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Modulation of plant cytokinin levels in the *Wolbachia*-free leaf-mining species *Phyllonorycter mespilella*

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Abstract

As phytohormones lie at the very core of molecular mechanisms controlling the plant physiology and development, they have long been hypothesized to be involved in insect-induced plant manipulations. Cytokinins (CKs) are phytohormones now widely recognized to be utilized by leaf-mining and gall-inducing insects in the control of the physiology of their host plant. In some leaf-mining moth species, larvae can supply the hormones themselves, bacterial symbionts contributing to the production of CKs. Our objective was to investigate whether closely related leaf-miner species sharing the same ecological niche but differing in their *Wolbachia* infection status develop similar strategies to manipulate their host plant. An extensive identification and quantification of CKs has been used to elucidate physiological patterns relevant for the plant–insect interactions. Our results show that modulation of plant CK levels is impaired in the *Wolbachia*-free leaf-mining moth *Phyllonorycter mespilella* (Hübner) (Lepidoptera: Gracillariidae) contrasting with results previously observed in the closely related moth species *Phyllonorycter blancardella* (Fabricius) that produce and deliver CKs to the plant through an intricate interaction with *Wolbachia*. Our study suggests that mechanisms underlying colonization of the host plant and adaptation to fluctuating environmental conditions are different between the two leaf-miner species and that *P. mespilella* larvae most likely do not produce CKs. This species rather only buffers the degradation of CKs naturally occurring during the senescence of leaves leading to few active CKs being maintained at a sufficiently high level to induce and maintain a ‘green island’ phenotype (photosynthetically active green patches in otherwise senescing leaves). This study further provides converging experimental evidence pointing toward the influence of bacterial symbionts in the ability of leaf-mining moths to control the physiology of their host plant with consequences for their ecology and evolutionary diversification.

Introduction

The ability of phytophagous insects to exploit plant resources requires them to address the nutritional mismatch between what plants provide and what insects require (Schoonhoven et al., 2005; Behmer, 2009; Raubheimer et al., 2009; Body et al., 2013). It also requires them to avoid plant direct and indirect defenses

(Schoonhoven et al., 2005). Strategies used include insect manipulation of host plant physiology resulting in the inhibition of the plant immune responses (Dussourd, 2003; Despres et al., 2007; Felton & Tumlinson, 2008; Bruessow et al., 2010) and/or plant-manipulation resulting in improved nutritional benefits for the parasitic herbivore at the expense of the plant (Awmack & Leather, 2002; Lieutier et al., 2017). Some of the most spectacular insect-induced plant manipulations are the strong reprogramming of host plant development leading to new plant structures, such as galls (Price et al., 1987; Stone & Schönrogge, 2003). These insect-generated shelters provide insects with protection against natural enemies and

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abiotic factors but also an enhanced nutritional environment (Stone & Schönrogge, 2003). Other phenotypes, such as ‘green islands’ (photosynthetically active green patches in otherwise senescing leaves) induced by several leaf-mining insects, are also clear examples of plant manipulation that bears numerous similarities with gall-inducers with respect to the possible mechanisms involved in their production and their insect-induced effects on plants (Giron et al., 2016).

Plant manipulation by herbivorous insects is tightly linked with their ability to influence or even control the plant phytohormonal balance (Erb et al., 2012; Giron et al., 2013; Zhang et al., 2016). Phytohormones are key regulators of plant growth and developmental processes and modulate plant responses to their biotic and abiotic environment (Pieterse & Dicke, 2007; Erb et al., 2012; Vanková et al., 2014). It is thus not surprising that they have been the target of insect herbivores during the course of evolution allowing them to successfully colonize and exploit various host plants (Schultz & Appel, 2004). For example, Colorado potato beetle larvae deliver bacteria in their saliva to their host plant which decreases jasmonic acid (JA) and JA-responsive anti-herbivore defenses due to an accumulation of salicylic acid (SA) via a negative crosstalk between SA and JA (Chung et al., 2013). This manipulation lures the host plant to induce an anti-bacterial rather than an anti-insect response. Several studies on Hessian fly and other gall-inducers such as *Eurosta solidaginis* (Fitch) and *Gnorimoschema gallaesolidaginis* Riley suggest that insects are somehow elevating concentrations of auxin (AUX) in attacked tissues resulting in the induction of a nutritive tissue on which larvae feed (Mapes & Davies, 2001a; Tooker & De Moraes, 2011a,b; Tooker & Helms, 2014). Cytokinins (CKs) are phytohormones widely recognized to be involved in plant manipulation by gall-inducers and leaf-miners (Giron et al., 2013, 2016; Tooker & Helms, 2014). These phytohormones regulate many plant growth and developmental processes and interact with AUX in a complex manner that varies according to their precise spatial distributions within plant tissues (Costacurta & Vanderleyden, 1995; Pernisová et al., 2011; Schaller et al., 2015). Increased CK levels can favor cell division but also nutrient translocation toward the insect’s feeding site and delay leaf senescence thus contributing to gall induction and a ‘green island’ phenotype (Mok & Mok, 2001; Balibrea-Lara et al., 2004; Walters & Mcroberts, 2006; Walters et al., 2008; Giron et al., 2013; Schaller et al., 2015). Studies on the pteromalid wasp *Trichilogaster acaciaelongifoliae* (Froggatt), the psyllid *Pachypsylla celtidismamma* (Fletcher), the tephritid fly *E. solidaginis*, and the maize orange leafhopper, *Cicadulina bipunctata* (Melichar), report high concentrations of CKs

