth *Phyllonorycter blancardella* modifies the cytokinin (CK) profile of mined leaf-tissues, and the insect symbiotic bacteria *Wolbachia* is involved in the plant manipulation to the benefit of the insect host. To gain a deeper understanding into the possible origin and dynamics of CKs, we conducted an extensive characterization of CKs in larvae and in infected apple leaves. Our results show the enhanced CK levels in mines, both on green and yellow leaves, allowing insects to control their nutritional supply under fluctuating environmental conditions. The spatial distribution of CKs within the mined leaves shows that hormone manipulation is strictly limited to the mine suggesting the absence of CK translocation from distant leaf areas toward the insect feeding site. Mass spectrometry analyses reveal that major CK types accumulating in mines and larvae are similar to what is observed for most gall-inducers, suggesting that strategies underlying the plant manipulation may be shared between herbivorous insects with distinct life histories. Results further show that CKs are detected in the highest levels in larvae, reinforcing our hypothesis that CKs accumulating in the mines originate from the insect itself. Presence of bacteria-specific methylthio-CKs is consistent with previous results suggesting that insect bacterial symbionts contribute to the observed phenotype. Our study provides key findings toward the understanding of molecular mechanisms underlying this intricate plant–insect–microbe interaction.

Key words cytokinins; insect bacterial symbionts; leaf-miners; phytohormones; plant–insect–microbe interactions; plant manipulation

Introduction

In the last decades, jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) have been described as major plant hormones regulating plant responses to biotic and abiotic stresses (Clarke *et al.*, 2000; Ferrari *et al.*, 2007; Bari & Jones, 2009; Santner *et al.*, 2009; Erb *et al.*, 2012; Coolen *et al.*, 2016; Großkinsky *et al.*, 2016). Besides these well-characterized phytohormones, cytokinins (CKs) have

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recently re-emerged as important players regulating evolutionary trade-offs between plant growth and defense (Herms & Mattson, 1992; Giron *et al*., 2013) or plant defense and reproduction (van der Krieken *et al*., 1990; D'Aloia *et al*., 2011). Because CK-mediated effects on plant physiology include modulation of plant defense, allocation of resources, inhibition of senescence and regulation of cell division, they are a target for both insects and microbes to disrupt the plant defensive response and/or to withdraw plant resources for their own benefit (Mok & Mok, 2001; Sakakibara, 2006; Giron *et al*., 2013). It is now clear that both microbes and plants can synthesize CKs, and emerging data strongly suggest that insects may produce such regulators either directly or indirectly thanks to their association with endosymbiotic bacteria (Kaiser *et al*., 2010; Giron & Glevarec, 2014; Tooker & Helms, 2014; Zhang *et al*., 2016). This suggests that insects could be the sources of phytohormones—rather than simply manipulating the plant phytohormonal balance/signaling—allowing them to hijack the plant machinery for their own benefit and giving rise to intricate plant–microbe–insect interactions.

Production of CKs has been demonstrated in gall-inducing bacteria (e.g., Stes *et al*., 2011, 2013), nodulating bacteria (e.g., Frugier *et al*., 2008; Kisiala *et al*., 2013) and plant-associated fungi and viruses (e.g., Walters *et al*., 2008; Baliji *et al*., 2010; Morrison *et al*., 2017). Additionally, CKs have been detected in the body, saliva or accessory glands of insects suggesting their ability to produce and deliver these effectors to the plant (Giron *et al*., 2013; Bartlett & Connor, 2014; Tooker & Helms, 2014). They have been found in several gall-inducing insect species (Ohkawa, 1974; Mapes & Davies, 2001b; Dorchin *et al*., 2009; Straka *et al*., 2010; Tooker & De Moraes, 2011a, 2011b; Yamaguchi *et al*., 2012; Giron *et al*., 2013; Tanaka *et al*., 2013), in the apple-tree leaf-miner (Engelbrecht *et al*., 1969; Body *et al*., 2013) and in the labial glands of several leaf-miners including the birch green miner (Engelbrecht *et al*., 1969; Engelbrecht, 1971). However, the evolutionary origin of CKs involved in plant–insect interactions is still unclear due to a lack of an extended biochemical characterization of CKs involved under various environmental conditions and in different areas of the attacked plant.

Previous experiments in the *Malus domestica*/*Phyllonorycter blancardella* leaf-mining system have shown that insects induce "green islands," which are characterized by photosynthetically active green patches in otherwise senescing leaves. Results obtained using a targeted enzyme-linked immunosorbent assay (ELISA), allowing the characterization of a limited number of CKs, demonstrated that these leaf areas are enriched in CKs and that insects can manipulate their nutritional microenvironment not only on yellow but also on green leaves (Giron *et al*., 2007; Body *et al*., 2013). An extensive characterization of how the leaf-miner *P. blancardella* modulates the plant CK profile and how this might interfere with the plant global hormonal balance was recently conducted but only on green leaves (Zhang *et al*., 2016). Major phytohormones and transcriptional activity of plant cells in contact with *P. blancardella* were monitored and compared to those of control unmined leaf tissues. This showed that the level of CK active forms strongly increased in mined zones (specifically *t*Z-type CKs) and that CKs which accumulated in the mined area do not originate from the plant CK-biosynthetic pathway (Zhang *et al*., 2016). Several lines of evidence also showed that all *P. blancardella* larvae were infected with *Wolbachia* therefore suggesting that CKs are likely to originate from microbial symbionts (Giron *et al*., 2007; Kaiser *et al*., 2010; Body *et al*., 2013; Gutzwiller *et al*., 2015).

The objective of this study was to conduct an extensive identification and quantification of CKs in both green and yellow leaves of *M. domestica* infested by *P. blancardella* taking into account temporal and spatial aspects of the interaction. CKs were also characterized in larvae to investigate their possible contribution to the production of CKs. Our extensive CK profiling includes *cis*-type CKs and 2-Methylthio (2-MeS)-derivatives. *Cis*-type CKs are a group of CKs that have often been ignored compared to *trans*-type CKs mainly due to the lack of appropriate analytical methods (Schäffer *et al*., 2015). The ability to produce *cis*-CKs has been identified in plant species but also in several pathogens including bacteria (Scarbrough *et al*., 1973) and fungi (Strzelczyk *et al*., 1989). While *cis*-CKs potentially play a role in plant resistance, those produced by pathogens could in contrast possibly contribute to the successful development of the pathogens. Bacteria-produced *cis*-CKs were shown to accumulate in *Arabidopsis* tissues infected by *Rhodococcus fascians* for which they correlated with the proliferation and symptom maintenance of the pathogen (Pertry *et al*., 2009). Insects could also potentially use *cis*-CKs in combination with other CKs to manipulate the plant physiology as suggested by the high levels of *cis*-Zeatin (*c*Z) found in the larval body of a galling aphid (Straka *et al*., 2010). 2-MeS-CKs comprise a group of hydrophobic CK derivatives that have also been neglected due to their usually low quantity in plant tissues and because they are generally regarded as mere *t*RNA degradation products, but with no known source biochemical pathway and as yet unknown physiological significance for the plant. When detected in plant tissues, they are often considered to be of endophytic origin (Ajitkumar & Cherayil, 1988; Petry