in galls (Mapes & Davies, 2001a,b; Dorchin et al., 2009; Straka et al., 2010; Tokuda et al., 2013). In leaf-miner insects, studies on the nepticulid moths *Ectodemia argyropeza* (Zeller), *Stigmella argyropeza* (Zeller), *Stigmella argentipedella* (Zeller), *Stigmella* spp., and the gracillariid moth *Phyllonorycter blancardella* (Fabricius) show large accumulation of CKs in ‘green islands’ (Engelbrecht et al., 1969; Engelbrecht, 1971; Giron et al., 2007; Body et al., 2013; Zhang et al., 2016).

How herbivores manipulate plant signaling still remains to be discovered for the great majority of them. Whether or not insects can be the source of CKs found at high levels at the insects feeding sites – rather than simply manipulating plant phytohormonal biosynthesis/signaling – remains a challenging question to be answered in most systems. However, significant amounts of phytohormones have been found in the saliva, body and accessory glands of some insect species suggesting that insects may also directly produce relevant levels of phytohormones or other effectors with mimicking functions (Giron et al., 2013; Bartlett & Connor, 2014; Tooker & Helms, 2014; Zhang et al., 2017). In sawflies (Hymenoptera: *Pontania* spec.) that induce galls on *Salix japonica* Thunb., high CK levels have been detected in larvae (Yamaguchi et al., 2012). Interestingly, females can also play a role in gall induction or growth by injecting fluids during oviposition that are rich in phytohormones. CKs and their precursors were found at high concentrations in adult female accessory glands (Yamaguchi et al., 2012). CKs have been detected in the body, saliva or accessory glands of several other gall-inducing (Ohkawa, 1974; Mapes & Davies, 2001b; Dorchin et al., 2009; Straka et al., 2010; Tooker & De Moraes, 2011a,b; Giron et al., 2013; Tanaka et al., 2013) and leaf-mining insect species (Engelbrecht et al., 1969; Engelbrecht, 1971; Body et al., 2013; Zhang et al., 2017), suggesting their ability to produce and deliver these effectors to the plant (Giron et al., 2013; Bartlett & Connor, 2014; Tooker & Helms, 2014; Zhang et al., 2017).

Plant manipulation by insects sometimes involves associations with one or more symbiotic partners, and insect symbionts are now recognized as key partners in plant–insect interactions (Frago et al., 2012; Biere & Bennett, 2013; Giron et al., 2013, 2017; Sugio et al., 2014; Berasategui et al., 2017). There is also growing evidence that insect-associated microbes are active players in the ability of insects to modulate the plant hormonal balance to the benefit of their insect host (Kaiser et al., 2010; Body et al., 2013; Sugio et al., 2014; Zhang et al., 2016, 2017; Giron et al., 2017). Metabolic profiling targeted to the bacterial chemical signatures (Zhang et al., 2017), and comparisons between insects deprived of their endosymbiotic bacteria through antibiotic treatments (Kaiser et al., 2010)

or between distinct populations that do or do not harbor endosymbionts (Barr et al., 2010) have provided key indirect evidence of the role of symbionts in the modulation of plant phytohormones. However, without labeling experiments, artificial rearing media, or knockout mutants it often remains difficult to distinguish conclusively between plant-, insect-, and symbiont-derived hormones. Therefore, whether hormones involved in plant manipulation by insects originate from the plant, the herbivorous insect, or its symbiont(s) is usually unknown (see for an exception Yamaguchi et al., 2012).

Previous experiments in the *Malus domestica* Borkh./*P. blancardella* leaf-mining system have shown that insects induce 'green islands'. These leaf areas are enriched in CKs, and CKs accumulated in the mined area do not originate from the plant CK-biosynthetic pathway (Zhang et al., 2016). Several lines of evidence also indicated that insects contain large amounts of CKs and phytohormones accumulated in the mines originate from the insect itself (Body et al., 2013; Zhang et al., 2017). Insect bacterial symbionts (*Wolbachia*) contribute to the observed phenotype (Giron et al., 2007; Kaiser et al., 2010; Body et al., 2013; Gutzwiller et al., 2015) likely synthesizing CKs delivered to the plant (Zhang et al., 2017), including bacteria-specific methylthio-CKs (2-MeS-CKs).

The objective of the present study was to conduct an extensive identification and quantification of CKs in both green and yellow leaves of *M. domestica* infested by *Phyllonorycter mespilella* (Hübner), a closely related species of *P. blancardella*, sharing the same ecological niche but not harboring *Wolbachia*. This study was designed to investigate whether or not closely related species sharing the same ecological niche develop similar strategies to manipulate their host plant, especially regarding the alteration of CK levels. Comparing phytohormonal alterations induced by two closely related species that differ in their *Wolbachia* infection status could help to shed light on the influence of bacterial symbionts on the modulation of the plant phytohormonal balance.

Materials and methods

Biological material

Phyllonorycter mespilella is a polyvoltine leaf-mining microlepidopteran of apple trees, closely related to *P. blancardella* (Gutzwiller et al., 2015). The two species are morphologically cryptic (F Dedeine & D Giron, unpubl.). They share the same ecological niche and can often be found on the same trees, shoots, or even leaves. The development of larvae is similar to that of *P. blancardella* and divided into five instars (Pottinger & Leroux, 1971; Body et al., 2015). The first three instars (L1–3) that

feed on interstitial fluids are fluid feeders. During this period, larvae define the outline of the mine by separating the two leaf integuments. The last two instars that consume the lower and upper parenchyma are tissue-feeders, which result in the formation of feeding windows on a characteristic tentiform-shaped mine (Pottinger & Leroux, 1971; Djemai et al., 2000; Body et al., 2015). Similar to *P. blancardella* and other leaf-mining species, *P. mespilella* manipulate the host plant to induce 'green islands'.

Both green and yellow leaves infested by L4 larvae (only one mine per leaf) and unmined (an adjacent neighboring leaf of the same color as the infested leaf) leaves were simultaneously collected in the field between 09:00 and 10:00 hours in autumn (November) on *M. domestica* apple-trees, in a biologically managed orchard in La Chapelle-aux-Naux, France (47°19'09"N, 0°25'42"E). Unmined green and yellow leaves were used as a control. Leaves with intermediate phenotypes (mix of green and yellow tissues) were not collected in order to compare two distinct contrasted environments. The nutritional content of yellow leaves is significantly decreased and cannot meet the insect nutritional requirements but the closely related leaf-mining moth *P. blancardella* was shown to maintain a nutritional homeostasis in mined tissues (Body et al., 2013). This allows the insect to maintain a favorable nutritional environment in an otherwise degenerating context (Giron et al., 2007). Field observations and phytohormone quantification show reduced levels of abscisic acid favoring the suppression of abscission during the critical larval feeding stage (Zhang et al., 2016). Mined areas (M) were dissected on ice following the exact outline of the mine and were stored at –80 °C until further analysis. Leaf-miner insects and frass were removed from the mine. Ipsilateral tissues (leaf tissues on the same side of the main vein as the mine: U1), and contralateral tissues (leaf tissues on the opposite side of the main vein and of the mine: U2) were also dissected (Giron et al., 2007). Adjacent unmined leaves were used as a control (C). Each leaf sample was ground with a mortar and a pestle in liquid nitrogen after lyophilization (Bioblock Scientific Alpha 1–4 LD plus lyophilizator) in order to have an extra-fine leaf powder (Body et al., 2013).

Species identification and infection status of the leaf-miner insects

Genomic DNA was extracted from leaf-miner larvae using the NucleoSpin Tissue XS kit (Macherey-Nagel, Hoerd, France) following the manufacturer's instructions. Each DNA sample was used for two different polymerase chain reactions (PCR) using cytochrome c oxidase subunit 1 (COI) and *Wolbachia* surface protein (*wsp*) primers for species identification and determination of the infection status, respectively. The primers used in this study were

LEP-F1 and LEP-R1 (Hebert et al., 2005) for COI amplification and *wsp*81f and *wsp*691r (Braig et al., 1998) for *wsp* amplification. COI amplified products were then sequenced in both directions at Eurofins Genomics (Ebersberg, Germany). Species identification was performed using the 'species'-level identification function of the BOLD ID Engine (Ratnasingham & Hebert, 2007) based upon aligned COI sequence data and infection status was verified by agarose gel electrophoresis on *wsp* amplified products (Kaiser et al., 2010). As a positive control, PCR amplification of an insect gene (EF1-a) was always performed to ensure the quality of DNA and that lack of amplification of bacterial genes was not owing to manipulation errors. As a positive control, PCR amplifications of *Wolbachia* genes from infected insects were performed at the same time to ensure the efficiency of the various primers used for detection. All insects collected were tested for the presence or absence of *Wolbachia* ($n = 22$ in green leaves, $n = 12$ in yellow leaves) and corresponding leaf tissues were used to obtain the plant hormonal profile. In *Phyllonorycter* spec., *wsp* was found to be a reliable marker for *Wolbachia* infection leading to consistent results when compared with data obtained with other *Wolbachia* markers (Braig et al., 1998; Kaiser et al., 2010; Gutzwiller et al., 2015; F Dedeine & D Giron, unpubl.).

LC/MS analysis of plant hormones

The analysis of plant hormones was carried out following the protocol developed by Dobrev & Kaminek (2002) and Dobrev & Vankova (2012). In brief, for each sample ($n = 22$ in green leaves, $n = 12$ in yellow leaves, for each area M, U1, U2, and C), an aliquot of 100 mg fresh weight was transferred into a microcentrifuge tube; 500 μ l of cold extraction buffer [methanol/water/formic acid at 15/10/5 (vol/vol/vol), -20 °C] was added to each tube as well as a mixture of stable isotope labeled internal standards (10 pmol) (Table 1). After incubation for 30 min at -20 °C, each extract was centrifuged at 17 000 rpm and the supernatant collected. A second extraction of the residue was performed for each sample to ensure that all the target compounds were collected as much as possible. The two supernatants were pooled for each sample and evaporated in a vacuum concentrator (Alpha RVC, Christ). Sample residues were dissolved into 0.1 M formic acid and applied to mixed mode reverse phase-cation exchange SPE column (Oasis-MCX; Waters SAS, Guyancourt, France), in order to remove as much as possible of interfering substances from the extract without losing significant amounts of target compounds. The hormone fraction eluted with 0.35 M NH_4OH in 70% methanol contained CKs, including CK precursors [CK nucleotides (CK NTs): iPNT, tZNT, DZNT, and cZNT], transport forms [CK