et al., 2009; Tarkowski *et al*., 2010). 2-MeS-CKs have been demonstrated to be essential components of plant–microbe interactions (Pertry *et al*., 2009). In contrast to classical CKs, the 2-MeS-CKs produced by *R. fascians* were not degraded by the *Arabidopsis* CK dehydrogenases (CKXs) machinery allowing for an accumulation of CKs in infected tissues and bacterial persistent effects. Additionally, the production of specific bacterial 2-MeS-CKs that are less active but also less toxic than other CKs may allow bacteria to avoid deleterious effects on plant development and CK-mediated plant defenses (Pertry *et al*., 2009). While 2-MeS-CKs have not been investigated in any plant–insect interactions, we previously hypothesized that 2-MeS-CKs are potentially important for gall-inducing and leaf-mining insects (Giron & Glevarec, 2014). The constant presence of the insect at a localized feeding site would need the accumulation of specific CKs to facilitate persistent benefits to the insect, thus 2-MeS-CKs could play an important role toward reaching this goal because of their biological properties. 2-MeS-CKs are produced in high quantities by bacteria and some 2-MeS-CKs seem to be strictly bacteria-specific CKs (Skoog & Armstrong, 1970; Ajitkumar & Cherayil, 1988; Mok & Mok, 1994). Characterization of 2-MeS-CKs could thus help clarify whether insect bacterial symbionts directly contribute to the production of CKs involved in the plant–insect interaction investigated in this work.

Materials and methods

Biological material

Phyllonorycter blancardella (Fabricius, 1781) (Lepidoptera: Gracillariidae) is a polyvoltine leaf-mining microlepidopteran of apple trees. The larva establishes and maintains a permanent "feeding area" for its development. The first 3 instars (L1–L2–L3) that feed on interstitial fluids are fluid-feeders. L1–L3 larvae define the outline of the mine by separating the 2 leaf integuments. The last 2 instars (L4–L5) that consume the lower and upper parenchyma are tissue-feeders (Body *et al*., 2015). Their consumption of the upper palisade cells of the leaf results in the formation of feeding windows (Pottinger & LeRoux, 1971; Djemaï *et al*., 2000). The insect suppresses the plant defense system, modifies the leaf physiology to create green islands on yellow leaves and inhibits senescence in order to maintain an appropriate food supply (Body, 2013). In this system, endosymbiotic bacteria associated with insects play a key role in the plant–insect interaction (Giron *et al*., 2007; Kaiser *et al*., 2010; Body *et al*., 2013; Gutzwiller *et al*., 2015). Both green and yellow mined (only 1 mine per leaf at the L4–L5 tissue feeders instar) and unmined (an adjacent neighboring leaf) leaves were collected in the field between 09:00 a.m. and 10:00 a.m. in autumn (November) on *Malus domestica* apple trees, in a biologically managed orchard. Leaf tissues were dissected on ice following the exact outline of the mine, frozen immediately in liquid nitrogen, and then stored at −80 °C until analysis of CK profiles ($n = 5$ for each leaf area). Leaf-mining insects and frass were removed from the mine. Leaf tissues on the same side and different side of the main vein and the adjacent healthy leaves were used as controls and marked as U1 (unmined ipsilateral tissues), U2 (unmined contralateral tissues), and C (control) respectively. Larvae were also frozen immediately in liquid nitrogen and stored at −80 °C for further CK analyses ($n = 3$ for each leaf area). All leaf and insect samples were ground with a mortar and a pestle in liquid nitrogen after lyophilization (Bioblock Scientific Alpha 1–4 LD plus lyophilizator).

Extraction and purification of CKs

A modified protocol described by Quesnelle and Emery (2007) and Farrow and Emery (2012) was used for CK extraction. The freeze-dried leaf and larvae samples were resuspended in extraction buffer Bieleski #2 (CH_3OH : H_2O : HCOOH [15 : 4 : 1, v/v/v]), spiked with 10 ng of each of the deuterated internal standard CKs, (OlChemim Ltd., Olomouc, Czech Republic; Table 1), and homogenized (ball mill, RetschMM300; 5 min, 25 Hz) at 4 °C with zirconium oxide grinding beads (Comeau Technique Ltd., Vaudreuil-Dorion, Canada). The samples were allowed to extract passively overnight (approximately 12 h) at −20 °C. Pellets were removed by centrifugation (Thermo Scientific; Model Sorvall ST16, Ottawa, Canada; 10 min at 10 000 r/min), the obtained samples were re-extracted with 1 mL extraction buffer at −20 °C for 30 min. The pooled supernatants were dried in a speed vacuum concentrator at 35 °C.

Extraction residues were reconstituted in 1 mL of 1 mol/L formic acid (pH 1.4) to ensure complete protonation of all CKs. Each extract was purified on a mixed mode, reverse-phase, cation-exchange cartridge (Waters; Oasis MCX 6 cc; 150 mg, Mississauga, ON, Canada). Cartridges were activated with 5 mL of HPLC grade methanol and equilibrated using 5 mL of 1 mol/L formic acid (pH 1.4). After equilibration, each sample was loaded and washed with 5 mL of 1 mol/L formic acid (pH 1.4). CKs were eluted based

Table 1 Cytokinins (CKs), scanned by liquid chromatography-positive electrospray ionization tandem mass spectrometry (HPLC-(ESI+)–MS/MS). Deuterated internal standards purchased from OlChemim Ltd. (Olomouc, Czech Republic), were used for the analysis.