ribosides (CK Rs): iPR, tZR, DZR, and cZR], active CKs [free bases (CK FBs): iP, tZ, DHZ, and cZ], reversible storage forms [CK O-glucosides (CK OGs): tZOG, tZROG, DZOG, DZROG, cZOG, and cZROG], but also 2-methylthio-derivatives (2-MeS-Z and 2-MeS-ZR). Fractions were evaporated to dryness in vacuum concentrator and dissolved into 30 μ l 10% methanol. An aliquot (10 μ l) from each fraction was separately analyzed on high-performance liquid chromatography (HPLC) (Ultimate 3000; Dionex, Hemel Hempstead, UK) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP; Applied Biosystems, Foster City, CA, USA) set in selected reaction monitoring mode. Mass spectrometer was run on electrospray ionization positive mode (ESI+). Quantification of hormones was done using isotope dilution method with multilevel calibration curves ($r^2 > 0.99$). Data processing was carried out with ANALYST v.1.5 software (Applied Biosystems).

Statistical analysis

Statistical analyses were performed using IBM SPSS v.19 (IBM, Armonk, NY, USA). The Shapiro–Wilk test was used to test whether data were normally distributed. Non-parametric statistics (Kruskal–Wallis test followed by all pairwise multiple comparisons) were used as data were not normally distributed even after log-transformation.

Results

Insect species identification and detection of endosymbionts

Cloning and sequencing of the corresponding products showed that the insects identified were *P. mespillela*, not infected with *Wolbachia*. We successfully amplified the *wsp* gene for insect-infected controls (*P. blancardella*, *P. hostis*, and *P. anceps*) but all *P. mespillela* individuals tested ($n = 22$ in green leaves, $n = 12$ in yellow leaves) were not infected with *Wolbachia*. This is consistent with earlier results (Gutzwiller et al., 2015) and a larger-scale survey conducted on more than 100 individuals from four localities across an ecological gradient (F Dedeine & D Giron, unpubl.).

CK content decreases in mined tissues

In total, 17 CKs that belonged to four isoprenoid types of CKs (tZ, cZ, iP, and DZ) and their methylthio derivatives were identified in both green and yellow leaves (Table 2). Identified CKs included precursors (CK NTs: iPNT and tZNT), transport forms (CK Rs: iPR, tZR, DZR, and cZR), active forms (CKFBs: iP, tZ, DZ, and cZ), reversible storage forms (CK OGs: tZOG, tZROG, DZOG, DZROG, cZOG, and cZROG), but also one 2-methylthio-derivative (2-MeS-ZR). tZ was the only type of CKs that was

Table 1 Cytokinins (CKs) quantified by liquid chromatography-positive electrospray ionization tandem mass spectrometry [HPLC-(ESI+)-MS/MS]

Isoprenoid cytokinins				Labeled CK standard
Nucleotides (CKNTs)	1	<i>t</i> ZNT	<i>Trans</i> -zeatin riboside-5'-monophosphate	² H ₅ - <i>t</i> ZRMP
	2	<i>c</i> ZNT	<i>Cis</i> -zeatin riboside-5'-monophosphate	
	3	DZNT	Dihydrozeatin riboside-5'-monophosphate	² H ₃ DZRMP
	4	iPNT	N ⁶ -isopentenyladenosine-5' monophosphate	² H ₆ -iPRMP
Ribosides (CKRBs)	5	<i>t</i> ZR	<i>Trans</i> -zeatin riboside	² H ₅ <i>t</i> ZR
	6	<i>c</i> ZR	<i>Cis</i> -zeatin riboside	
	7	DZR	Dihydrozeatin riboside	² H ₃ -DZR
	8	iPR	N ⁶ -isopentenyladenosine	² H ₆ -iPR
Free bases (CKFBs)	9	<i>t</i> Z	<i>Trans</i> -zeatin	² H ₅ - <i>t</i> Z
	10	<i>c</i> Z	<i>Cis</i> -zeatin	
	11	DZ	Dihydrozeatin	² H ₃ -DZ
	12	iP	N ⁶ -isopentenyladenine	² H ₆ iP
Glucosides (CKGCs)	13	<i>t</i> ZOG	<i>Trans</i> -zeatin-O-glucoside	² H ₅ - <i>t</i> ZOG
	14	<i>c</i> ZOG	<i>Cis</i> -zeatin-O-glucoside	
	15	DZOG	Dihydrozeatin-O-glucoside	² H ₇ DZOG
	16	<i>t</i> ZROG	<i>Trans</i> -zeatin-O-glucoside riboside	² H ₅ - <i>t</i> ZROG
	17	<i>c</i> ZROG	<i>Cis</i> -zeatin-O-glucoside riboside	
	18	DZROG	Dihydrozeatin-O-glucoside riboside	² H ₇ DZROG
	19	<i>t</i> Z9G	<i>Trans</i> -zeatin-9-glucoside	² H ₅ - <i>t</i> Z9G
	20	<i>c</i> Z9G	<i>Cis</i> -zeatin-9-glucoside	
	21	DZ9G	Dihydrozeatin-9-glucoside	² H ₃ -DZ9G
Methylthiols (2-MeS-CKs)	22	2MeSZ	2-Methylthio- <i>trans</i> -zeatin	² H ₅ MeSZ
	23	2MeSZR	2-Methylthio- <i>trans</i> -zeatin riboside	² H ₅ MeSZR
	24	2MeSiP	2-Methylthio-N ⁶ -isopentenyladenine	² H ₆ MeSiP
	25	2MeSiPA	2-Methylthio-N ⁶ -isopentenyladenosine	² H ₆ MeSiPR