Isoprenoid cytokinins	Labeled CK standard
Nucleotides (CKNTs)	
1. *Trans*-zeatin riboside-5'-monophosphate (*t*ZNT)	$^2H_5$[9RMP]Z
2. *Cis*-zeatin riboside-5'-monophosphate (*c*ZNT)	
3. Dihydrozeatin riboside -5'-monophosphate (DHZNT)	$^2H_3$[9RMP]DHZ
4. N^6-isopentyladenosine-5' monophosphate (iPNT)	$^2H_6$[9RMP]iP
Ribosides (CKRBs)	
5. *Trans*-zeatin riboside (*t*ZR)	$^2H_5$[9R]Z
6. *Cis*-zeatin riboside (*c*ZR)	
7. Dihydrozeatin riboside (DHZR)	$^2H_3$[9R]DHZ
8. N^6-isopentyladenosine (iPR)	$^2H_6$[9R]iP
Free bases (CKFBs)	
9. *Trans*-zeatin (*t*Z)	$^2H_3$DHZ
10. *Cis*-zeatin (*c*Z)	
11. Dihydrozeatin (DHZ)	
12. N^6-isopentyladenine (iP)	$^2H_6$iP
Glucosides (CKGCs)	
13. *Trans*-zeatin-O-glucoside (*t*ZOG)	$^2H_5$ZOG
14. *Cis*-zeatin-O-glucoside (*c*ZOG)	
15. Dihydrozeatin-O-glucoside (DHZOG)	$^2H_7$DHZOG
16. *Trans*-zeatin-O-glucoside riboside (*t*ZROG)	$^2H_5$ZROG
17. *Cis*-zeatin-O-glucoside riboside *c*ZROG	
18. Dihydrozeatin-O-glucoside riboside (DHZROG)	$^2H_7$DHZROG
19. *Trans*-zeatin-9-glucoside (*t*Z9G)	$^2H_5$Z9G
20. *Cis*-zeatin-9-glucoside (*c*Z9G)	
21. Dihydrozeatin-9-glucoside (DHZ9G)	$^2H_3$DHZ9G
Methylthiols (2-MeS-CKs)	
22. 2-Methylthio-*trans*-zeatin (2MeSZ)	$^2H_5$MeSZ
23. 2-Methylthio-*trans*-zeatin riboside (2MeSZR)	$^2H_5$MeSZR
24. 2-Methylthio-N^6-isopentyladenine (2MeSiP)	$^2H_6$MeSiP
25. 2-Methylthio-N^6-isopentyladenosine (2MeSiPA)	$^2H_6$MeSiPR
Aromatic cytokinins	Labelled CK standard
26 Benzyloaminopurine (BA)	$^2H_7$BA
27 Benzyloaminopurine riboside (BAR)	$^2H_7$BAR

on their chemical properties. The nucleotide fraction (CKNTs) was eluted using 5 mL of 0.35 mol/L ammonium hydroxide, free bases (CKFBs) retain on the column based on charge and hydrophobic properties and, thus, these were eluted last using 5 mL of 0.35 mol/L ammonium hydroxide in 60% methanol. All samples were evaporated to dryness in a speed vacuum concentrator at 35 °C, and stored at −20 °C.

CKNTs were dephosphorylated using 3 units of bacterial alkaline phosphatase (12 μL) in 1 mL of 0.1 mol/L ethanolamine-HCL (pH 10.4) for 12 h at 37 °C (Emery *et al*., 2000). The resulting ribosides (CKRs) were brought to dryness in a speed vacuum concentrator at 35°C. Samples were reconstituted in 1.5 mL double distilled water for further purification on a reversed-phase C18 column (Canadian Life Sciences; C18/14, 3 cc, 500 mg; Peterborough, ON, Canada). Columns were activated using 3 mL HPLC grade methanol and equilibrated with 6 mL double distilled water. The samples were loaded onto the C18 cartridge and allowed to pass through the column by gravity. The sorbent was washed with 3 mL of double distilled water and analytes were eluted using 1.25 mL HPLC grade methanol. All sample eluents were dried in a speed vacuum concentrator at 35 °C and stored at −20 °C until further processing.

Prior to LC-MS/MS analysis, all dried CK samples were reconstituted in 1.5 mL of starting conditions buffer (CH_3COOH : CH_3CN : ddH_2O [0.08 : 5.0 : 94.92, vol/vol/vol]).

CKs quantification and analysis

Hormones were identified and quantified by electrospray ionization, liquid chromatography-tandem mass spectrometry, HPLC-(ESI+)–MS/MS, (Shimadzu LC10ADvp HPLC connected to an Applied Biosystem SCIEX QTRAP 5500 Quadrupole Mass Spectrometer, Foster City, CA, USA). A 20 μL sample volume was injected on a Luna reversed-phase C18 column (Phenomenex; 3 μm, 150 × 2.1 μm, Torrance, CA, USA) and CKs were eluted with an increasing gradient of 0.08% acetic acid in acetonitrile (A) mixed with 0.08% acetic acid in double distilled water (B) at a flow rate of 0.28 mL/min. The initial conditions were 5% A and 95% B, changing linearly in 12 min to 95% A and 5% B. Conditions remained constant for 5 min, and then immediately returned back to initial conditions for 12 min. The effluent was introduced into the electrospray source (source block temperature of 700 °C), using conditions specific for each CK and analysis was obtained by multiple reaction monitoring (MRM) of the protonated intact CK molecule $[M+H]^+$ and the specific production.

Data analysis

Hormone analysis for each of the tested tissue types was performed in 5 (leaf samples) or 3 replications (larvae samples). All the obtained data were analyzed using Analyst (v 1.6.2) software (AB SCIEX, Framingham, MA, USA) to calculate peak area. Quantification was achieved through isotope dilution analysis based on recovery of ^{2}H-labelled internal standards.

CK data were analyzed using Statistica 8.0 software. The Shapiro–Wilk test was used to test if data were normally distributed. As data were not normal, we transformed them using a sqrt[log(x+1)] function so a parametric test could be used. MANOVA analysis was performed to determine whether the overall CK signature was differing between groups. For MANOVA, we used the Pillai's test statistic, which is considered to be the most robust to violations of assumptions. As follow-ups to the multivariate MANOVA test, univariate ANOVAs were used to determine which individual variables differ between groups. Where significant effects of hormone were observed, *post hoc* comparisons were performed by Fisher LSD test ($P < 0.05$; df = 36). Additionally, a Principal Component Analysis was conducted using the hormone data obtained from HPLC-MS analysis of each tested tissue type to visualize specific patterns potentially not revealed by statistical analyses performed (data not shown).

Results

CK content increases in mined tissues with distinct patterns in green and yellow leaves

In total, 16 CKs that belong to 4 isoprenoid types of CKs (iP, *t*Z, DZ, and *c*Z-type CKs—see Table 1 for abbreviations) are identified in both green and yellow leaves (Figs. 1 and 2). Identified CKs include precursors (CK nucleotides [CKNTs]: iPNT, *t*ZNT, DZNT, and *c*ZNT), active forms (CK ribosides [CKRBs]: iPR, *t*ZR, DZR, and *c*ZR; CK free bases [CKFBs]: iP, and *t*Z), and reversible storage forms (CK glucosides [CKGCs]: *t*ZOG, *t*ZROG, DZOG, DZROG, *c*ZOG, and *c*ZROG). 2-methylthio derivatives (2-Me-SZ and 2-Me-SZR) are also identified in both green and yellow leaves (Fig. 5). The overall CK signature differed significantly between leaf areas (MANOVA: $F_{9,31} = 3.57$, $P < 0.001$, $n = 40$). Mined areas are enriched in CKs both on green and yellow leaves with higher levels of CKs observed on yellow leaves (Fig. 1A). The specific CK composition also differs between mines on green and on yellow leaves (Fig. 1B) with a lower amount of precursors and a slightly larger amount of active forms in mines on yellow leaves. High levels of CK storage forms are observed both on green and yellow leaves. Spatial distribution of CKs within infected leaves shows that CK alterations are strictly restricted to the mined area (Fig. 1A) and that CK profiles of control leaf tissues (C) are similar to those of adjacent (U1) and distant (U2) areas. The PCA analysis confirmed these results highlighting clearly distinct groups between mined areas and uninfected tissues (C, U1, and U2) that are combined in a single group (data not shown).

Overall, *t*Z-type CKs are the CKs undergoing the highest modulation in infected leaves. They significantly accumulate in mines compared to unmined or control tissues both on green and yellow leaves. This is the only type of CK that is present in all 4 CK fractions (CKNTs, CKRBs, CKFBs, and CKGCs) (Fig. 2). The presence of mines in green leaves resulted in a statistically significant increase in the levels of t*Z* derivatives. The concentration of CK precursor *t*ZNT is characterized by a 2 fold increase in mines while active forms *t*ZR and *t*Z are about 3 times more abundant in the mines compared to controls. Storage forms *t*ZOG and *t*ZROG that could be reactivated by β-glucosidases are respectively about 2 and 20 times more abundant in the mine compared to controls. Interestingly, in yellow leaves *t*ZNT, *t*ZR, and *t*Z are detected only in mined tissues. Storage forms *t*ZOG and *t*ZROG accumulate respectively about 2 and 28 times more in the mines compared to controls. Compared to mines on green leaves, mined tissues on yellow leaves show lower amounts of *t*Z but larger concentrations of *t*ZR and *t*ZROG.

A very different pattern emerged for *c*Z-type CKs. While mines are characterized by an overall accumulation of CKs, *c*Z type-CKs are either not affected or decreased in mined tissues. *c*ZNT is not present in mines on yellow leaves, and *c*ZR and *c*ZROG are not detected in mines both on green and yellow leaves. *c*Z is not detected in any leaf tissues.

Only active forms of iP-type CKs accumulate in mines with distinct patterns between green and yellow leaves (Fig. 2). In green tissues, iP is about 2 times more abundant in mines compared to control leaves while the quantities of iPNT and iPR are not affected by the presence of the leaf-mining larvae. In yellow leaves, iP can only be detected in mined zones but iPR is also affected with concentrations about 11 times higher in mined leaf tissues than in unmined tissues. Compared to mines on green leaves, mined tissues on yellow leaves show lower amounts of iPNT and iP but higher concentrations of iPR.

DZ-type of CKs show very similar patterns both on green and yellow leaves, with mined areas

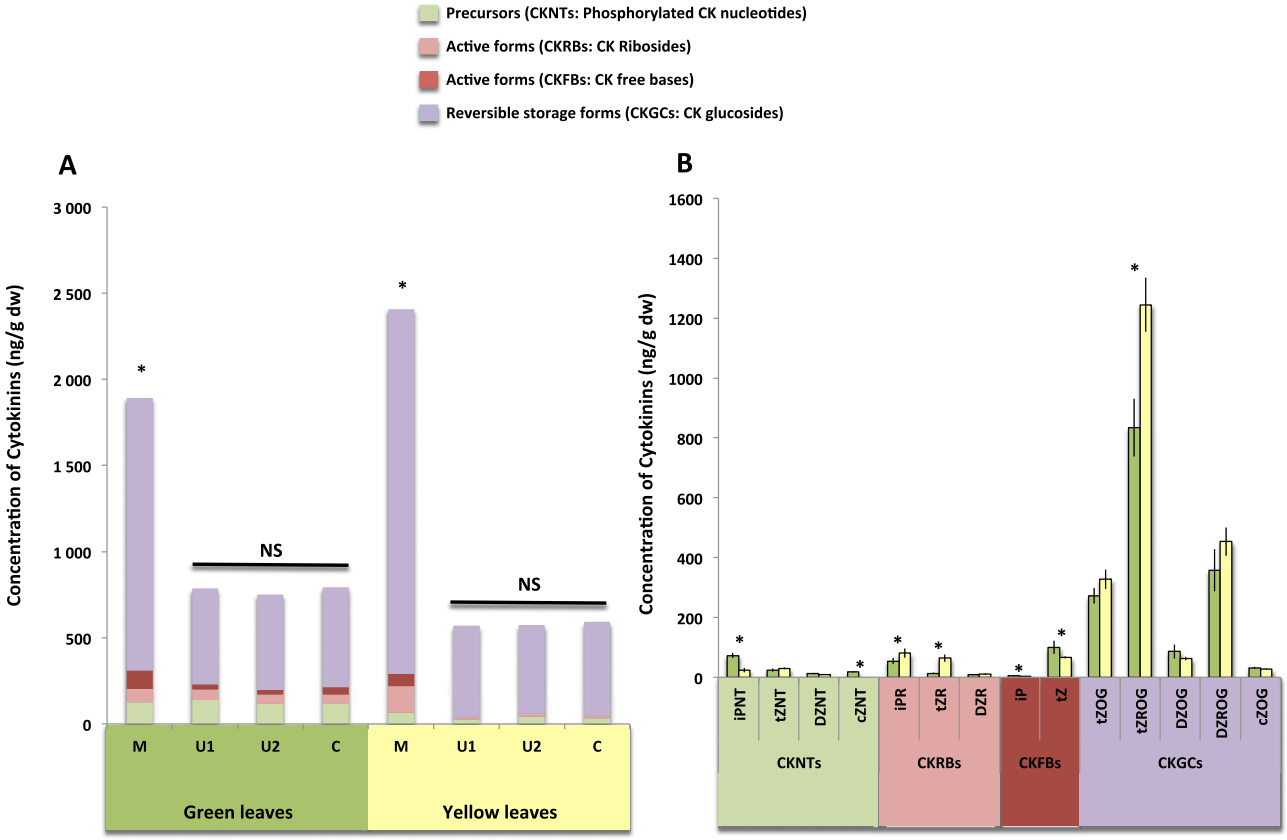

Fig. 1 (A) CK concentrations in green and yellow leaves in mined (M) and unmined plant tissues (unmined ipsilateral tissues U1, unmined contralateral tissues U2 and control tissues C). Light green = CK nucleotides (CKNTs: iPNT, *t*ZNT, DZNT, and *c*ZNT); light red = CK active forms (CK ribosides) (CKRBs: iPR, *t*ZR, DZR, and *c*ZR); dark red = CK free bases (CKFBs: iP and *t*Z); purple = CK reversible storage forms (CK glucosides) (CKGCs: *t*ZOG, *t*ZROG, DZOG, DZROG, *c*ZOG, and *c*ZROG). (B) Specific CK composition identified in mines on green (green bars) and yellow leaves (yellow bars). Data are presented as mean ± SEM. Statistical differences are indicated by asterisks. See Table 1 for abbreviations.

accumulating DZR and DZROG with similar amounts for mines on green and mines on yellow leaves (Fig. 2). In green leaves, DZR is about 3 times and DZROG 2 times more abundant in mines compared to controls while they are 10 times and 2 times more abundant in mines on yellow leaves. Interestingly, DZNT is only detected in mines on yellow leaves, whereas it is not present in unmined and control tissues. DZ was not detected in any leaf tissues.