Analysis used deuterated internal standards for CK identification, purchased from OlChemim (Olomouc, Czech Republic).

observed in all CK fractions (CK NTs, CK Rs, CK FBs, and CK OGs). The overall CK signature differed between leaf areas (Kruskal–Wallis test: $P < 0.001$; $n = 136$). Overall, the feeding activity of leaf-mining larvae induced a two-fold decrease in CKs in mines compared to control leaves both on green and yellow leaves (total CKs; green leaves, in mines: 225.30 ± 9.34 pmole per gDW (mean \pm SE), in controls: 455.20 ± 22.70 ; yellow leaves, in mines: 111.09 ± 3.43 , in controls: 246.76 ± 8.82 ; Figure 1). The total amount of CKs in mines on yellow leaves was half the total amount of CKs in mines on green leaves (Kruskal–Wallis test: $P < 0.001$).

Distinct patterns could be observed on green vs. yellow leaves (Figure 2). In green leaves, lower amounts were observed for all CKs (Kruskal–Wallis test: $P < 0.001$) in mines, except for *t*ZR, *t*Z, and *t*ZROG levels, that remained constant. Only *t*Z and *t*ZROG levels showed a slight trend toward an accumulation in mines. In yellow leaves, there was a global decrease in CKs in mines (Kruskal–Wallis test: $P < 0.001$), but the analysis of the CK composition revealed specific patterns for iPR, *t*ZR, and *t*Z

that had higher levels than controls in the mine. iPNT, *t*ZNT, iP, DZ, and DZROG showed a slight decreased trend (not significant), and DZR and *t*ZROG remained constant (Figure 2). Finally, *c*ZR, *c*Z, *t*ZROG, DZOG, *c*ZOG, and *c*ZROG amounts, all decreased in mines (Kruskal–Wallis test: $P < 0.001$).

Modulation of CK amounts differed strongly between *P. mespillela* and *P. blancardella* (Table 2). Whereas *P. blancardella* larvae induced a 2.5-fold increase in mined tissues on green leaves and a four-fold increase in mines on yellow leaves (Zhang et al., 2017), CK amounts in mines induced by *P. mespillela* showed a two-fold decrease compared to controls both on green and yellow leaves. Except for a few specific CKs, the CK composition in mines induced by *P. mespillela* on green vs. yellow leaves was relatively similar (Table 2).

Discussion

Whereas high concentrations of CKs have been found in leaves infected by *P. blancardella* (Giron et al., 2007; Body

Table 2 Mean (\pm SEM) cytokinin (CK) concentration (pmole g⁻¹ fresh weight) in mined apple leaves and their comparisons with control leaves following attack by *Phyllonorycter mespilella* (n = 22 in green leaves, n = 12 in yellow leaves) and *P. blancardella*. CK levels in mines, compared to controls, increased (+), decreased (-), or stayed the same (0). Slight decrease (not significant) is indicated by '(-)', slight increase (not significant) by '(+)'. Data on *P. blancardella* were obtained from Zhang et al. (2017)

K categories	CKs	Green leaves			Yellow leaves		
		CK concentrations <i>P. mespilella</i>	Levels in mines compared to controls		CK concentrations <i>P. mespilella</i>	Levels in mines compared to controls	
			<i>P. mespilella</i>	<i>P. blancardella</i>		<i>P. mespilella</i>	<i>P. blancardella</i>
CK NTs	iPNT	0.51 \pm 0.08a	-	0	0.25 \pm 0.05b	0 (-)	0
	tZNT	0.26 \pm 0.07a	-	+	0.34 \pm 0.11a	0 (-)	0
	DZNT			+			0
	cZNT			+			-
CK Rs	iPR	1.19 \pm 0.15a	-	+	1.20 \pm 0.26a	+	+
	tZR	2.08 \pm 0.36a	0	+	5.05 \pm 0.93b	+	+
	DZR	1.22 \pm 0.21a	-	+	1.61 \pm 0.24a	0	+
	cZR	0.22 \pm 0.03a	-	-	0.31 \pm 0.04a	-	-
CK FBs	iP	0.48 \pm 0.12a	-	+	0.50 \pm 0.22a	0 (-)	0
	tZ	5.42 \pm 0.60a	0 (+)	+	6.12 \pm 1.20a	+	0
	DZ	0.41 \pm 0.09a	-		0.54 \pm 0.16a	0 (-)	
	cZ	0.30 \pm 0.08a	-		0.24 \pm 0.08a	-	
CK OGs	tZOG	16.40 \pm 1.64a	-	+	10.84 \pm 1.63b	-	+
	tZROG	171.66 \pm 18.20a	0 (+)	+	61.63 \pm 7.14b	0	+
	DZOG	4.17 \pm 0.27a	-	+	3.52 \pm 0.40a	-	+
	DZROG	11.33 \pm 0.96a	-	+	9.95 \pm 0.83a	0 (-)	+
	cZOG	0.40 \pm 0.04a	-	0	0.58 \pm 0.07a	-	0
2-MeS-CKs	cZROG	8.49 \pm 0.78a	-	-	8.41 \pm 1.02a	-	-
	2-Me-SZ			0			0
	2-Me-SZR	38.22 \pm 4.44a	-	+	49.29 \pm 7.19a	-	-

Means within a row followed by different letters indicate significant difference between green vs. yellow leaves (Kruskal–Wallis test; $P < 0.001$).

et al., 2013; Zhang et al., 2016) and several other leaf-mining moths (Engelbrecht et al., 1969; Engelbrecht, 1971), infestation by *P. mespilella* draws a very different picture. The overall CK content in mined areas strongly decreases both on green and yellow leaves compared to control areas. Increased levels of CKs can only be observed in mines on yellow leaves for three CK active (or easily hydrolysable to active) forms (iPR, tZR, tZ), most likely due to the strong decrease in CKs in non-infested leaf areas resulting from the senescing program, rather than a CK accumulation in the mine per se. Indeed, iPR and tZ levels in mines remain constant when leaves are turning yellow, whereas they decrease strongly in unmined areas. tZR displays a slightly different pattern with an increased concentration in mines from yellow leaves compared to mines from green leaves.

Increased levels of iPR, tZR, and tZ in mines from yellow leaves can allow insects to induce a 'green island' and keep an appropriate nutritional supply in a degenerating context. This strategy is similar to *P. blancardella* but

underlying mechanisms are most likely different. First, *P. blancardella* larvae produce and deliver CKs to the plant, especially in yellow leaves, inducing an accumulation of CKs in mines (Zhang et al., 2017). For *P. mespilella*, somehow larvae most likely only maintain the CK content of mines, thereby enabling insects to overtake the plant senescing program. Whether the increased level of tZR in mines from yellow leaves compared to mines from green leaves results from activation of a plant or insect CK biosynthetic pathway, or simply from metabolic conversions from other CK compounds or CK translocations from other leaf areas, remains to be established. Second, CK profiling indicates distinct patterns between *P. blancardella* and *P. mespilella* (absence of DZ and cZ in *P. blancardella*; absence of DZNT, cZNT, and 2-MeS-Z in *P. mespilella*). Third, characterization of CKs in apple leaves attacked by *P. blancardella* indicates that mines are enriched in CKs both on green (2.5-fold increase compared to control) and yellow leaves (4-fold increase compared to control) (Zhang et al., 2017), whereas higher

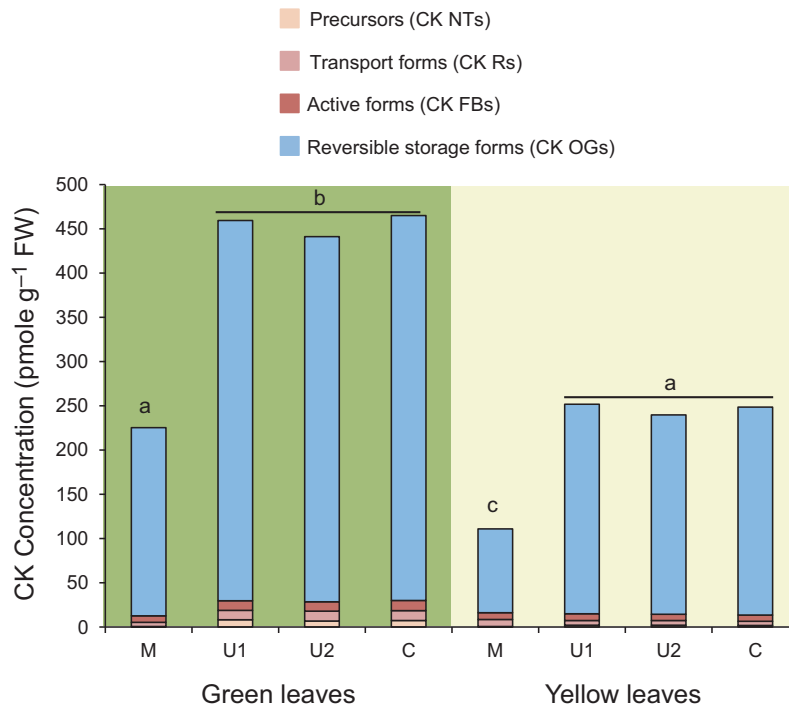


Figure 1 Cytokinin (CK) metabolites quantified (pmole g⁻¹ dry weight) in green and yellow apple leaves (n = 22 in green leaves, n = 12 in yellow leaves). Levels of four functional groups of CKs [nucleotides (CK NTs): iPNT and tZNT], transport forms [ribosides (CK Rs): iPR, tZR, DZR, and cZR; see Table 1 for abbreviations], active forms [free bases (CK FBs): iP, tZ, DZ, and cZ], and reversible storage forms [O-glucosides (CK OGs): tZOG, tZROG, DZOG, DZROG, cZOG, and cZROG] in *Phyllonorycter mespilella* mined (M) and unmined (U) plant tissues (ipsilateral, U1; contralateral, U2), and in control tissues (C). Columns capped with the same letter are homologous groups (Kruskal–Wallis test followed by pairwise multiple comparisons).