High concentration of CKs are found in larvae with contrasted profiles on green and yellow leaves

High concentrations of CKs are found in larvae both on green and yellow leaves but with larger quantities in yellow leaves (Fig. 3). All types of CKs can be found in larvae, from precursors to active and storage forms (Fig. 3); however, larvae primarily contain CKRBs and CKFBs (Fig. 4) contrasting with leaf tissues that mainly contain CKGCs (Fig. 1). All iP- and *t*Z-type CKs (except for *t*ZOG) found in leaf tissues are also found in larvae. Interestingly, no *c*Z-type CKs are detected in larvae while they are detected in plant tissues. Larvae from mines on yellow leaves contain higher levels of CKs with a strong increase of active forms (both CKRBs and CKFBs) compared to larvae from green leaves. Strong alterations are observed for *t*Z that show an 8 fold higher concentration in larvae from mines on yellow leaves compared to larvae from mines on green leaves (Fig. 3), while *t*Z concentrations are shown to be lower in mined tissues of yellow leaves compared to mined tissues on green leaves (Fig. 2). Interestingly, other *t*Z-types of CKs (*t*ZNT, *t*ZR, and *t*ZROG) also occur in higher concentrations in larvae

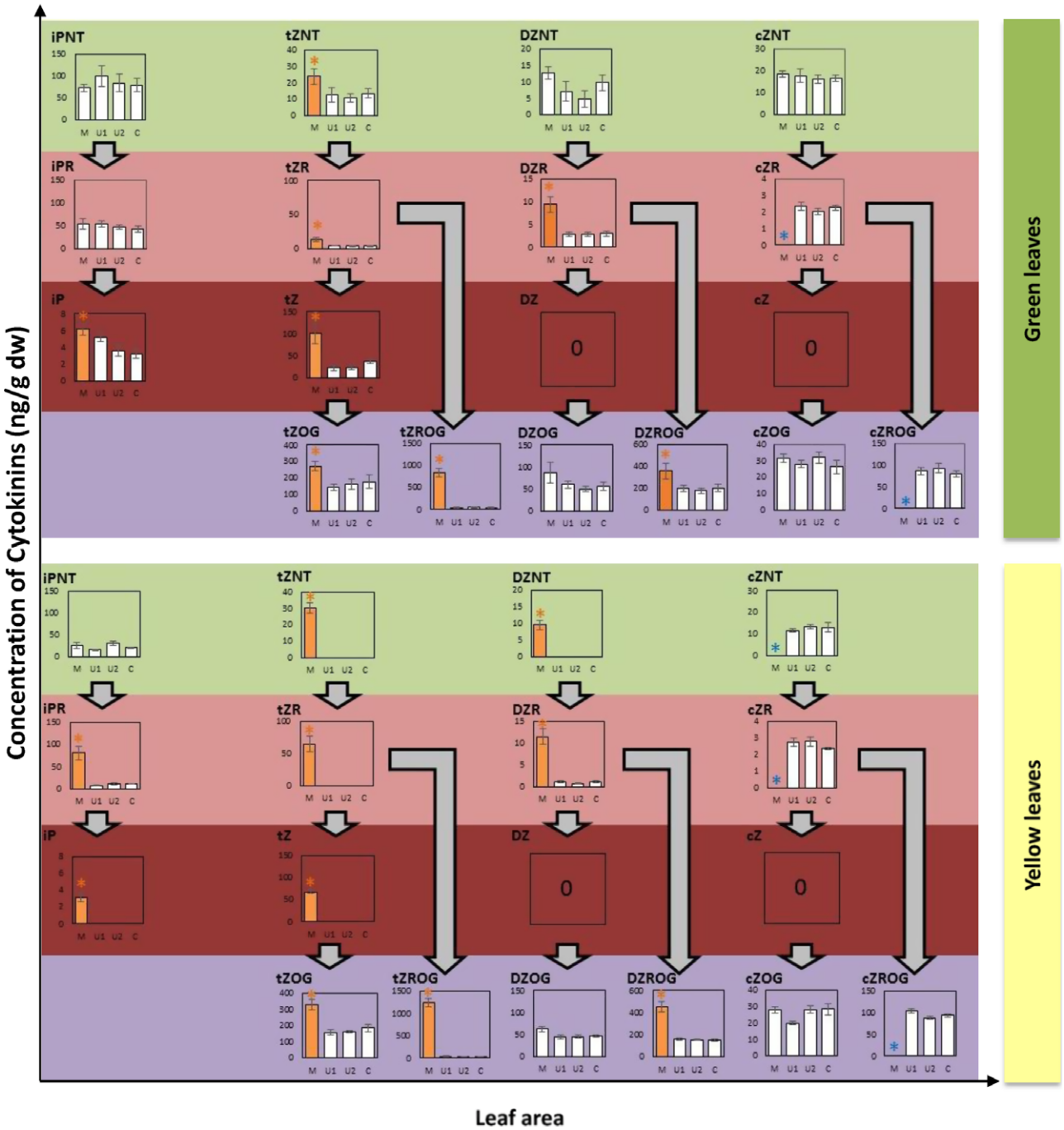

Fig. 2 Changes in CK levels in green and yellow leaf tissues of mined (M) and unmined areas (unmined ipsilateral tissues U1, unmined contralateral tissues U2 and control tissues C). Metabolic flows between precursors (light green), active forms (light and dark red) and reversible storage forms (purple) are indicated by large arrows based on current model of CK biosynthesis pathways (Mok & Mok, 2001; Sakakibara, 2006; Spíchal, 2012). Data are presented as mean ± SEM. Statistical differences are indicated by asterisk (orange asterisks indicate a significant increase while blue ones indicate a significant decrease). Orange bars shown significant increases of CK concentrations. See Fig. 1 for background colors and Table 1 for abbreviations.