levels of CKs are only observed for iPR, tZR, and tZ on yellow leaves for *P. mespilella*. Taken together, these results suggest that mechanisms underlying the plant–insect interaction differ between the two leaf-miner species, and that *P. mespilella* larvae most likely do not produce CKs and only buffer the degradation of CKs occurring during the senescence of apple tree leaves. This hypothesis is backed-up by the overall homeostasis of the CK content of mined areas in green and yellow leaves.

The benefits of decreased CK levels in mined areas on green leaves for the plant or for the insect are difficult to estimate because CKs are plant hormones that play a key role in numerous plant physiological processes, including plant morphology, plant defense, leaf senescence, and source–sink relationships (Mok & Mok, 2001; Sakakibara, 2006; Giron et al., 2013). It may well be a plant defense response (Giron et al., 2013; Naseem et al., 2014) or an insect-mediated modulation of CKs toward an optimal steady CK profile to the benefit of the insect. However, the maintenance of functional green tissues on yellow leaves is of considerable ecological value to the development of the larvae as it allows the insect to maintain a favorable

nutritional environment in an otherwise degenerating context providing the leaf-miners with the required nutrients for completing their development before winter (Body et al., 2013, 2015). This also enables the larvae to allow potentially for an additional generation of insects (Kaiser et al., 2010).

Bacterial symbionts have been hypothesized to contribute to the production of CKs, by *P. blaucardella* especially through the synthesis of specific 2-MeS-CKs (Zhang et al., 2017). Several other lines of evidence also suggested that CKs are likely to originate from microbial symbionts and *Wolbachia* was identified as a key candidate (Giron et al., 2007; Kaiser et al., 2010; Body et al., 2013; Gutzwiller et al., 2015). Interestingly, *P. mespilella* do not host *Wolbachia* and modulation of CK levels is impaired compared to *Wolbachia*-infected *P. blaucardella*. Antibiotic treatments have previously shown that *Wolbachia*-free *P. blaucardella* larvae lose their ability to induce CK-mediated phenotypes and that only larvae harboring bacterial symbionts contain significant amounts of CKs that are not plant-derived (Kaiser et al., 2010; Body et al., 2013). In the Western corn rootworm (*Diabrotica virgifera*

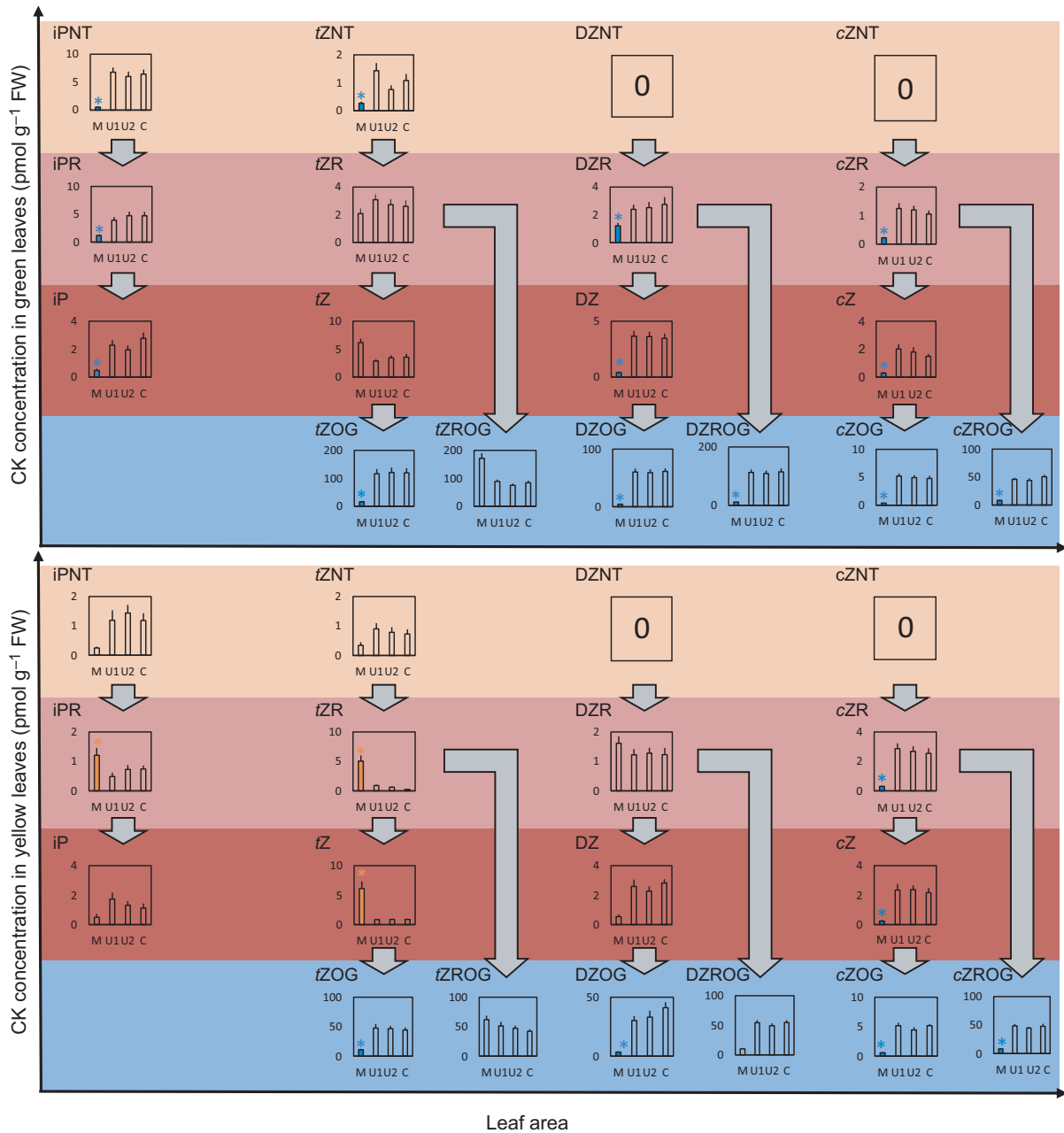


Figure 2 Changes in mean (+ SEM) cytokinin (CK) levels (pmole g⁻¹ dry weight) in green (upper panel) and yellow (lower panel) apple leaf tissues (n = 22 in green leaves, n = 12 in yellow leaves) of *Phyllonorycter mespillella* mined (M) and unmined (U) areas (ipsilateral tissues, U1; contralateral tissues, U2), and control tissues (C). Metabolic flows between precursors (CK NTs, light orange; see Table 1 for abbreviations), transport forms (CK Rs, light red), active forms (CK FBs, dark red), and reversible storage forms (CK OGs, blue) are indicated by large arrows based on a current model of CK biosynthesis pathways (Mok & Mok, 2001; Sakakibara, 2006; Spíchal, 2012). Asterisks indicate statistical differences (orange asterisks indicate an increase, blue asterisks indicate a decrease).

virgifera LeConte), *Wolbachia* was also shown to alter the plant physiology by suppressing defense-related gene expression in maize plants but *Wolbachia*-free populations failed to do so (Barr et al., 2010; see Robert et al., 2013; for

contradictory results suggesting that the observed effects of symbionts can be context dependent). Consistent with previous studies, our results suggest that bacterial symbionts may be strictly required to allow leaf-mining larvae

to produce and deliver CKs to the plant. Manipulative experiments or investigation of several populations that differ in their *Wolbachia* infection status will now be required to validate this hypothesis in *P. mespilella* and to identify how the absence of *Wolbachia* is involved in the metabolic capacities of *P. mespilella* larvae and their ability to modulate the phytohormonal profile of the mine. Comparisons of life-history traits and population dynamics in *P. mespilella* and *P. blancardella* are expected to display marked differences due to the inability of *P. mespilella* larvae to induce a large accumulation of CKs in mines therefore most likely compromising their ability to fully control their nutritional supply (Body et al., 2015) and the plant immune response (Body et al., 2013) in both green and yellow leaves.

Conclusion

Our study demonstrates that modulation of plant CK levels is largely impaired in the *Wolbachia*-free leaf-mining moth *P. mespilella* and that accumulation of active CKs is strictly restricted to mines on yellow leaves. This contrasts with results previously observed in the closely related *P. blancardella* that shared common plant alteration patterns with other leaf-miners and gall-inducer species. These results suggest that mechanisms underlying the plant-insect interaction differ between the two leaf-miner species and that *P. mespilella* larvae most likely do not produce CKs. Additionally, an extensive identification and quantification of phytohormones was required to reveal plant alterations that are relevant to the ecology of the leaf-mining larvae, the total CK content being lower in mines on both green and yellow leaves. Finally, our understanding of how CKs might regulate plant biotic interactions and how they interact with other phytohormones has been mostly derived from studies on model plant species such as *Arabidopsis thaliana* (L.) Heynh. and *Oryza sativa* L. (Kazan & Manners, 2009; Naseem et al., 2014). Too much generalization will neglect the involved complexity in a particular biotic interaction, especially when it involves a tripartite relationship between a plant, an insect, and bacterial symbionts. As a consequence, elucidating molecular mechanisms underlying the observed decrease in CKs in leaf tissues attacked by *P. mespilella* and estimating their fitness consequences for the insect will require further investigations. However, converging experimental lines of evidence pointing toward the influence of bacterial symbionts in the ecology and evolutionary diversification of leaf-mining moths demonstrate the excitement that surrounds these investigations and the promise they hold for a fuller understanding of plant-biotic interactions.

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