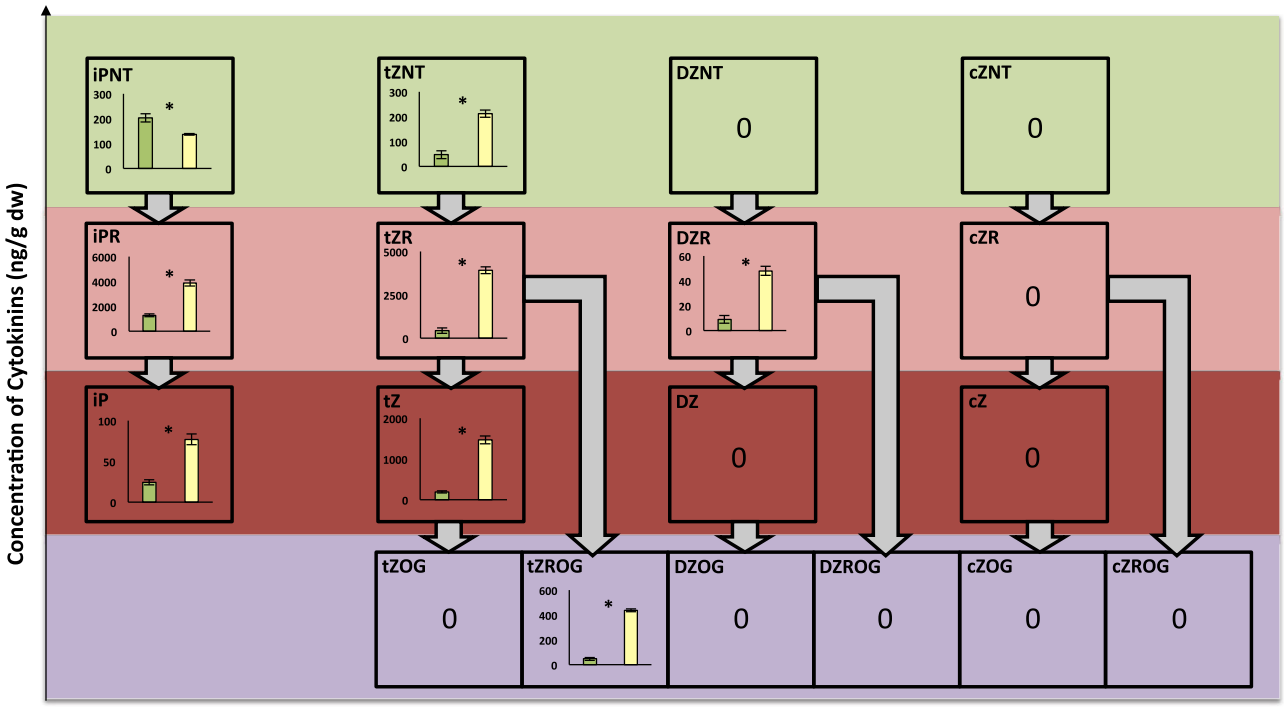

Fig. 3 Concentrations of CKs in larvae from green (green bars) and yellow leaves (yellow bars). Metabolic flows between precursors (light green), active forms (light and dark red), and reversible storage forms (purple) are indicated by large arrows based on current model of CK biosynthesis pathways (Mok & Mok, 2001; Sakakibara, 2006; Spíchal, 2012). Data are presented as mean ± SEM. Statistical differences are indicated by asterisk. See Fig. 1 for background colors and Table 1 for abbreviations.

from mines on yellow leaves compared to larvae from mines on green leaves. Larvae contain the highest levels of both total CKs and total *t*Z-type CK levels both in green and yellow leaves (Fig. 4).

High concentration of 2-MeS-CKs can be found both in larvae and leaves but with differences between these tissue types

High concentrations of 2-MeS-CKs are found both in larvae (Fig. 5A) and leaf tissues (Fig. 5B). Very high amounts of 2-MeS-CKs are detected in larvae, especially those isolated from green leaves. In larvae, identified 2-MeS-CKs include typical plant and microbial 2-MeS-CKs (2-Me-SZ and 2-Me-SZR) but also specific prokaryotic 2-MeS-CKs (2-MeS-iPA). High concentrations of 2-MeS-CKs are also detected in all leaf tissues (2-Me-SZ and 2-Me-SZR). Interestingly 2-MeS-iPA is not detected in any leaf tissue although it was present in larvae. Only 2-Me-SZR is specifically altered in mined tissues with distinct patterns in green and yellow leaves. Overall 2-Me-SZR concentrations are higher in yellow leaves but with lower levels in mined areas compared to control, while the opposite is observed on green leaves.

Discussion

Cytokinins are plant hormones involved in numerous plant–biotic interactions. An increase in CK concentration is commonly observed after insect or pathogen attack, suggesting that these molecules play a pivotal role in the profound reconfiguration of the plant primary and secondary metabolism observed in infested plants. These phytohormones can be associated with plant-induced defense but they have also been suggested to be used by arthropods and pathogens to hijack the plant metabolism, control its physiology and/or morphology and successfully invade the plant (Giron *et al*., 2013). The exact origin of CKs that accumulate in infested plant areas is mostly unknown, especially in the case of plant-manipulating insects such as leaf-miners and gall-inducers (Giron *et al*., 2013; Bartlett & Connor, 2014; Tooker & Helms, 2014; Giron *et al*., 2016).

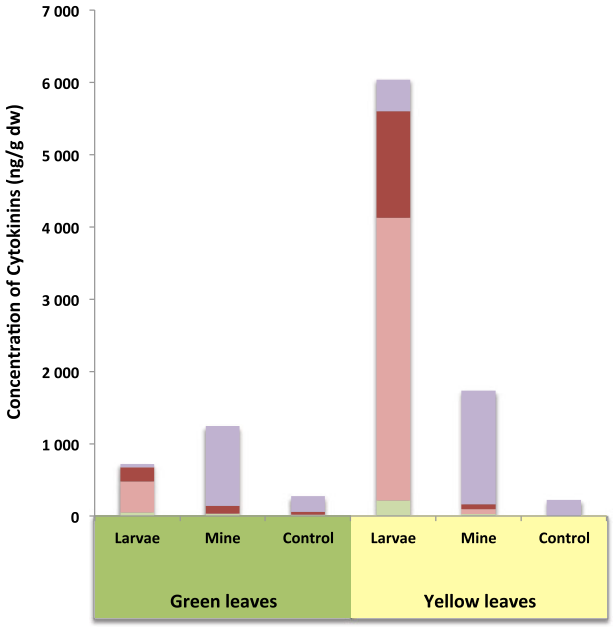

Fig. 4 Levels of *t*Z-type CKs in larvae, and in mined and control leaf tissues both on green and yellow leaves. Light green = *t*ZNT; light red = *t*ZR; dark red = *t*Z; purple = *t*ZOG and *t*ZROG.

While iP- and *t*Z-type CKs predominantly originate from the methylerythritol phosphate (MEP) pathway, *c*Z-type CKs are derived from the mevalonate (MVA) pathway (Mok & Mok, 2001; Sakakibara, 2006). Our data show that both pathways are likely to be active in the plant. However, in mined areas the strong decrease of *c*ZNT (in yellow leaves) and *c*ZR (in green and yellow leaves) suggest that the MVA pathway is inhibited in response to the feeding activity of larvae. Alternative hypotheses suggest a degradation of *c*ZNT and *c*ZR into glucoside storage forms and/or that *c*Z-type CKs are converted to fuel the observed increase in *t*Z-type CKs (Vreman *et al*., 1974; Bassil *et al*., 1993; Yonekura-Sakakibara *et al*., 2004). The steady levels of *c*ZOG and the decreased levels of *c*ZROG in mines, along with transcriptomic data available on this system (Zhang *et al*., 2016) rule out the degradation hypothesis, since genes related to CK inactivation processes (*cis*Zeatin-O-glucosyltransferase and β-glucosidase) are not differentially expressed between mined and unmined leaf areas (Zhang *et al*., 2016). Currently there is no compelling evidence for enzyme-facilitated isomerization that would support the conversion hypothesis. A formal demonstration of the inhibition of the MVA pathway in mined areas remains to be determined and any implications for the plant–insect interaction investigated.

*c*Zs have long been thought to be biologically inactive or with lower activities compared to iP- and *t*Z-types CKs which are generally considered to be the active natural CKs (e.g., Gyulai & Heszky, 1994; Gajdošová *et al*., 2011). Very little data are available on *c*Z-type CKs and experimental proof of their possible functions in plant–biotic interactions remains lacking. In addition to their potential role in abiotic stress responses and pathogen resistance, *c*Z-type CKs presumably also play a role in defence metabolite accumulations after herbivore attack (Schäffer *et al*., 2015). However, the ability to produce *c*Z-type CKs has been identified in several pathogens and very high levels have been found in larvae of the galling

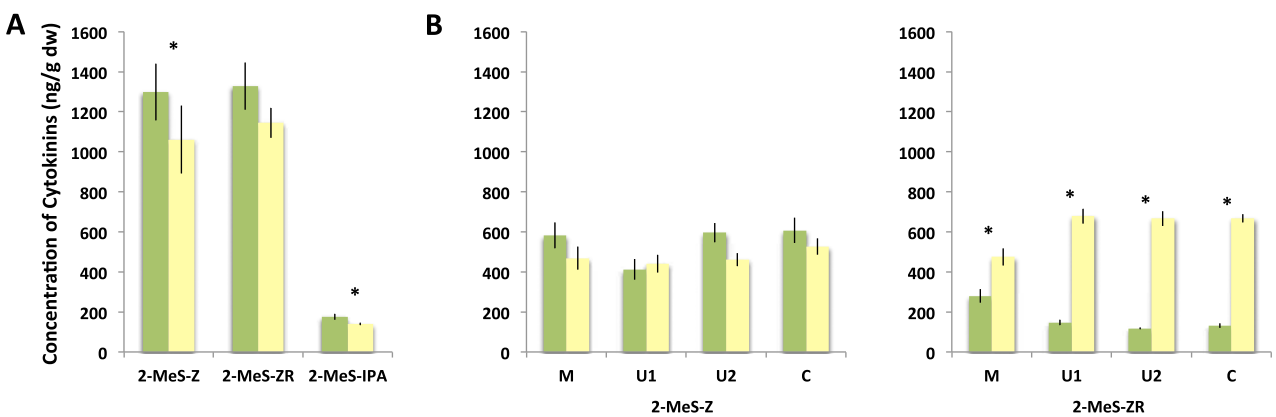

Fig. 5 Concentration of 2-methylthio-CKs in larvae and leaf tissues. (A) Concentrations of 2-MeS-CKs in larvae from green and yellow leaves. (B) Concentrations of 2-MeS-CKs in green and yellow leaf tissues in mined (M) and unmined areas (unmined ipsilateral tissues U1, unmined contralateral tissues U2 and control tissues C). Data are presented as mean ± SEM. Statistical differences are indicated by asterisks. Green bars = data from green leaves; yellow bars = data from yellow leaves. 2-Methylthio-*trans*-zeatin riboside (2-MeS-ZR), 2-Methylthio-*trans*-zeatin (2-MeS-Z), 2-Methylthio-N^6-isopentyladenosine (2-Mes-iPA).

aphid *Pachypsylla celtidis* (e.g., Pertry *et al*., 2009; Straka *et al*., 2010) suggesting that they might be also involved in bacteria- or insect-induced gall formation (Schäffer *et al*., 2015). Contrasting with the hypothesis that *c*Z-type CKs could be key factors in the strategy developed by plant-manipulating insects to control their host–plant (Kaiser *et al*., 2010; Schäfer *et al*., 2015), *P. blancardella* larvae completely lack these types of compounds both on green and yellow leaves. This result, combined with the strong decrease of *c*ZR in mined areas, call for a deeper experimental investigation of the functional role played by *c*Z-type CKs in mediating plant-insect interactions. It also suggests that different insects may use different strategies.

The spatial distribution of CKs within infected leaves shows that CK alterations are restricted to the mined area and that CK profiles of control tissues are similar to those of unmined areas of infected leaves. Absence of CK gradients suggests that CKs accumulated in the mined areas are unlikely to be transported from other parts of the leaf/plant. Previous results on green leaves demonstrated that expression of CK-related genes contrasts with CK accumulation patterns in mined leaf areas, which strongly suggest that CKs accumulated in the mined area originate mostly from the insect itself rather than being produced by the plant (Zhang *et al*., 2016). High levels of CKs found in larvae both on green and yellow leaves are consistent with this hypothesis, and insects most likely produce and deliver CKs to the plant as a strategy to create a favorable nutritional environment. Overall, CK types found in larvae are similar to CKs found in leaf tissues. However, in mined tissues, the most abundant CKs are glucoside storage forms whilst larvae contain negligible amounts of CKGCs (besides *t*ZROG). Glucoside storage forms are known to be resistant to degradation by CK dehydrogenase enzymes, and they can be reactivated by β-glucosidases (Mok *et al*., 2000). Therefore, they can help maintain insect-induced effects over the entire lifecycle of the insect (Zhang *et al*., 2016). Additionally, plant molecular activity is potentially acting to reduce the CK active pool in a possible attempt to regulate the flow of CKs provided by the insect thus contributing to the observed high levels of CKGCs (Zhang *et al*., 2016). Interestingly, insects mainly contain active CK forms (either as CK ribosides or CK free bases) that could potentially strengthen their impact on the plant physiology, with the conversion of a fraction of these CKs into CKGCs happening later in the mine. High levels of iP, iPR, *t*Z, *t*ZR, and *t*ZROG and absence of *c*Z-type CKs in larvae strongly suggest that the MEP pathway is very active in larvae while the MVA pathway is not.

Higher levels of CKs are observed in mines and larvae on yellow leaves compared to green leaves. This contrasts with the strong decrease of CKs in senescing leaves and suggests that the production of CKs by larvae is further enhanced in yellow leaves to potentially compensate for an otherwise senescent environment. Lower levels of iP and *t*Z in mines on yellow, compared to green leaves, is likely due to the very high metabolic demand required by yellow leaves to maintain a green-island phenotype during leaf senescence. Higher levels of all *t*Z-type CKs in larvae from yellow leaves compared to larvae from green leaves suggests that insect benefits from plant- and insect-borne CKs on green leaves, but shifts on yellow leaves to (almost) exclusively insect-borne CKs produced through the MEP pathway. This is further supported by the great quantity of active CKs (both CKRBs and CKFBs) in larvae (especially on yellow leaves) and higher levels of CKs in larvae compared to mined tissues.

An extensive characterization of CKs reveals that *t*Z-type CKs are the main compounds that are modulated in infected green and yellow leaves. This was also observed in many other studies on gall-inducing and nodulating bacteria (Jameson, 2000; Sakakibara *et al*., 2005; Pertry *et al*., 2009; Choi *et al*., 2011; Kisiala *et al*., 2013; Giron & Glevarec, 2014) as well as on gall-inducing insects that reported high concentrations of *t*Z (Mapes & Davies, 2001a,b; Dorchin *et al*., 2009; Straka *et al*., 2010; Tokuda *et al*., 2013; Bartlett & Connor, 2014; Tooker & Helms, 2014). Therefore, alteration of *t*Z-type CKs appears to be a common tool shared by phylogenetically distant organisms to invade host plants. Elevation of *t*Z-type CK concentrations and the high biological activity of *t*Z contribute to the alteration of the plant phytohormonal balance, potentially playing a key role in the development and maintenance of galls and green-islands (Giron *et al*., 2007; Kaiser *et al*., 2010; Yamaguchi *et al*., 2012; Schaller *et al*., 2015). Increased *t*Z-type CK levels can also favor nutrient translocation toward the insect's feeding site, inhibition of leaf senescence and mitigation of direct and indirect plant defenses (Giron *et al*., 2013).

In the challenge to determine the exact origin of CKs involved in plant manipulation by insects, one should keep in mind that microbial partners associated with insects may be involved. There is growing evidence that microorganisms are important "hidden players" in insect–plant interactions and microbial symbionts can directly or indirectly affect the plant by interfering with the plant signaling pathways (Frago *et al*., 2012; Biere & Bennett, 2013; Giron *et al*., 2013; Sugio *et al*., 2015; Giron *et al*., 2017). Curing the apple tree leaf-miner *P. blancardella* of its endosymbiotic bacteria *Wolbachia* resulted in the loss of the CK-induced green-island phenotype on

apple tree leaves and also resulted in the absence of detectable CKs in larvae compared to the nontreated controls (Kaiser *et al*., 2010; Body *et al*., 2013). Several other lines of evidence also suggested that these phytohormones are likely to originate from microbial symbionts (Giron *et al*., 2007; Kaiser *et al*., 2010; Body *et al*., 2013; Gutzwiller *et al*., 2015). Based on the recent studies of plant–bacteria systems and on the few data available for insects, it has been hypothesized that plant-manipulating insects may use bacterial symbionts as secret weapons to produce CKs including specific 2-MeS-CKs that will help insects to overtake plant gene expression, defeat the CK-degrading capacity of the plant and cause persistent effects on the plant cellular machinery (Giron & Glevarec, 2014). To the best of our knowledge, these 2-MeS-CKs have not been investigated in any plant–insect interaction so far.

In the literature, 2-MeS-CKs are often considered to be of endophytic origin (Ajitkumar & Cherayil, 1988; Petry *et al*., 2009; Tarkowski *et al*., 2010). It is very likely that environmental microorganisms colonized leaves, explaining the presence of 2-MeS-CKs in control leaf tissues. While 2-MeS-CKs are usually found in low quantities in plant tissues (Tarkowski *et al*., 2010; Spíchal, 2012), our results show that they can significantly contribute to the plant CK pool. 2-MeS-CKs have been demonstrated to be essential components of plant–microbe interactions (Thimmappaya & Cherayil, 1974; Armstrong *et al*., 1976; Pertry *et al*., 2009; Kisiala *et al*., 2013). In *Bradyrhizobium* sp., bacterial 2-methylthio derivatives of *t*Z and iP (2-MeS-*t*Z and 2-MeS-iP) are not sufficient to allow nodule organogenesis, but they positively contribute to the induction of symbiotic nodule development in *Aeschynomene* plants, and can activate *in vitro* the legume CK receptors (Podlešáková *et al*., 2013). In *Arabidopsis* infected by *R. fascians*, the high accumulation of CKs at the infection site due to the constant presence of the bacteria and continuous production of CKs is most likely counterweighed by the production of less active but less toxic and less degradable 2-MeS-CKs in addition to other CKs (iP, *t*Z, and *c*Z) (Pertry *et al*., 2009; Tarkowski *et al*., 2010; Spíchal, 2012). Our study shows that mines contain significant amounts of 2-MeS-CKs including 2-MeS-ZR that strongly accumulates in mines on green leaves. Interestingly 2-MeS-ZR was previously found to be characteristic of CKs produced by plant-associated microorganisms (Greene, 1980). Intriguingly, amounts of 2-MeS-ZR are lower in the mined areas on yellow leaves compared to other parts of the infested leaves and controls. In our attempt to test the hypothesis that bacterial symbionts may contribute to the direct production of CKs by insects it is worth noticing that 2-MeS-CKs (2-MeS-Z and 2-MeS-ZR) were found in high levels in insects both on green and yellow leaves. Moreover, a specific type of 2-MeS-CKs (2-MeS-iPA) was found only in insect samples. 2-MeS-iPA is a predominant and specific form of CK found in prokaryotes (Skoog & Armstrong, 1970). It is still unclear whether all 2-MeS-CKs can be considered as exclusively bacteria-specific compounds but the relative proportion of 2-MeS-CKs and their specific nature are known to be characteristic to certain microorganisms. 2-MeS-CKs potentially contribute to allow persistent effects, while avoiding CK degradation by the plant, deleterious effects on plant development and CK-mediated plant defenses (Ajitkumar & Cherayil, 1988; Pertry *et al*., 2009). This appears to be highly relevant for gall-inducing and leaf-mining insects that develop for an extended period of time at a localized feeding site (Giron & Glevarec, 2014) and further supports the hypothesis that CKs of bacterial origins are most likely involved in the interaction between *P. blancardella* and its host–plant.

Conclusion

The extensive identification and quantification of CKs in both green and yellow leaves of *M. domestica* infested by *P. blancardella* are consistent with the idea that leaf-mining insects produce and deliver CKs to the plant especially in yellow leaves, thereby enabling insects to overtake the plant senescing programme. Manipulation of the plant by the insect is limited to the feeding area and occurs mainly through a modulation of specific pathways of CK biosynthesis with a common strategy shared by arthropods and plant-associated microorganisms. Our study further suggests that bacterial symbionts of the insect may contribute to the production of CKs through the synthesis of specific 2-MeS-CKs. Functional tests are necessary to validate the possible function of the various CKs and the specific role of each partner in this intricate plant–insect–microbe interaction.

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Disclosure

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